

2005

# Kidney Development in *Eleutherodactylus coqui* With Relation to Edema Syndrome

Seung Yun Lee

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**Kidney Development in *Eleutherodactylus coqui***  
**With Relation to Edema Syndrome**

A Thesis  
Presented to the Bayer School of Natural and Environmental Sciences  
Department of Biological Sciences

In partial fulfillment of the requirements  
For the Degree of Masters of Science

By  
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## Abstract

Vertebrates develop embryonic kidneys before the final adult kidney. Particularly for organisms with aquatic larvae such as frogs, the first embryonic kidney, the pronephros is essential for the larvae's survival. Those larvae with defective or without pronephroi exhibited pronounced edema. Edema is severe swelling of a body part as a result of fluid retention. To date, although the embryonic kidneys have been extensively studied in various model organisms, it has not been studied in *Eleutherodactylus coqui*. *E. coqui* is a direct developing frog and lacks a tadpole phase in its life cycle. I examined the kidney development in *E. coqui* using endogenous alkaline phosphatase staining and histology. From the histology, I found that *E. coqui* embryos develop a pronephros followed by the mesonephros in normal development. The endogenous alkaline phosphatase staining was a novel finding and a good marker for early pronephric development in *E. coqui*. The kidney development in *E. coqui* embryos exhibiting edema was also studied. The key characteristic in the edematous embryos was the large lumens of the tubules. The edematous embryos also had poor limb development. I speculate that the edema in the *E. coqui* embryos was due to a defective pronephros, however, it is a correlation in the scope of this study and other causal possibilities are considered such as defects in the lymphatic system, the circulatory system and the skin. The possible relations between limb and kidney development is also discussed.

## **Acknowledgements**

I begin by thanking my parents and brother for their unconditional love and support throughout my life. In addition to my family, I must thank my friends outside of Duquesne, Lorraine, Powen, Carolyn, Nezia, Shareena, Edlyn, Cenie, Hye Jin and Ting who were another great source of love and support during my graduate studies. In addition, I also thank Carolyn for being so generous in creating the wonderful kidney illustrations in this thesis.

I also thank everyone in the Elinson Lab for all the great times we've shared. Dr. Elinson has been the most wonderful advisor, being encouraging and supportive throughout my tribulations. My deepest gratitude to you Dr. Elinson for giving me the opportunity to work in your lab, for believing in my capabilities and for your patience. The Elinson Lab members have been wonderful. I thank Kim for her help with all of my questions. I thank Michelle for her friendship and support. I thank Srikanth for his support and encouraging talk, especially during the writing of the thesis. Lastly, I thank everyone, Pete, Uma, Stephen, Zach, Kelly and Oscar for their support and our fun times in and out of the lab.

I would like to thank my friends and colleagues at Duquesne, Jen Gabel, Shane, Becky, Dawn, Shelby, Katie, Jade, Laila, Eddie, Matt Guyaux, and Jed for their support and friendship. I also thank Phil and Jeff for their help with lab materials. I will always remember and cannot thank enough the late Dr. Weisberg for his kindness, support and encouragement through my times of hardship. I also thank Mary Ann from the Dean's Office for finding me support through the last stretch of my Duquesne career, Pam and Judy from the Biology office for their kindness and help with my various requests.

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# Introduction

## I. Biphasic vs. Direct Development

The biphasic mode of development occurs throughout the animal kingdom. This mode of development involves the fertilized egg first developing into a feeding and motile larva. At the end the larval stage, metamorphosis occurs which gives rise to the adult form. Among the frogs, the anuran amphibians, many undergo biphasic development; however, there are also those that undergo direct development. Direct development is the mode of development in anuran amphibians in which there is no tadpole stage. The terrestrial life style correlates with trends towards direct development (Duellman and Trueb, 1986). If water is not always present for feeding tadpoles to hatch into, direct development allows such adverse environmental conditions to be overcome by ultimately producing a terrestrial adult at hatching. Thus, it can be viewed as an extreme developmental adaptation to environment. The continued reduction in larval period with accompanied accumulation of developmental modifications may have eventually led to direct development (Callery *et al.*, 2001). Since metamorphosis could occur in a non-aquatic environment, there would have been no selective advantage to retaining feeding larva or tadpole-specific structures. A selectively neutral character may have been retained if it was involved in pleiotropic roles in other morphogenetic events or the larval structure may have been adapted to a new developmental mode.

## II. *Eleutherodactylus coqui*

The genus *Eleutherodactylus* contains approximately 500 species which are all direct developers (Hanken, 1999), and *Eleutherodactylus coqui* is the particular species of

direct developing frogs of interest in this thesis. *E. coqui* frogs are an entirely terrestrial species, native to Puerto Rico, which now have become abundant in Hawaii. An *E. coqui* mating pair produces a clutch of 30 eggs each month in a laboratory colony setting (Elinson *et al.*, 1990). In their natural environment, the eggs are incubated by the father for approximately three weeks. Then, little free-living froglets emerge from jelly capsules (Townsend and Stewart, 1985).

*E. coqui* have large eggs which are approximately 3.5 mm in diameter. That size is twenty times the size of a *Xenopus laevis* egg. Only 3% of the egg volume is the animal cytoplasm while the rest is the vegetal yolk. The increase in vegetal cytoplasm has likely led to expansion of the endoderm used solely for nutrition. The increased nutritive endoderm alters the development of the body wall in which a secondary coverage of the yolk occurs (Elinson and Fang, 1998), an event which does not occur in biphasic frogs. This expansion of the body wall is similar to the spreading of the chorion to enclose the yolk in chicks and may represent a way that evolved to accommodate the large uncleaved yolk mass. The *E. coqui* egg still follows the typical amphibian yolk cleavage pattern in having complete divisions. This is in contrast to the large yolky eggs of amniotes in which the yolk is not cleaved. In amniotes, following gastrulation, the yolk is surrounded by growth of extraembryonic tissues (Elinson, 1987; Elinson and Fang, 1998)

The development of the *E. coqui* embryo is very interesting because there are tadpole-specific features which have been lost and early development of adult structures (Elinson *et al.*, 1990). The *E. coqui* embryo lacks tadpole features such as horny mouth parts, an adhesive organ called the cement gland, lateral line organs, and a coiled gut. The

tadpole's long gut enables it to digest fibrous plant matter. Likewise, only rudimentary gills are present in *E. coqui*. The failure of the cement gland (Fang and Elinson, 1996) and lateral line formation (Schlosser *et al*, 1999) is due to the lack of response of the ectoderm to inducing signals in *E. coqui*. This means that the genes needed to develop tadpole structures are absent in *E. coqui*; however, transplant experiments show that *E. coqui* embryos are still able to send out inductive signals for both cement glands and lateral line organs. While lacking tadpole features, there is early and simultaneous appearance of all four limbs, which occurs at late larval stages in biphasically developing frogs (Callery and Elinson, 2000). In *X. laevis*, the hindlimb buds appear first at stage 46, 4 days into development just before the tadpole begins to feed. Forelimb buds do not appear until stage 48, 3.5 days later (Nieuwkoop and Faber, 1994). The *E. coqui* embryo also develops a large membranous tail which is not used for swimming; but is thought to have a respiratory function. Lastly, an egg tooth develops prior to hatching. This egg tooth may be homologous to the tadpole teeth. The egg tooth of *E. coqui* is a structure in place of the hatching gland in biphasic embryos and is similar to the egg tooth in chicks.

### **III. Kidney development**

#### **A. Vertebrate kidney development**

All vertebrates have distinct embryonic and adult kidneys (Goodrich, 1930; Burns, 1955; Saxén, 1987). In amphibians and fish, the first functional embryonic kidney is the pronephros. Well-developed pronephroi are present in all fish and amphibians while birds and most mammals have poorly developed pronephroi. The adult kidney in amphibians and fish is called variously the mesonephros or the opisthonephros. The term

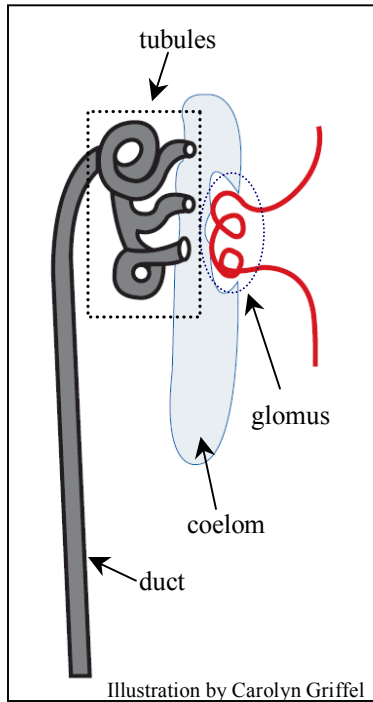


Figure i: Schematic diagram of pronephros

opisthonephros is sometimes used to distinguish it from the transient mesonephros of amniotes. The metanephros is the adult kidney in birds and mammals.

The pronephros is essential for survival in organisms with aquatic larvae such as frogs. It produces dilute urine that allows the animals to maintain water balance. If the pronephros is not functional, aquatic larvae die rapidly from edema (Howland, 1921). Edema is severe swelling of a body

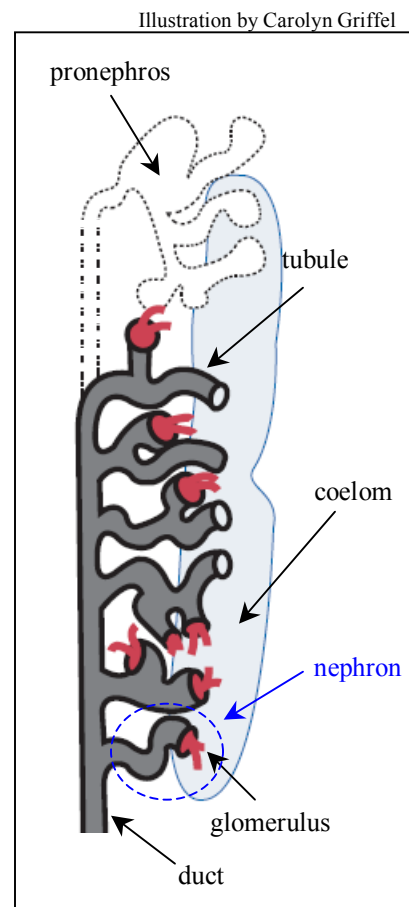
part as a result of fluid retention. The pronephros is simple and forms within a day or two of fertilization. The entire pronephros is in essence a single large nephron. The nephron is the functional unit of the kidney which is responsible for the purification and filtration of the blood. The pronephros can be divided into three parts: the glomus, the tubules, and the duct, in which each has particular functions (Fig. i). The job of the glomus is filtration. The glomus is a tuft of capillaries which filters the blood as would the glomeruli in a nephron; however, the filtrate is deposited into a cavity rather than the Bowman's space. The cavity is the coelom, also known as the body cavity, in some cases. In others, a dorsal subcompartment of the coelom is called the nephrocoel, and in others the pericardial cavity is used. The tubules perform resorptive and excretory functions. The tubules have distinct proximal and distal segments in which solute resorption and waste excretion occur in the proximal segment and water resorption occurs in the distal segment. The urine passes down from the distal tubule to the pronephric duct and

ultimately to the cloaca. The duct disposes urine and assists in the induction of the adult kidneys in a range of organisms from amphibians to mammals (Burns, 1955; Saxén, 1987). The amphibian pronephros also functions as a hematopoietic organ, and is a major site of myeloid cell differentiation. The hematopoietic function is taken over by the mesonephros during metamorphosis.

The mesonephros has a more complex organization. It consists of a linear sequence of nephrons linked to the nephric duct (Fig. ii). The

glomerulus is the glomus renamed. The glomus becomes the glomerulus when it becomes surrounded by the Bowman's capsule and is now internal. This means the filtration unit is directly integrated into the kidney tubule. While the mesonephros is the terminal kidney in amphibians and fish, the degree of development of the mesonephros in amniotes is linked to the form of placental development. In some organisms, such as the pig, the fetal and maternal tissues are opposed and the embryo has a large, well-developed mesonephros that remains until the adult kidney becomes functional. In other organisms such as

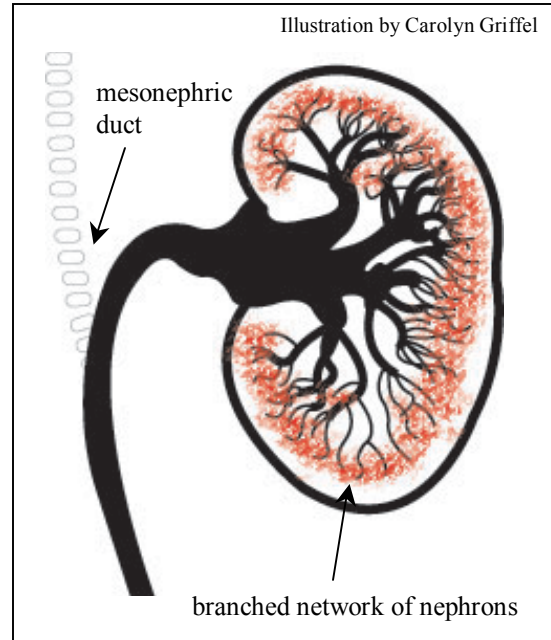
rodents and primates, the maternal epithelium breaks down and bathes the intervillous spaces of the fetal epithelium directly with blood, allowing for a more efficient supply of



**Figure ii:** Schematic diagram of mesonephros

nutrients and removal of wastes. In such cases, the embryonic kidney is less complex and often degenerates before the metanephros is formed (Bremer, 1916; Witschi, 1956).

The metanephros is the final kidney that develops in all amniotes, from reptiles to humans. It is the most complex of kidneys. As opposed to the linear organization of the nephrons in the mesonephros, a branched architecture with arborized networks of nephrons is present in the metanephros (Fig. iii). The functions of the metanephros are to eliminate waste, regulate blood fluid volume and solute levels, control blood pH, produce endocrine hormones and modify some metabolites.



**Figure iii:** Schematic diagram of metanephros

### **B. *Xenopus laevis* kidney development**

In the model amphibian *X. laevis*, the South African clawed frog, a part of the pronephros first begins to function at stage 31, which is approximately a day and a half after fertilization at 22° to 24°C under laboratory conditions (Nieuwkoop and Faber, 1994). By stage 35/36, two days post-fertilization, a rich blood supply to the pronephros has developed. At stage 37/38, the entire pronephros has become functional. From stage 37 to stage 47, the complexity of the pronephros increases by coiling of its tubules and collecting tube. The tubes become thicker, and the lumina widen. The tadpole begins to feed after five days at stage 47 and stops during metamorphosis which begins after

approximately 17 days at stage 51. Metamorphosis in many biphasic frogs is triggered by the thyroid hormone and its interaction with its receptors (Tata, 1996). Starting at stage 53, approximately 24 days into development when the fore and hindlimbs have foot paddles, the pronephros begins to degenerate. From stage 58 and beyond, the pronephros is no longer functional. At this stage, the forelimbs are broken through the skin, and all three claws are present in the hindlimb. The climax of metamorphosis is through stage 59 to stage 65; which lasts eight days (Leloup and Buscaglia, 1977). The pronephros has completely disappeared at stage 64, at which the embryo has become a metamorphosed frog with a little bit of the tail remaining.

While the pronephros develops and later degenerates, the adult kidney of amphibians, the mesonephros also begins to form. At stage 39, the first mesonephric cells appear. At stage 48, the first 6 to 8 pairs of mesonephric tubes extend and begin to coil. Some of the tubes also become functional. Unlike the pronephros, the mesonephros degenerates and then reorganizes. First signs of degeneration in the mesonephros occur at stage 55. The mesonephros reaches full length at stage 58. By stage 62, new mesonephric tubes and glomeruli are formed. (Nieuwkoop and Faber, 1993)

Development of the *X. laevis* pronephros has been studied with antibodies specific to the pronephros (Vize *et al.*, 1995). The antibody 3G8 recognizes the pronephric tubules starting at stage 31. By stage 38, the extended and coiled structures of the tubules are positive for 3G8. The antibody 4A6 recognizes the pronephric duct and the stain first appears at stage 38. Solid duct staining was observed by stage 43. Staining with the 4A6 antibody overlaps slightly with the point where the 3G8 antibody staining stops. The



antibodies allowed better differentiation between the tubules and duct in the later stages. This differentiation had been difficult because the duct coils underneath the pronephric tubules and the structures appear very similar.

In addition to the kidney specific antibodies, over 200 genes have been examined by *in situ* hybridization and approximately 30 genes have been confirmed to have specific expression during the development of the pronephros (Brändli, 1999). Specifically, *Lim-1*, which encodes a LIM class homeodomain may be essential for the development of the entire urogenital system (Shawlot and Behringer, 1995) and *Pax-2*, a paired-box transcription factor has been identified to be necessary for elongation and/or maintenance of the nephric duct (Torres *et al.*, 1995; Favor *et al.*, 1996). *HNF-1 $\beta$* , *Pax-8*, and *Lim-1* are the earliest markers for the pronephros (Demartis *et al.*, 1994; Taira *et al.*, 1994; Heller and Brändli, 1999). Likewise, gene expression in the pronephros has been reported for the following genes *Pax-8*, *Delta-1*, *Wnt-4*, *Iro-3*, *Sal-1*, *WT-1*, and *Msr* (Heller and Brändli, 1997, 1999). A brief summary of gene expression in the pronephros is presented in Table 1.

### **C. *Eleutherodactylus* kidney development**

Kidney development in several species of the genus *Eleutherodactylus* has been studied. The studies were all done prior to Townsend and Stewart's (1985) staging table for *E. coqui*, so there is no standardized staging. Fortunately, Townsend and Stewart provide a table to compare the various staging between authors and their frogs. Staging according to Townsend and Stewart's table is noted 'TS'.

**Table 1.** Genes expressed in the pronephros.

<i>Type of gene product</i>	<i>Gene name</i>	<i>Location of expression</i>	<i>Reference</i>
Transcription factors	<i>Emx-1</i>	-	Pannese <i>et al.</i> , 1997
	<i>ets-2</i>	-	Meyer <i>et al.</i> , 1997
	<i>HNF-1<math>\beta</math></i> (LFB-3)	pronephric tubules, duct	Demartis <i>et al.</i> , 1994
	<i>Iro-3</i>	pronephric tubules	Bellefroid <i>et al.</i> , 1998
	<i>Lim-1</i>	pronephric tubules, duct	Taira <i>et al.</i> , 1994
	<i>Pou-2</i>	pronephric duct	Witta <i>et al.</i> , 1995
	<i>Sal-1</i>	pronephric duct	Hollemann <i>et al.</i> , 1996
	<i>WT-1</i>	pronephric capsule	Carroll and Vize, 1996; Semba <i>et al.</i> , 1996
	<i>XFD-11</i>	-	Koster <i>et al.</i> , 1998
	<i>Id-2</i>	pronephric duct	Wilson and Mohun, 1995; Gawantka <i>et al.</i> , 1998
Secreted factors	<i>BMP-7</i>	pronephric tubules	Wang <i>et al.</i> , 1997
	<i>gremlin</i>		Hsu <i>et al.</i> , 1998
	<i>VEGF</i>	pronephric capsule	Cleaver <i>et al.</i> , 1997
	<i>WIF</i>	-	Hsieh <i>et al.</i> , 1999
	<i>Wnt-4</i>	pronephric tubules, duct	Saulnier <i>et al.</i> , 2002
Cell surface receptors	<i>integrin-<math>\alpha</math>6</i>	pronephric duct	Lallier <i>et al.</i> , 1996
	<i>frizzled-3</i>	pronephric tubules	Shi <i>et al.</i> , 1998
Na <sup>+</sup> /K <sup>+</sup> ATPase Pump		pronephric tubules, duct	Uochi <i>et al.</i> , 1997
Transporters	<i>SGLT, SLC5A, SLC6A14, SLC7A8, NKCC2, ROSIT</i>	pronephric tubules	Zhou and Vize, 2004; Zhou and Vize, 2005a, b
Chloride conductance channel	<i>CIC-K</i>	pronephric distal tubule, duct	Vize, 2003

Lynn (1942) reports a detailed description of the embryology of *E. nubicola* in which the embryos were staged according to the number of days prior to hatching, designated ‘- number’ (days). The descriptions are based on observations from numerous embryos that were fixed, embedded, sectioned and stained. At -23 days (equivalent to TS 4 of *E. coqui*), there is an early sign of the pronephros indicated by the thickening of the somatic layer of the lateral mesoderm in the anterior body region. By -21 days (TS 5), definite pronephric tubules have developed, illustrated by the histological sections. At -20 days (TS 6), three nephrostomes are present in the pronephros and a well developed glomerulus projects into the body cavity. At -11 days (TS 12), the pronephros reaches its maximum development; however, it is present at hatching as a compact organ lacking nephrostomes.

