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Eggshell calcium regulates calcium transport protein expression in an oviparous snake

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EGGSHELL CALCIUM REGULATES CALCIUM TRANSPORT PROTEIN EXPRESSION IN AN OVIPAROUS SNAKE

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By

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Abstract

One hypothesis to explain the high incidence of independent evolutionary transitions from oviparity to viviparity among squamate reptile (snake and lizard) lineages proposed that embryonic development is independent of eggshell calcium. Recent research on embryonic squamate calcium nutrition does not support this hypothesis as at least 25% of the calcium in hatchling oviparous squamates is extracted from the shell. An alternative hypothesis is that shell calcium supplements calcium from yolk in oviparous squamates, but is not obligatory for embryonic development. We tested this hypothesis by physically peeling the outer layers of the shell, and thus removing the calcium, early in development of *Pantherophis guttatus* (corn snake) eggs. The hypothesis alternative was supported by experimental results showing that survivorship to hatching did not differ for peeled eggs compared to intact eggs. Yet hatchlings from peeled eggs were shorter (273.6 ± 3.4 vs. 261.0 ± 3.7 mm, $p=0.0028$, $n=16$), lighter (6.36 ±0.22 vs. 5.75 ± 0.23 g, $p=0.0158$, $n=16$), and had reduced calcium (40.8 ± 1.7 vs. 30.5 ± 1.8 mg, $p<0.001$, $n=16$), which could impact hatchling fitness in the wild. An additional hypothesis that embryos detect and respond to calcium availability was tested by assaying the two tissues involved in embryonic calcium acquisition for changes in expression of calcium transport protein (by immunoblotting) following shell calcium removal. The yolk sac, involved in yolk calcium transport, had no detectable change in the developmental expression of calbindin-D28K, a marker for calcium transport activity. However, the chorioallantois, involved in shell calcium transport, showed reduced calbindin-D28K expression relative to samples from intact eggs, suggesting

eggshell calcium regulates chorioallantoic calcium transport activity. The findings of this study suggest that evolution of viviparity is enhanced by a mechanism for detection and mobilization of eggshell calcium in oviparous embryos that also functions in the uterine environment.

Introduction

A 70 kg human has 1260 g of calcium. Humans (regardless of size or age) have about the same concentration of calcium in their bodies. Over 98% of that calcium is in the skeleton, and the remaining calcium is used in processes like muscle contraction and nerve conduction (McCarthy and Kumar, 1999). Calcium is essential in all vertebrates. However, no vertebrate begins life as an egg with the high calcium concentration it will have as an adult. For the neonate to have all the calcium it needs, calcium must be acquired during embryonic development. In *Pantherophis guttatus* (corn snakes), the majority of embryonic calcium uptake occurs in the developmental stages just before hatching (Stewart et al., 2004).

Among vertebrates, the calcium required for the formation of the skeleton can come from one or more of four sources, depending on reproductive mode. Yolk is a major source of calcium for oviparous, or egg-laying, vertebrates. However, the yolk of most oviparous vertebrates is not sufficiently provisioned to provide all needed calcium to complete development. In oviparous terrestrial vertebrates, the eggshell is a source of additional calcium. In some, like archosaurs and turtles, the eggshell provides most of the calcium used by the developing embryo, and in some birds 90% of hatchling calcium comes from the eggshell (Packard 1994). In squamates (snakes and lizards), most of the calcium acquired by the embryo comes from the yolk and is supplemented to varying degrees by eggshell calcium (Stewart and Ecay, 2010). Viviparous reptiles and mammals—those that birth live young—can acquire additional calcium from the mother through the placenta. Bony fish and

amphibians, which lay their eggs in water, do not have a calcified eggshell; they obtain calcium from their surrounding environments.

Throughout the course of vertebrate evolution, about 140 independent transitions from oviparity to viviparity have occurred, and over 100 of these transitions have taken place in squamate species. It is not yet known, however, why this occurs more frequently in squamates than in other vertebrate lineages. It is suspected the transition is constrained by calcium utilization patterns during embryonic development.

Although based on limited observation, one of the original hypotheses spurring this research was that eggshell calcium is not necessary for the growth and development of oviparous squamate embryos (Packard et al., 1977). This hypothesis, while provocative, has never been directly tested. As mentioned, hatchling squamates acquire a larger percentage (often over 50%) of their calcium from the yolk than other reptiles, but recent studies show that squamates do acquire calcium from the eggshell as well (Packard 1994; Stewart and Ecay, 2010). Though not a direct test of the calcium independence hypothesis, this finding suggests that eggshell calcium is more important than the hypothesis of Packard et al. (1977) predicts. To conduct a more direct test, the calcium crystal layer from the outside of the eggshell was removed (through peeling), making them more easily comparable to developing embryos of viviparous squamates. Embryos were then sampled at late developmental stages and at hatching to assess growth, calcium content, and expression of calcium transport proteins.. Although the embryos survived to hatching, they were smaller than the hatchlings from the intact eggs and

had reduced calcium overall. This finding indicates that embryonic growth is sensitive to available calcium and, as size contributes to fitness in natural settings, can have an impact on the eventual survival of the hatchling, which may too suggest that eggshell calcium is more important than the hypothesis of Packard et al. (1997) predicts.

Eggs of all oviparous amniotes share a set of common structures. Surrounding and containing the embryo is the eggshell. The eggshell is formed in the oviducts shortly after fertilization and is composed of multiple layers of fibrous protein, and coating the outside of the eggshell is a layer of calcium carbonate crystals. Early in development the fused chorion and allantois membranes grow out to surround the embryo. The chorioallantois lines the inner surface of the shell and surrounds the yolk sac, amnion, and embryo. In amniotes, extraembryonic calcium (i.e., eggshell or placental) is transported to the embryo through the blood vessels of this membrane. As mentioned, the yolk is a major source of calcium (in addition to other nutrients) for the developing embryo. These nutrients are then transported through blood vessels in the yolk sac membrane to the embryo.

Several mechanisms of epithelial calcium transport have been described in other tissues. These are well studied in the kidney and intestinal epithelial cells of chickens and mammals, though less is known about calcium transport mechanisms in the extraembryonic membranes of snakes. The protein calbindin-D28K is a component in one such mechanism of epithelial calcium transport. Calbindin-D28K acts as a cytosolic buffer and intracellular transporter for calcium in transporting epithelial cells. When calcium enters the cell, it binds to calbindin-D28K, which then

diffuses across the cytosol. A plasma membrane calcium ATPase (PMCA) releases the calcium from calbindin-D28K and pumps it out of the cell, so that it is free to diffuse in the allantoic blood vessels to be carried to the growing embryo.

Vitamin D maintains whole body calcium by regulating calcium transport in the gut and kidneys. When the amount of calcium in the blood is low, parathyroid hormone is produced. Parathyroid hormone increases 1-α-hydroxylase activity, which catalyzes the final step in the activation of vitamin D (McCarthy and Kumar, 1999). The active form of vitamin D alters gene expression in the intestine and the kidney, inducing synthesis of calcium transport proteins (such as calbindin-D28K). This facilitates intestinal absorption of dietary calcium and reabsorption of filter calcium by the kidney. Both activities raise blood calcium levels toward normal. It is hypothesized that vitamin D plays a similar role regulating calcium transport in amniote embryonic development.

Because the hypothesis of Packard et al. (1977) is not supported by data gathered after removing eggshell calcium, an alternative hypothesis is needed. One possibility is that there is a mechanism allowing squamates to transition from the utilization of eggshell calcium in oviparity to the utilization of placental calcium in viviparity more easily than non-squamates. In other calcium transporting systems like chicken intestine and kidney, it has been shown that a calcium deficiency results in the up-regulation of calcium transport proteins (Bar et al., 1990, 1998; Meyer et al., 1992). We hypothesized that calcium transport proteins in developing corn snake embryos are regulated similarly. The removal of the eggshell calcium will create a demand for calcium by the embryo, and the expression of calcium transport

proteins will increase as a result. Because the chorioallantois is involved in the transport of calcium from these external sources, it is likely to show some evidence of any such mechanisms. For the purposes of this study, we have compared calcium transport protein expression by the chorioallantois of peeled versus intact eggs. The results indicate that the chorioallantois is sensitive to its environment. When lacking the external calcium from the eggshell, notable changes occur in calbindin expression.

Materials and Methods Draft

Paired samples (peeled versus intact) of yolk sac and chorioallantoic tissues from *Pantherophis guttatus* were analyzed at different developmental stages by immunoblotting to determine expression of calcium transport proteins (calbindin-D28K, carbonic anhydrase II, and PMCA). Actin immunoblotting was used as a control for sample loading into gels and electroblotting transfer efficiency. Protein expression was quantified using densitometry, and these readings were analyzed statistically.

Sampling

Eggs used in this study came from *Pantherophis guttatus* research colonies at East Tennessee State University and Trinity College (Hartford, CT). Eggs were obtained shortly after oviposition and incubated over moist vermiculite at 26°C, as previously described (Stewart et al., 2004). With external morphology as an indicator, embryonic stage was determined by sampling one egg from each clutch (Zehr, 1962). Half the eggs from each clutch had the outer layers of their eggshells peeled shortly after oviposition to remove more than 90% of the eggshell calcium. The remaining unpeeled eggs served as the control group.

