The development of the chicken skull and the histology of some of its sutures

by

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Abstract

The use of differing definitions of homology has led to controversies about the identity of bony elements in vertebrate skulls. An example is the controversy concerning the identity of the avian frontal, which has been assigned that name based on its location and shape. However, developmentally, it has been suggested to be a fusion of the frontal and parietal (i.e. frontoparietal). To assist in resolving this controversy, a search for a suture in the gap between the two ossification centres of the chicken's frontal was attempted. The production of a staging table of the chicken skull revealed a putative suture would be found between stages HH36 and 37. Histological examination of the developing frontal failed to find any evidence of a frontal-parietal suture, leading to the conclusion that the avian frontal is a single element formed from two ossification centers and is not a frontoparietal.

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Chapter 1: Introduction

The emerging field of evolutionary development, or evo-devo for short, combines research into evolutionary and developmental change derived from ontogenetic studies of phylogenetically relevant extant taxa (Arthur, 2011). Evolutionary development has helped in understanding the nature of phenotypic variation within and between species (Abzhanov et al., 2004; Rebeiz et al., 2009; Arthur, 2011). Evolutionary development has also revealed the constraints on phenotypic variation whose components change in frequency over time during evolution (Arthur, 2011). This constraint is referred to as developmental drive (Arthur, 2011) and allows for certain variants to evolve, but not others (Young et al., 2014). The capacity of evolutionary development to help in understanding phenotypic variation at the population and species levels plays an important role in micro- and macroevolutionary studies. However, evolutionary development has sometimes caused revisions in our understanding of the nature of the evolution and relationship between taxa. For example, evolutionary development has revealed that evolutionary novelties, such as feathers, arise through modification of the regulation of genes that control the development of ancestral structures, such as scales (Harris et al., 2002). Such modification of ancestral traits to produce derived traits has led to novel evolutionary concepts, such as deep homology. Deep homology refers to similarity in the gene regulatory pathways that give rise to them, in taxa that are phylogenetically very distant from each other, such as the eye within Animalia (Shubin et al., 2009).

The concept of deep homology exemplifies one of the problems that evolutionary development has presented to the field of evolutionary biology; namely, identifying

homologs. The developmental definition of homology could define two structures as homologous that might conflict with other definitions, such as the morphological definition. For example, the avian frontal is defined based on its location and shape (Romer, 1956), whereas some recent comparative developmental data has cast some doubt on that appropriateness of its name (e.g., Evans and Noden, 2006). The frontal in mouse and the axolotl develop from a population of mesenchymal embryonic cells called neural crest cells, while the parietal develops from a population of mesenchymal embryonic cranial mesodermal cells (Jiang et al., 2002; Maddin et al., 2016). In chickens, however, the frontal is derived from both types of embryonic cells – neural crest cells rostrally and mesodermal cells caudally (Evans and Noden, 2006) – raising the hypothesis that the ancestral frontal and parietal fused to form a compound frontoparietal during the evolution of Aves (Evans and Noden, 2006). Furthermore, it implied that the putative parietal is a postparietal (Maddin et al., 2016), which is thought to have been lost in archosaurs (Fabbri et al., 2017). On the other hand, a recent geometric morphometric comparative analysis (Fabbri et al., 2017) argued that the frontal in birds is not a frontoparietal based on two lines of evidence: the positions of the frontal and parietal dorsal to the fore-and midbrain, respectively, is conserved within Reptilia, as is the frontal-parietal suture being dorsal to the region between the fore- and midbrain. Smith-Paredes et al. (2018) later argued against the frontoparietal hypothesis using comparative cranial ossification sequence data of *Alligator mississippiensis*. There they argued that the posterior part of the alligator frontal is homologous to the postocular bone (i.e., the postfrontal), because the posterior ossification centre of the avian frontal resembles the postocular ossification of the alligator (Smith-Paredes et al., 2018).

The importance of resolving the homology of the avian frontal element stems from its contribution to our understanding of bird evolution. Birds belongs to a lineage of dinosaurs called maniraptoran theropods (Brusatte et al., 2015). During the evolution of theropods the size of the brain (especially the cerebrum) increased dramatically towards the avian lineage (Balanoff et al., 2013). The increase is suspected to be necessary for flight capability and increased visual acuity (Balanoff et al., 2013), which are associated with the cerebrum (Medina and Reiner, 2000). The increase in the cerebrum size probably led to the expansion of the size of the frontal given that it always occurs dorsal to it (Fabbri et al., 2017). The expansion might have occurred through the increase in the contribution of mesodermal cells to the developing frontal bone. This interpretation is based on the presence of a neural crest and mesodermal boundary within the avian frontal (Evans and Noden, 2006), which occurs between the frontal and parietal in mammals and axolotls (Jiang et al., 2002; Maddin et al., 2016), and may be an example of the phenomenon of developmental system drift (DSD).

Developmental system drift describes the divergence in the formation of the same structure in two different taxa (True and Haag, 2001). For example, Piekarski et al. (2014) found that the axolotl frontal bone is derived from the mandibular stream of neural crest cells, and the parietal is derived from mesodermal cells. On the other hand, the *Xenopus* frontoparietal is derived from three different streams of neural crest cells: mandibular, hyoid and branchial (Piekarski et al., 2014). This developmental divergence indicates the flexibility in the development of structures, while conserving the complement of components of the vertebrate skull. Thus, if the frontal is further shown to be only a frontal (not a frontoparietal) then this implies that the avian frontal element

didn't fuse with the parietal during the evolution of birds, but instead changed the way in which it develops.

To further resolve the issue of the homology of the avian frontal, I have taken a novel approach which is to search for evidence of a suture between the two ossification centres of the avian frontal bone. Sutures are cranial joints that appear between cranial elements (Maloul et al., 2014). I hypothesize that if the avian frontal is a fusion of previously discrete frontal and parietal bones, a suture would appear between them during development. To test this hypothesis, it was first necessary to determine which developmental stages capture the development of the frontal in the chicken. As the current staging table for chicken is restricted to external morphology (i.e. that of Hamburger and Hamilton, 1992), a complementary staging table describing events in the development of the skull is needed. In Chapter 2, a developmental series of chicken, spanning stages HH35 to hatching of Hamburger and Hamilton (1992), was collected and cleared and stained for bone. This led to the identification of stages that capture frontal formation. Additionally, an ossification sequence was derived, as well as documentation of previously unknown variation in the development of the chicken skull. This new complementary staging table will benefit the field of avian embryology in general, as it provides approximate time points for the formation of all cranial elements and sutures.

Based on the data pertaining to the timing of bone formation obtained in Chapter 2, a comparison of the histology of the gap between the two ossification centres that form the avian frontal with that of three different contexts in the skull was conducted in Chapter 3. The first is a known suture of the chicken skull represented by the nasal-frontal, frontal-parietal and parietal-supraoccipital suture. The second is an early fusing

suture, represented by the interpremaxillary. The third is a presumptive region of bone formation in which no suture is known to occur, represented by the caudal region of the presumptive frontal bone. If histological features of a suture were found in the gap between ossification centres, even features resembling a fusing suture, this would suggest two distinct bone elements comprise the avian frontal and the frontoparietal hypothesis would be supported. Furthermore, the gap would not histologically resemble a region of presumptive bone of a single ossification. Alternatively, if the gap between the two ossification centres lack a suture's histological features and instead resemble a region of presumptive bone formation, the frontoparietal hypothesis would not be supported.

The results of the analyses conducted within this thesis reject the hypothesis that the avian frontal is a compound element. No evidence of sutural structures was observed within the ossifying frontal bone, and it instead resembled the region of presumptive bone. These results are discussed as they related to establishing the homology of the avian frontal, and considerations for future research on the topic and that of homology assessments in general is provided.

Chapter 2: Development of the chicken skull: a complement to the external staging table of Hamburger and Hamilton (HH)

2.1 Abstract

Embryonic staging tables provide a standard of developmental stages that can be used by individual investigators and provide approximate time points for the study of developmental phenomena. Surprisingly, a staging table of the development of the avian skull remains lacking, despite the presence of a plethora of studies on the avian skull and its role as a model species in developmental research. A detailed photographic staging table of the osseous portion of the chicken skull is presented here, from cleared and stained stages spanning HH35 to hatching. This table documents the development of most of the cranial elements in the chicken skull from the start of ossification until the element takes its final shape. The table shows that the elements of the lower jaw and ventral side of the skull begin ossifying before the skull roof. The table shows that most elements take roughly five days to reach their final shape, while some elements takes nine days, such as the frontal. The specific timing of events in frontal bone formation is determined, as its pattern of development may impact recent discussions of the putative homology of the element. The timing of ossification of the frontal appears to vary greatly between stages HH36 to 38, with the two-separate ossification centres appearing variably between stages HH36 and 37 and fusing by stage HH38. The obtained results lead to a number of hypotheses about chicken skull development and also provide a timeframe for the study of suture formation.