At -18 days (TS 7), a small mesonephric tubule opening into the Wolffian duct, also called the nephric duct, in its central part is discernable. By -10 days (TS 13), the mesonephros exceeds the pronephros in size. At the time of hatching, the pronephros is still present with glandular secretion and the mesonephros is almost of definitive size. The degeneration of the pronephros correlates with other bodily changes, such as growth of limbs and changes in pigment pattern from embryo to frog, occurring at its usual place in the metamorphic pattern. When biphasic frogs undergo metamorphosis, the following changes occur: hindlimbs grow rapidly, the pigment pattern changes from that of the tadpole to that of the adult frog, the gills, tail, and intestine undergoes extensive resorption, and the larval mouth part is replaced by adult structures.

## **2. *Eleutherodactylus augusti latrans***

The barking frog, *Eleutherodactylus augusti latrans*, is found in Southern Arizona, parts of New Mexico to Texas and parts of Mexico including along the Pacific coast. At a stage equivalent to TS 10-12, large pronephroi are present in the anterior region. Several nephrostomes are developed (Valett and Jameson, 1961).

## **3. *Eleutherodactylus martinicensis***

The whistling frog, *Eleutherodactylus martinicensis*, is found in Barbados and other islands in the Caribbean. At stage II (TS 4), the primordia of the pronephros are present. The region is small and limited to the extreme cranial portion of the nephrogenic mesoderm. Only two tubules are present. At stage III (TS 5), the pronephros has expanded and the duct has formed. There are four tubules which are extensively dilated, coiled and form a compact organ. At stage IV (TS 7), the pronephros starts to regress, and the mesonephric tubules appear. By stage V (~ TS 9), only two pronephric tubules retain their lumen. More mesonephric tubules continue to develop, show dilation and coiling. At stage VI (~ TS 11), several mesonephric tubules have developed and they open medially into the pronephric duct (Adamson, 1960).

The emergence of the pronephros in *E. martinicensis* occurs at approximately the same time as it does in *E. nubicola*. However, the pronephros regresses earlier in *E. martinicensis* at TS 7 than in the other two frogs. For *E. nubicola*, its pronephros does not reach maximum development until TS 12 and *E. a. latrans* has large pronephroi at TS 10-12. The mesonephros of *E. nubicola* and *E. martinicensis* begins to develop at the same stage. Overall, the *Eleutherodactylus* pronephros develops early in development

and may remain until the embryo hatches. The development of the mesonephros overlaps with the presence of the pronephros.

#### **D. Edema**

There have been a few studies in which occurrences of edema in developing embryos were found to be due to defects in the developing kidney. Surgical removal of the pronephros in the spotted salamander, *Ambystoma punctatum* (Howland, 1921) or a genetic lesion in zebrafish, *Danio rerio* (Drummond, 1998) which compromised pronephric function led to severe edema and ultimately death in the developing embryos. Edema has also been observed in haploid embryos (Rafferty, 1961)

When the pronephric rudiments were removed from both sides of the *Ambystoma* embryo, the most conspicuous condition was pronounced edema, particularly in the anteroventral region (Howland, 1921). Even when interruptions to the development of the pronephros were less radical, as in removal of segments of the pronephros as opposed to the entire pronephros, embryos exhibited the same symptoms. When only one pronephros was removed, the remaining pronephros exhibited hypertrophy, enlargement of the cells already present. The walls of the hypertrophied tubules were thinner and the cell were often flattened and elongated. The lumens of the hypertrophied tubules were also remarkably larger than that of an unoperated control specimen. When only one of the pronephroi were removed, edema was absent while hypertrophy of the remaining pronephros occurred. The tubules were thicker with thinner walls and the length was longer.

In zebrafish, eighteen independent recessive mutations affecting pronephric development were isolated (Drummond *et al.*, 1998) from a large-scale ENU mutagenesis screen for development mutants (Driever *et al.*, 1996). The unifying phenotype of all the mutants was the appearance of fluid-filled cysts in the region of the normal pronephros. All homozygous pronephric mutants died by 5 to 6 days post-fertilization with the larvae becoming grossly edematous. The largest group of mutants manifest cysts soon after hatching, develop severe edema, and display an axis curvature defect. Since heart function appeared to be normal in the mutant homozygotes, the edema is assumed to be due to loss in pronephric function and failed osmoregulation (Drummond *et al.*, 1998). Edema can occur due to heart defects and/or heart failure because a weak heart is unable to pump hard enough to keep up with the cycle of sending and receiving the blood from the rest of the body. The blood in which the heart was unable to pump out back ups and seeps out of the blood vessels into other tissues, such as the lungs.

Haploid frog (*Rana pipiens*) embryos were generated by fertilizing eggs with sperm that were irradiated with UV light. One of the notable symptoms of haploidy was edema which was often severe. When transplantation experiments were done with diploid larvae receiving a haploid pronephros and vice versa, diploid larvae with a haploid pronephros displayed an edematous, retarded development and limited survival which was similar to unoperated haploid larvae (Rafferty, 1961). In haploid embryos of *X. laevis*, Fox and Hamilton (1964) concluded that the edema in the embryos is a result of excess water flowing through the ectoderm rather than renal failure because the embryonic kidneys were hypertrophied.

Edema in humans can be categorized based on the primary location of swelling. Some of the main categories are the following. Angioedema is swelling of the skin and can be caused by an allergic reaction. Lymphedema is accumulation of the lymphatic fluid in the interstitial tissue which results in swelling in various parts of the body. Lymphedema occurs when lymphatic vessels are missing, impaired, damaged or when lymph nodes are removed. Pulmonary edema is the condition where fluid fills into the lungs instead of air. This prevents the lung from absorbing oxygen. Often, heart problems cause pulmonary edema. Other causes are illnesses such as pneumonia, exposure to certain toxins and medication and exercising or living at high elevations.

(<http://www.nlm.nih.gov/medlineplus/edema.html>)

#### **E. Alkaline phosphatase activity in the developing kidney**

Alkaline phosphatase (AP) is commonly conjugated to secondary antibodies and molecular probes. Its activity is easily visualized using various substrates. Endogenous AP activity can also be easily visualized using the same substrates. Endogenous AP activity is present in the pronephric duct in axolotl, *Ambystoma mexicanum*, at the tailbud stage (Zackson and Steinberg, 1988). Zackson and Steinberg (1988) speculated that AP acts as a cell guidance associated molecule responsible for migration of the pronephric duct and cranial neural crest cells. The expression pattern of AP in *Xenopus* embryos is similar to that in the axolotl (J. Drawbridge, Rider College, pers. comm. Aug 4, 2005). Endogenous AP is also present on the apical surfaces of pronephric duct cells of zebrafish embryos (Drummond *et al*, 1998).

AP activity in the pronephros of various organisms does not appear to be necessary for development of the excretory system. In mice, a multi-gene family has 3 loci encoding alkaline phosphatases. They are designated embryonic, intestinal and tissue-non-specific (TNS) alkaline phosphatases (Terao and Mintz, 1987; Manes *et al.*, 1990; Narisawa *et al.*, 1994). TNS AP is expressed in numerous tissues which included bone, liver, kidney, testis, fibroblasts, macrophages and many other lower level cell types (McComb *et al.*, 1979). When AP is expressed ubiquitously in the mouse embryo, the development of the embryo is unaffected (Skynner *et al.*, 1999). In an opposite case, when embryonic AP was knocked out, no obvious phenotypic abnormalities were present (Narisawa *et al.*, 1997). When TNS AP was knocked out, abnormalities were present. There was abnormal bone mineralization and central nervous system defects in neonates in which there was aberrant development of the lumbar nerve roots. There were also disturbances in the intestinal physiology, increased apoptosis in the thymus, and abnormal spleens. Nevertheless, most organ systems, including the kidney, developed fine (Narisawa *et al.*, 1997).

#### **IV. Thesis Objectives**

To date, there has been no study on the development of the kidney of *E. coqui*. In this thesis, I intend to examine normal kidney development in *E. coqui* embryos using endogenous alkaline phosphatase staining and histology. Then, kidney development in embryos exhibiting edema to various degrees will be examined using the same techniques in order to infer whether the edema is related to abnormal kidney development.



## Materials and Methods

### I. *E. coqui* embryos and staging

#### A. Embryos

All *E. coqui* embryos were obtained from natural matings in the laboratory colony consisting of adult frogs that had been caught in the wild. The adults were collected in Puerto Rico under the permit issued by the Departamento de Recursos Naturales and in Hawaii under the Injurious Wildlife Exports Permits issued by the Department of Land and Natural Resources. After fertilization occurred, the eggs were removed from the guarding male. The eggs were placed in a plastic Petri dish with a piece of filter paper, moistened with 20% Steinberg's solution. The embryos were examined daily to document their developmental progress. The Petri dish chamber was kept moist by adding more 20% Steinberg's solution as needed. When embryos reached a desired stage, the chosen embryos were separated. The selected embryos were submerged in 20% Steinberg's solution for a few hours to allow the jelly capsule to swell with fluid. Then the jelly layers and the fertilization membrane were removed using watchmaker's forceps.

All Steinberg's solutions were made from two separate 20X stock solutions which were kept in 4°C. Steinberg's Stock A is 1.16M NaCl, 13mM KCl, 17mM MgSO<sub>4</sub>·7H<sub>2</sub>O, and 6.7mM CaNO<sub>3</sub>. Steinberg's Stock B is 100mM Tris, pH adjusted to 7.4 with HCl. To make the 20% solution, 10ml of Stock A and 10ml of Stock B was brought up to a liter with deionized (dI) H<sub>2</sub>O.

## **B. Staging**

For those embryos with normal development, the stage of development was determined according to Townsend and Stewart's (1985) staging table. Each stage is denoted 'TS number'.

For the embryos that displayed the edema syndrome, there was difficulty in staging due to lack of any previously documented studies and due to the variance in severity of the syndrome in the different embryos. Growth was greatly retarded in the embryos exhibiting the edema syndrome when comparing the edematous embryos to the normally developing embryos in the same clutch. Initially, staging was based on hindlimb development according to Townsend and Stewart since the hindlimb was not as severely affected by the edema syndrome. This staging could be misleading, however, depending on the severity of the syndrome exhibited in the embryo. Therefore, the approximate age of the edematous embryos was also determined using the date in which the clutch was collected and from the clutch's developmental progress notes that were kept in the laboratory.

## **II. Fixation**

All embryos were removed from their jelly capsules prior to fixation using one of the following fixatives.

### **A. MEMFA**

MEMFA (100mM MOPS, 2mM EGTA, 1mMgSO<sub>4</sub>, 3.7% formaldehyde, pH 7.4) was prepared fresh using 1 part 10X MEM stock solution (1M MOPS, 20mM EGTA,

10mMgSO<sub>4</sub>, autoclaved), 1 part 37% formaldehyde stock solution, and 8 parts dI H<sub>2</sub>O. The dejellied embryos were placed into glass vials containing MEMFA, and fixed overnight with gentle rocking on the Nutator at room temperature. The next morning, two 30 minute (min) 100% ethanol washes were done at room temperature. After the second ethanol wash, the embryos were stored in fresh 100% ethanol at -20°C until used for further assays. (Harland, 1991) This fixation method was used primarily for detection of alkaline phosphatase activity.

### **B. Smith's fixative**

For fixation using Smith's fixative, equal parts of two stock solutions were mixed fresh for use (Rugh, 1965). Stock Smith's A is 1% potassium dichromate (K<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub>) and is a bright orange solution. Stock Smith's B is a 5% acetic acid, 7.4% formaldehyde solution. The dejellied embryos were placed into glass vials containing Smith's fixative for 1 to 2 days in the dark. The glass vials were covered with foil and gently rocked on the Nutator at room temperature. The originally orange solution became a greenish yellow color after the embryos were fixed. After fixation, the embryos were rinsed twice for 30 min each with dI H<sub>2</sub>O. The rinsed embryos were then preserved and stored in 1.5% formaldehyde solution at room temperature. This fixation method was used for histology.

### **C. Bouin's fixative**

For fixation using Bouin's fixative, the pre-made fixative was purchased from LabChem Inc. The fixative can also be made in the lab (Rugh, 1965). The dejellied embryos were placed in the yellow colored Bouin's fixative in a glass vial at room

temperature. Parafilm was used to thoroughly seal the vials because the picric acid may explode if it becomes dry. This fixative serves as a preservative, so the embryos were not transferred to a preservative after fixation. Fixed embryos were kept in Bouin's solution until further study. This fixation method was also used for histology.

### **III. Alkaline phosphatase activity stain – modified immunohistology protocol**

This alkaline phosphatase activity stain protocol is a modified version of the immunohistology protocol which uses an alkaline phosphatase substrate for detection of the secondary antibody. All embryos used for this assay were fixed in MEMFA and preserved in 100% ethanol at -20°C.

First, the embryos were rehydrated through an ethanol series consisting of 10 min washes in each of the following ethanol solutions: 100% ethanol, 95% ethanol, 75% ethanol/25% PBS (1X phosphate buffered saline: 137mM NaCl, 2.7mM KCl, 10mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8mM KH<sub>2</sub>PO<sub>4</sub>), 50% ethanol/50% PBS, 25% ethanol/75% PBS, and finally 100% PBS. Following rehydration, the embryos were permeabilized with four 30 min washes in 0.1% Triton-X in PBS. After permeabilization, the embryos were washed with a blocking solution (10mg/ml BSA (bovine serum albumin, Sigma) in 0.1% Triton-X in PBS) for 30 min. In the original immunohistology protocol, BSA is used to block non-specific sites. Then, the embryos went through four 1 hour washes in PBT (2mg/ml BSA in 0.1% Triton-X in PBS) which is the step to remove any residual non-bound antibodies remaining in the embryo in the original protocol. The embryos were prepared for the alkaline phosphatase substrate by three 5 min washes in alkaline phosphatase buffer

(100mM Tris pH 9.5, 50mM MgCl<sub>2</sub>, 100mM NaCl, 0.1% Tween 20). Half of the embryos were also washed with alkaline phosphatase buffer with levamisole (5mM) freshly added. Levamisole is used to block endogenous alkaline phosphatase activity in the original protocol. After buffering in alkaline phosphatase buffer, BM Purple AP Substrate (Roche) was added to the embryos for approximately 30 min. A light aquamarine blue stain begins to appear in the pronephric region after approximately 15 minutes. Another alkaline phosphatase substrate, NBT/BCIP, was tried. NBT/BCIP is 4.5µl NBT (4-nitro blue tetrazolium chloride (Roche), 75mg/ml stock in 70% DMF (N, N-dimethyl formamide), stored at -20°C) and 3.5µl BCIP (5-bromo-4-chloro-3-indolyl phosphate (Roche), stored at -20°C) in 1ml alkaline phosphatase buffer. This stain appeared faster, but there was much more background staining in older embryos, and thus was not as effective as BM Purple.

After alkaline phosphatase activity became apparent, the embryos were fixed with 3.7% formaldehyde in TBS (Tris buffered saline: 150mM NaCl, 50mM Tris HCl pH 7.4) or with MEMFA overnight with gentle rocking on the Nutator.

After fixation of the stain, embryos were cleared to better visualize the stain pattern. The embryos went through a methanol series to dehydrate and prepare them for infiltration with the organic clearing solution. The methanol series was as follows: 30 min washes in 100% PBS, 30% methanol/70% PBS, 50% methanol/50% PBS, 70% methanol, 95% methanol and two 30 min washes in 100% methanol. Then the embryos were placed into the clearing solution BABB (benzyl alcohol: benzyl benzoate:: 1:1) in a watch glass. Using a glass container was important because BABB dissolves the plastic

well plates and the solution becomes cloudy. The embryo may also get stuck to the bottom of the plate as the plastic dissolves in the organic solution.

#### **IV. Embedding and Sectioning**

The embryos were fixed with either Smith's fixative or Bouin's fixative for embedding and sectioning.

##### **A. Embedding**

The embedding procedure begins with a dehydration series to prepare the fixed embryos for the paraplast. The dehydration series is as follows: two 10 min washes with dI H<sub>2</sub>O, one 30 min wash with 50% ethanol, followed by two 30 min washes with 70% ethanol. In the case for Bouin fixed embryos, instead of the two 30 min 70% ethanol washes, several 30 min washes with 70% ethanol solution with 2% NH<sub>4</sub>OH, prepared from a 28% NH<sub>4</sub>OH stock, were done until the wash solution was clear and no longer yellow at the end of the wash period. The NH<sub>4</sub>OH washes remove the picric acid, and usually six to eight washes were necessary before the ethanol wash solution was clear. After the 70% ethanol wash, one 20 min 90% ethanol wash followed by two 20 min 100% ethanol washes were done. The total time for the 90% and 100% ethanol washes was kept under 1 hour.

Next, the embryos were left in amyl acetate overnight or longer to thoroughly remove any water that may be remaining in the tissues. After amyl acetate, the embryos were cleared with toluene in the fume hood. The toluene was changed three times over a 30 min period.

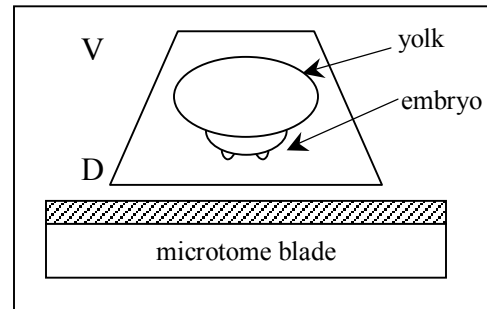
The cleared embryos were placed individually into wells of a ceramic 12-well plate containing melted paraplast in the 60°C incubator. The ceramic well plates worked well. They insulated the heat better and kept the paraplast melted during transfers in which the incubator temperature drops due to the incubator door being open. This was also easier than transferring the embryos out of the glass vials as had been previously done in the lab. The embryos were transferred from toluene into fresh paraplast using trimmed plastic transfer pipettes. This needed to be carefully done so as not to damage the very brittle embryos. Approximately every 30 min, the embryos were carefully transferred into wells with fresh paraplast. The embryos were placed into fresh paraplast three times.

To embed the paraplast infiltrated embryos, melted paraplast was poured into paper boats constructed from index cards. The boats were placed outside of the incubator at room temperature. The embryos were placed into the melted paraplast in the paper boats. This transfer needed to be done quickly before all of the paraplast solidified in the paper boat. The embryos were oriented before the paraplast completely solidified using heated dissecting needles. The paper boats now containing the embedded embryos were kept on the cold surface of the Tissue-Tec II apparatus to allow the paraplast to complete solidify quickly.

## B. Sectioning

The paraplast blocks containing the embryos were trimmed into trapezoid shapes using a razor blade and mounted onto wooden blocks.

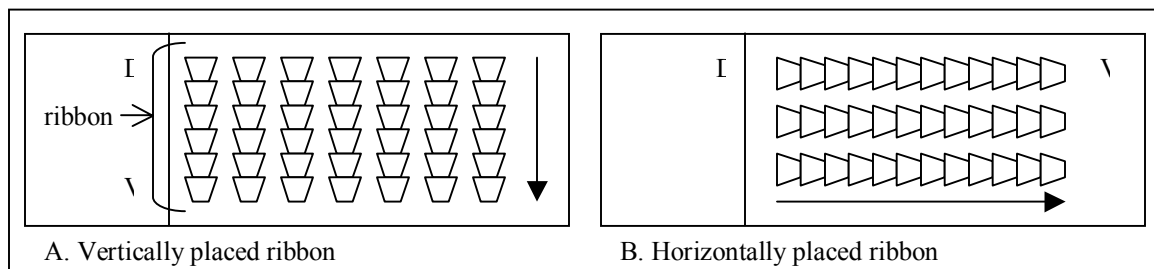
Sections of 10 $\mu$ m thickness were made using the Spencer 820 Microtome. The trimmed paraplast block containing the embedded embryo was



**Figure 1:** Orientation of the block face relative to the microtome blade. V; ventral side, D; dorsal side

positioned in the microtome so that the microtome blade cuts through the dorsal side of the embryo first (Fig. 1). Thus, the dorsal side of the embryo was the wider side of the trapezoid.