Eggs were sampled for yolk sac and chorioallantois tissues in pairs (one peeled egg with one intact egg from the same clutch) at different stages through development. For stage 36, which lasts three times longer than most other stages, samples were taken in early, middle, and late stage 36. Tissue samples were placed in cell lysis buffer (Sigma) at a volume to tissue weight ratio of 3:1 and frozen at -80°C for later use.

Samples were thawed and additional cell lysis buffer was added for homogenization (1 X the original volume of buffer for chorioallantois and yolk sac stage 28 and earlier, 2 X the original volume of buffer for later stage yolk sac) in 1.5 mL microcentrifuge tubes. Glass beads were added, and a cell disrupter was used to homogenize the tissues (3 x 5 minutes at 4°C). Homogenized samples were centrifuged for ~1 minute at 15,000 rpm to pellet beads and homogenization resistant tissue fragments. Supernatants were transferred to new tubes and samples (5-10 µL) taken for protein assay. If not used immediately, samples were frozen at -80°C. The Pierce BSA Protein Assay microtiter plate protocol was used to determine the protein concentration of each sample.

Electrophoresis and Immunoblotting

SDS-polyacrylamide gels (5-20% gradient) were prepared (or precast Novex NuPAGE® 4-12% Bis-Tris gels were used). Samples were diluted to 2.5-5.0 µg protein/µL with 2X electrophoresis sample buffer (ESB), and gel lanes loaded with 25-50 µg homogenate protein (Laemmli, 1970). The gels were electrophoresed for approximately 45 minutes at a constant voltage of 200 V. Some gels were stained in 0.25 % Coomassie blue overnight to visualize protein bands formed in the electrophoretic separation. Novex Sharp Pre-Stained Protein Standards were used to identify sample proteins.

For electroblotting, gels were placed in blotting buffer (192mM Glycine + 25mM Tris (pH 8.3) + 10% methanol + 0.01% SDS) for 30 minutes and transferred to PVDF membranes (Millipore) with a constant current of 200 milliAmps for 2.5

hours (Towbin et al., 1979). Electroblotted proteins were visualized by staining in 0.1% Ponceau S and the position of molecular weight standards marked in pencil.

Subsequent to electroblotting, PVDF membranes were washed in blocking buffer (5% nonfat dry milk, 2% horse serum, 0.05% Tween-20, 0.02% sodium azide in Tris-buffere saline (TBS; pH 7.4)) for 1 hour and incubated in a primary antibody solution overnight in at 4°C or for 4 hours at room temperature with constant agitation. Primary antibody dilutions (in blocking buffer) used in this study were 1:50,000 rabbit polyclonal anti-corn snake calbindin-D28K (developed by the lab, Ecay and Stewart, unpublished), 1:500 mouse monoclonal (2A2-1) anti-chick carbonic anhydrase II (Dr. Paul Linser, Whitney laboratory, University of Florida, St. Augustine, Florida), 1:2,000 mouse monoclonal (clone 5F10) anti-human PMCA (Sigma, A7952), and 1:50,000 mouse monoclonal (clone C4) anti-chicken gizzard actin (Millipore, MAB 1501). The membranes went through a series of washes (3 x 5 minutes blocking buffer and 3 x 5 minutes blocking buffer). Then the membranes were incubated in a secondary antibody solution (ECL™ anti-rabbit IgG, horseradish peroxidase-linked whole antibody from donkey and ECL™ anti-mouse IgG, GE Healthcare horseradish peroxidase-linked whole antibody from sheep) for 2 hours. Then the membranes went through another series of washes (2 x 5 minutes blocking solution, 2 x 5 minutes TBS/Tween-20 solution, and 2 x 5 minutes TBS).

Quantification and Statistical Analysis

The PVDF membranes were covered in 1:1 Millipore chemiluminescence reagent and exposed to x-ray film. This procedure allowed for a visualization of immune complexes. Exposed and developed films were digitized on a flatbed

scanner (Epson Perfection V500 Photo) and quantified using densitometry (Un-Scan-It Gel™ 5.3 digitizing software, Silk Scientific).

Subsequent rounds of immune-detection were achieved by stripping the PVDF membranes of the first round of primary and secondary antibodies in TBS/Tween-20 solution and stripping solution (50 mM Glycine pH 2.75 + 0.2% SDS + 0.1% Tween-20). They were then run through a second round of immunoblotting with a different set of antibodies. The typical order of antibody reactions was carbonic anhydrase II followed by calbindin-D28k and finally actin.

Densitometry readings were statistically analyzed using the SAS 9.1 program with a mixed model analysis of variance and Scheffe's multiple comparisons test. Actin densitometry readings were used as a covariate. Estimates of densitometry (quantified expression) produced by this analysis were relativized and compared in bar graphs. Expression in paired intact and peeled samples were compared with one another at each individual developmental stage (36E, 36L, and 37), then all three stages were compared to one another.

Results

The effect of peeling on calbindin-D28K expression in yolk sac membrane was tested. No difference in expression between yolk sac samples from peeled and intact eggs was observed. Figure 1 shows calbindin expression in yolk sac membrane samples from peeled and intact eggs at stages 34, 36E, and 36L. These results confirm prior observations that calbindin expression in corn snake yolk sac membrane is unchanged by eggshell peeling, shown in Figure 2 (Ross et al., 2012). Carbonic anhydrase II and PMCA are not expressed at detectable levels in yolk sac membrane (Ecay and Stewart, unpublished).

The chorioallantoic membrane expresses all three proteins of interest. Increased expression of calcium transport proteins coincides with increased calcium mobilization late in development (Ecay et al., 2004). Therefore, samples from these later stages (36E, 36L, and 37) were chosen for immunoblotting analysis. Samples from both the embryonic and abembryonic poles at stage 37 were also analyzed to determine if there are any regional differences in calcium transport protein expression.

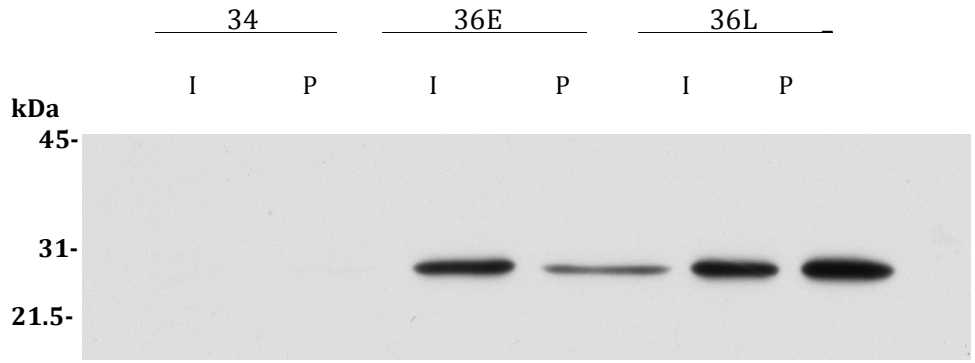

Figure 1. Protein expression in paired yolk sac tissue samples at stages 34, 36E, and 36L. Calbindin expression did not differ significantly between peeled (P) and intact (I) eggs.

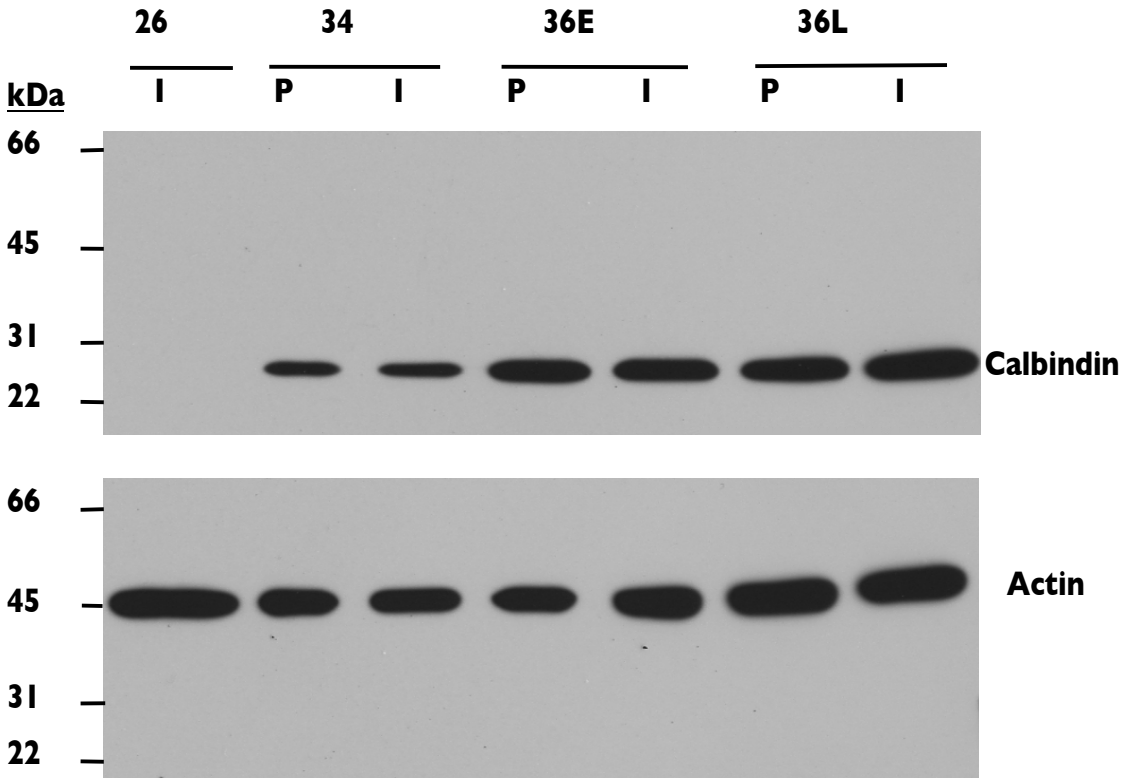

Figure 2. Protein expression in paired yolk sac tissue samples at stages 26, 34, 36E, and 36L. Calbindin expression did not differ significantly between peeled (P) and intact (I) eggs. Actin was used as a control.