2.2 Introduction:

The study of chicken development has played a crucial role in understanding organismal development and, more recently, evolution (Davey and Tickle, 2007; Bhullar et al., 2015). Heart formation was one of the first developmental processes to be examined in chickens, followed by the discovery of germ layers (Pander, 1817), organizing centres (Saunders, 1948), developmental patterning, determination of fate-maps, and a large number of other important developmental discoveries (Stern, 2005; Davey and Tickle, 2007). More recently, studies of chicken development have illuminated evolutionary questions of homology (Evans and Noden, 2006), developmental constraint (Young et al., 2014), heterochrony (Bhullar et al., 2012), heterometry (Abzhanov et al., 2004) and heterotopy (Bhullar et al., 2015), which together have advanced our understanding of bird skull evolution and diversity. These discoveries were made possible by the ease of obtaining chicken eggs from farms, incubation of eggs until hatching and manipulation of embryos (Kain et al., 2014). Ease of manipulation of chicken embryos is possible because of the capacity to open the eggshell and observe the developing embryos without sacrificing it (Kain et al., 2014).

The kinds of manipulations that can be performed on developing chickens include the transplantation of a piece of embryonic tissue, such as a piece of neural tube, and implanting it into another embryo (Fish and Schneider, 2014). This technique allowed the determination of the developmental origin of feather pigmentation (Willier and Rawles, 1940) and skull bones (Couly, 1993). Another technique that is allowed by the ease of access to chicken embryo is bead implantation (Hashimoto et al., 1999). Beads are microscopic spheres that can uptake a particular protein and then be injected into an

embryo (Gerlach et al., 2015). This technique has been used to examine embryonic cell migration and regions of gene expression (Abzhanov et al., 2004). These features of chicken development have led to the species being a standard that is used in staging the development of other birds such as turkey, duck, goose, Guinea Fowl (Sellier et al., 2006), Japanese quail (Ainsworth et al., 2010) and Darwin's finches (Abzhanov et al., 2004).

Recently, studies of chicken skull formation have contributed to the understanding of organismal evolution, which is part of the field of evolutionary developmental biology, or evo-devo for short. Chicken skulls form from two populations of embryonic undifferentiated cells: cranial neural crest (CNCCs) and paraxial mesodermal cells (PMCs) (Evans and Noden, 2006). Both of these cell populations migrate to the craniofacial region of the embryo, which is anterior to the developing heart, and undergo proliferation and differentiation into bone, cartilage, muscle (Evans and Noden, 2006). The craniofacial region of the chick embryo is composed of six components: forebrain vesicle, midbrain vesicle, hindbrain vesicle, mandibular arch, and hyoid arches along with the ectodermal region surrounding them (Twigg and Wilkie, 2015).

Cranial neural crest cells, like other NCCs, form during a stage in development called neurulation (Mayor and Theveneau, 2013) at the dorsal apex of the neural tube. After the formation of neural crest cells form, they begin migrating to the ventral side of the embryo (Mayor and Theveneau, 2013). At the anterior end of the neural tube the three vesicles of the brain form: forebrain, midbrain and hindbrain (Suzuki and Vanderhaeghen, 2015). Cranial neural crest cells migrate from the dorsal side of the brain

vesicles to the preotic region and branchial arches (mandibular arch, hyoid and branchial) in four streams that are called preotic, mandibular, hyoid and branchial (Hall, 1999; Santagati and Rijli, 2003). Cranial neural crest cells of the mandibular stream migrate to the mandibular arch, and the hyoid stream migrates to the hyoid arch (Santagati and Rijli, 2003). After arriving to these arches, they begin to proliferate below the ectoderm and turn the arches into swellings known as prominences (Parada and Chai, 2015). These prominences form the facial region of the skull (Sperber et al., 2010). The mandibular arch gives rise to the maxillary and mandibular prominences, of which the maxillary fuses with two other prominences the lateral nasal and the frontonasal prominences (Sperber et al., 2010). The lateral nasal prominences appear on the lateral side of the embryonic face between the eyes and nasal pits, while the frontonasal prominence forms at the most rostral side of the embryonic face (Hu and Marcucio, 2009). These prominences form from the migration and proliferation of preotic CNCC stream to the facial region (Hall, 1999; Santagati and Rijli, 2003). The fusion of the frontonasal, lateral nasal and maxillary prominences gives rise to the upper jaw (Linde-Medina and Newman, 2014). Specifically, the CNCC maxillary stream gives rise to the maxillaries, palatines, vomers, jugals, quadratojugals, rostroparasphenoid, squamosals, quadrates and pterygoids (Helms and Schneider, 2003); whereas the preotic CNCCs give rise to the premaxillaries, mesethmoid, prefrontal, nasals and supraocular ossification of the frontal (Hall, 1999; Santagati and Rijli, 2003).

The other population of embryonic cells that form the skull bones are PMCs (Evans and Noden, 2006). The mesoderm is an inner layer of the embryo, positioned between the ectoderm and endoderm, that forms during gastrulation (Kimelman and

Griffin, 1998). The mesoderm is divided into many regions, including the paraxial mesoderm that gives rise to the cranial paraxial mesoderm and the somites (Evans and Noden, 2006; (Chal and Pourquié, 2009) The anterior part of the paraxial mesoderm, the cranial paraxial mesoderm, occurs directly ventrolateral to the mid- and hindbrain (Evans and Noden, 2006). The mesenchymal cells of the paraxial mesoderm migrate dorsally to the skull roof and the caudal end of the head region (Evans and Noden, 2006). These mesenchymal cells give rise to the postorbital ossification of the frontal, basisphenoid, sellaparasphenoid, orbitosphenoid, parietal, exoccipital, and supraoccipital (Evans and Noden, 2006).

Mesenchymal cells, whether CNCCs or PMCs, give rise to bone tissue in the skull using one of two different processes called intramembranous and endochondral ossification. Intramembranous ossification is a process in which disconnected mesenchymal cells condense with each other and differentiate into osteoblasts (Berendsen and Olsen, 2015). Osteoblasts secrete an organic bone matrix progenitor called osteoid that contains collagen (Scott-Savage and Hall, 1979). Subsequently, a calcium matrix is deposited inside the osteoid in the form of plates and rods (trabeculae) that then traps osteocytes within it. The resulting structure is then referred to as an ossification centre (Saladin and McFarland, 2018). An ossification centre grows via osteoprogenitors at the osteogenic front to form a bone element (Jin et al. 2016). On the other hand, endochondral ossification begins with the formation of a cartilaginous model of the bone, which begins with the differentiation of mesenchymal cells to chondrocytes (Mackie et al., 2008). After the formation of the cartilaginous model, bone ossification begins in the midshaft and epiphyseal regions (Mackie et al., 2008). In these regions

chondrocytes transition to hypertrophic chondrocytes, which undergo cell death and are replaced by osteoblasts that deposit the bone matrix (Mackie et al., 2008).

Much of what is known about chicken skull development relies on the use of the Hamburger and Hamilton staging table (Hamburger and Hamilton, 1992), which is based on developmental events of the external anatomy. An embryonic staging table is a description of a series of features that appear in the embryo of an animal during its development from fertilization to hatching. The usefulness of a staging table is in the standard of developmental stages that can then be applied by individual investigators, regardless of the rate of embryonic growth, which can vary for different reasons (e.g., temperature, genetic differences, timing of incubation, etc.) (Hamburger & Hamilton, 1992). A staging table also provides relative time points that researchers can use to study a specific developmental process, such as somite formation (e.g. somitogenesis at stage HH6; Hamburger & Hamilton, 1992). Given the usefulness of staging tables for a variety of applications, the lack of a staging table pertaining to events in the development of the chicken skull is puzzling. Previous studies have described chicken skull formation (Jollie, 1957; Maxwell, 2008; Maxwell and Harrison, 2008), but did not provide detailed photographs or comprehensively describe the development of each element up to hatching. For example, Maxwell (2008) compared the sequence of the ossification of cranial bones in chicken, turkey and quail without referring each ossification event to a stage or providing a photographic series. Similarly, Jollie (1957) provided descriptions of the ossification of cranial elements with some illustrative Figures, but only mentioned the duration of incubation for reference. However, given that the rate of development can vary across chicken eggs, the incubation period does not always produce repeatable

results (Hamburger & Hamilton, 1992). Moreover, the number of replicates was not mentioned in these earlier studies leaving knowledge of intraspecific variation incomplete. For example, Maxwell (2008) and Jollie (1957) reported a single, large ossification centre for the frontal bone in chicken. On the other hand, Erdmann (1940) and Smith-Paredes et al. (2018) reported the occurrence of two ossification centres – one supra- and one postorbital. It is not known whether these discrepancies are due to temporal differences in ossification, which may lead to an observer missing a short window of time when the centres are separate. This potential problem warrants the need for replicates in determining the number of ossification centres in developing bones, such as the frontal.