Section ribbons were floated onto dI H<sub>2</sub>O on slides coated with aminoalkyl silane



**Figure 2:** Two possible placement orientations of section ribbons on a slide.

(Sigma), which were on a slide warmer at approximately 45°C. Placing the ribbon vertically (Fig. 2A), allowed for easier visualization later under the microscope. In this way, the embryo's dorsal side was facing up and not sideways which is what happens if the ribbon was placed horizontally (Fig 2B). More water was added onto the slide gradually as more ribbons were added to the slide. If the slide was completely covered with water, the ribbons moved around when trying to place the ribbon and made it



difficult to position the ribbons. The water allows expansion of the section, giving better adherence to the slide. Each embryo was not sectioned all at once. Sectioned short ribbons were placed onto the slide as sectioning proceeded. This also allowed for easier placement of the ribbons. Serial sections were done, and each slide contained approximately six to eight ribbon strips with each strip containing six to nine sections per strip depending on the trimming and the size of the embryo. Once section ribbons filled a slide, the water was kept on the slide for approximately 15 min. Then, the excess water was removed using a glass Pasteur pipette. There would still be residual water on the slides, so the slides were left on the slide warmer overnight to dry completely and to allow the sections to adhere to the slide.

## **V. Hematoxylin and Eosin staining**

Each solution in the following procedure was in a Wheaton glass staining dish. The rectangular dishes were set up in a series, so that the slides were just carried through each solution in a removable glass slide rack.

The slides with the paraplast embedded sections were first carried through three washes of 100% xylene in the fume hood for 10 min each to remove the paraplast. Next the slides went through an ethanol series to rehydrate the sections: two 5 min washes in 100% ethanol, two 5 min washes in 95% ethanol, one 5 min wash in 70% ethanol, one 5 min wash in 50% ethanol and lastly 5 min in 100% dI H<sub>2</sub>O. The slides were stained in Harris Hematoxylin solution (Fisher). The manufacturer's protocol recommended staining in the hematoxylin solution for only 8 to 10 min. The short staining time was

insufficient, however, since the stain was faint. A 30 min staining time was found to be more effective. Since Harris Hematoxylin is a regressive stain, the slides could be kept in the differentiating solution slightly longer if the 30 min overstained them. After 30 min in hematoxylin, the slides were rinsed briefly in tap water until the water no longer had any trace of stain. Then, the stained slides went into the differentiation solution (1% HCl in 70% ethanol) for 5-10 seconds. The slides were dipped in the solution a few times until the dark purple Hematoxylin stain was not as strong. The slide was briefly viewed under the microscope to determine if more destaining was needed. It is suggested to destain in the differentiating solution until the cytoplasm has only a faint stain but the sharp nuclear stain remains (Clopton, 2004). After the destain, the slides were rinsed in running tap water for 10 min by having the Wheaton glass stain dish at an angle under the running faucet so that the water flowed over. Next, the slides went into blueing solution (0.3%  $\text{NH}_4\text{OH}$ ) for 30-90 seconds followed by Eosin Y stain solution (10g Eosin Y, 1ml acetic acid, brought up to 500ml with dI  $\text{H}_2\text{O}$ ) for 10 min. For this stain as well, recommended staining time was only 2 min; however, the longer stain time was more effective. After the eosin stain, the stained slides went through an ethanol dehydration series: two 2 min washes in 95% ethanol and two 2 min washes in 100% ethanol. In the fume hood, the slides went through one 2 min wash in ethanol: xylene:: 1:1, followed by two 2 min washes in 100% xylene. The last xylene wash could be longer without damaging or reducing the stain. The slides were removed from the last xylene wash, and the excess xylene was absorbed using paper towels. The sections were mounted with Permount (Fisher) and a glass cover slip. The Permount was allowed to solidify overnight in the fume hood.

## **VI. Imaging**

The stained sections were examined using the Leitz compound microscope. The pronephric alkaline phosphatase activity was examined using the Leica MZ6 dissecting microscope. Images were photographed using the QImaging Retiga 1300 digital camera which could be connected to either microscope. The photographs were then processed with the QCapture software and Adobe Photoshop.

# Results

## I. Normal and edematous *E. coqui* embryos

### A. Normal *E. coqui* embryos

Embryos of *E. coqui* were staged according to the table of Townsend and Stewart (1985). In normal development, all four limb buds appear by TS 4 (Fig. 3). As development progresses, the limb buds elongate and become little limbs with foot paddles at TS 7 (Fig. 3). Starting at TS 8, the digits of the feet become evident, and the embryos begins to resemble a little frog with a large membranous tail and big belly. The white yolk is approximately half covered by the body wall by late TS 9 (Fig. 3) and will become almost fully covered by TS 11 (Fig. 3). The toes of both fore and hindlimbs are distinct and the irises of the eyes are black at TS 11 (Fig. 3). Pigmentation in the skin starts to appear at TS 6. By late TS 10, the pigmentation is quite dark on the torso of the embryo, and it becomes difficult to see any structures underneath. Each stage is equivalent to approximately a day of development at  $23^{\circ}\text{C} \pm 1^{\circ}\text{C}$ .

### B. *E. coqui* embryos with edema

Edema is defined as swelling of an organ or tissue due to accumulation of excess fluid. There are many root causes. In *E. coqui* embryos that are raised in the lab, it was common to observe a few embryos in a clutch that developed poorly and became severely swollen. At times, entire clutches would consist of edematous embryos.

In the early stages of development, the edema syndrome was not very apparent. When the normal embryos were approximately TS 4 and TS 5 with all four limb buds, it became possible to pick out the ones beginning to develop the edema syndrome. In the

early edematous embryo, one of the key features was the absence of the forelimb buds (Fig. 4A) and in some cases, swelling was seen in these early embryos as well. The rest of the embryo resembles a normal TS 5 embryo with prominent hindlimb buds. In the severe cases, the forelimbs never appeared (Fig. 4C); however, in less severe embryos, small forelimb buds appeared later on (Fig. 4B). Only looking at the limbs, the embryo did not look much older than a TS 6 embryo (Fig. 3), but the pigmentation of the irises was closer to a TS 7 embryo (Fig. 3). As these edematous embryos developed, the pigmentation on their body also became darker (Fig. 4C) as would occur in normal development. Blood clots were often seen in edematous embryos as well as the swelling on the ventral side of the embryo in the abdomen area (Fig. 4C). In those embryos that developed forelimbs, the forelimbs were quite small even at a later stage in development (Fig. 4D). Embryos with edema not only developed abnormally, but development in general was retarded. Other features such as the tail remained quite small in the edematous embryos.

In the severely edematous embryos, the observations were based primarily on previously fixed specimens. The edema syndrome was not as severe when I was collecting the specimens. In comparison to the early and intermediate edematous embryos, in the severely edematous embryos more severe swelling was first evident. In the younger embryo with severe edema, the embryo resembled a ball with a head, two stubs of feet and a tail sticking out (Fig. 5A). The embryo had no forelimb buds and its hindlimb buds were small. In an older severely edematous embryo (Fig. 5B), very small forelimb buds appeared, and the embryo was severely swollen. The view from the posterior side of the embryo exhibits the large accumulation of fluid in the embryo's

body. The yolky tissue was slightly stretched. In a normal embryo, the embryo would be close to hatching at approximately 18 days after fertilization, but the edematous embryo's development was very much behind. A similar extent of swelling was also present in a slighter younger embryo (Fig. 5C). The yolk tissue was not stretched in this embryo, but the embryo was very severely swollen throughout its entire body. The most extreme cases of edema were those embryos in which the yolk tissue was very stretched and ruptured through the body wall (Fig. 5D). Some embryos also had very tiny forelimb buds and small hindlimbs with such severe swelling (Fig. 5D).

## **II. Pronephric development indicated by alkaline phosphatase activity**

### **A. Rationale – Immunohistochemistry control**

In *X. laevis*, the pronephros is the first embryonic kidney in development. A functional pronephros is essential for proper development, and there has already been much research on the embryonic organ. In particular, there have been antibodies developed specifically against the *X. laevis* pronephros (Vize *et al*, 1995). Since antibodies are often used cross species, the anti-*Xenopus* pronephros antibodies were used in hopes of detecting the pronephros in *E. coqui*.

Immunohistochemistry was tried using the known antibodies against *X. laevis* pronephric tubules (3G8) and duct (4A6) (Vize *et al*, 1995) with an alkaline phosphatase conjugated secondary antibody on both *X. laevis* and *E. coqui* embryos. There were no positive results in the *X. laevis* embryos, but there was positive alkaline phosphatase staining in the pronephric region of *E. coqui* embryos in both *E. coqui* experimental and control groups. There were two experimental groups in which each experimental group

was treated with one of the anti-kidney antibodies and secondary antibody, while the control group was treated with only the secondary antibody. This positive staining in a control group suggested that there was endogenous alkaline phosphatase activity in the pronephric region of *E. coqui* embryos. Accordingly, endogenous alkaline phosphatase activity was assayed by modifying the immunohistochemistry protocol without using any antibodies.

### **B. Alkaline phosphatase activity in limbs and spinal column**

Alkaline phosphatase activity was detectable in the limbs and the spinal column of *E. coqui* embryos. Alkaline phosphatase stain in the bone/cartilage began in the middle of the spinal column of TS 6 embryos (Fig. 6C). Staining began in the middle of the upper forelimb and both upper and lower hindlimb of TS 9 embryos (Fig. 7B). By TS 11, there was strong alkaline phosphatase stain in the entire fore and hindlimbs (Fig. 7D). The staining pattern beyond TS 11 was not determined.

### **C. Alkaline phosphatase activity in pronephros of normal embryos**

In the normal TS 4 embryo, a small area of blue alkaline phosphatase (AP) stain was present by the forelimb bud area (Fig. 6A). There was some dark blue background stain on the surface of the snout area, eyes and future backbone area. In the normal TS 5 embryo, the area of the AP stain was larger and darker by the forelimb buds (Fig. 6B). There also was slightly more background stain on the surface of the entire embryo. In the normal TS 6 embryo, the aquamarine blue colored AP stain was larger and in the same area as in the earlier embryos. The area would now be considered the shoulder area (Fig. 6C). The staining was not a solid patch of stain, but rather, there appeared to be a pattern.

After removing some of the tissue surrounding the stain, coiling of the pronephric tubules was visualized more clearly (Fig. 8A). There was some dark blue colored background stain on the surface of the embryo. In the normal TS 7 embryo, the size of the AP stain in the shoulder area was approximately the same as it was in TS 6 (Fig. 6D). As was done with the TS 6 embryo, the tissue surrounding the stained pronephros was removed and coiling of the tubules was visualized in the TS 7 embryo (Fig. 8B).

In the normal TS 8 embryo, the AP stain continued to appear in the shoulder area (Fig. 7A). Any pattern in the stain was difficult to see due to the presence of more pigmentation in the embryo's skin. In the normal late TS 9 embryo, the AP stain was fainter in the shoulder area (Fig. 7B). Likewise it was more difficult to see the stain because the pigmentation in the skin was darker. A close up image of the area was difficult to capture as well because of the location. In the normal TS 10 embryo, the AP stain in the shoulder area was still present (Fig. 7C). To get a better view, some of the outer pigmented skin layer was removed to better visualize the stain (close up of Fig. 7C).

At TS 11, the AP stain no longer seemed to be present (Fig. 7D). Either the stain was much fainter, or perhaps due to the darker pigmentation of the skin, the stain was not as easily visualized. To overcome the problem of dark pigmentation in the skin, early and late stage embryos were bleached. No AP stain was present; however, in all of the bleached embryos. And thus, the staining was not determined for stages beyond TS 11 because the pigmentation in the skin is quite dark in the later stages which make it difficult to see any staining of structures underneath.



The AP stain appeared despite the embryos being exposed to the endogenous AP inhibitor, levamisole (Fig. 6A, B). The degree of AP stain in the embryos without exposure to levamisole was not significantly different to the stain in embryos treated with levamisole. At times, the AP stain was darker in the levamisole treated embryos and at other times it was the opposite.

The total number of embryos examined for AP staining in normal *E. coqui* embryos is summarized in Table 2. AP stain was present in all embryos examined for stages TS 4 through TS 7. For stages TS 8 through TS 10, AP stain was seen in most but not all of the embryos examined.

#### **D. Alkaline phosphatase activity in pronephros of edematous embryos**

Once the staining pattern in the normal embryos was known, the staining in edematous embryos was examined. In an early edematous embryo; eTS 4/5 - 5 days old, with no forelimb buds, the AP stain was present in what would be the forelimb bud area but the size of the stain was much smaller (Fig. 9A) than the staining in a normal TS 5 embryo (Fig. 6B). In a slightly older embryo; 7 days old, AP stain was present in what would be the forelimb bud area but the staining was quite faint (Fig. 9B). The development of the embryo itself was very retarded in comparison to a normal embryo (Fig. 9C) from the same clutch. In another edematous embryo which was approximately 7 days old with no forelimbs, the AP stain was present in what would be the forelimb bud area (Fig. 9D). The size of the area of stain was small as well. In an older embryo which was approximately 10 days old with no forelimbs, the AP stain was present in what

would be the shoulder area; but the stain was faint and no pattern of the stain could be determined (Fig. 9E).

In another edematous embryo which was approximately 10 days old but with forelimbs (Fig. 10A), the AP stain was in the shoulder area and similar to the stain present in a normal TS 6 embryo. However, a normal embryo from the same clutch at the time of fixation was a TS 8 embryo which exhibited less AP stain (Fig. 10B) as had been seen in normal TS 8 embryos. In an older edematous embryo with small forelimbs, AP stain was present in the shoulder area more distinctly only on one side (Fig. 10C). In the normal embryo from the same clutch at the time of fixation was a TS 11 embryo (Fig. 10D) and it shows no AP stain as had been in other normal TS 11 embryos.

There was a difference between the edematous specimens in that some of the embryos examined had been fixed in August, 2004, and stored for a year before staining, while others were fixed more recently, in June, 2004 and then stained. The longer storage period, however, does not seem to be a cause for the decreased stain in the edematous embryos. In particular, recently fixed edematous embryos showed little (Fig. 9B) or no (Fig. 10E) AP stain in the pronephric area, while a normal embryo fixed at the same time from the same clutch (Fig. 9C, 10F) shows definite AP staining.

The total number of embryos examined for AP staining in edematous *E. coqui* embryos is summarized in Table 3.

### **III. Histology**

#### **A. Pronephros of normal *E. coqui* embryos**

In addition to the whole mount visualization of the pronephros via the alkaline phosphatase stain, serial histological sections were made and studied. In order to make comparisons in the edematous embryos, the histology of normal kidney development was first examined.

In a normal TS 4 embryo, openings in the mesodermal tissue were present at approximately the region of the forelimb bud (Fig. 11A). Under higher magnification, it was evident that there was a beginning of organization of the cells surrounding the openings (Fig. 11B). In a normal TS 5 embryo, pronephric tubules were evident near the forelimb bud (Fig. 11C). The cells were organized in a circular manner, as a cross section of a tube would be, and these cells and structures were easily distinguishable from the other cells in the section (Fig. 11D). In a normal TS 6 embryo, more pronephric tubules were present, and the area of the pronephros was bigger (Fig. 11E) than in TS 5 (Fig. 11C). The openings were larger and blood cells were present around the tubules (Fig. 11F). In sections from a more posterior part of the embryo, the early signs of the developing mesonephros were present in a group of clustered cells located ventral to the notochord (Fig. 11G). The cells of the mesonephros were smaller and more tightly organized (Fig. 11H) than were in the pronephros.

In a normal TS 7 embryo, the size of the pronephros was approximately the same (Fig. 12A) as in TS 6 (Fig. 11E). In a section from a more posterior part of the embryo, the mesonephros at TS 7 continued to be in the early stages of development (Fig. 12C). The

specific organization of the cells was difficult to determine at a higher magnification; however, the groups of cells were distinct from the rest (Fig. 12D). In a normal TS 8 embryo, the area of the pronephros was larger, and there were bigger lumens in the pronephric tubules (Fig. 12E). The shape of the cells was the same as in the previous stages; however, the overall tubule structures were larger (Fig. 12F). The mesonephros was now more distinct at TS 8 and both mesonephroi were located just ventral to the notochord (Fig. 12G). The organization of the cells was easily distinguishable under higher magnification (Fig. 13H). The individual cells were difficult to resolve; however, there seemed to be one to two very tightly packed tubules.

In a normal TS 9 embryo, the size of the pronephros was approximately the same as TS 8 (Fig. 13A). The cells were organized in a similar manner as in previous stages; however some of the tubules were not hollow in the lumen. There was a diffuse pink stain in the lumen (Fig. 13B). The mesonephros was more developed and more easily distinguishable from the rest of the structures of the embryo (Fig. 13C). The mesonephric cells were very tightly organized without much of a lumen (Fig. 13D). In a normal TS 10 embryo, the epithelium of the pronephric tubules was thinner (Fig. 13E). The pronephric cells were slightly smaller and blood cells surrounded the tubules (Fig. 13F). The mesonephros was now larger and the mesonephric tubules were more distinct (Fig. 13G). Blood cells surrounded the mesonephric tubules. The mesonephric tubules were less tightly packed and the lumens of the tubules were visible (Fig. 13H).

In a normal TS 11 embryo, the pronephros was slightly smaller than at TS 10. The lumens of the pronephric tubules were smaller as well (Fig. 14A). Under higher

magnification, the pronephric cells were somewhat swollen (Fig. 14B). The mesonephros was larger with a more elongated overall shape (Fig. 14C). The mesonephric tubules were more tightly organized than in TS 10 (Fig. 14D). In a normal TS 12 embryo, the pronephros was approximately the same size as in TS 11 (Fig. 14E). Some of the lumens of the pronephric tubules were not hollow and contained a material which diffusely stained (Fig. 14F). The mesonephros was approximately the same size and shape as in TS 11 (Fig. 14G); however, the mesonephric tubules were less tightly organized (Fig. 14H) than in TS 11 (Fig. 14D).

In a normal TS 13 embryo, the pronephros was slightly smaller (Fig. 15A) than it was in TS 12. There was more space between the pronephric tubules, and some of the lumens of the pronephric tubules were not hollow (Fig. 15B). The mesonephros was smaller (Fig. 15C) than in TS 12 and less tightly packed. The mesonephric cells resembled the pronephric cells, and there was space between the mesonephric tubules (Fig. 15D). In a normal TS 14 embryo, the pronephros was more tightly organized (Fig. 15E). The boundaries between the tubules were less distinct. The lumens of the tubules were not hollow but contained some material that stained pink (Fig. 15F). The mesonephros was smaller (Fig. 15G) but the organization of the tubules (Fig. 15H) was similar to TS 13.

In a normal TS 15 embryo, the pronephros was very tightly packed and small (Fig. 16A). There was hardly any space between the tubules and the lumens of the tubules were minimal (Fig. 16B). The mesonephros (Fig. 16C) was approximately the same size as in TS 14. There was a rich blood supply adjacent to the mesonephros (Fig. 16D).

## **B. Pronephros of edematous *E. coqui* embryos**

Two sets of edematous *E. coqui* embryos were examined. One set was fixed recently in Smith's fixative. The other set was fixed a few years ago in Bouin's fixative. The embryos which were fixed in Smith's fixative displayed less severe cases of edema and most embryos had small forelimbs. The embryos that were fixed in Bouin's fixative exhibited more severe cases of the edema syndrome and did not have any forelimbs.

In an early edematous embryo; approximately 5.5 days post-fertilization, a few pronephric tubules were present (Fig. 17A). There were fewer tubules present in the edematous embryos than in a normal embryo of the same age, TS 5 (Fig. 11C). The cells of the pronephric tubules had a smaller nucleocytoplasmic ratio in the edematous embryo (Fig. 17B). In another early but more severely edematous embryo, approximately 6.5 days post-fertilization, there were structures that appeared to be early pronephric tubules (Fig. 17C). Under higher magnification, organization of cells was evident but it was not a mature pronephric tubule (Fig. 17D). In an older edematous embryo, approximately 8 days post-fertilization, the most notable difference compared to a normal embryo was that the lumens of the tubules were very large (Fig. 17E). The diameter of the lumen of one of the tubules was almost equal the size of the forelimb bud. The epithelium of the large tubules was very thin and the individual cells of the epithelium were difficult to distinguish (Fig. 17F). There was no mesonephros in the embryo.