Figure 3 illustrates the typical results in an immunoblotting analysis of chorioallantoic membrane samples from peeled and intact eggs at three embryonic stages from one clutch of eggs lain by a single female. Each of the four panels show specific protein expression in the same chorioallantois samples on the same PVDF membrane. These samples came from paired eggs (one peeled with one intact) at three developmental stages (36E, 36L, and 37). The antibodies were stripped after each film was developed so that different antibodies could be used.

The first panel of Figure 3 shows PMCA expression. Though PMCA immunoblots of chorioallantois in three other squamate species—*Virginia striatula*, *Pseudemoia pagenstecheri*, and *Zootoca vivipara*--have produced consistent results in other studies, attempts to produce similar immunoblots from corn snake samples have not been as successful (Fregoso et al., 2012; Stinnett et al., 2011; Stewart et al., 2011). The immunoblot showing PMCA expression in the first panel of Figure 3 is not representative of PMCA expression in all samples. Aggregate data for PMCA in all analyzed samples shows no significant change in expression as a result of eggshell peeling (Figure 4).

The second panel of Figure 3 shows carbonic anhydrase II expression. Carbonic anhydrase II expression also does not differ between samples as a result of peeling (Figures 5-9). No differences between embryonic and abembryonic samples at stage 37 were found (Figures 7 and 8). The pattern of expression is consistent through all three stages (Figure 9).

The third panel of Figure 3 shows calbindin-D28K expression. Calbindin-D28K expression in samples from peeled eggs is significantly less than that in

samples from intact eggs (Figures 10-14). Differences in calbindin-D28K expression from peeled and intact eggs are similar in embryonic and abembryonic chorioallantois samples (Figures 12 and 13). The changes in expression of calbindin-D28K in chorioallantoic samples from peeled eggs are consistent through all three stages shown (Figure 14).

The fourth panel of Figure 3 shows actin expression. Actin expression is consistent in all samples. Actin is used as an internal control for sample loading into gels and electroblotting transfer efficiency. Actin expression is used to normalize the expression of other proteins for statistical analysis.

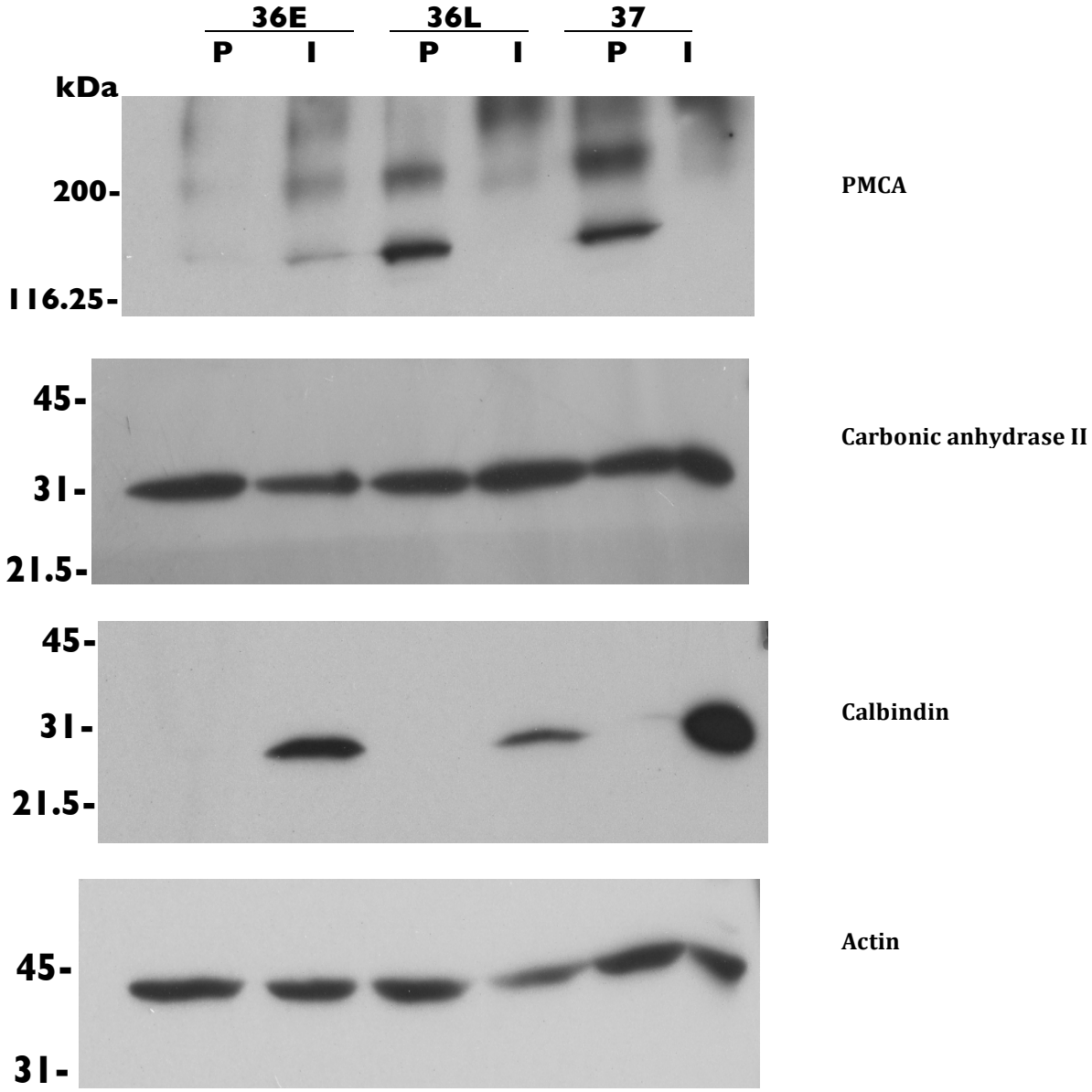

Figure 3. Protein expression in paired chorioallantoic tissue samples at stages 36E, 36L, and 37. Calbindin expression differs significantly between peeled (P) and intact (I) eggs. Samples from peeled eggs express far less calbindin than samples from intact eggs. PMCA and CAII do not show any change in samples from peeled eggs. Actin was used as a control.