Resolution between conflicting observations like these is important, as they may shed light on issues such as the identity of the avian frontal bone, which has been brought into question (Evans and Noden, 2006; Maddin et al., 2016). This is because of its unique developmental origin, where the supraocular ossification centre of the frontal is derived from neural crest cells and the postorbital ossification centre is derived from paraxial mesodermal cells (Evans and Noden, 2006; Noden, 1982;1984, Couly, 1993). In mammals, the frontal is derived only from neural crest cells, whereas the parietal is derived from mesodermal cells (Jiang et al., 2002). This pattern has led some authors to suggest that the avian postorbital ossification should be regarded as homologous to the mammalian parietal (Evans and Noden, 2006; Maddin et al., 2016). Determining whether or not the postorbital ossification centre represents an ancestrally discrete bone, such as the parietal, can be aided by determining whether or not a suture between the two frontal ossification centres is present. The reason for such a suggestion is that bones develop

sutures between one another (Zhao et al., 2015). Thus, if the frontal ossification centres are two, previously separate bones then a suture might form between them. However, in order to find this suture, the approximate stage at which these two ossifications form is needed first, so that detailed analyses of frontal formation can be performed. Here I present such a staging table for the skull of chicken.

Materials and Methods:

2.2.1 Specimen Collection

Thirty white, leghorn chicken (*Gallus domesticus*) eggs were obtained from Canadian Food Inspection Agency. Eggs were incubated at 38°C at 50% humidity in a Styrofoam incubator (Hova-Bator Genesis 1588, Incubator Warehouse, Idaho) in accordance with Carleton University's animal care policies. Eggs were maintained under these conditions for eight days, at which time sampling of embryos began. The sampling began at this point because skull ossification is known to begin at that time (Abzhanov et al., 2007). Sampled embryos were staged according to Hamburger and Hamilton stages (HH; Hamburger and Hamilton 1992). The sample included stages HH35 to HH45, the latter of which represents the last stage before hatching and the final stage of collection permitted by our animal care clearance. Most stages were sampled twice (refer to appendix B) with the exception of stages HH36 and HH37. Stages HH36 and HH37 were sampled with 63 specimens due to the relevance of these stages to frontal formation.

Once extracted from the eggs, the heads were isolated and the eyes and skin were removed (from some specimens) to accelerate the clearing process. Specimens were fixed in 10% formalin solution (Fisher Scientific, #SF1004) overnight at room temperature.

After fixation the heads were placed in tap water for 30 minutes and moved through an ethanol dehydration series (25%, 50%, 75%, 100%). The duration that each specimen spent in each ethanol solution varied by stage, but each head was moved to a higher concentration of ethanol when it sunk to the bottom of the ethanol filled tube. Specimens were then moved directly into the whole-mount clearing and staining protocol outlined below.

2.2.2 Whole-mount clearing and alizarin red staining

Whole-mount clearing and alizarin red staining for bone was carried out using the protocol of Beckett (2015). Specimens were dehydrated and placed directly from 100% ethanol into a solution of 1% potassium hydroxide (KOH) and 3% hydrogen peroxide (H_2O_2) (48 mL of 1% KOH in water with 1.5 mL H_2O_2) overnight, followed by a rinse the next morning in tap water for half an hour. Specimens were then placed in 3% sodium tetraborate solution for 24hrs. The next morning, specimens were stained in a solution of 1% KOH with 1 mg/ml Alizarin Red S (# CAS 130-22-3, from Fisher Scientific) for a further 24hrs. Following this, digestion of soft tissue was performed by submerging the specimen in a solution of 1% trypsin (Thermofisher, #27250018) in 2% sodium tetraborate (49.5 mL of 2% sodium tetraborate with 0.5 g of trypsin), until the specimen was soft and almost transparent. The length of time for clearing each specimen differed depending on the stage and ranged from 1- 5 days. The specimens were then moved through a series of solutions of glycerol and KOH in water (20% glycerol in 1% KOH, 40% glycerol in 1% KOH). The timing differed among different stages, but the head was moved to a higher concentration when structural integrity seemed to be weakening.

Finally, the specimens were placed in a solution of equal parts 70% glycerol in 1% KOH and 70% ethanol for storage and analysis.

2.2.3 Specimen photography

Specimens were moved into a 50 mL beaker containing equal parts 70% glycerol in 1% KOH and 70% ethanol and placed on an LED light pad with a fibre optical illuminator (Gaomon, Sarasota). The dorsal, lateral and ventral sides of the skulls were photographed using an Olympus digital camera with an Olympus Zuiko 12-50 mm lens on a tripod.

2.3 Results:

2.3.1 Hamburger and Hamilton Stage 35:

Skull ossification is known to begin at this stage (Abzahnov, 2007). The initial ossifications are sparse and appear distant from each other without articulations between them. Five elements appear at this stage: the nasal, pterygoid, squamosal and quadratojugal. When viewed dorsally the nasal is the most prominent ossification and appears as a small oval shaped ossification in the lateral preorbital region of the skull (Figure 2.1A). At the caudal margin of the skull, the squamosal appears (viewed laterally) as a small, round sheet-like ossification in the ventrolateral postorbital region of the skull (Figure 2.1B). Rostral to the squamosal, the quadratojugal appears as a thin, long ossification that has a thick, round caudal margin and a tapering rostromedial margin, when viewed ventrally (Figure 2.1B, C). Medial to the quadratojugals, the pterygoid forms as a faint, thin midshaft ossification that points rostromedially. The pterygoid and

nasals, however, do not always seem to form at this stage, because the other replicate did not have either of those elements.

2.3.2 Hamburger and Hamilton Stage 36:

At this stage the skull appears larger with most of the elements of the beak beginning to ossify and the continued growth of the elements from stage 35. Furthermore, eight new elements appear at stage 36: the jugal, maxillary, palatines, frontal, prefrontal, premaxillary, quadrate and rostroparasphenoid (Fig. 2). The most prominent elements at this stage are the nasals, palatines, pterygoids and squamosals.

From a dorsal view of the skull the premaxillary appears as a thin, laterally - extending element that forms caudal to the tip of the beak. Caudal to the premaxillary, the nasals have acquired the shape of two right-angle triangles pointing in opposite directions to each other when viewed dorsally. The nasal has a dorsal apex that points towards the supraorbital region and a lateral apex that points approximately caudally towards the upper jaw. Lateral to the nasals, the prefrontal is a preorbital element that intrudes