In an edematous embryo which was approximately 10 days post-fertilization, the pronephric tubules were even larger and there seemed to be fewer tubules compared to a

normal embryo as well (Fig. 18A). As in the 8 day edematous embryo, the epithelium of the large tubules was thin (Fig. 18B). Early mesonephros was present in this edematous embryo (Fig. 18C). The structure and shape of the mesonephric cells (Fig. 18D) were similar to those in a normal TS 8 embryo (Fig. 12G). At eTS 8, approximately 10.5 days post-fertilization, a severely edematous embryo only exhibited a few pronephric tubules (Fig. 18E). The tubules were small and but contained normal pronephric cells (Fig. 18F). In a section from a more posterior area of the embryo, a very small mesonephros was present (Fig. 18G). The shape and size of the cells resembled normal mesonephric cells; however, the cells were not tightly packed (Fig. 18H).

In an eTS 9/10 edematous embryo, 11 days post-fertilization, there were more pronephric tubules and the pronephros resembled a normal one (Fig. 19A); however, the cells of the pronephric tubules had a smaller nucleo-cytoplasmic ratio (Fig. 19B). The lumens were larger than in a normal embryo. The mesonephros of the edematous embryo resembled a normal mesonephros but the lumens were larger (Fig. 19C). The cells were less tightly packed (Fig. 19D).

In the severely edematous embryo at 18 days post-fertilization, the pronephros was similar to those in the early edematous embryos, in which the lumens were large (Fig. 20A). The cells were like normal pronephric cells, but their boundaries were unclear in the pronephros on the one side (Fig. 20B). In the oldest severely edematous embryo; 20 days post-fertilization, the pronephric tubules were large (Fig. 20C). The cells of the tubules on the one side resembled normal pronephric cells surrounding large lumens. As seen previously, the very large tubule on the other side had a thin epithelium (Fig. 20D).

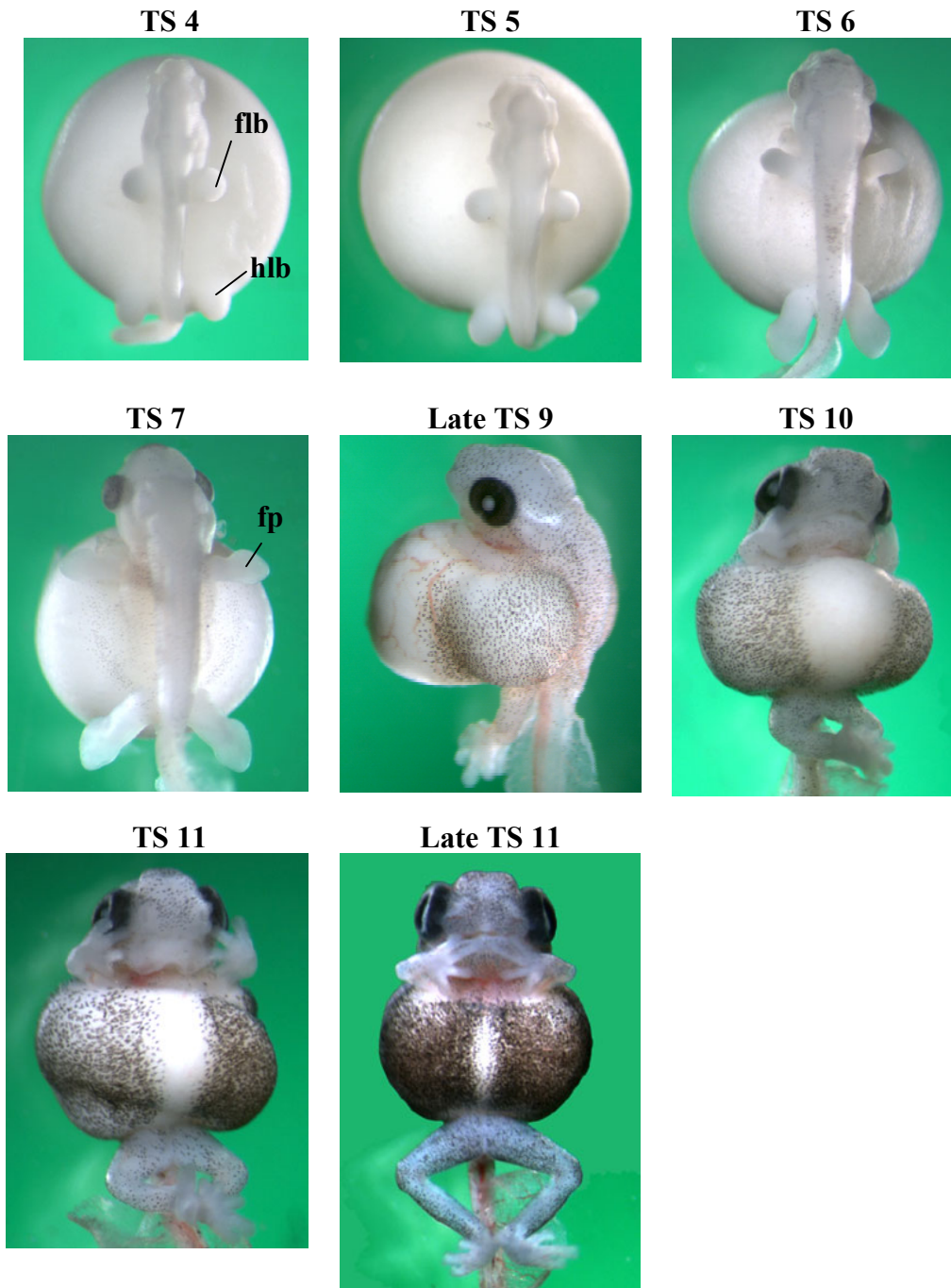
This embryo had a small mesonephros (Fig. 20E). The cells of the larger tubules resembled pronephric cells (Fig. 20F). The remaining cells, which were more closely packed, were more like mesonephric cells (Fig. 20F).

Overall, the pronephros of the edematous embryos contained large tubules with thin epithelia. The mesonephros also contained large tubules. In the more severely edematous embryos, the pronephros was less developed with few tubules, as was the mesonephros.



**Figure 3:** Normal *E. coqui* embryos. The stages are assigned according to Townsend and Stewart (1985) and designated TS. The yolk width is approximately 3.5 mm in the early stages; TS 4 through TS 7. The length of the embryo reaches approximately 5 mm from head to the beginning of the tail at TS 11.

flb: forelimb bud. fp: foot paddle. hlb: hindlimb bud.



**Figure 3**

**Figure 4:** *E. coqui* embryos exhibiting early and intermediate edema syndrome. All embryos were MEMFA fixed. A) Early edema - ~ 5 days post-fertilization. Dorsal view. No forelimb buds were present (dotted circles) while hindlimb were obvious. B) Early edema- ~ 7 days post-fertilization. Lateral view. Small forelimb buds were seen (white arrow). C) Intermediate edema - ~ 11 days post-fertilization. Forelimbs were absent (dotted circles), although hindlimbs were present. There was swelling in the abdominal area. Blood clots were present in the area where the forelimb would be (dotted circle). D) Intermediate edema - ~ 12 days post-fertilization. Small but short forelimbs were present (black arrow). There was severe swelling in the abdominal area.

bct: blood clot. flb: forelimb bud. hlb: hindlimb bud.

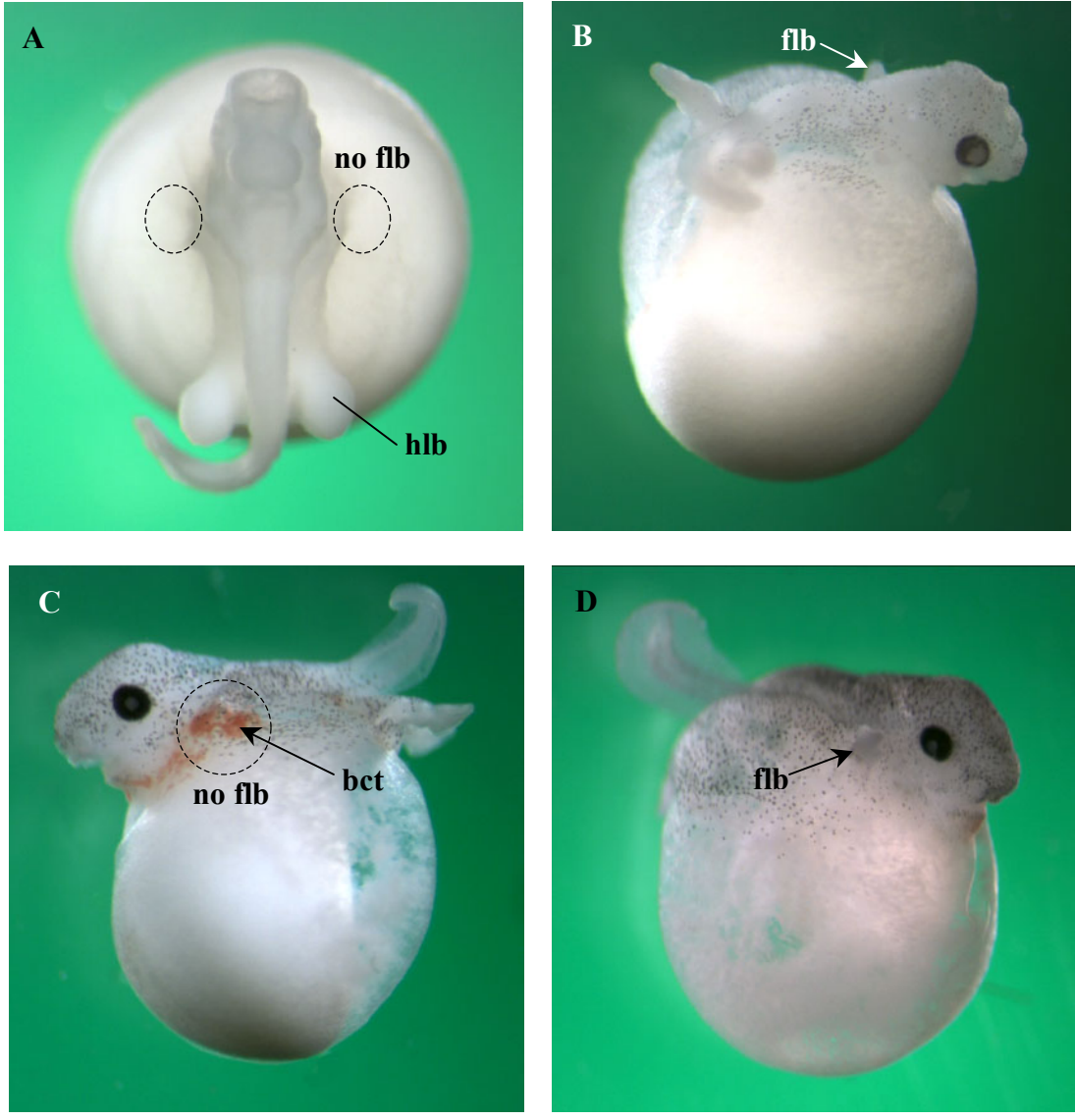
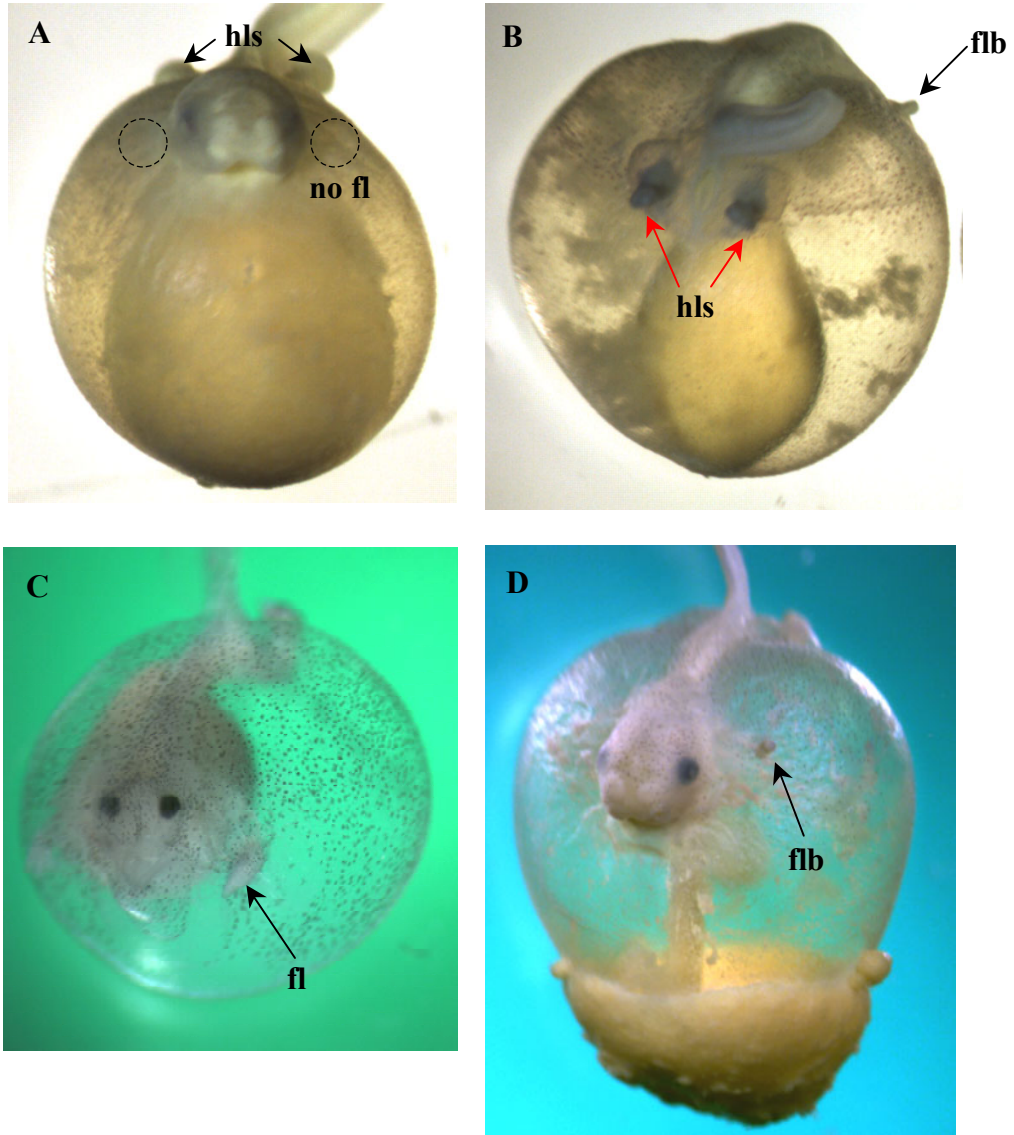


Figure 4

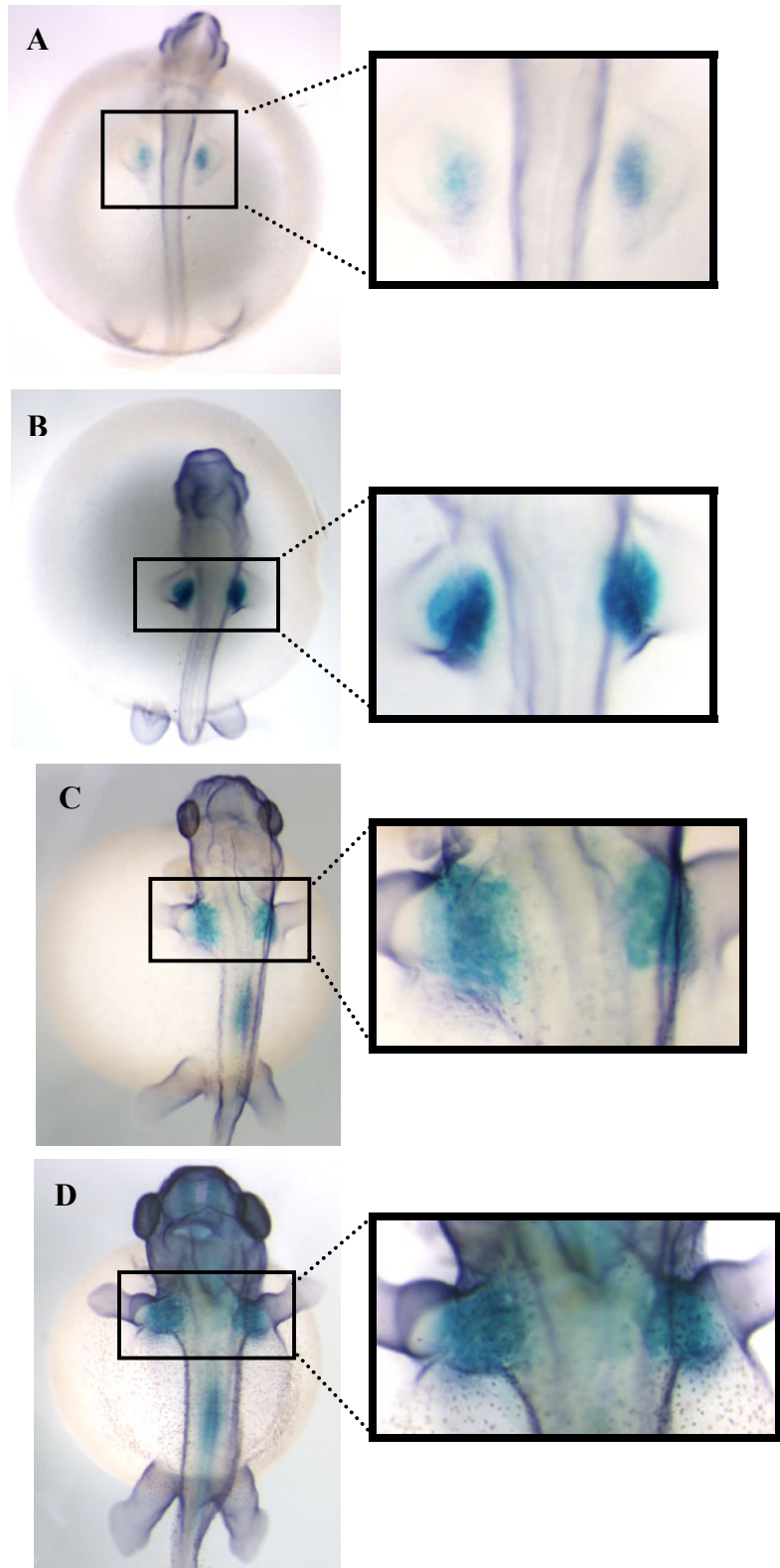
**Figure 5:** *E. coqui* embryos exhibiting severe edema. The embryos of (A), (B), and (D) were fixed in Bouin's fixative while the embryo of (C) was alive. A) ~ 10.5 days post-fertilization. Anterior view. No forelimbs were present (dotted circles) and the hindlimbs were small (black arrows). There was severe swelling in the entire embryo. B) ~ 18 days post-fertilization. Posterior view. Small forelimb buds (black arrow) and small hindlimbs (red arrows) were present. Growth was very retarded for the age of embryo and there was severe swelling of the entire embryo. C) ~14 days post-fertilization. Forelimbs were small (black arrow). There was severe swelling in the entire embryo. D) ~ 18 days post-fertilization. There was severe swelling in the entire embryo. Very small forelimb buds were present (black arrow) and development was retarded for all limbs. The yolk tissue was stretched, and it ruptured through the body wall.

fl: forelimb. flb: forelimb bud. hls: hindlimbs.