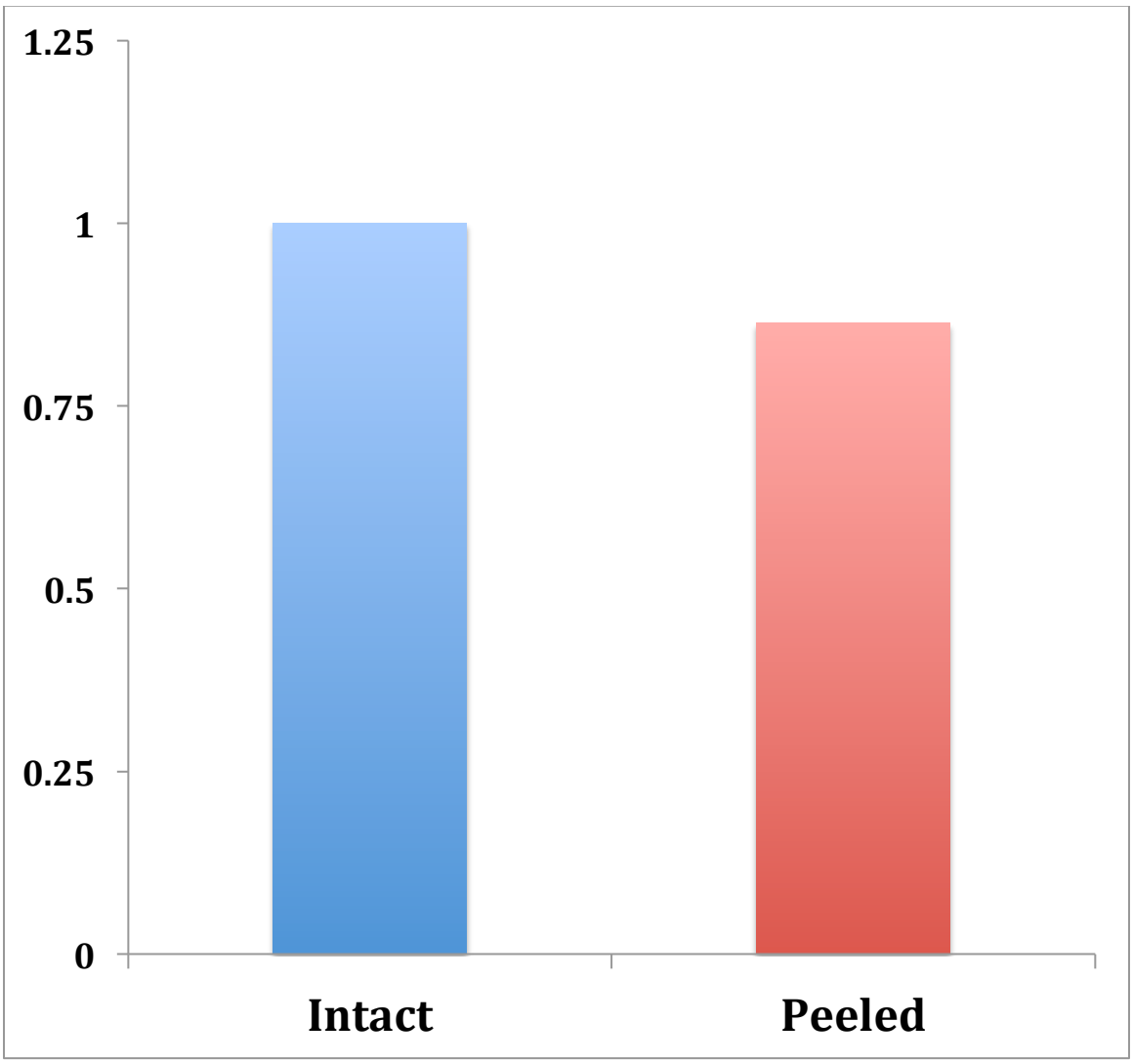

Figure 4. Relative expression of PMCA in embryonic chorioallantois from peeled and intact eggs at embryonic stage 37.
p = 0.7377 n = 7 paired samples from 3 females

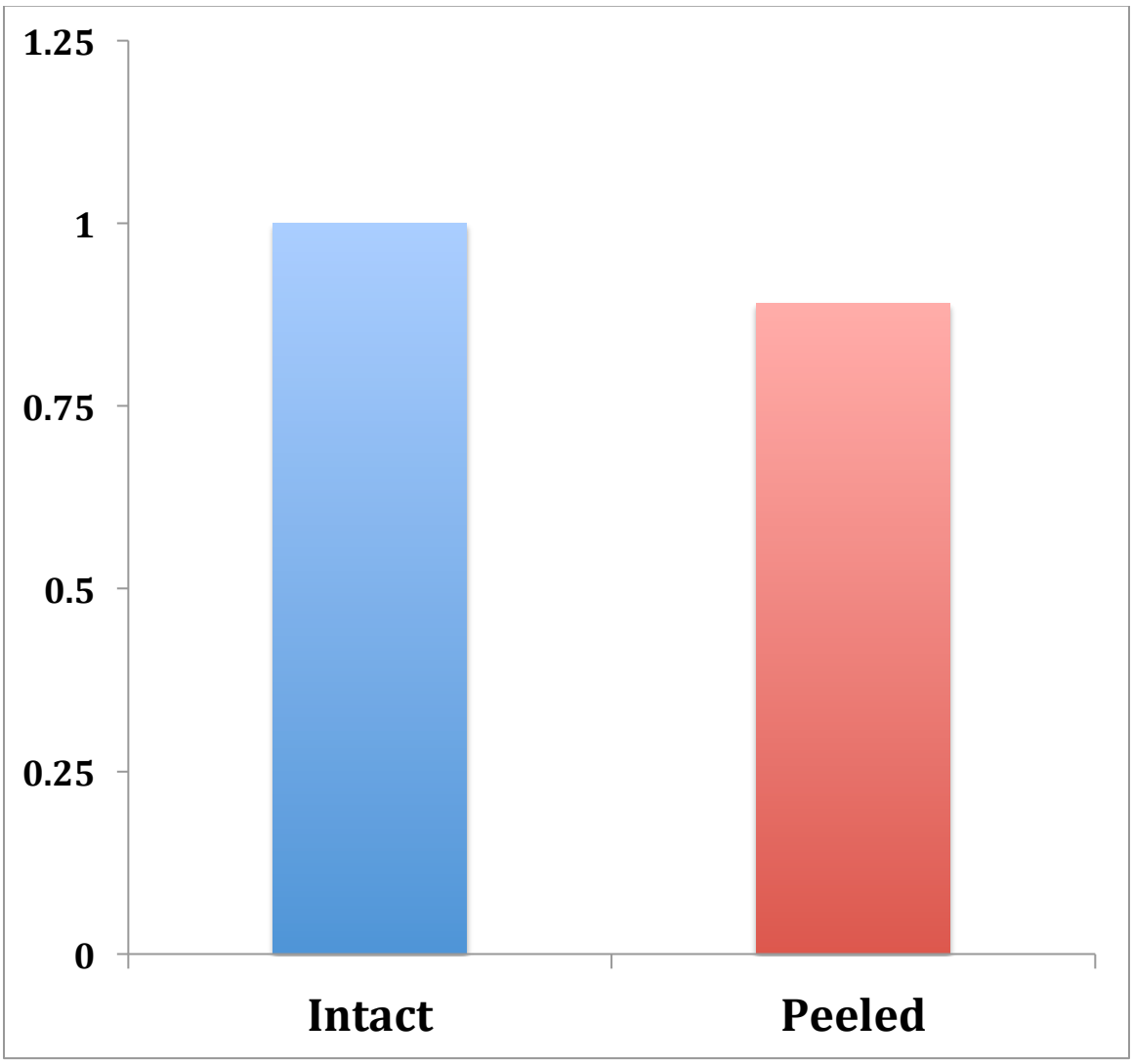

Figure 5. Relative expression of carbonic anhydrase II in embryonic chorioallantois from peeled and intact eggs at embryonic stage 36E.
p = 0.4377 n = 5 paired samples from 5 females

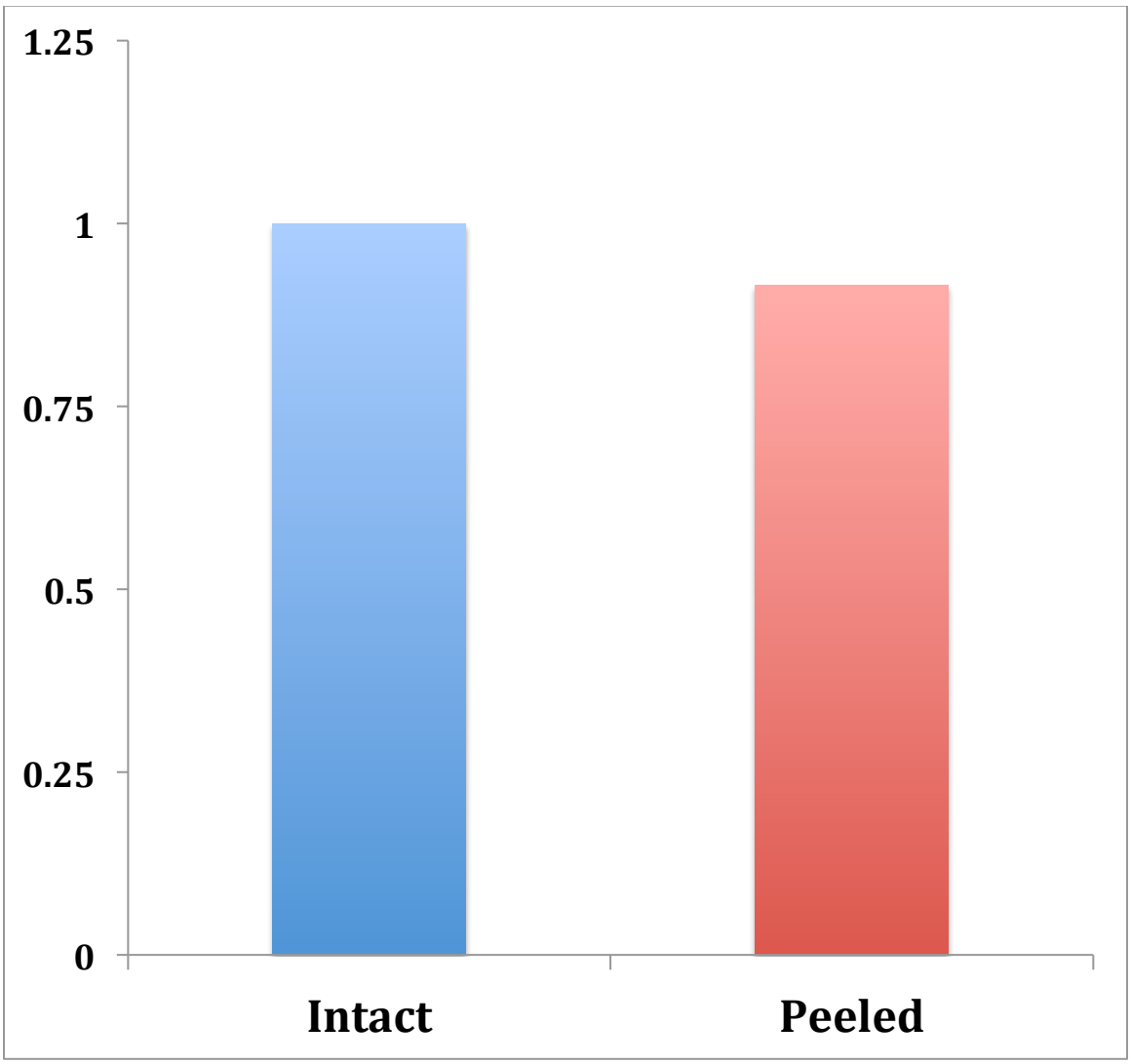

Figure 6. Relative expression of carbonic anhydrase II in embryonic chorioallantois from peeled and intact eggs at embryonic stage 36L.
p = 0.4663 n = 6 paired samples from 6 females