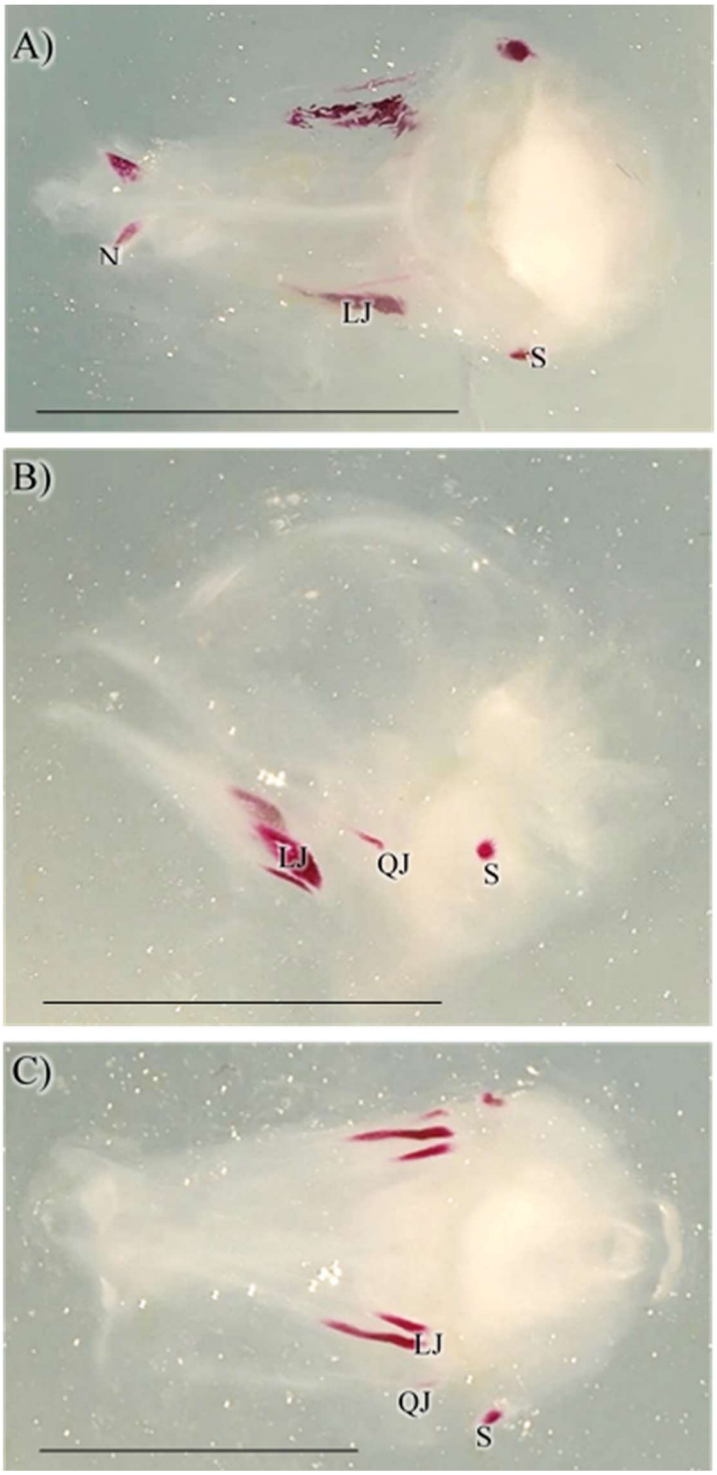

Figure 2.1: Whole-mount cleared and stained chicken skull at stage HH35. The skulls are shown from a dorsal (A), left lateral (B) and ventral (C) views. The pterygoid is too faint to been seen. The rostral side of the skull is on the left. Scale bar is 1 cm. Abbreviations: LJ: Lower jaw, N: Nasal, QJ: Quadratojugal and S: Squamosal.

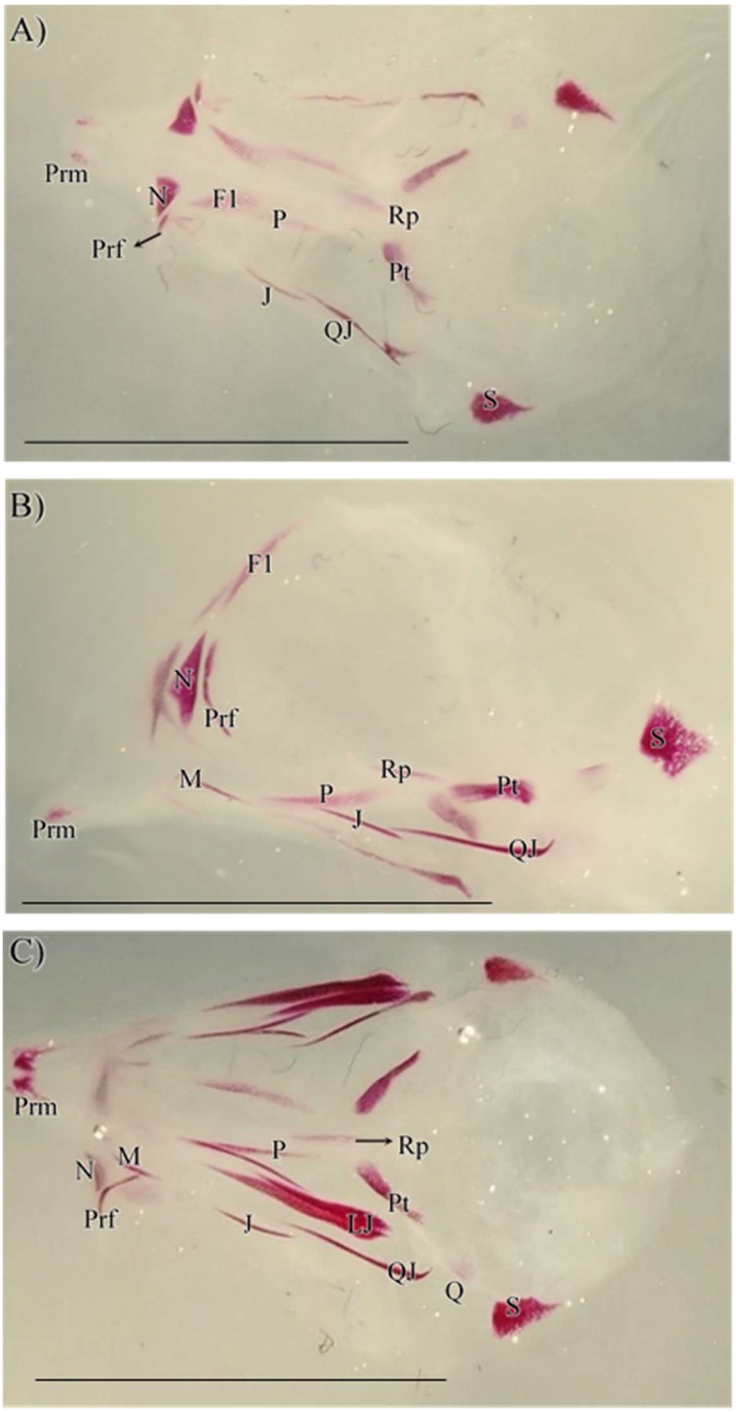

Figure 2.2: Whole-mount cleared and stained chicken skull at stage HH36. The skulls are shown from a dorsal (A), left lateral (B) and ventral (C) views. The rostral side of the skull is on the left. Scale bar is 1 cm. Abbreviations: F1: Supraoccular ossification of the frontal, J: Jugal, LJ: Lower jaw, M: Maxillary, N: Nasal, P: Palatine, Prf: Prefrontal, Prm: Premaxillary, Pt: Pterygoid, Q: Quadrate, QJ: Quadratojugal, Rp: Rostroparasphenoid and S: Squamosal.

laterally above the plane of the orbit. The prefrontal appears either as a singular element (in 2 speciemens) or as two separate ossifications (in one specimen) - a laterally wide, dorsal one and a thin, spike-shaped, ventrocaudally-pointing one. Ventral to the prefrontal, the maxillary appears (viewed laterally) as a faint, triangular-shaped element with three rods emanating from its apices; a very short, dorsally-extending rod that approaches the nasals, a slightly longer, rostrally-extending rod approaching the premaxillary, and a long, caudolaterally-extending rod that approaches the jugal. The jugal appears as a thin, long element on the lateral side of the upper jaw, extending towards the quadratojugal. The quadratojugal has further elongated rostrally at this stage, so as to extend ventral to the jugal, whereas its caudal side forms a ventrally-facing curved margin (Figure 2.2A & B).

On the supraorbital dorsal side of the skull, the supraocular ossification centre of the frontal, the one that forms above the interorbital septum. It is wide caudally and tapers rostrally as it approaches the nasal (Figure 2.2A). The squamosal appears rectangular in shape caudoventral to the orbit, directed along the dorsorostral-ventrocaudal axis, when viewed laterally. Medial to the squamosal, the quadrate bone appears as a mid-shaft ossification pointing rostromedial, when viewed ventrally. Rostral to the squamosal, the pterygoid further ossifies along its rostral and caudal ends, thus increasing its length and bringing it closer to the palatine and quadrate. Medial to the pterygoid, the rostroparasphenoid forms as a laterally thin, dorsoventrally thick element that is rostrocaudally oriented. Lateral to the rostroparasphenoid, the palatine forms as a dorsoventrally flat ossification that has a lateral curvature (when viewed ventrally), which extends in the rostrocaudal direction occupying most of the palatal region. (Figure 2.2C).

2.3.3 Hamburger and Hamilton Stage 37:

At Hamburger and Hamilton Stage 37, the elements of the beak and the ventral region of the skull appear larger than HH 36. The most prominent ossifications are those of the frontal, nasal, palatine, prefrontal, premaxillary, pterygoid and squamosal, however, the latter four elements do not appear in all specimens at this stage in development (Figure 2.3A). Moreover, the basisphenoid, exoccipitals and sellaparasphenoid first appear at this stage.