**Figure 5**

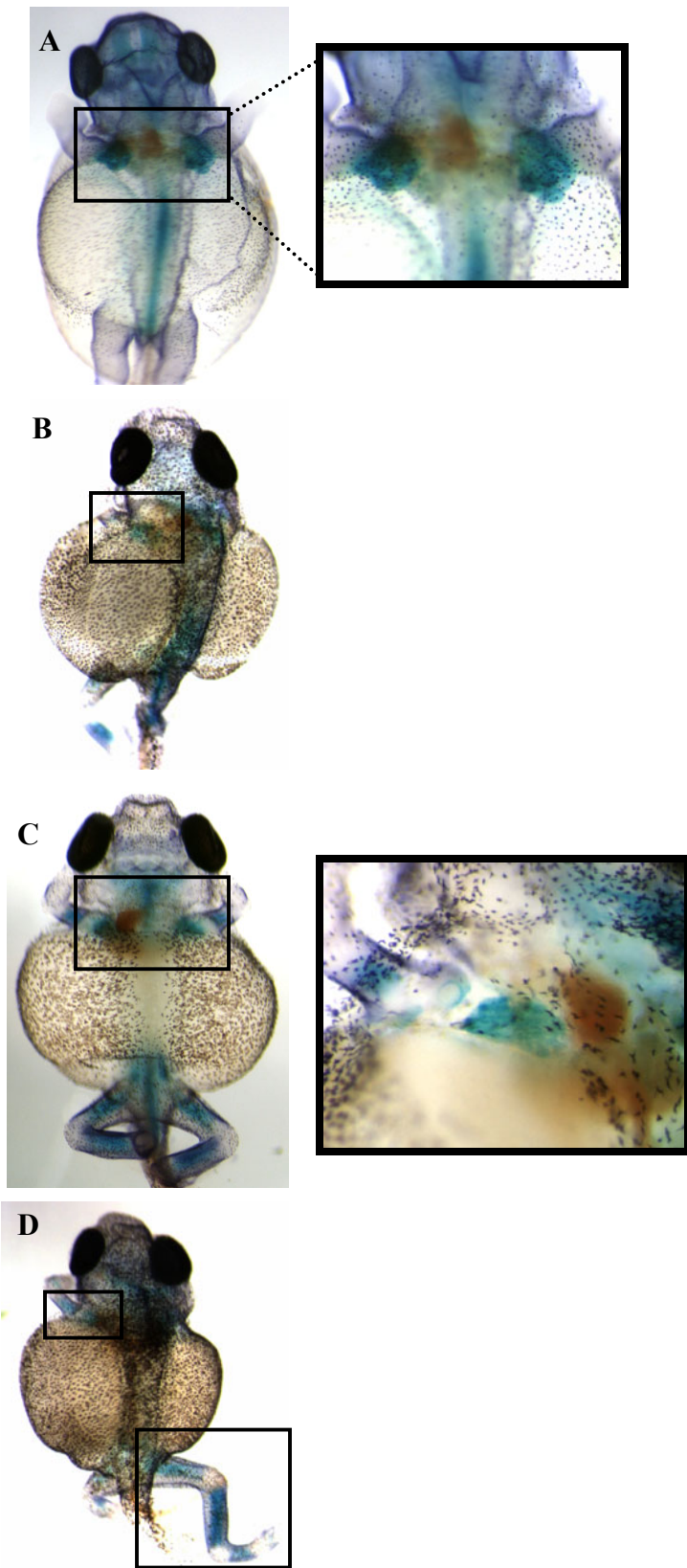
**Figure 6:** Endogenous alkaline phosphatase (AP) activity in normal early (TS 4-7) *E. coqui* embryos. All images were taken after clearing the embryos. A) TS 4 embryo. AP stain was present in an area by each forelimb bud, despite treatment with levamisole. B) TS 5 embryo. AP stain was larger in the area of the forelimb buds than in TS 4, despite treatment with levamisole. C) TS 6 embryo. AP stain in the forelimb bud/shoulder area was quite prominent. AP stain also began to appear in the spinal column. D) TS 7 embryo. AP stain in the shoulder area was approximately the same as in the TS 6 embryo. The stain in the spinal column has now extended more posteriorly. There was stain in the brain as well.



**Figure 6**



**Figure 7:** Endogenous alkaline phosphatase (AP) activity in normal later (TS 8-11) *E. coqui* embryos. All images were taken after clearing the embryos. A) TS 8 embryo. AP stain in the shoulder area was prominent. The stain in the spinal column was present slightly more anterior as well as extending more posterior, down to the beginning of the tail. B) Late TS 9 embryo. AP stain in the shoulder area was less prominent but remained apparent. It was more difficult to see the stain due to the darker pigmentation in the outer skin. The stain was present in the middle of the upper forelimbs and both the upper and lower hindlimbs. C) TS 10 embryo. AP stain in the shoulder area was less prominent but remained apparent. A higher magnification of the area is shown on the right. This was after some of the pigmented skin layer was removed before photographing the image. Stain was present in the upper forelimbs and the both the upper and lower hindlimbs. D) TS 11 embryo. AP stain in the shoulder area was no longer visible. Stain was present through the entire length of both fore and hindlimbs.

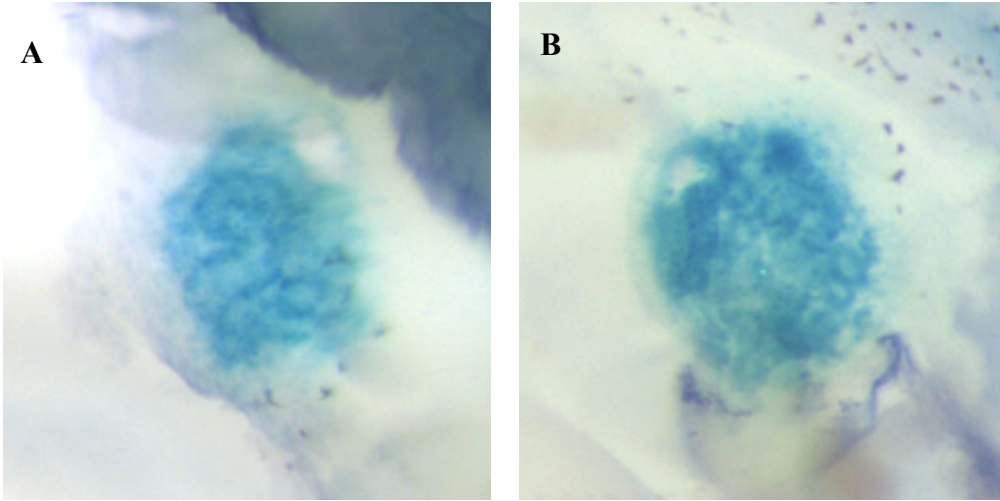


**Figure 7**

**Table 2.** Alkaline phosphatase activity in pronephros of normal *E. coqui* embryos

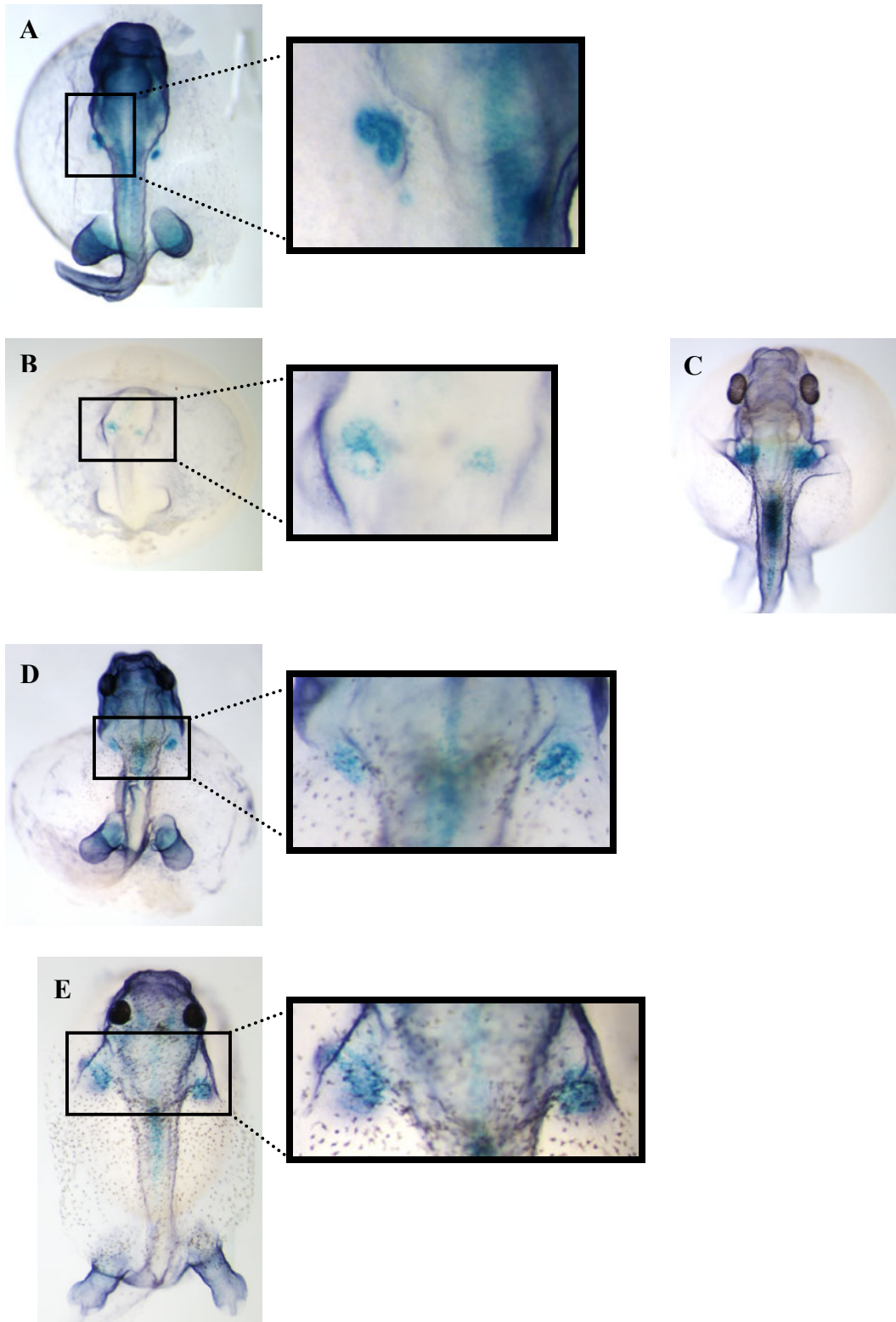
<i>Stage</i>	<i>Number of embryos with alkaline phosphatase activity</i>	<i>Total number of embryos examined</i>
TS 4	10	10
TS 5	12	12
TS 6	12	12
TS 7	22	28
TS 8	7	9
TS 9	3	6
TS 10	9	11
TS 11	0	5

**Figure 8:** Coiling of the pronephric tubules in normal *E. coqui* embryos stained for alkaline phosphatase (AP) activity. All images were taken after clearing the embryos. The tissues surrounding the pronephros were removed to better visualize the coiling that can be seen. A) AP staining of the pronephros in a TS 6 embryo. B) AP staining of the pronephros in a TS 7 embryo.



**Figure 8**

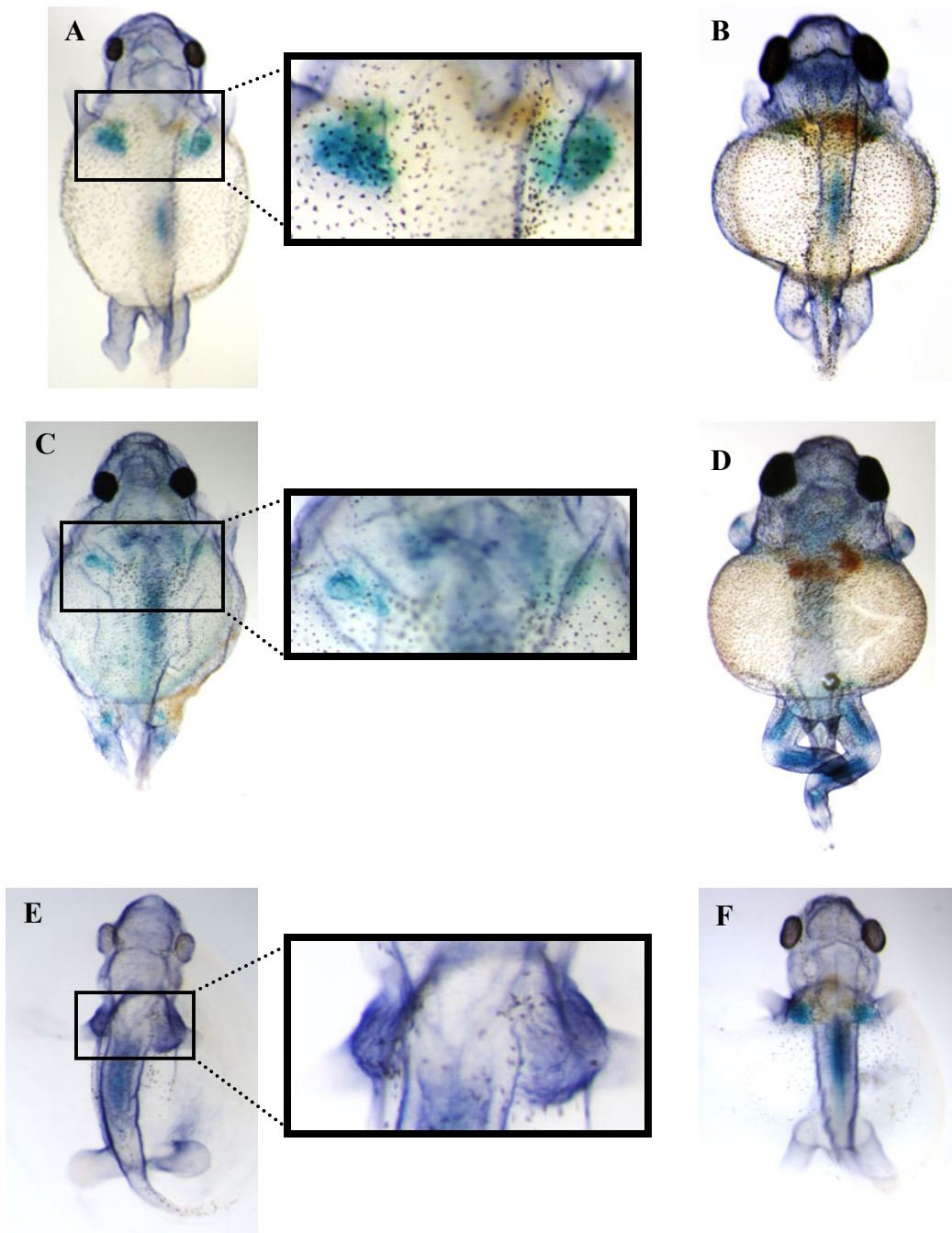
**Figure 9:** Endogenous alkaline phosphatase (AP) activity in early *E. coqui* embryos with edema. All images were taken after clearing the embryos. Specimens in (A), (D), and (E) were fixed August, 2004, stored in ethanol at -20°C and stained June, 2005. Specimens in (B) and (C) were fixed in June, 2005, stored in ethanol at -20°C and stained in June, 2005. A) eTS 4/5 embryo - 5 days post-fertilization. No forelimb buds were present, and AP stain was present in the forelimb bud area. B) eTS 4/5 embryo - 7 days post-fertilization. All limb buds were very small. AP stain was present in the forelimb bud areas. C) Normal TS 6 embryo of the same clutch at the time of fixation as (B) served as a reference. Dark AP stain was present in the shoulder areas. D) eTS 6 embryo - 7 days post-fertilization. No forelimb buds were present. AP stain was present in forelimb bud area. Faint AP stain was present in the spinal column. E) eTS 7 embryo - 10 days post-fertilization. No forelimbs were present. AP stain was present in the shoulder areas. Faint AP was present in the spinal column.



**Figure 9**

**Figure 10:** Endogenous alkaline phosphatase (AP) activity in older *E. coqui* embryos with edema. All images were taken after clearing the embryos. Specimens in (C) and (D) were fixed August, 2004, stored in ethanol at -20°C and stained June, 2005. Specimens in (A), (B), (E) and (F) were fixed in June, 2005, stored in ethanol at -20°C and stained in June, 2005. A) eTS 7 embryo - 7 days post-fertilization. Small forelimbs were present. AP stain was present in the shoulder area. Stain was present in middle part of spinal column. B) Normal TS 8 embryo of the same clutch at the time of fixation as (A) served as a reference. C) eTS 9 embryo - 13 days post-fertilization. Small forelimbs were present. Light AP stain was present in the shoulder area. Stain was present in the spinal column and in the upper hindlimbs. D) Normal TS 11 embryo of the same clutch as (C) served as a reference. E) eTS 5 embryo - 7 days post-fertilization. Both fore and hindlimbs buds were present. No AP staining was present in the pronephric area. Only background stain was present. F) Normal TS 6 embryo of the same clutch at the time of fixation as (E) served as a reference.





**Figure 10**

**Table 3:** Alkaline phosphatase activity in pronephros of edematous *E. coqui* embryos

<i>Stage; Age of embryo in days (d)</i>	<i>Number of embryos with alkaline phosphatase activity</i>	<i>Total number of embryos examined</i>
eTS 4/5; 5d no forelimb	1	2
eTS 4/5; 5d small forelimb bud	2	2
eTS 4/5; 7d delayed limb bud growth	4	4
eTS 6; 7d no forelimb	2	2
eTS 7; 10d no forelimb	1	2
eTS 7; 9d small forelimb	5	5
eTS 7; 7d (normal TS 8)	2	2
eTS 8; 11d small forelimb	4	5
eTS 9; 13d	2	2

**Figure 11:** Histology of embryonic kidney(s) in normal *E. coqui* embryos (TS 4-6).

Histological sections (10  $\mu\text{m}$ ) of Smith fixed, paraplast embedded embryos were stained with Harris hematoxylin and Eosin Y. A) TS 4. In approximately the region of the forelimb bud on one side, openings were present (box). B) Higher magnification of openings in (A). Organization of cells was evident. C) TS 5. The pronephric tubules were evident on both sides of the embryo near the forelimb bud. D) Higher magnification of some of the tubules boxed in (C). The cells were organized in a circular manner and easily distinguishable from the other cells. E) TS 6. More pronephric tubules were present. F) Higher magnification of the pronephros boxed in (E). The openings are larger and blood cells were present around the tubules. G) TS 6. Early signs of the developing mesonephros in a section more posterior in the embryo. H) Higher magnification of the mesonephros boxed in (G). The cells were smaller and more tightly organized.

flb: forelimb bud. bc: blood cells. n: notochord.