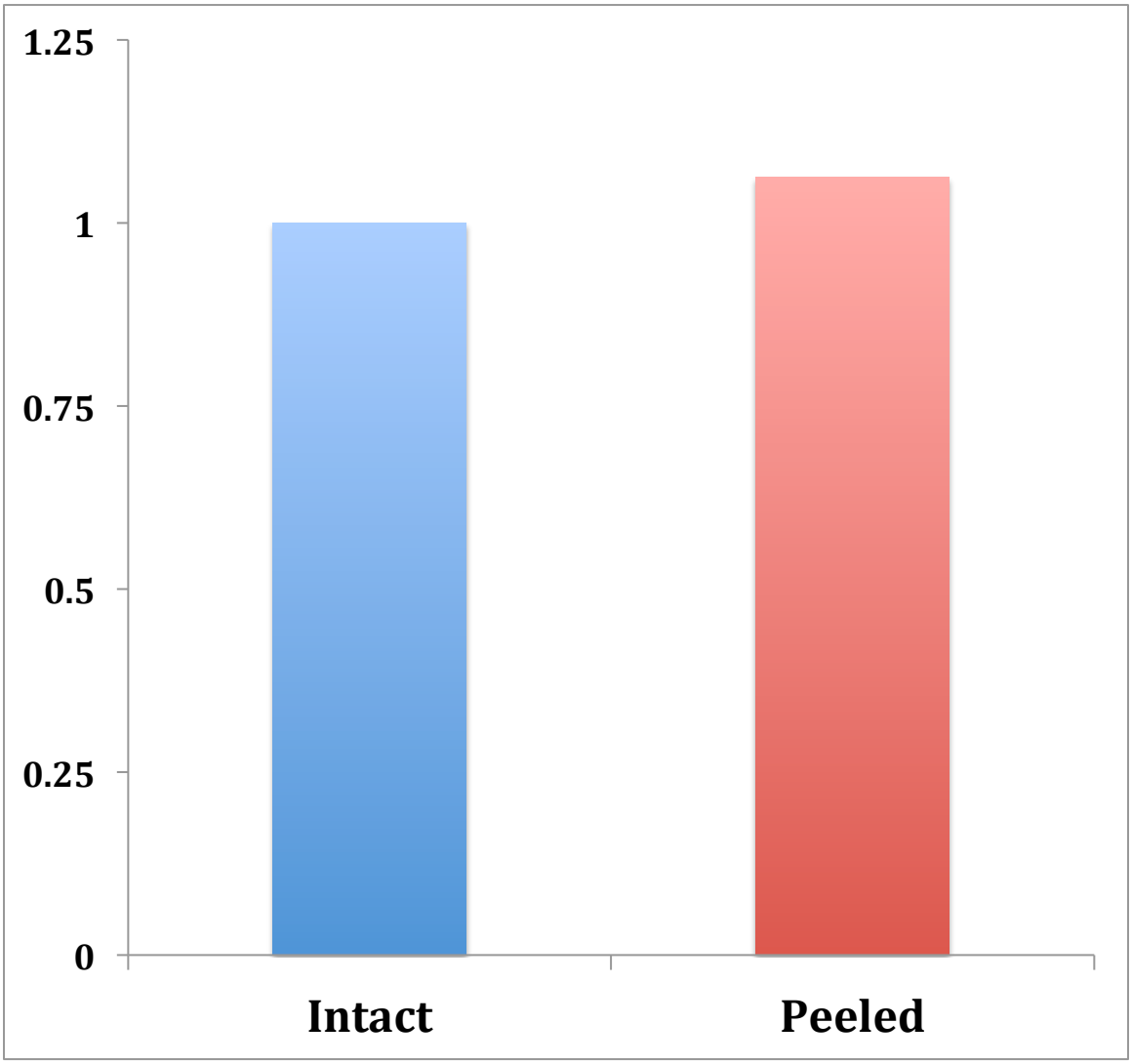

Figure 7. Relative expression of carbonic anhydrase II in embryonic chorioallantois from peeled and intact eggs at embryonic stage 37.
p = 0.8032 n = 12 paired samples from 8 females

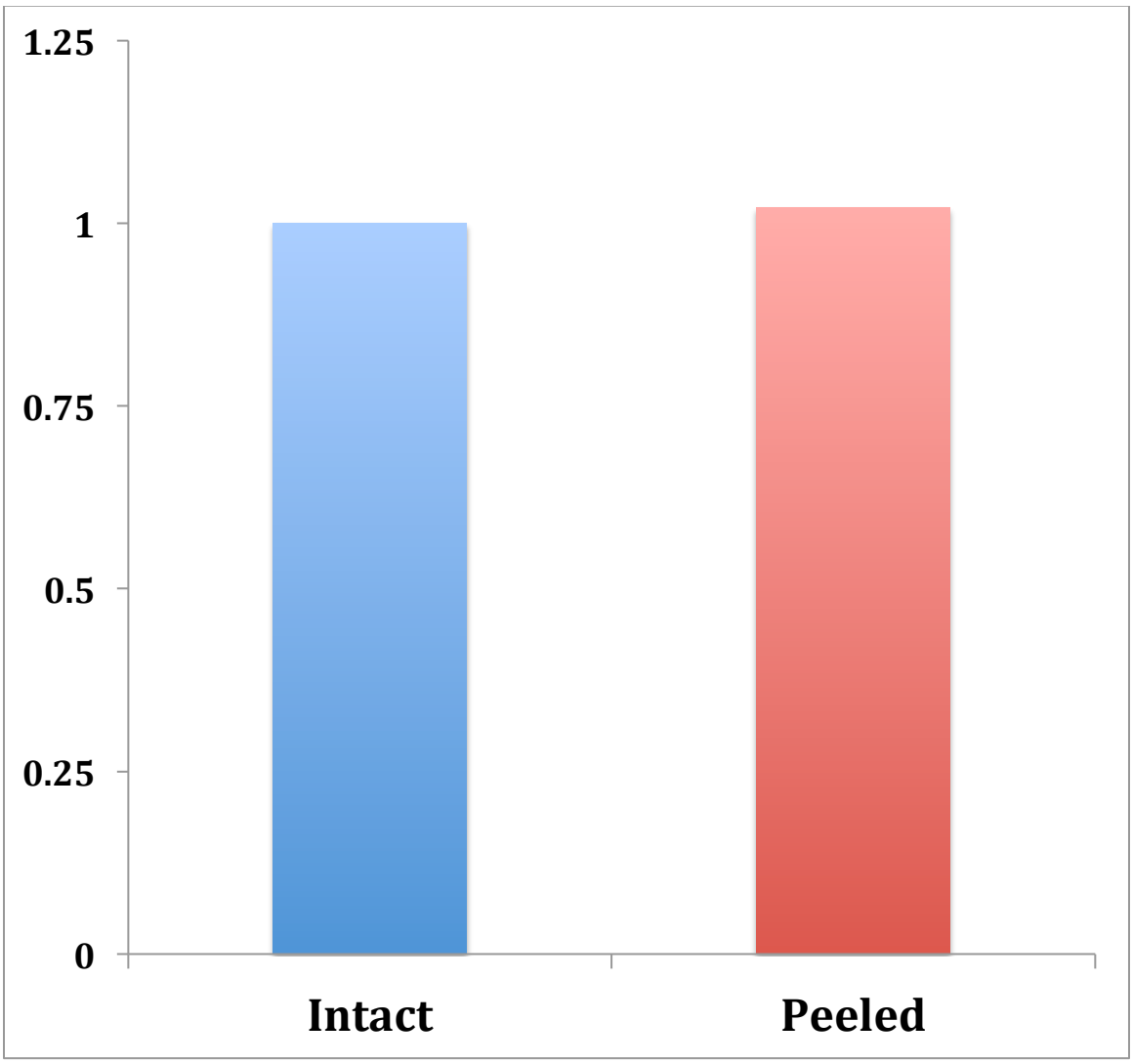

Figure 8. Relative expression of carbonic anhydrase II in abembryonic chorioallantois from peeled and intact eggs at embryonic stage 37.
p = 0.9360 n = 7 paired samples from 3 females