The premaxillary develops a medial process that extends dorsocaudally, giving the element a slight V-shape when viewed dorsally. The tip of the premaxillary point rostrally and slightly medially, whereas their processes point caudally. This orientation makes the presumptive interpremaxillary suture wider caudally than rostrally. Caudal to the premaxillary, the nasal appears as the most prominent dorsal ossification with a caudally-pointing rod-like process that almost reaches the maxillary when viewed laterally. Another shorter rod-like process extends dorsally towards the frontal. Lateral to the nasal is the prefrontal that is now dorsally and caudally expanded. Ventral to the prefrontals, the maxillary develops a fourth mediocaudally-directed process that extends towards the palatine when viewed ventrally. Dorsal to the maxillary, the frontal has developed a postorbital ossification centre that appears as a convex, sheet-like ossification that points laterally and tapers rostrally when viewed dorsally (Figure 2.3A). The two ossification centres of the frontal are variably linked (Figure 2.4A) or unlinked (Figure 2.4B&C) at this stage in different specimens (refer to section 2.3.11). Moreover, one specimen of this stage showed bilateral asymmetry of the frontal ossification centres with both present appeared

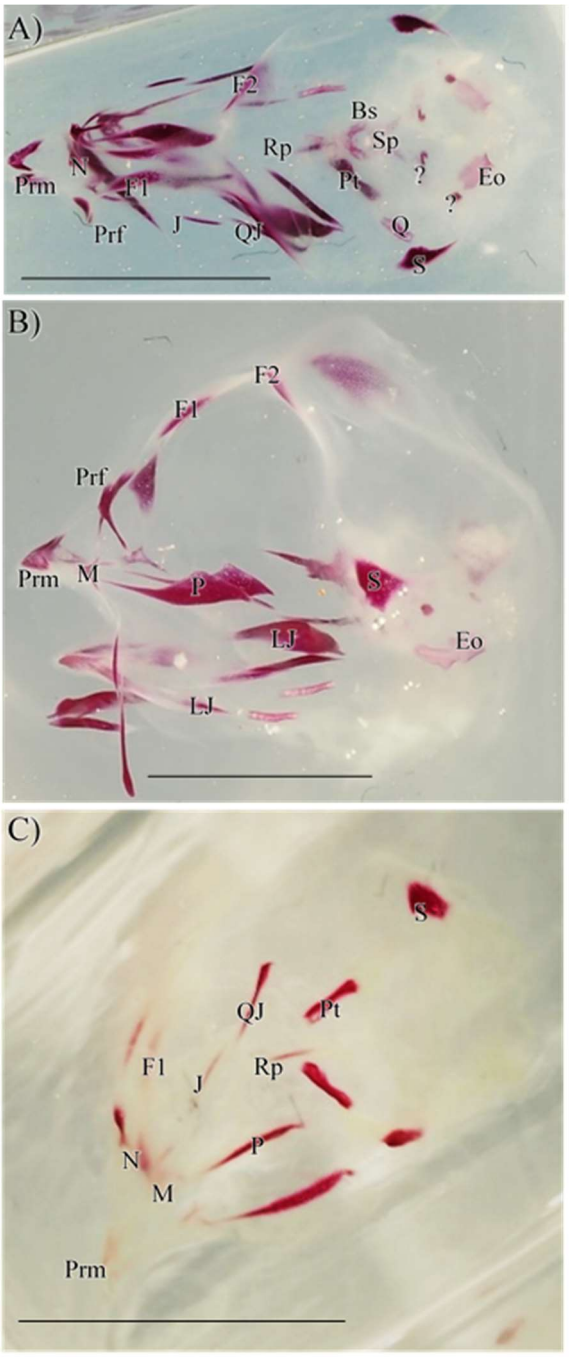

Figure 2.3: Whole-mount cleared and stained chicken skull at stage HH37. The skulls are shown from a dorsal (A), left lateral (B) and ventral (C) views. The rostral side of the skull is on the left. Scale bar is 1 cm. Abbreviations: Bs: Basisphenoid, Eo: Exoccipital, F1: Supraoccular ossification of the frontal, F2: Postorbital ossification of the frontal, J: Jugal, LJ: Lower jaw, M: Maxillary, N: Nasal, P: Palatine, Prf: Prefrontal, Prm: Premaxillary, Pt: Pterygoid, Q: Quadrate., QJ: Quadratojugal, Rp: Rostroparasphenoid, Sp: Sellaparasphenoid and S: Squamosal.

on the right side of the skull, but only the supraocular ossification center appearing on the left side (Figure 2.4B).

On the ventral side of the skull, the most prominent ossifications are the squamosal, pterygoid and palatine (Figure 2.3). The palatine develops a long, thin, sheet-like extension that extends dorsally from its caudal surface. The dorsal-facing sheet originates deep in the body of the palatine and tapers towards its caudal tip. Lateral to the palatine, the jugal appears as a thin, infraorbital element that ossifies from the middle and extends rostrocaudally towards the maxillary and the quadratojugal. The quadratojugal forms caudally to the jugals and has a thick, rounded caudal margin and a thin rostral margin that tapers towards the jugals. A rostrocaudally oriented gap separates the jugal and quadratojugal, which is interpreted as the presumptive jugal-quadratojugal suture. The squamosal appears larger at this stage and is the only element that could be seen from the lateral and ventral views of the skull (Figure 2.3B & C).

Medial to the squamosals, the rostroparasphenoid has undergone considerable growth since the previous stage. The knife-shaped rostral margin has extended further rostrally and expanded laterally. Caudal to the rostroparasphenoid, the basisphenoid appears as a cup-shaped element with a narrow rostral margin and a wide, laterally-bifurcating, caudal margin. The basisphenoid appears fused to the rostroparasphenoid. The sellaparasphenoid forms caudal to the basisphenoid. It is identified as a thin, sheet-like ossification with paired rostral and caudal processes giving the element a bow tie-shape. At the caudal margin of the skull, rostral to the foramen magnum, the exoccipital forms as a flat, oval-shaped ossification, with a long rostrolateral axis.

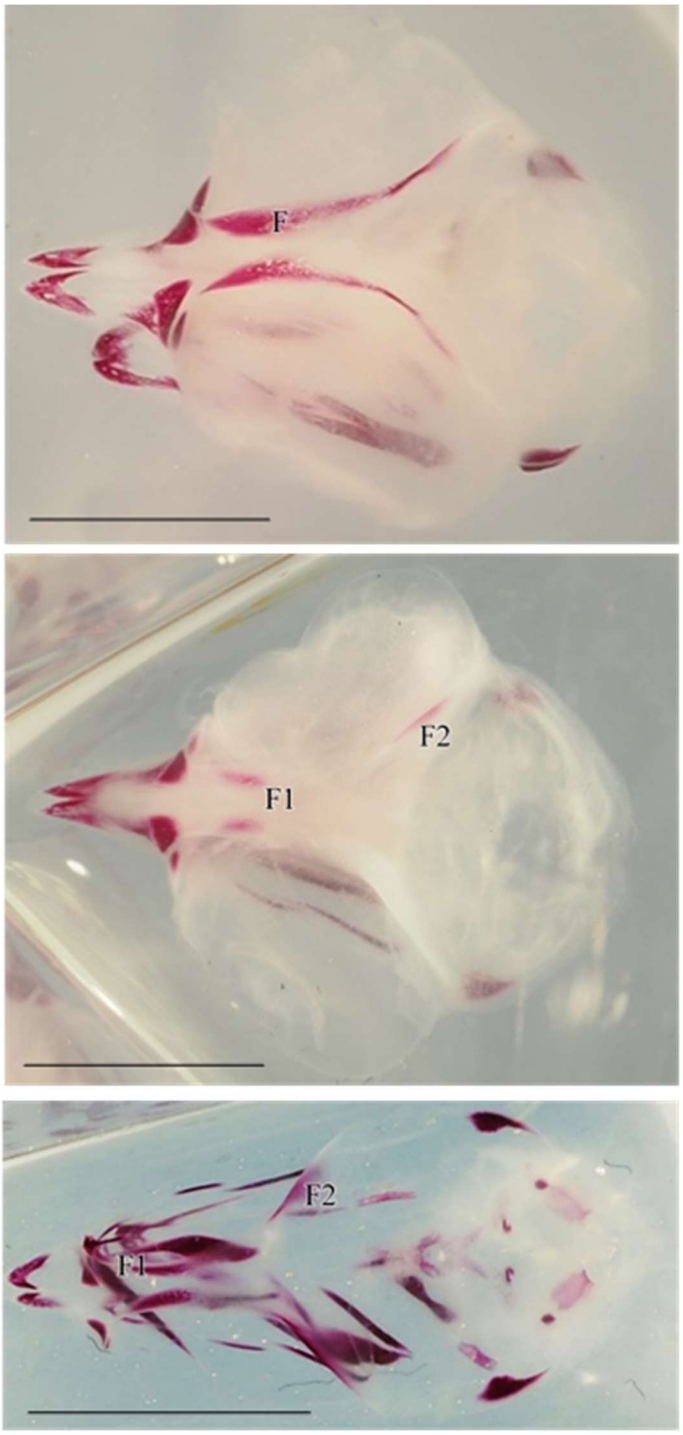

Figure 2.4: Intraspecific variation in frontal development at stage HH37 in *Gallus domesticus*. The frontal is identified a single ossification (a and b bottom), or two separate ossification centres (b top and c). The rostral side of the skull is on the left. Scale bar is 1 cm. Abbreviations: F: Frontal, F1: Supraoccular ossification of the frontal and F2: Postorbital ossification of the frontal.