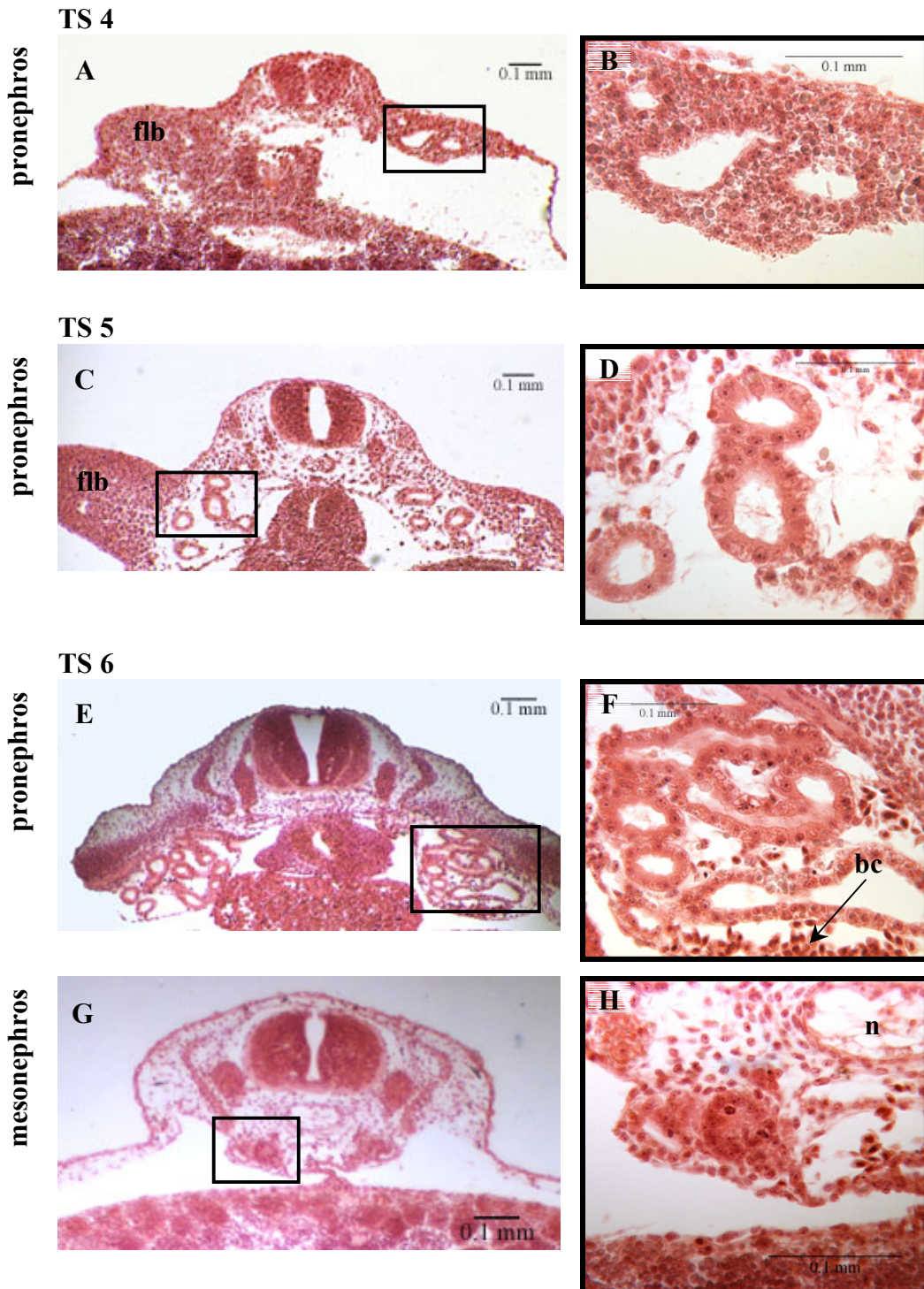
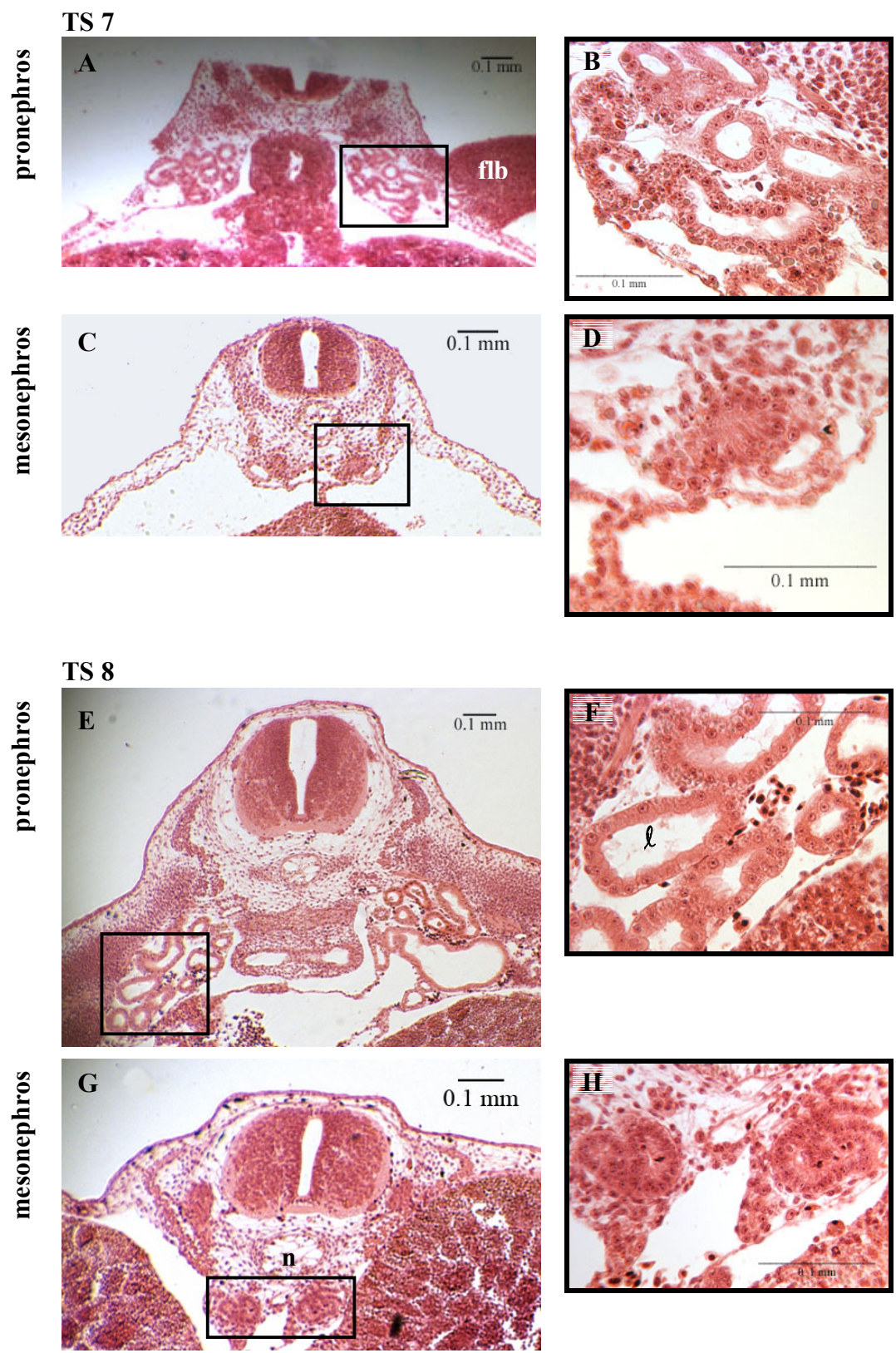


Figure 11

**Figure 12:** Histology of embryonic kidneys in normal *E. coqui* embryos (TS 7, 8). A) TS 7. The size of the pronephros was approximately the same as in TS 6. B) Higher magnification of the pronephros boxed in (A). The organization of the cells looks similar to TS 6. C) TS 7. The mesonephros continued to be in the early stages of development. D) Higher magnification of the mesonephros boxed in (C). The specific organization of the cells was difficult to determine. E) TS 8. The area of the pronephros was larger and there were bigger lumens in the pronephric tubules. F) Higher magnification of the pronephros boxed in (E). The shape of the cells were the same as in the previous stages, however the overall structures were larger. G) TS 8. The mesonephros was now more distinct. H) Higher magnification of mesonephroi in (G). The organization of the cells was clear.

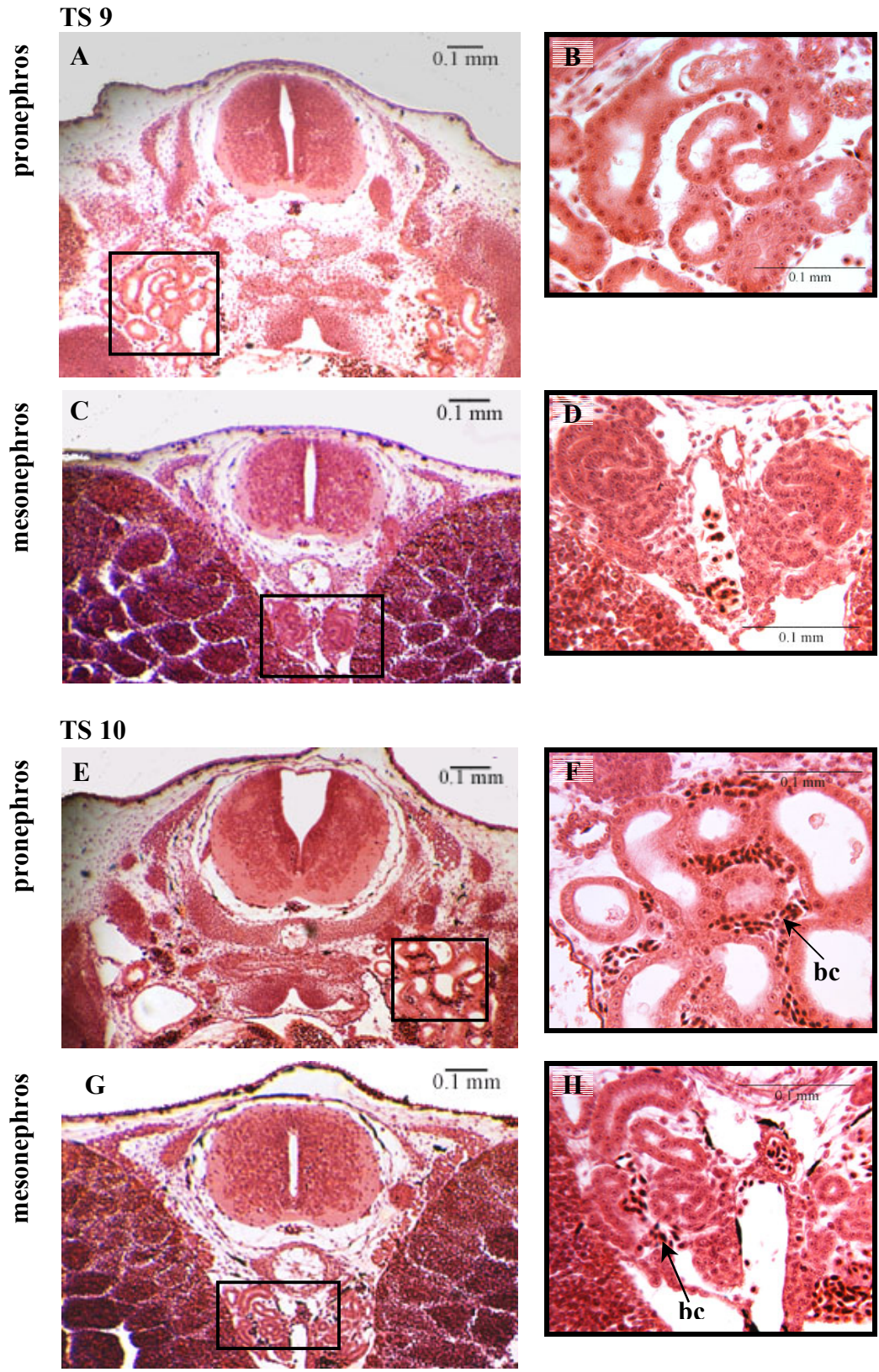
ℓ: lumen. n: notochord.



**Figure 12**

**Figure 13:** Histology of embryonic kidneys in normal *E. coqui* embryos (TS 9, 10). A) TS 9. The size of the pronephros was approximately the same as TS 8. B) Higher magnification of the pronephros boxed in (A). The cells were organized in a similar manner as in previous stages; however, some of the tubules were not hollow in the lumen. There was a diffuse pink stain. C) TS 9. The mesonephros was more developed and more easily distinguishable from the rest of the structures of the embryo. D) Higher magnification of the mesonephroi in (C). The mesonephric cells were very tightly organized without there being much of a lumen. E) TS 10. The epithelium of the pronephric tubules was thinner. F) Higher magnification of the pronephros boxed in (E). The pronephric cells were slightly smaller. G) TS10. The mesonephros was now larger and the mesonephric tubules were more distinct. H) Higher magnification of the mesonephros in (G). Blood cells surrounded the mesonephric tubules. The mesonephric tubules were less tightly packed.

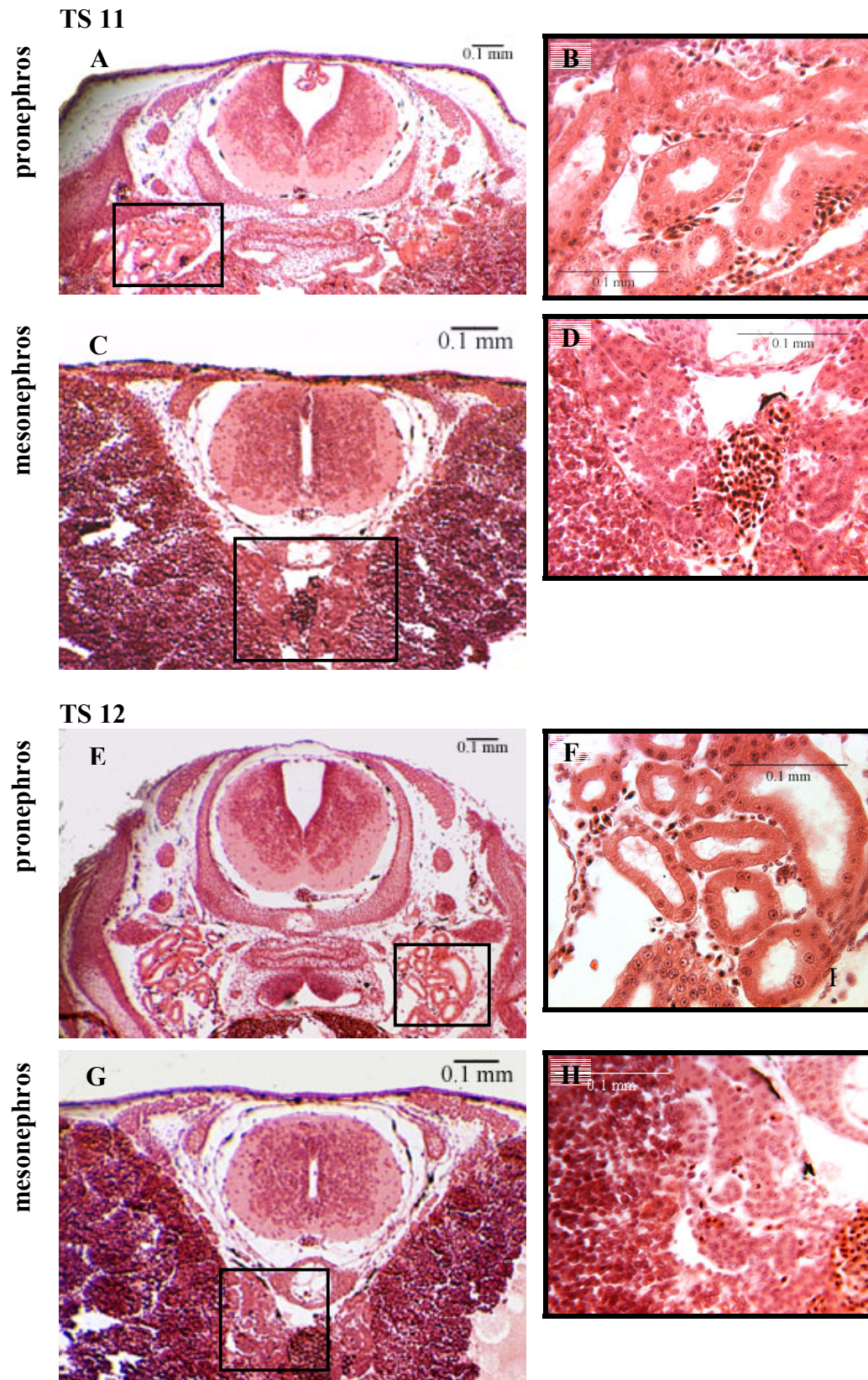
bc: blood cells.



**Figure 13**



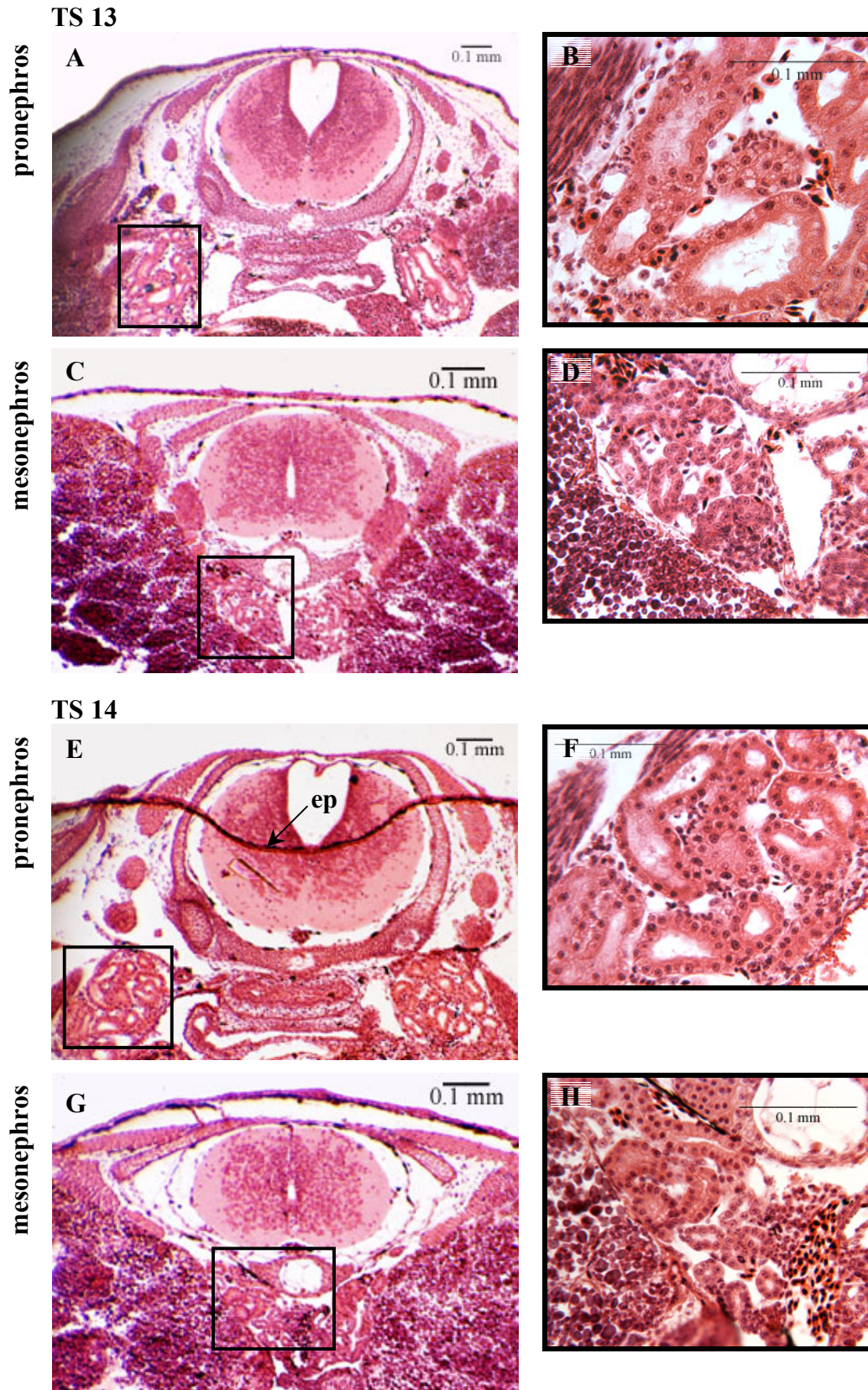
**Figure 14:** Histology of embryonic kidneys in normal *E. coqui* embryos (TS 11, 12). A) TS 11. The lumens of the pronephric tubules were smaller than in TS 10. B) Higher magnification of the pronephros boxed in (A). The pronephric cells had a smaller nucleo-cytoplasmic ratio. C) TS 11. The mesonephros was larger. D) Higher magnification of the mesonephroi in (C). The mesonephric tubules were more tightly organized than in TS 10. E) TS 12. The pronephros was approximately the same size as in TS 11. F) Higher magnification of the pronephros boxed in (E). Some of the lumens of the pronephric tubules were not hollow. G) TS 12. The mesonephros was approximately the same size as in TS 11. H) Higher magnification of the mesonephros boxed in (G). The mesonephric tubules were less tightly organized than in TS 11.