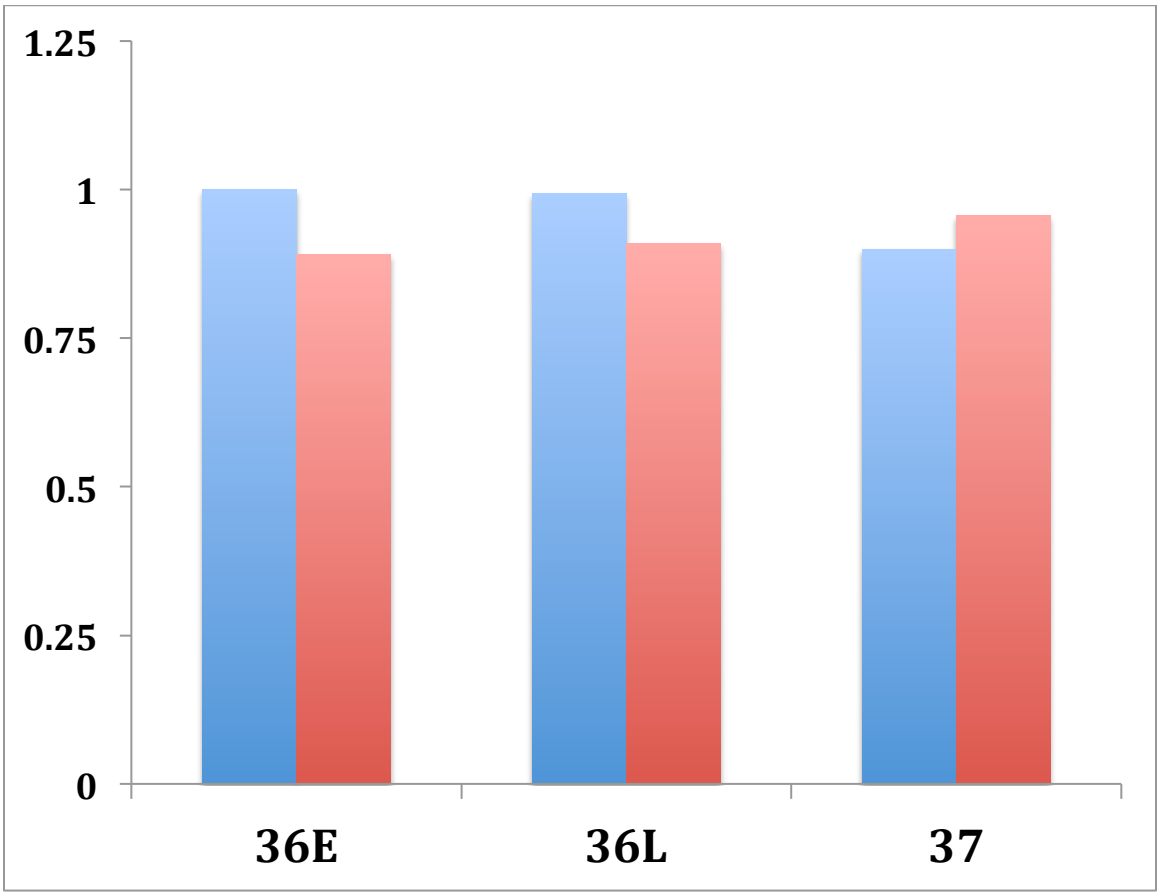

Figure 9. Relative expression of carbonic anhydrase II in embryonic chorioallantois from peeled and intact eggs at embryonic stages 36E, 36L, and 37.
36E: p = 0.4377 n = 5 paired samples from 5 females
36L: p = 0.4663 n = 6 paired samples from 6 females
37: p = 0.8032 n = 12 paired samples from 8 females

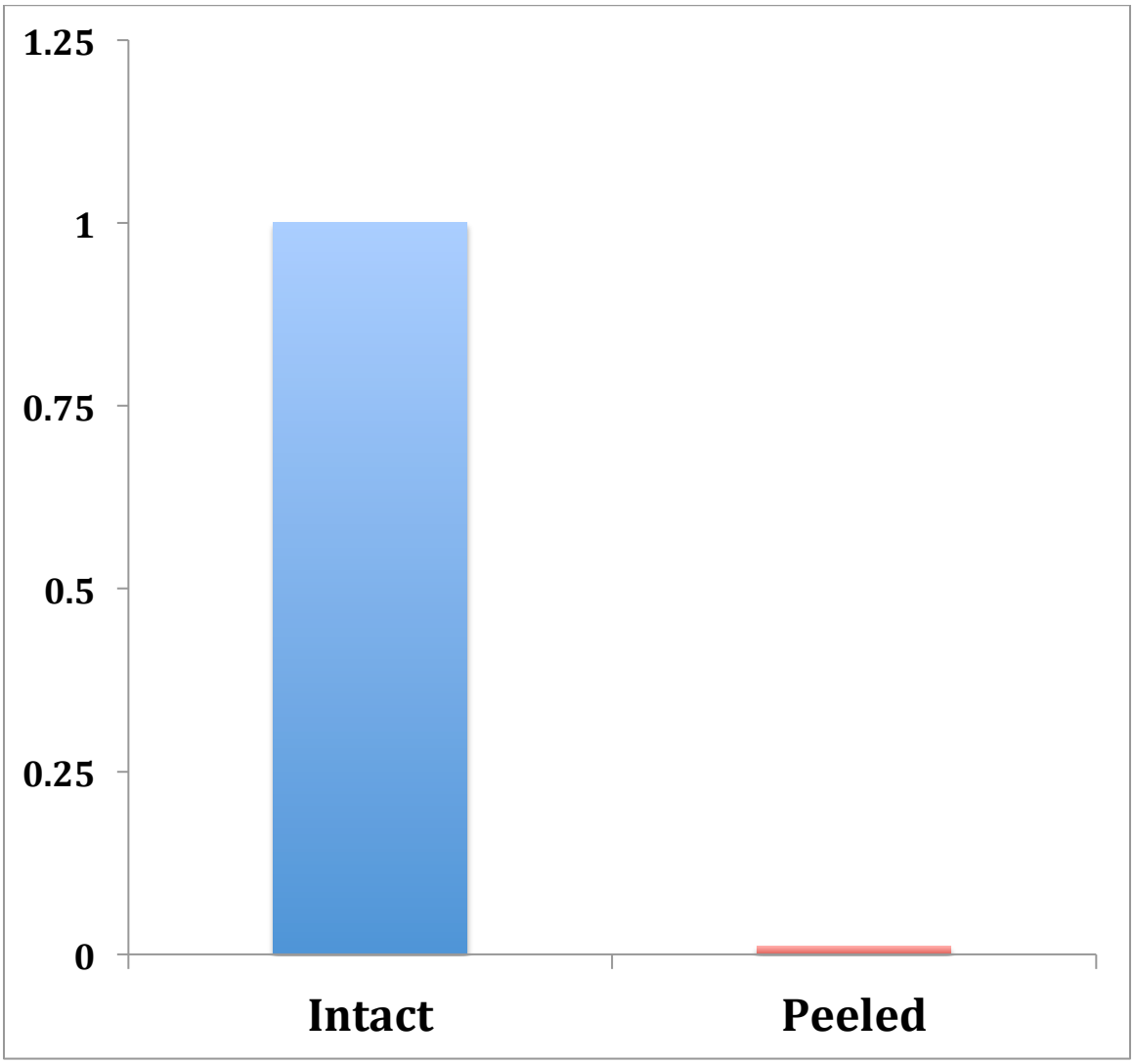

Figure 10. Relative expression of calbindin in embryonic chorioallantois from peeled and intact eggs at embryonic stage 36E.
p = 0.1358 n = 5 paired samples from 5 females