However, the latter four elements only appeared in one of the three specimens at this stage in development (Figure 2.3A).

2.3.4 Hamburger and Hamilton Stage 38:

At this stage the ossifications are very pronounced, covering most of the beak and ventral regions of the skull, with the ventral surface elements have begun ossifying. Elements of the caudal margin of the braincase are unossified as of yet.

The expanded apex of the premaxillary now defines the beak tip in lateral profile, and the margins of the cavity between the two processes of the premaxillary clearly demarcate the dorsomedial and rostral sides of the nasal cavity when viewed laterally. The medial process extends medially to contact the nasal and possibly forming the premaxillary-nasal suture when viewed dorsally. However, the lateral process of the premaxillary develops a small bifurcation at its apex. A third process of the premaxillary appears medially to the palatine bones extending caudally, when viewed from a ventral view. The nasal, however, is much larger than it was in the previous stage and has extended from the dorsal to the ventral sides of the beak, curving laterally. The rostrolateral margin of the nasal, when viewed dorsally, clearly defines the caudal margin of the nasal cavity. Moreover, the rostral-most tip of the nasal tapers into a thin wedge that comes near the medial process of the premaxillary. Ventral to the nasal, the body of the maxillary ossifies, so as to appear as a sheet-like extension dorsal to the palatine bones. However, dorsal to maxillary, the frontal has expanded medially, and extended to the ventrocaudal margin of the orbit, approaching the squamosal. The expansion of the frontal leads to the thinning of the interfrontal suture supraorbitally, and the appearance of a wider caudal margin of the frontal. However, due to the laterally convex shape of the frontal, the

interfrontal suture appears wider at its rostral and caudal margins than its middle region. Caudal to the frontal, the squamosal extends dorsally to approach the frontal, and forms a socket that faces the rostromedial position of the quadrate bone (Figure 2.5A&B).

On the ventral side of the skull, the palatine extends further rostrally to reach the ventromedial process of the premaxillary. Caudal to the palatine, the quadrate and pterygoid continue to ossify along their rostral and caudal margins and appear longer than in stage 37. Medial to quadrates, all the components of the sphenoid complex appear to have fused with each other. Moreover, the rostral facing end of the rostroparasphenoid has expanded further towards the mid-palatal region. As for the basisphenoid, it becomes more pronounced and appears to have fused with the sellaparasphenoid. The sellaparasphenoid has also become more pronounced with its caudal processes appearing longer than its rostral processes. Caudal to the sellaparasphenoid, the exoccipital have expanded laterally and rostrally (Figure 2.5C).

2.3.5 Hamburger and Hamilton Stage 39:

At Hamburger and Hamilton Stage 39, the skull appears slightly larger than the previous stage. New ossifications occurring at this stage include those for the dorsolateral ossifications of the braincase, those surrounding the foramen magnum, and those for the orbitosphenoid, parietal, supraoccipital, and the vomer. As for the rest of the elements, their ossifications appear thicker than in the previous stage.

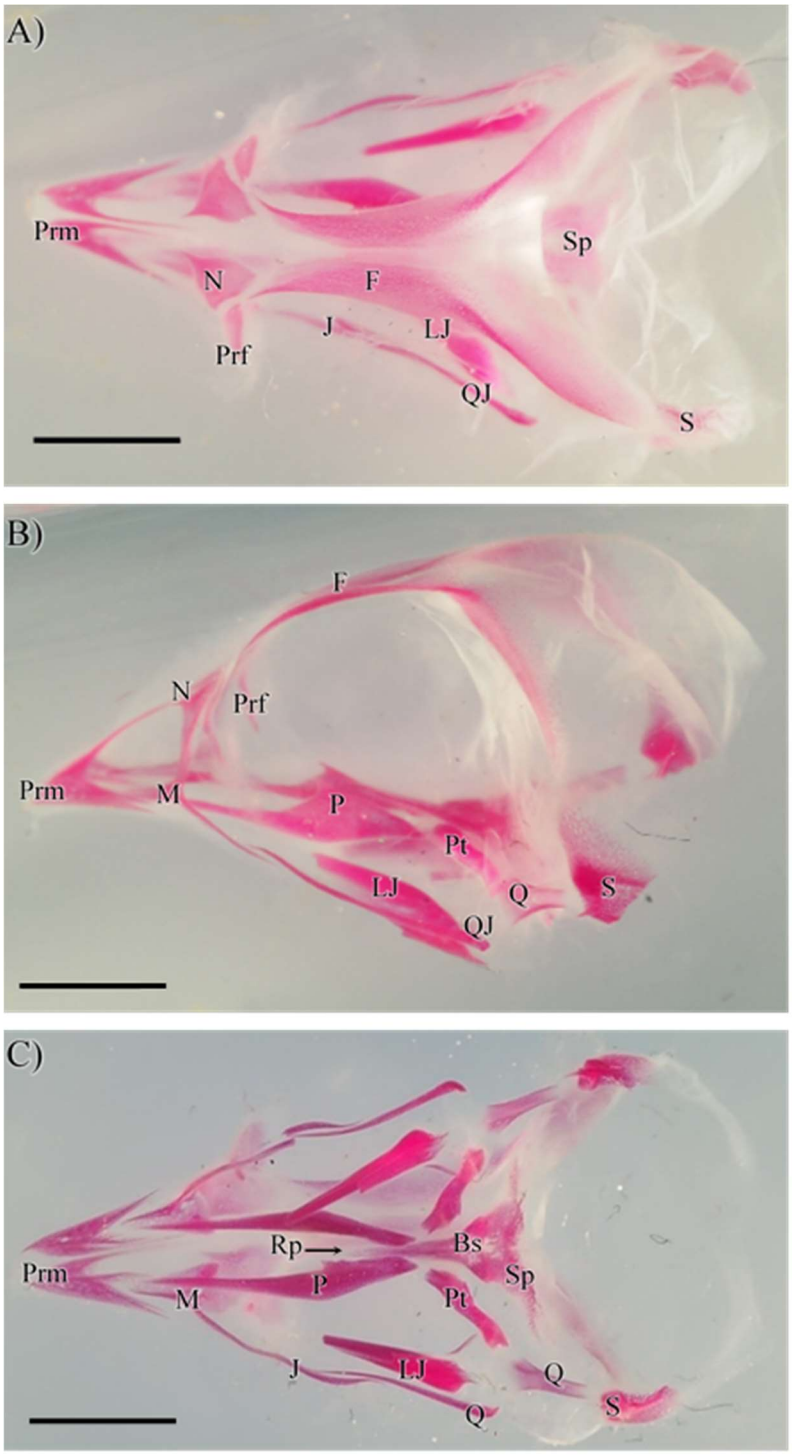

Figure 2.5: Whole-mount cleared and stained chicken skull at stage HH38. The skulls are shown from a dorsal (A), left lateral (B) and ventral (C) views. The rostral side of the skull is on the left. Scale bar is 1 cm. Abbreviations: Bs: Basisphenoid, F: Frontal, J: Jugal, LJ: Lower jaw, M: Maxillary, N: Nasal, P: Palatine, Prf: Prefrontal, Prm: Premaxillary, Pt: Pterygoid, Q: Quadrate, QJ: Quadratojugal, Rp: Rostroparasphenoid, Sp: Sellaparasphenoid and S: Squamosal.