**Figure 14**

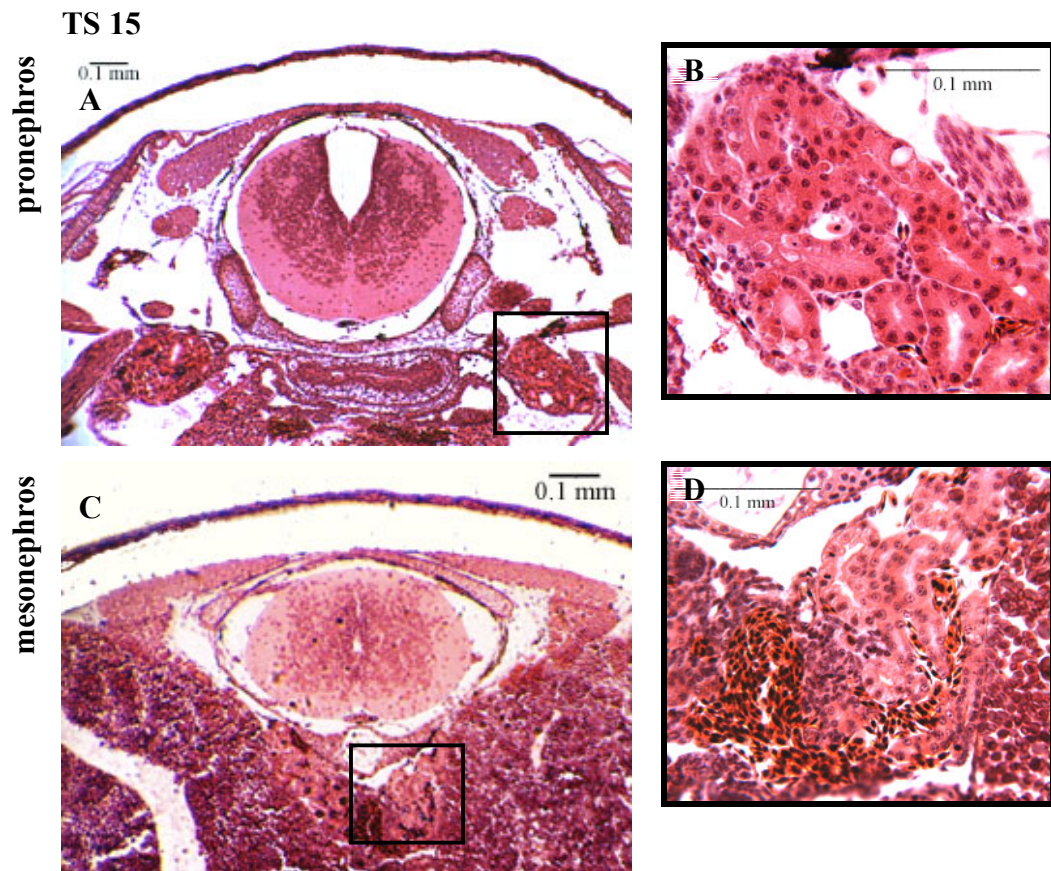
**Figure 15:** Histology of embryonic kidneys in normal *E. coqui* embryos (TS 13, 14). A) TS 13. The pronephros was slightly smaller than it was in TS 12. B) Higher magnification of the pronephros boxed in (A). There was more space between the pronephric tubules and some of the lumens of the pronephric tubules were not hollow. C) TS 13. The mesonephros was smaller than in TS 12 and less tightly packed. D) Higher magnification of the mesonephros in boxed (C). The mesonephric cells resembled the pronephric cells. There was space between the mesonephric tubules. E) TS 14. The pronephros was more tightly organized. The dark line in the middle is the epidermis that folded over when the section was placed on the slide after sectioning. F) Higher magnification of the pronephros in boxed (E). The boundaries between the tubules were less distinct. The lumens of the tubules were not hollow. G) TS 14. The mesonephros was small. H) Higher magnification of the mesonephros boxed in (G). The organization of the tubules was similar to TS 13.

ep: epidermis.



**Figure 15**

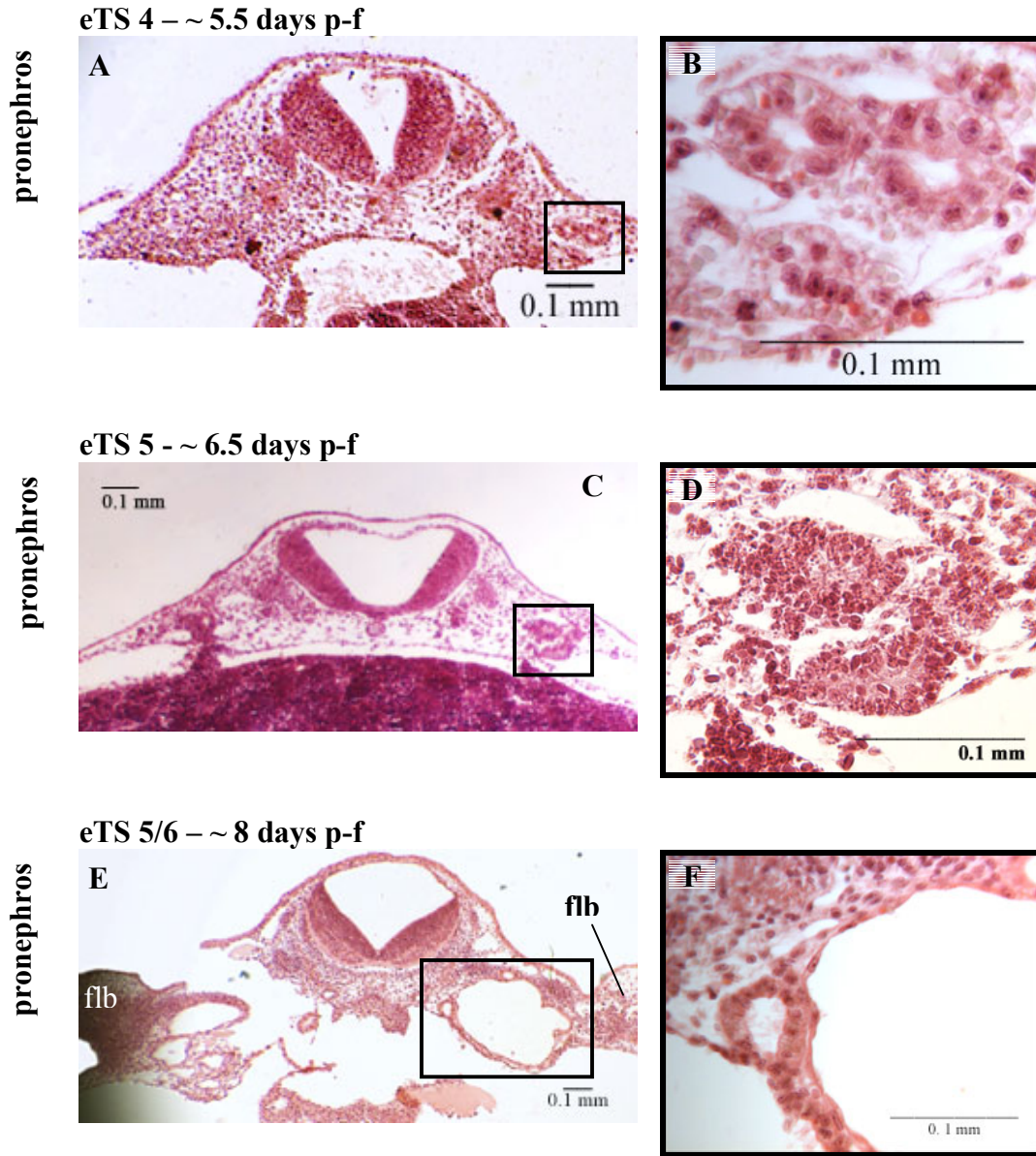
**Figure 16:** Histology of embryonic kidneys in a normal *E. coqui* embryo (TS 15). A) TS 15. The pronephros was very tightly packed and small. B) Higher magnification of the pronephros boxed in (A). There was hardly any space between the tubules. The lumens of the tubules were minimal. C) TS 15. The mesonephros was approximately the same size as in TS 14. D) Higher magnification of the mesonephros boxed in (C). There was a rich blood supply adjacent to the mesonephros.



**Figure 16**

**Figure 17:** Histology of embryonic kidneys in early edematous *E. coqui* embryos. The embryos of (A), (B), (E), and (F) were fixed in Smith's fixative while the embryo of (C) and (D) was fixed in Bouin's fixative. A) eTS 4 embryo without forelimb buds - ~ 5.5 days post-fertilization (p-f). There were fewer pronephric tubules than in a normal TS 5 embryo. B) Higher magnification of (A). The cells appeared to have a smaller nucleocytoplasmic ratio than those of normal embryos. C) eTS 5 - ~ 6.5 days post-fertilization embryo without forelimb buds. There were early signs of pronephric tubules. D) Higher magnification of (C). Individual pronephric tubule cells were very difficult to distinguish. E) eTS 5/6 embryo with small forelimb buds - ~ 8 days post-fertilization. Pronephric tubules were present near the forelimb buds. The lumens of the pronephric tubules were very large. F) Higher magnification of (E). The epithelium of the enlarged pronephric tubules was very thin.

flb: forelimb bud

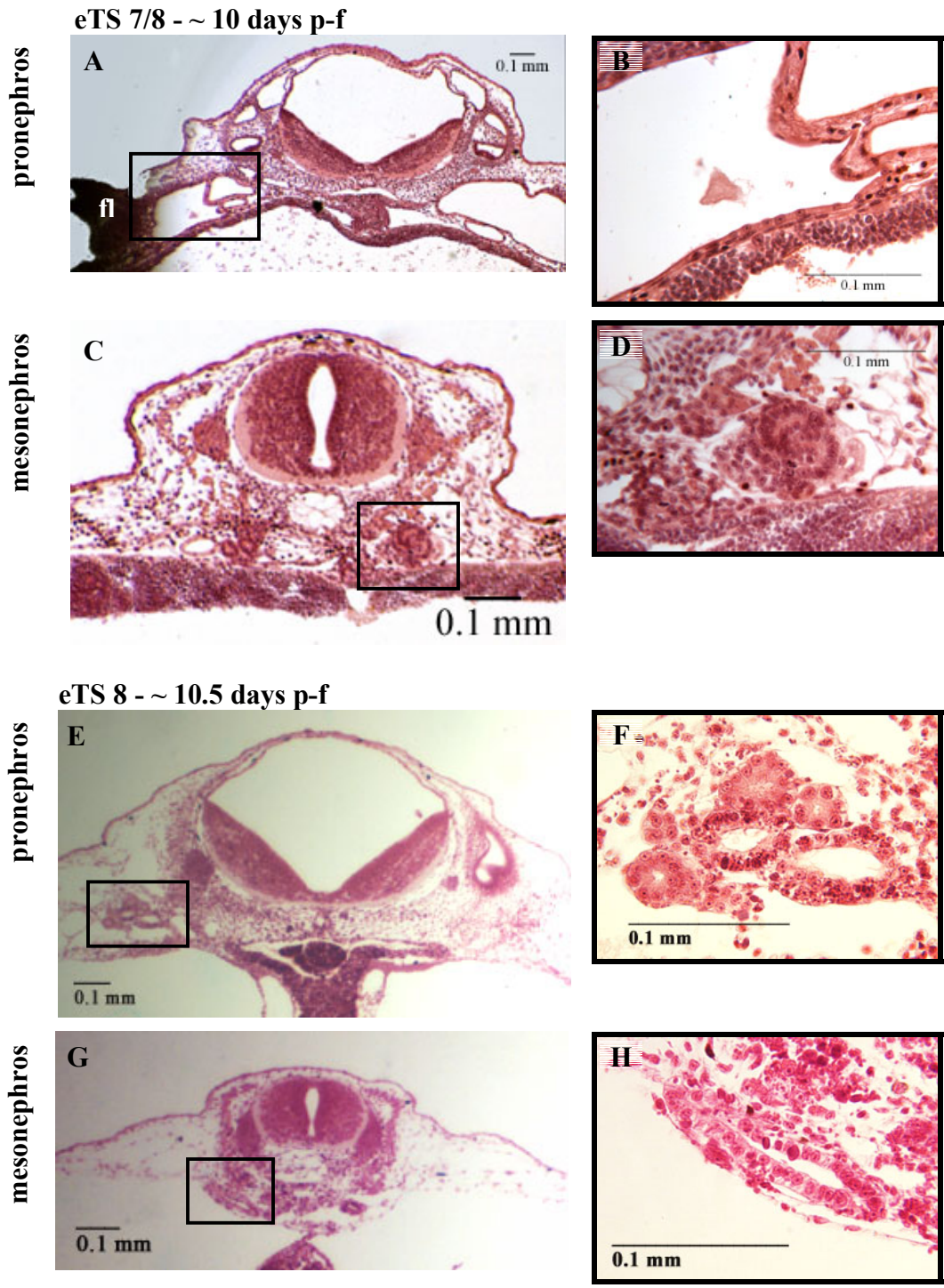


**Figure 17**



**Figure 18:** Histology of embryonic kidneys in older edematous *E. coqui* embryos. The embryos of (A), (B), (C), and (D) were fixed in Smith's fixative while the embryos of (E), (F), (G), and (H) were fixed in Bouin's fixative. A) eTS 7/8 embryo with small forelimbs - ~ 10 days post-fertilization. The pronephric tubules were present near the forelimb and were very large. B) Higher magnification of (A). The epithelium of the pronephric tubules was very thin and individual cells were difficult to distinguish. C) eTS 7/8 embryos with small forelimbs - ~ 10 days post-fertilization. There were a few mesonephric tubules. D) Higher magnification of the mesonephros boxed in (C). The structure and shape were similar to the mesonephros present in normal TS 8 embryos. E) eTS 8 - ~ 10.5 days post-fertilization embryo without forelimbs. There were a few pronephric tubules. F) Higher magnification of (E). The cells resembled those of pronephric cells of normal embryos. G) eTS 8 - ~ 10.5 days post-fertilization embryo without forelimbs. A single mesonephric tubule was present. H) Higher magnification of (G). The shape and size of the cells were similar to mesonephric tubule cells of normal embryos; however, the cells were not as tightly packed.

fl: forelimb.



**Figure 18**

**Figure 19:** Histology of embryonic kidneys in 11 day old edematous *E. coqui* embryos. The embryo was fixed in Smith's fixative. A) eTS 9/10 embryo with small forelimbs - ~ 11 days post-fertilization. The lumens of the pronephric tubules were large. There were several pronephric tubules. B) Higher magnification of the pronephros boxed in (A). The pronephric tubule cells were larger than in normal embryos. C) eTS 9/10 embryo with small forelimbs - ~ 11 days post-fertilization. The lumens of the mesonephric tubules were large. D) Higher magnification of the mesonephros boxed in (C). The mesonephric tubule cells were similar to those seen in normal embryos however less tightly packed.

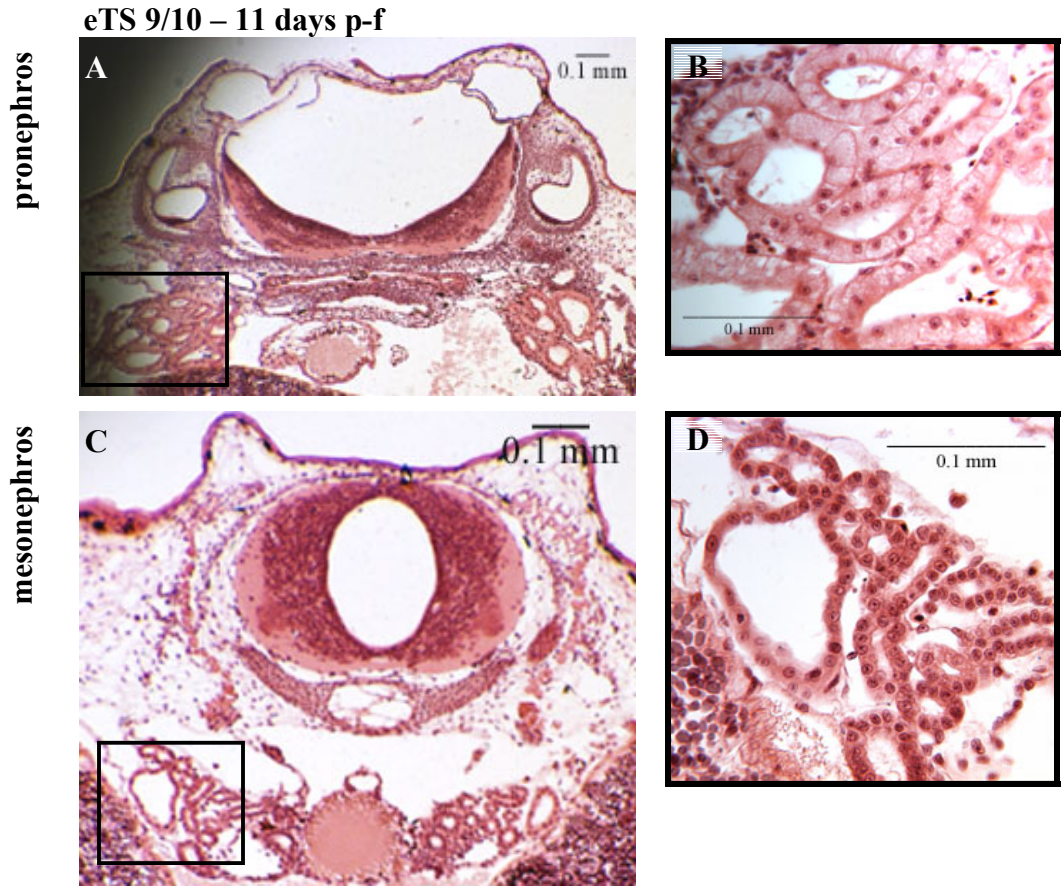
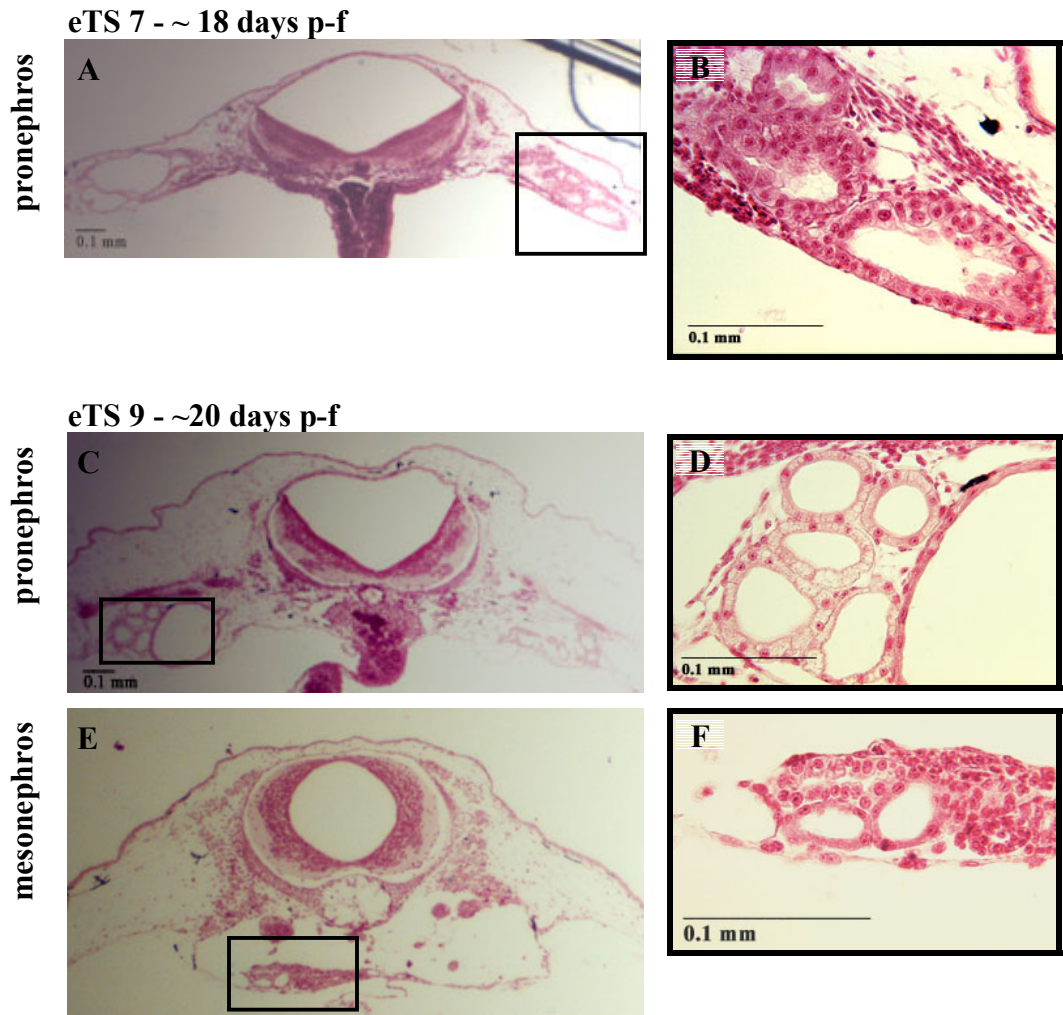


Figure 19

**Figure 20:** Histology of embryonic kidneys in older but severely edematous *E. coqui* embryos. All embryos were fixed in Bouin's fixative. A) eTS 7 - ~ 18 days post-fertilization. The pronephric tubules have very large lumens. B) Higher magnification of the pronephros boxed in (A). The boundaries of each of the tubules were not clear. The cells look similar to the pronephric cells present in normal embryos. C) eTS 9 - ~20 days post fertilization embryo. Large pronephric tubules were present. D) Higher magnification of (C). The cells of the smaller tubules resembled normal tubule cells with large lumens. The epithelium of the one very large tubule on the right was much thinner. E) eTS 9 - ~20 days post-fertilization embryo. There were mesonephric tubules in the more posterior region of the embryo. The lumens of the tubules were large. F) Higher magnification of (E). The cells in the larger tubules resembled more closely to pronephric tubule cells, while the more closely packed cells were similar to normal mesonephric cells.