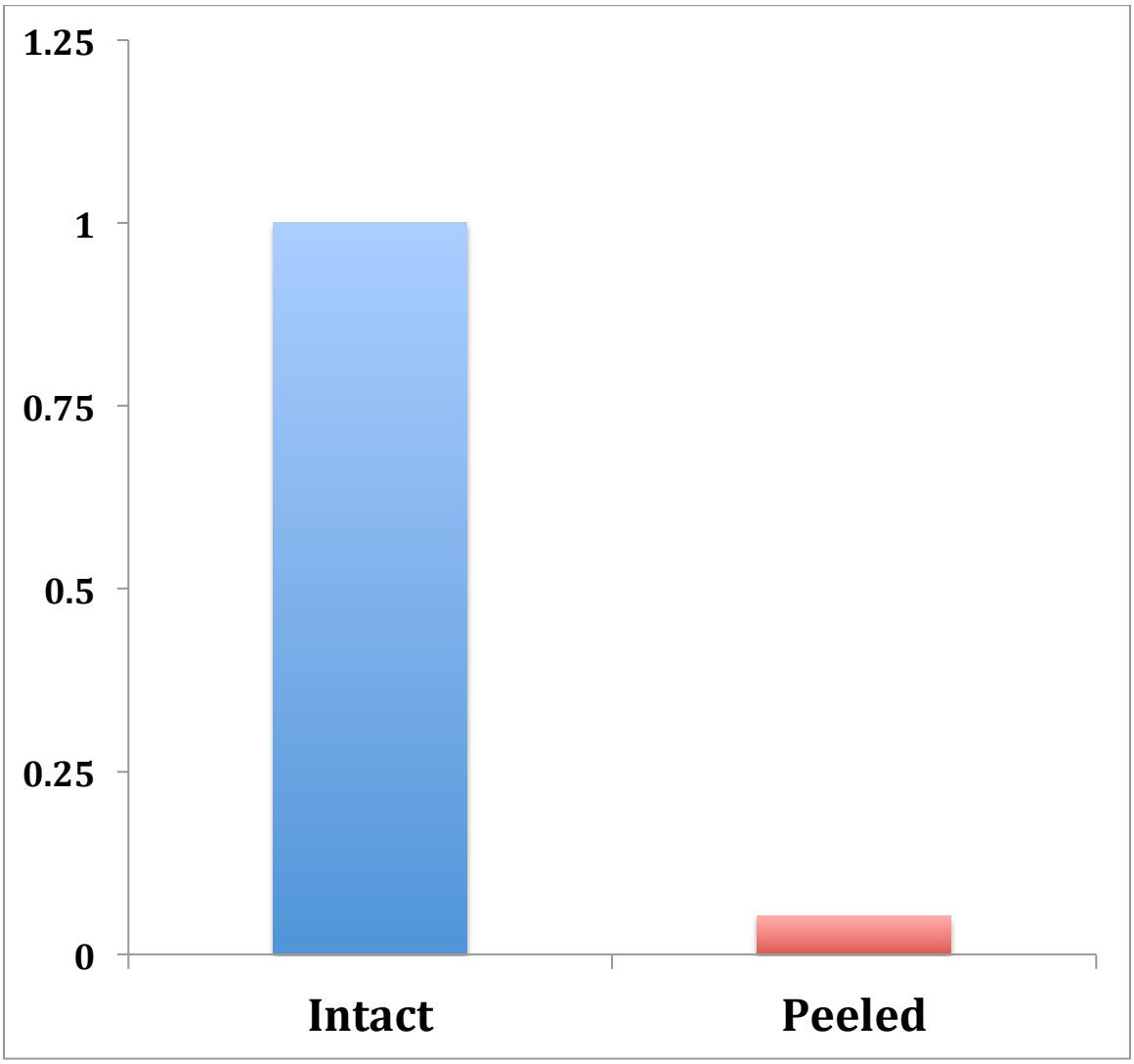

Figure 11. Relative expression of calbindin in embryonic chorioallantois from peeled and intact eggs at embryonic stage 36L.
p = 0.0258 n = 6 paired samples from 6 females

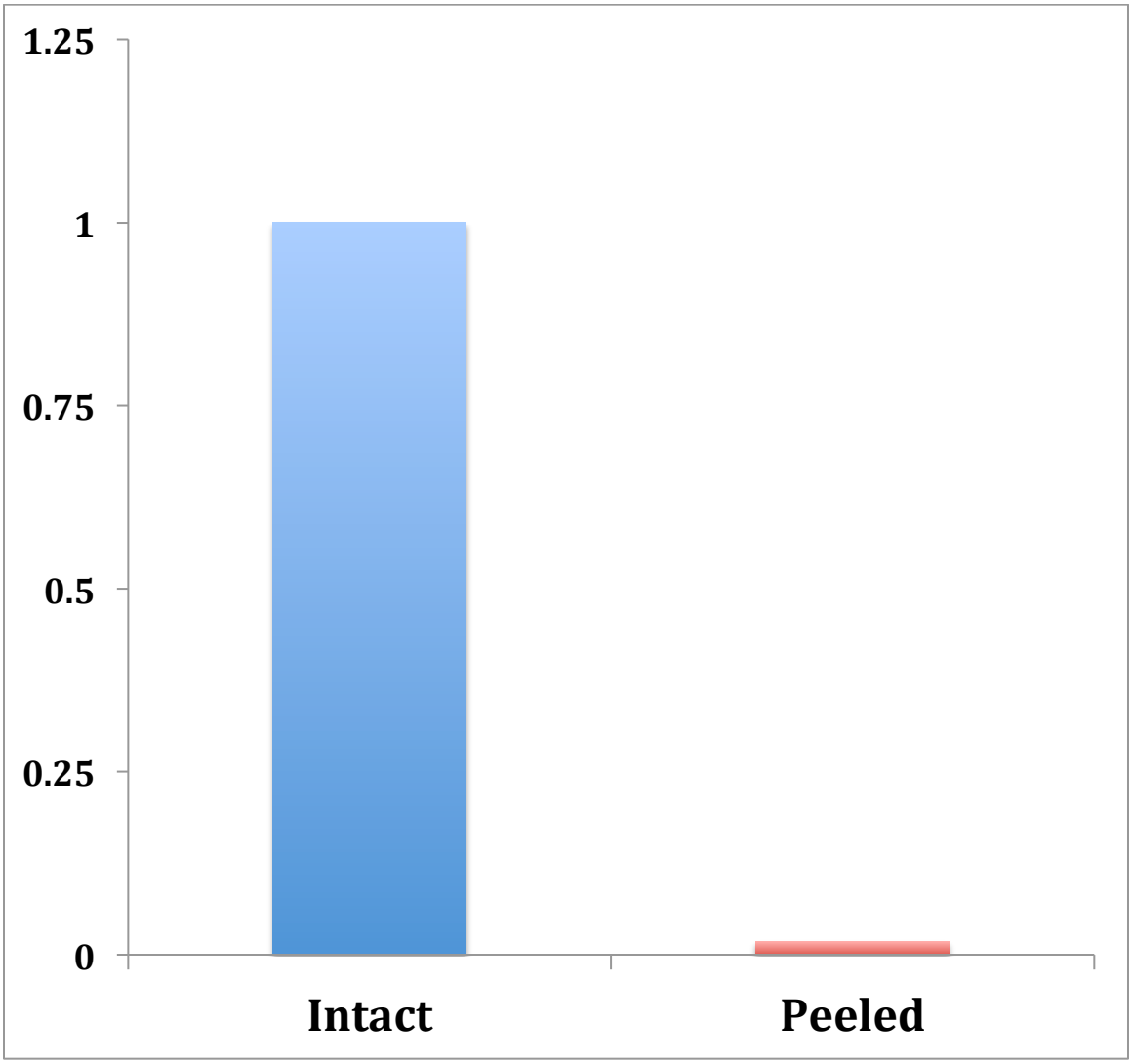

Figure 12. Relative expression of calbindin in embryonic chorioallantois from peeled and intact eggs at embryonic stage 37.
p = 0.0003 n = 12 paired samples from 8 females

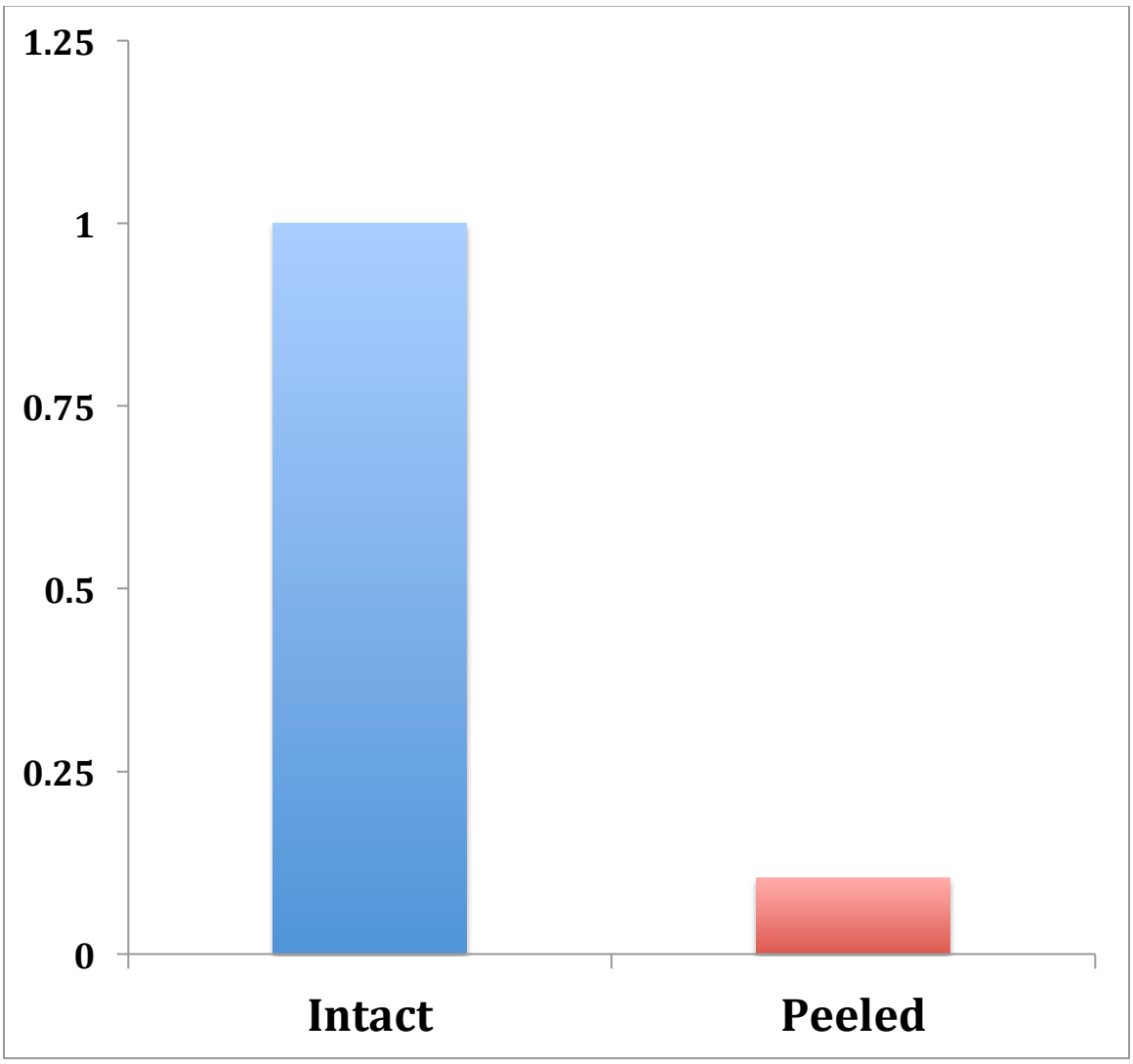

Figure 13. Relative expression of calbindin in abembryonic chorioallantois from peeled and intact eggs at embryonic stage 37.
p = <0.0001 n = 7 paired samples from 3 females