The premaxillaries fuse at their rostral apices while leaving a small, round unossified tip of the beak. All three processes of the premaxillary elongate in the caudal direction, which brings the ventromedial process near the palatine. Moreover, the dorsocaudal extension of the medial process brings it nearer to the nasal. The elongated nasal appears larger with a pointed dorsorostral apex. The internasal suture is long and sinusoidal. Caudal to the boney nasal, the frontal extends medially to almost cover the supraorbital region, except for the interfrontal suture region. The ossified dorsocaudal margin of the frontal is oriented mediocaudally, while the ventrocaudal margin comes near the orbitosphenoid (Figure 2.6A). The orbitosphenoid is visible for the first time as two ventromedially-oriented, sheet-like ossifications at the caudal surface of the orbit. The two ossifications have a thin extension connecting them, whereas the dorsal ossification does not seem to fuse with the frontal but extends ventral to it.

Caudal to the orbitosphenoid, the squamosal has extended dorsolaterally so as to form the lateral boundary of the braincase and comes in close proximity to the newly ossified parietal. The parietal appears as an undulating, sheet-like ossification at the dorsocaudal margin of the skull. This ossification appears optically denser at its caudal margin and thins rostrally. The parietal extends dorsally towards the skull median and extends below the squamosal on its lateral sides. The supraoccipital appears as the dorsocaudal-most element of the skull as a laterally-curved, sheet-like ossification. The dorsal and lateral sides of its ossification appear straight, whereas the medial surface appears mostly straight at the top and then curves laterally to accommodate the foramen magnum. The ventral surface, however, appears very small and straight (Figure 2.6B).

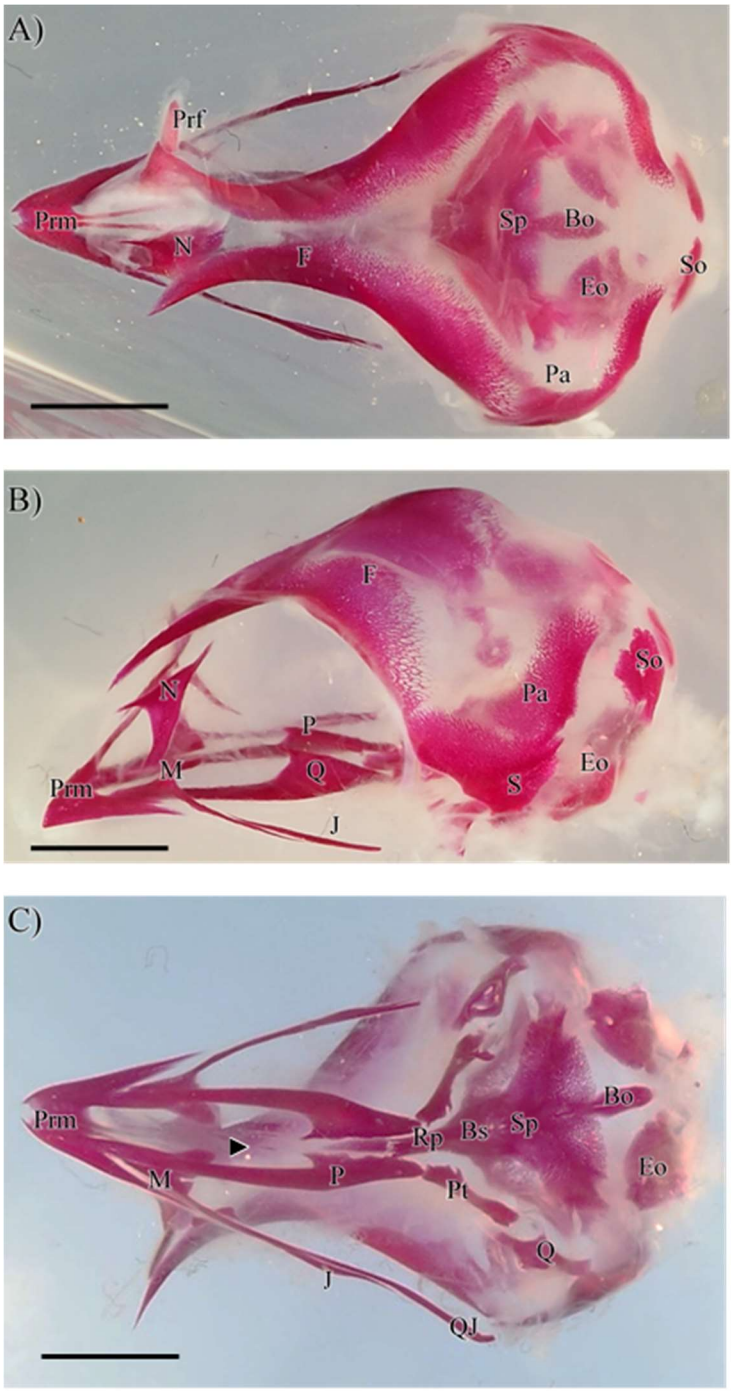

Figure 2.6: Whole-mount cleared and stained chicken skull at stage HH39. The skulls are shown from a dorsal (A), left lateral (B) and ventral (C) views. The rostral side of the skull is on the left. Scale bar is 1 cm. Abbreviations: Bo: Basioccipital, Bs: Basisphenoid, Eo: Exoccipital, F: Frontal, J: Jugal, M: Maxillary, N: Nasal, P: Palatine, Pa: Parietal, Prf: Prefrontal, Prm: Premaxillary, Pt: Pterygoid, Q: Quadrate, QJ: Quadratojugal, Rp: Rostroparasphenoid, So: Supraoccipital, Sp: Sellaparasphenoid and S: Squamosal.

On the ventral side of the skull, the exoccipital appears medial to the squamosal with most of its edges of a laterally rounded shape. The concave medial surface of the exoccipital curves dorsocaudally to accommodate the foramen magnum. Between the paired exoccipitals, the wide and elongate, medially positioned basioccipital extends from the sphenoidal complex to the rostral surface of the foramen magnum. Rostral to the basioccipital, the pterygoid and quadrate continue to ossify towards their epiphyseal ends. Rostral to the quadrate and medial to the palatine, the small, thin, dorsocaudally-oriented ossification of the vomer also appears at this stage (Figure 2.6C).

2.3.6 Hamburger and Hamilton Stage 40:

At Hamburger and Hamilton Stage 40, the skull looks very similar to stage 39 with a few changes in the braincase and palatal regions. Rostrally, the apices of the premaxillaries have extended further towards the rostral tip of the upper beak. Caudally, the postorbital portion of the frontal extends medially, leading to further overlapping of the squamosal on top of the frontal (Figure 2.7A&B). Ventral to the frontal, the orbitosphenoid expands laterally and forms processes that approach the squamosal. The processes of the ventral orbitosphenoid ossification deflect towards the dorsal orbitosphenoid ossification, which will form a fenestra between the two ossifications in later stages. Caudal to the orbitosphenoid, the parietal expands slightly in the rostral direction (Figure 2.7A). The other region of marked change from the previous stage is the rostromedial expansion of the vomer, which brings it near the caudomedial extension of the maxillary (Figure 2.7C).