**Figure 20**

## **Discussion**

### **I. Primary findings**

The histology of the pronephros and the mesonephros in normal *E. coqui* embryos was examined. The histology of the embryonic kidneys in edematous *E. coqui* embryos was also studied. The severity of edema varied considerably between embryos. In mild cases, there was just an overall swelling of the embryo and all of the limbs formed. In the severe cases, no forelimbs ever formed and all of development was very retarded. Development in general was retarded in the edematous embryo when compared to the normal embryos from the same clutch. There was a clear difference in histology between the normal versus the edematous embryos. The key characteristic in the edematous embryos was the large lumens of the tubules.

Endogenous alkaline phosphatase (AP) was discovered to be a marker for the developing pronephros in *E. coqui*. There was definite AP stain in the normal pronephroi in the early stages; TS 4 through TS 7. Starting at TS 8, the AP stain was more difficult to see and the stain was not as strong. In the late stages, stages beyond TS 11, the AP stain was not able to be determined due to the dark pigmentation of the skin. There was more stain in the normal compared to the edematous embryos.

### **II. Kidney development in *E. coqui* versus other *Eleutherodactylus* frogs**

Kidney development in *E. coqui* is as follows (Table 4). The first sign of pronephros is present at TS 4 as seen with AP staining and histology. The pronephros persists throughout the embryo's entire development, even until hatching. It is not known

however, when it is functional and whether it is still functional at hatching. The mesonephros first appears at TS 6. This is known only through histology because AP staining is only present in the pronephros.

The developmental progress of the *E. coqui* pronephros is similar to *E. nubicola* (Lynn, 1942) as presented in Table 4. The mesonephric development of *E. coqui* is also similar to *E. nubicola*. Lynn claims that the mesonephros is larger than the pronephros at -10 days (= TS 13) in *E. nubicola*; however, without morphometric analysis, it is difficult to tell if the mesonephros becomes larger in *E. coqui*.

Not much was reported regarding kidney development in *E. augusti latrans* (Valett and Jameson, 1961). The only comparison that can be made is that *E. augusti latrans* has a large pronephros like *E. coqui*, although 'large' is a relative term which was not well defined by Valett and Jameson.

The developmental progress of the embryonic kidneys in *E. martinicensis* (Adamson, 1960) in comparison to *E. coqui* is shown in Table 4. Adamson reports on the individual compartments of the pronephros, the pronephric duct, the pronephric tubules and the glomerulus (refer to schematic diagram in Fig. i). It is not clear how the specific number of tubules was determined, and thus I am unable to compare the numbers in *E. coqui*. The major difference in *E. martinicensis* is the early regression of the pronephros at the stage equivalent of TS 7. In *E. coqui*, the pronephros continues to develop and only becomes a very compact organ at hatching.



**Table 4.** Comparison of kidney development among *Eleutherodactylus* frogs

	<i>E. coqui</i>	<i>E. nubicola</i>	<i>E. martinicensis</i>
First signs of pronephros	TS 4	- 23 days (= TS 4)	Stage II (= TS 4)
Definite pronephric tubules	TS 5	- 21 days (= TS 5)	Stage III (= TS 5)
Maximum development of pronephros	~ TS 12	- 11 days (= TS 12)	n/a
Early mesonephros	TS 6, TS 7	- 18 days (= TS 7)	Stage IV (= TS 7)
Mesonephros is larger than pronephros	Difficult to tell	- 10 days (= TS 13)	n/a
Degeneration of pronephros	Remains present at hatching	Remains present at hatching	Begins at stage IV (= TS 7)

### III. Alkaline phosphatase as a marker for pronephros

The AP stain in the pronephros was a novel finding in *E. coqui*. Endogenous AP activity can be used as marker for the pronephros in the early stages in *E. coqui*. The AP stain showed a coiling pattern and seemed to only stain the pronephric tubules. The duct was not visible by AP staining. The increasing size of the AP stain area corresponds to development of pronephros as seen in the histology in the early stages; TS 4 to TS 7. The reason for the decrease in AP stain in the later stages, TS 8 and beyond, is unknown. Perhaps AP activity is no longer necessary in a fully developed pronephros. AP activity may be associated with the differentiation of the pronephros and the activity may cease once the pronephros is functional which is shown by the AP stain disappearing. Therefore, it would be useful to know when the pronephros becomes functional, if it becomes functional at all.

There was a definite difference present between normal and edematous embryos, with distinctly less AP stain in the pronephroi of the edematous embryos. This decrease in stain was not due to a longer storage period prior to staining. In recently fixed edematous embryos, there was little (Fig. 9B) or no (Fig. 10E) AP stain, while a normal embryo fixed at the same time from the same clutch (Fig. 9C, 10F) showed definite AP staining.

AP activity has shown to be a marker for the pronephros in other organisms such as the biphasic frog, *X. laevis* (Drawbridge, pers. comm.), axolotl, *A. mexicanum* (Zackson and Steinberg, 1988) and zebrafish, *D. rerio* (Drummond *et al.*, 1998). Thus, perhaps it is not so surprising that the same enzyme is present in the *E. coqui* pronephros and works as a pronephric marker. In relation to the necessity for alkaline phosphatase in the development of an organism, perhaps some of the abnormalities present in the edematous embryos can be explained by the decreased AP activity. In mice, the embryonic form of AP was not essential; however, when tissue-non-specific (TNS) AP was knocked out, the neonates had various abnormalities, such as severe epileptic seizures, poor coordination, no body fat, reduced muscle structure, a smaller spleen, impaired intestinal movement, and poor mineralization in bones (Narisawa *et al.*, 1997). Perhaps *E. coqui* AP is an ortholog to the TNS AP and the decrease in AP accounts for the poor kidney and limb development present in the edematous *E. coqui* embryos. Although the kidney was not one of the organs severely affected in the AP deficient mice, the other abnormalities in the mice can be correlated to the abnormalities in the edematous *E. coqui* embryos. It may be that AP activity is more necessary in the development of the kidney in *E. coqui* embryos.

It is unclear why levamisole did not fully inhibit endogenous AP activity as it did in the axolotl embryos from Jackson and Steinberg's study (1988). This may be due to the levamisole that was used being old and no longer effective.

The AP stain in the limbs confirms that the stain present in the area of the pronephros was indeed AP activity. Bone and cartilage formation has been previously studied (Hanken *et al.*, 1992) and the results are comparable to my AP staining. Hanken *et al.* (1992) report that at TS 7, cartilage is present in the femur, tibia and fibula, but not in the distal hindlimbs nor in the forelimbs. At TS 8, there is cartilage in the hindlimbs, proximal to the metatarsals and in the forelimbs proximal to the manus. At TS 9, there is cartilage in the forelimbs and hindlimbs from the limb girdles to proximal phalanges. Starting at TS 10, the cartilage is 'distinct' in all limbs. From my experiments, AP stain in the limbs starts in late TS 9. AP activity must not be active in the early cartilages since AP stain was not present in the limbs in the stages prior to TS 9.

#### **IV. Edema**

In this thesis, I present a definite correlation between the development of the pronephros and the occurrence of edema. In the pronephros of edematous *E. coqui* embryos, compared to normal embryos, there were overall fewer tubules. The histology showed that the epithelia cells were very thin in the tubules with large lumens. When the lumen was not so large, the cells still looked different from those in normal embryos in that they had a smaller nucleo-cytoplasmic ratio, making them look swollen. When the lumens are so large, there may not be enough pressure in the tubules to move the fluid through. This would slow the excretory process and lead to fluid building up, resulting in

edema. From the AP stain, the stained area was clearly smaller in the pronephros of the edematous embryos. In one edematous case, there was no AP stain. If the AP stain is indicative of the size of the pronephros, the edematous embryo has very small pronephroi, and they may not be large enough to accommodate the embryo's excretory need. The mesonephros was not always present in older edematous embryos and especially when the embryos were more severely swollen. The mesonephric cells in the edematous embryos were similar to normal mesonephric cells, and the mesonephric cells were smaller than the cells in the edematous pronephros. In some embryos, large lumens were present in the edematous mesonephros. The larger lumens may have made the mesonephros less effective, similar to the defective pronephros.

These observations indicate that the embryonic kidneys in the edematous embryos could be defective. Edema has been seen in various cases in other organisms in which the pronephros was involved. In amphibian larvae, the pronephros is a functioning organ. When both pronephroi were removed in the salamander, *A. punctatum* embryos, severe edema occurred (Howland, 1921). Edema occurred as well when parts of the pronephros were surgically removed in the *A. punctatum* embryos. When a single pronephros was removed, although edema was not seen, the remaining pronephros hypertrophied. In zebrafish, mutants were screened for defective pronephric development. These genetically mutant zebrafish embryos had fluid filled cysts in the region of the pronephros and exhibited gross edema (Drummond *et al.*, 1998). Drummond *et al* (1998) assumes that the edema is due to loss in pronephric function and failed osmoregulation since the heart function appeared to be normal in these mutant zebrafish embryos.

I speculate that the edema in the *E. coqui* embryos was due to a defective pronephros, however, it is a correlation in the scope of this study and other causal possibilities have to be considered such as defects in the lymphatic system, the circulatory system and the skin for reasons explained below.

The edema could be due to a defective lymphatic system. The lymphatic system is important in water balance in amphibians. It mediates fluid and solute return to the circulatory system during osmotic stress (Baustian, 1988; Hillman *et al.*, 1987; Jones *et al.*, 1992). Cranial lymphatic hearts form at stage Gosner 18 in *Rana esculenta*, the common water frog (Hoyer, 1905a, b), which is equivalent to the Nieuwkoop and Faber (1994) stage 26 in *X. laevis*. The lymph hearts are a series of contractile vesicles that pump the lymphatic fluid, the lymph, through the lymphatic system (Duellman and Trueb, 1986). The tadpole continues to develop an extensive lymphatic system throughout its development until metamorphosis. The lymph vessels are thin walled, arise from the mesoderm, and empty into large subcutaneous lymph sacs (Conklin, 1930). These sacs include all spaces between the skin and muscle and surrounding internal organs (Carter, 1979). The sacs are loosely separated by septa between the muscles and skin. Fluid is able to readily flow through the perforations in the septa. Lymph hearts are independent of the cardiovascular heart and also pump the fluid within the spaces (Deyrup, 1964). As fluid enters into the lymph sacs via the skin and other organs, the lymph is pumped into the posterior veins. A significant number of these veins supply blood to the peritubular vessels in the kidney. Then the kidney excretes the excess water without diluting the arterial blood (Feder and Burggren, 1992).

The importance of the lymphatic system has been demonstrated by death of the animal which had its lymph hearts destroyed (Baldwin *et al.*, 1990; Baustian, 1988). When embryos with their lymph hearts destroyed were in a hydrating environment, the animals exhibited increase in weight and edema (Foglia, 1941). The surrounding water contributed to the total amount of fluid present in the subcutaneous lymph sacs. The edematous *E. coqui* embryos may have dysfunctional lymph hearts which do not sufficiently pump the fluid that ultimately reaches the kidneys for excretion. Likewise, the lymph sacs may be defective and unable to retain the lymph properly. Fluid leaking into the body cavity may be resulting in edema. However, the lymphatic system of *E. coqui* has not been studied for this thesis.

The edema could be due to defective circulation. In humans, pulmonary edema, in which fluid fills the lungs, is the most severe manifestation of congestive heart failure ([www.emedicine.com](http://www.emedicine.com)). With a defective heart, blood will not be pumped and circulated throughout the body sufficiently and the blood may leak back into the organs. This reduced blood flow would mean that less blood is supplied to the kidney for excretion of any excess fluid in the body. There is poor circulation in the edematous *E. coqui* embryos as seen by blood clots on the surface of the embryos, although the beating heart was clearly visible in most cases. Perhaps because the circulation is poor, blood is pooling in areas, leading to the blood clots. The poor circulation may allow easier coagulation of the blood in blood vessels that may have minor injuries. In addition to the blood clots, the poor circulation may be resulting fluid retention in the edematous *E. coqui* embryos. More detailed examination of the cardiovascular system has not been done on *E. coqui* for this thesis.

Defective skin could also cause the edema syndrome. Frog skin is a stratified epithelium containing several different layers and is a major source of fluid entrance into the embryo. The skin is a like semi-permeable membrane. The osmotic flow of water is proportional to the osmotic gradient between the surrounding aquatic medium and the animal's body fluids in a healthy animal. When amphibians are placed in fresh water, water is taken up rapidly across the skin. Water uptake in dehydrated amphibians is also increased by the secretion of neurohypophyseal peptide hormones (Bentley, 1971). The nervous system, likewise, has influences on water uptake by the skin, independent of the neurohypophysis. Lesions in the midbrain greatly augmented water uptake (Adolph, 1934; Segura *et al.*, 1982). In adult terrestrial frogs, the pelvic region of the ventral skin is the primary region for water uptake. Various pharmacological agents increase or decrease water uptake as well. For example,  $\alpha$ -adrenergic agonist, phenoxygensamine, injected into the midbrain increases osmotic permeability and subcutaneous injection stimulates osmotic uptake (Segura *et al.*, 1982). Conversely, the  $\beta$ -adrenergic antagonist, propranolol blocks uptake of water (Yokota and Hillman, 1984).

There were no visible lesions or other obvious indications of a skin defect in the edematous *E. coqui* embryos. It is possible, however, that there are defects at a cellular level. The skin may no longer be semi-permeable and is allowing water to enter the embryo without control. Alternatively, the nervous system of these edematous embryos is disrupted and is unable to influence water uptake. A closer study of the *E. coqui* embryonic skin has not been conducted for this thesis.

## V. Relation between edema and limb development

In addition to the severe swelling in the edematous embryos, limb development was poor. The forelimb development was most affected in the edematous embryos, in which in severe cases of edema, the forelimb never develops. In tetrapod limb formation, three axes are under consideration. The proximal-distal axis is the shoulder to finger axis. The anterior-posterior axis is the thumb to pinky orientation. Lastly, the dorsal-ventral axis is the knuckle to palm sides. Induction of the early limb bud is initiated by fibroblast growth factor, FGF10. FGF10 is secreted by the lateral plate mesodermal cells. If FGF10 is present in a region where limbs do not normally form, extra limbs emerge (Ohuchi *et al.*, 1997; Sekine *et al.*, 1999). Wnt proteins stabilize the expression of FGF10. In addition to initiation of limb formation, FGF10 also induces the overlying ectoderm to form the apical ectodermal ridge (AER) which is the major signaling center for a developing limb. The AER synthesizes and secretes another FGF, FGF8, which feeds back positively to cause mesodermal cells to continue expressing FGF10. The AER enables the proximal-distal growth of the developing limb, expresses molecules which generate the anterior-posterior axis, and interacts with proteins that specify the dorsal-ventral axis. Sonic hedgehog protein is the main regulatory molecule in establishing the anterior-posterior axis. Sonic hedgehog initiates and maintains a gradient of proteins such as BMP2 and BMP7 (Laufer *et al.*, 1994; Kawakami *et al.*, 1996; Drossopoulou *et al.*, 2000). The dorsal-ventral axis is regulated in part by Wnt7a. Wnt7a induces the activation of Lmx1, a transcription factor that is essential for specifying dorsal cell fates in the limb.



Knowing the molecular pathways established in limb development, I wondered if there were any common molecular pathways in pronephric development. If such common molecular pathways existed, the misexpression of one and/or more of the genes involved could cause the defects in both limb and kidney development. Table 1 lists the genes expressed in the pronephros and BMP7 is a gene involved in both pronephros and limb development. In a recent study, Michos *et al* (2004) found that the BMP antagonist Gremlin is essential for the initiation of the epithelial-mesenchymal signaling interactions during limb and metanephric kidney organogenesis in mice. Gremlin mediated BMP antagonism was essential in inducing the initiation of ureter growth. Ureter growth is a key event in metanephric development. The ureter is a tubular structure that connects the kidney to the bladder, which is the organ that stores urine until urination. In early limb buds, Gremlin is required to establish a functional AER and the epithelial-mesenchymal feedback signaling that propagates sonic hedgehog. In addition to its involvement in metanephric development, Gremlin is expressed in the pronephros (Hsu *et al*, 1998) as well as BMP7 (Table 1).

## **VI. Future directions**

There still remain a few questions regarding the occurrence of edema in the *E. coqui* embryos. Although not explicitly recorded, there seemed to be fewer edematous embryos when the *E. coqui* were fed with vitamin supplements. If limb and kidney development is related, it should be noted that retinoic acid is critical for initiation of limb bud outgrowth. When synthesis of retinoic acid was blocked by the drug Disulphiram, limb bud initiation was prevented (Stratford *et al.*, 1996). Retinoic acid is synthesized in the

body from retinol, also known as vitamin A. Perhaps sufficient vitamin A in the *E. coqui* diet is necessary for correct limb development as well as proper kidney development. This can be tested by eliminating vitamin A in the *E. coqui* diet or by blocking the synthesis of retinoic acid using Disulphiram (Stratford *et al.*, 1996) and look for defects in limb and pronephric development. The retinoic acid receptors in the edematous embryos can be studied for any defects as well.

Edematous embryos have thus far only been reported to occur in laboratory colonies. Why does edema occur in laboratory colonies? Or is this phenomenon only noticed in the laboratory setting because embryos are more closely studied? It is possible that in a natural setting, the edematous embryos die off because they rarely hatched naturally from their jelly capsules in the lab. The older edematous embryos in my study were all dejellied early in their development. Likewise, when some of the less severely edematous embryos developed to a hatching morphology, they often had dysfunctional hindlimbs which do not bend making mobility limited. If this were the case in the wild, they would not only be easy prey but also be unable to sufficiently feed itself.

In this thesis, I provide an overview of the histology of the embryonic kidneys in *E. coqui* at each TS stage. I studied *E. coqui* kidney development at a macroscopic level, looking mostly at morphology. This overview establishes the framework for a more thorough analysis of each pronephric and mesonephric compartments: the duct, tubules and the glomus/glomerulus.

To date, there have been many molecular studies on embryonic kidney development in other organisms. It would be of importance to take a molecular approach to the embryonic kidney development in *E. coqui* for several reasons. To begin, it would be interesting to clone and examine the expression of *E. coqui* orthologs of *HNF-1 $\beta$* , *Lim-1* and *Pax-2*. The three genes are the earliest markers for the pronephros. Shawlot and Behringer (1995) speculated that *Lim-1* is essential for development of the entire urogenital system, so perhaps the edematous embryos have defective expression of *Lim-1*. Another gene of interest would be *Pax-2*, because Torres *et al* (1995) and Favor *et al* (1996) reported that it is necessary for duct elongation or maintenance in *X. laevis*. This is another gene that may show disrupted expression in the edematous embryos. In addition to the pronephric marker genes, it would be interesting to examine the expression of *Gremlin* and *BMP7*, genes involved in both limb and kidney development, to explore the link between the limb and pronephros development.

It would also be important to determine if and when the pronephros and mesonephros become functional in *E. coqui*. The pronephros may not be as important an organ in the direct developing *E. coqui*. The importance of the pronephros could be tested by removing the pronephroi in *E. coqui* embryos that are developing normally and observe for possible development of edema. If the embryos develop normally without their pronephroi, this would shed light on the possibility that the lymphatic system, circulatory system or the skin is involved. To test for the importance of the lymphatic system, the lymph hearts in *E. coqui* could be removed in normal embryos and the organisms' development of edema could be monitored. If the embryos develop normally without

lymph hearts as well, the circulatory system and the skin may play bigger roles in water regulation in *E. coqui*.

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