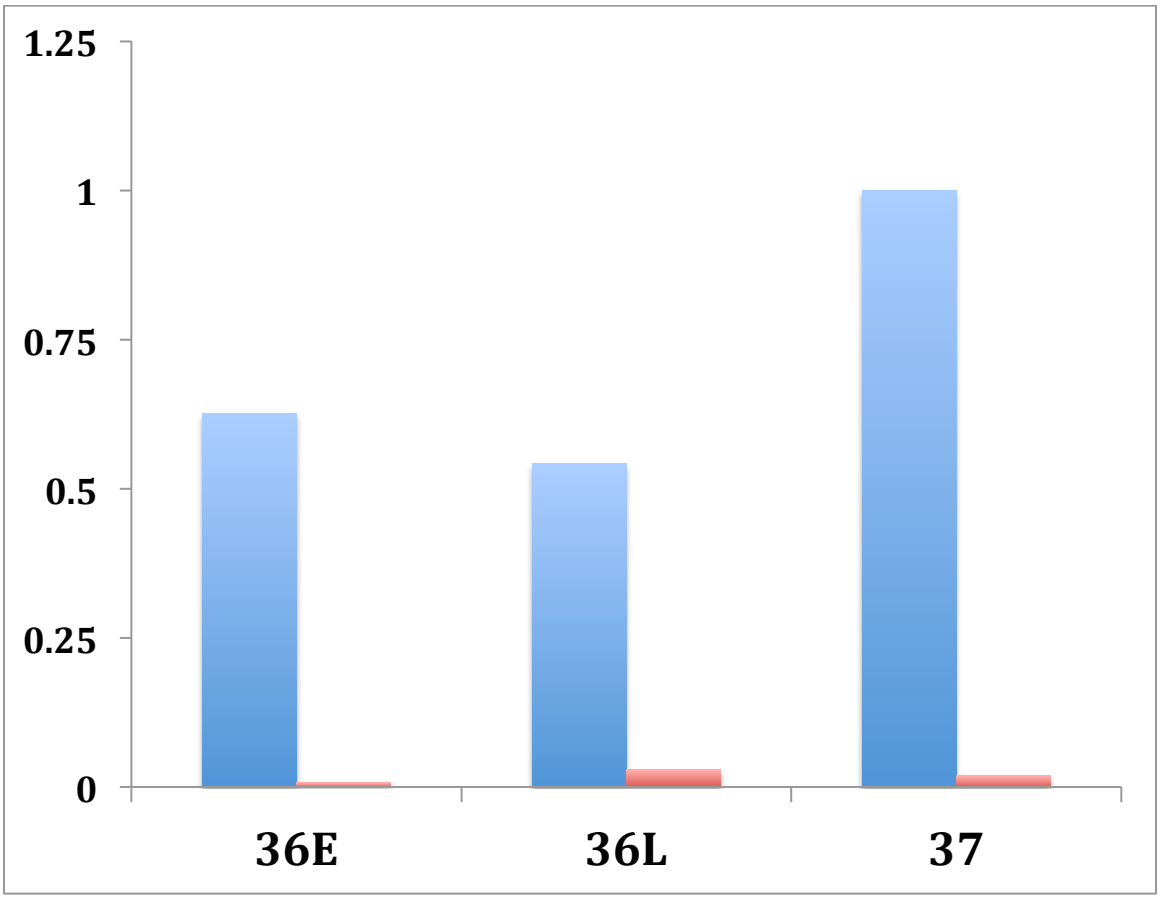

Figure 14. Relative expression of calbindin in embryonic chorioallantois from peeled and intact eggs at embryonic stages 36E, 36L, and 37.
36E: p = 0.1358 n = 5 paired samples from 5 females
36L: p = 0.0258 n = 6 paired samples from 6 females
37: p = 0.0003 n = 12 paired samples from 8 females

Discussion

We know from a previous study that removing eggshell calcium through peeling significantly impacts embryonic growth and development (Ross et al., 2012). Peeled eggs yield hatchlings that are shorter, lighter, and have less calcium than hatchlings from intact eggs. These findings indicate that eggshell calcium contributes to the growth and development of the embryo and are not consistent with the Packard et al. (1977) hypothesis that squamates make the evolutionary transition to viviparity so much more frequently than other oviparous groups because they are not as dependent on the eggshell for their embryonic calcium nutrition. It is possible that the growth changes caused by removing eggshell calcium could make it more difficult for the hatchlings from peeled eggs to compete for food and evade predators in natural settings, affecting their fitness unfavorably. If the differences caused by peeling were found to negatively impact the fitness of the hatchlings from peeled eggs in natural settings, then it might indicate that eggshell calcium not only contributes to embryonic growth but is also beneficial and necessary.

As stated in the introduction, there may instead exist a mechanism that allows for the frequent independent transitions from oviparity to viviparity in squamates. Such a mechanism might aid in the transition by allowing the developing embryo to use a placenta instead of an eggshell as a source of exogenous calcium, and it would likely involve the regulation of the proteins we know take part in the transport of eggshell calcium to the embryo. We hypothesized that the regulation of proteins involved in calcium transport in corn snake chorioallantois would be

similar to the regulation of calcium transport proteins in other calcium transporting systems that have been studied in the past (chicken kidney and intestinal epithelial tissues) (Bar et al., 1990, 1998; Meyer et al., 1992).

Samples from yolk sac membrane showed no difference in calbindin expression between peeled and intact eggs. This is not surprising, as the yolk sac membrane transports calcium from the yolk rather than the eggshell, and peeling the outer layers of the eggshell does not alter yolk calcium availability. No data exist for carbonic anhydrase II or PMCA in yolk sac because this tissue does not express these proteins at detectable levels for the methods used in this study.

The chorioallantois, however, is the transporting tissue for eggshell calcium. Removing the outer layers of the eggshell, and thus removing the bulk of the calcium from the eggshell, produces interesting changes that correspond with the shortage of transportable calcium. Peeling significantly affects calbindin-D28K expression in the chorioallantois. In our original hypothesis, we expected expression of calcium transport proteins to be regulated in such a way that it would increase when there was a demand for eggshell calcium (as it has been observed in other epithelial tissues) (Bar et al., 1990, 1998; Meyer et al., 1992). Instead, availability of transportable calcium from the eggshell appears to be a strong regulator, causing calbindin-D28K expression to decrease in the absence of eggshell calcium. Decreased expression in response to a demand for calcium is a novel pattern of regulation among calcium transporting epithelial tissues, and appears unique to calbindin-D28K in this instance. Carbonic anhydrase II shows no change in

expression when eggshell calcium is removed. These patterns are consistent in all stages sampled and in all clutches.

For PMCA, we have found that it is difficult to produce blots that are either clean or consistent to visualize expression in corn snake samples. This difficulty is likely due to inconsistencies in sampling. Multiple individuals collected samples. Everyone performs this process similarly, but it may differ minutely from person to person. PMCA expression may be more sensitive to these slight differences than either calbindin-D28K or carbonic anhydrase II, which could explain why it is the only one of these proteins that produced such inconsistent results. However, when all the data were combined, no significant difference in expression between samples from peeled and intact eggs was found.

What we can surmise from these results is that calbindin expression is sensitive to the availability of calcium in this system. However, carbonic anhydrase II and PMCA expression are regulated separately from calbindin. The function of calbindin is limited to facilitating calcium transport, and this result suggests that carbonic anhydrase II and PMCA have additional functions in chorioallantois beyond the support of eggshell calcium solubilization (carbonic anhydrase II) and calcium transport (PMCA).

We also began comparing protein expression at developmental stage 37 in chorioallantois at the embryonic and abembryonic poles of the egg to determine if these changes in calbindin-D28K expression are specific to a region of the calcium transporting membrane. However, there did not appear to be any localized

differences in the pattern of expression in either calbindin-D28K or carbonic anhydrase II.

Reintroducing exogenous calcium may provide more insight regarding the corn snake's reliance on eggshell calcium. We have started this process by incubating peeled and intact eggs in solutions of different calcium concentrations. We are interested in seeing if both hatchling size and calbindin expression can be rescued.

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