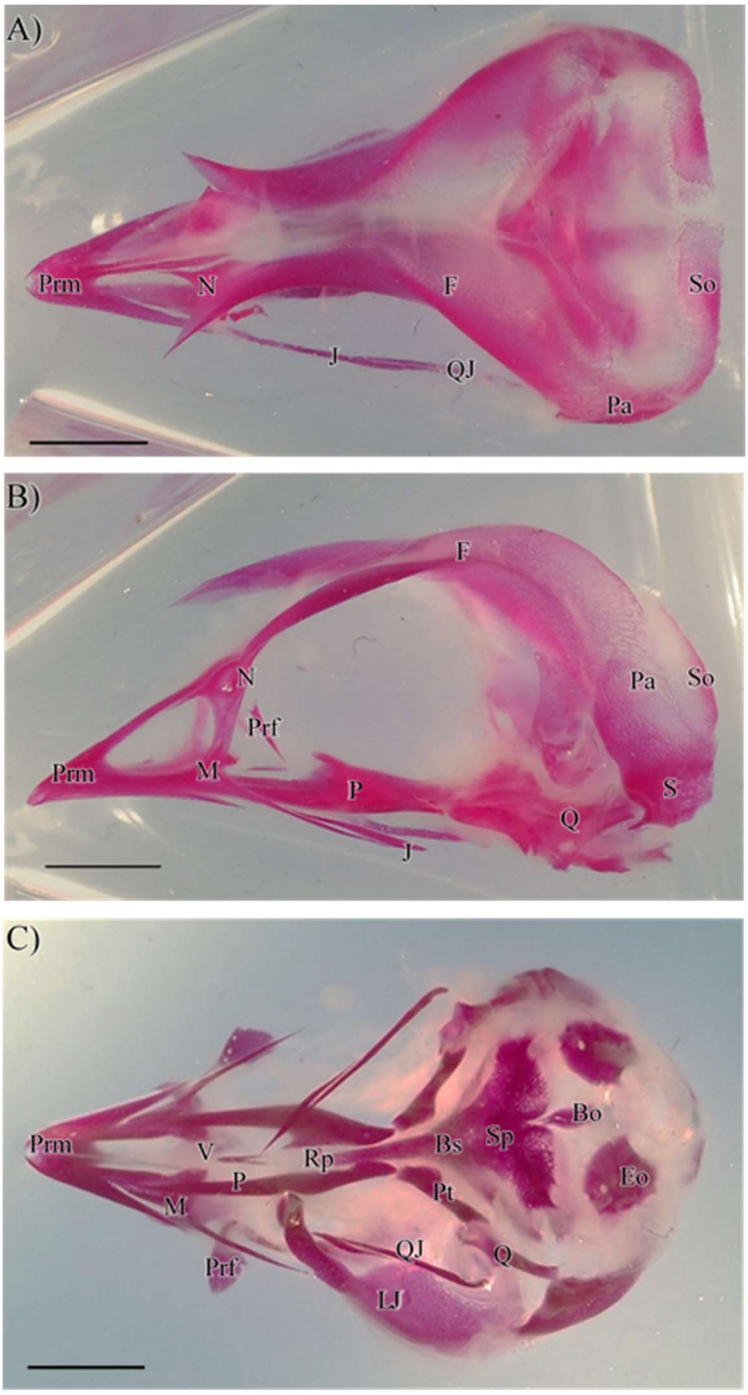

Figure 2.7: Whole-mount cleared and stained chicken skull at stage HH40. The skulls are shown from a dorsal (A), left lateral (B) and ventral (C) views. The rostral side of the skull is on the left. Scale bar is 1 cm. Abbreviations: Bo: Basioccipital, Bs: Basisphenoid, Eo: Exoccipital, F: Frontal, J: Jugal, LJ: Lower jaw, M: Maxillary, N: Nasal, P: Palatine, Pa: Parietal, Prf: Prefrontal, Prm: Premaxillary, Pt: Pterygoid, Q: Quadrate, QJ: Quadratojugal, Rp: Rostroparasphenoid, S: Squamosal, So: Supraoccipital, Sp: Sellaparasphenoid and V: Vomer.

2.3.7 Hamburger and Hamilton Stage 41:

At Hamburger and Hamilton Stage 41, the skull appears similar to the previous stage with the exception of appearing larger, with denser elements. A new ossification appears at this stage, the mesethmoid, and three elements have undergone recognizable changes in shape and size: the orbitosphenoid, supraoccipital and vomer.

The mesethmoid appears in the region of the interorbital septum, and in the gap between the dorsal ends of the frontal and the caudal margin of the nasal (Figure 2.8B). The mesethmoid appears as a vertical, thin, sheet-like element that doesn't articulate with any neighbouring elements. Dorsocaudally to the mesethmoid, the dorsalmost apices of the left and right frontals appear to be almost fused (Figure 2.8A). At the caudal margin of the orbit, the curved process of the ventral ossification of the orbitosphenoid has reached the dorsal process so as to form a fenestra. Caudal to the orbitosphenoid, the supraoccipitals appears almost fused to each other, forming a laterally-curved, flat, sheet-like ossification with round edges. The ventral side, however, has a strongly concave shape, which forms the dorsal surface of the foramen magnum. The vomer extends further rostromedially so as to appear as a rostrally pointed, V-shaped bone (Figure 2.8C).

2.3.8 Hamburger and Hamilton Stage 42:

At Hamburger and Hamilton Stage 42, the skull appears slightly larger than stage 41 with the expansion of the mesethmoid, frontal, and full development of the upper beak. The apices of the premaxillaries have fully formed at this stage such that the rostral tip of the beak appears fully ossified (Figure 2.9 A and C). Caudal to the premaxilla, the mesethmoid expands dorsally to approach the nasal and frontal (Figure 2.9B). The frontal

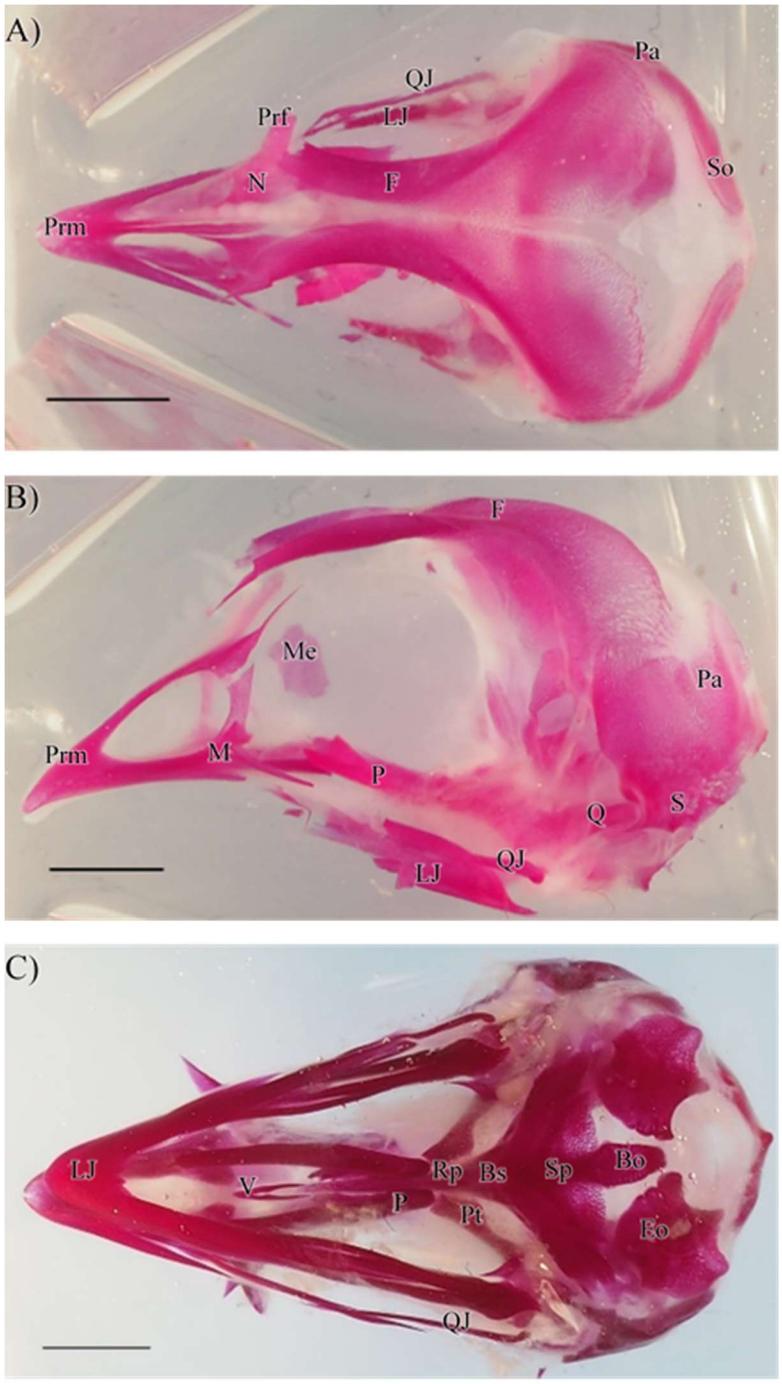

Figure 2.8: Whole-mount cleared and stained chicken skull at stage HH41. The skulls are shown from a dorsal (A), left lateral (B) and ventral (C) views. The rostral side of the skull is on the left. Scale bar is 1 cm. Abbreviations: Bo: Basioccipital, Bs: Basisphenoid, Eo: Exoccipital, F: Frontal, LJ: Lower jaw, M: Maxillary, Me: Mesethmoid, N: Nasal, P: Palatine, Pa: Parietal, Prf: Prefrontal, Prm: Premaxillary, Pt: Pterygoid, Q: Quadrate, QJ: Quadratojugal, Rp: Rostroparasphenoid, S: Squamosal, So: Supraoccipital, Sp: Sellaparasphenoid and V: Vomer.

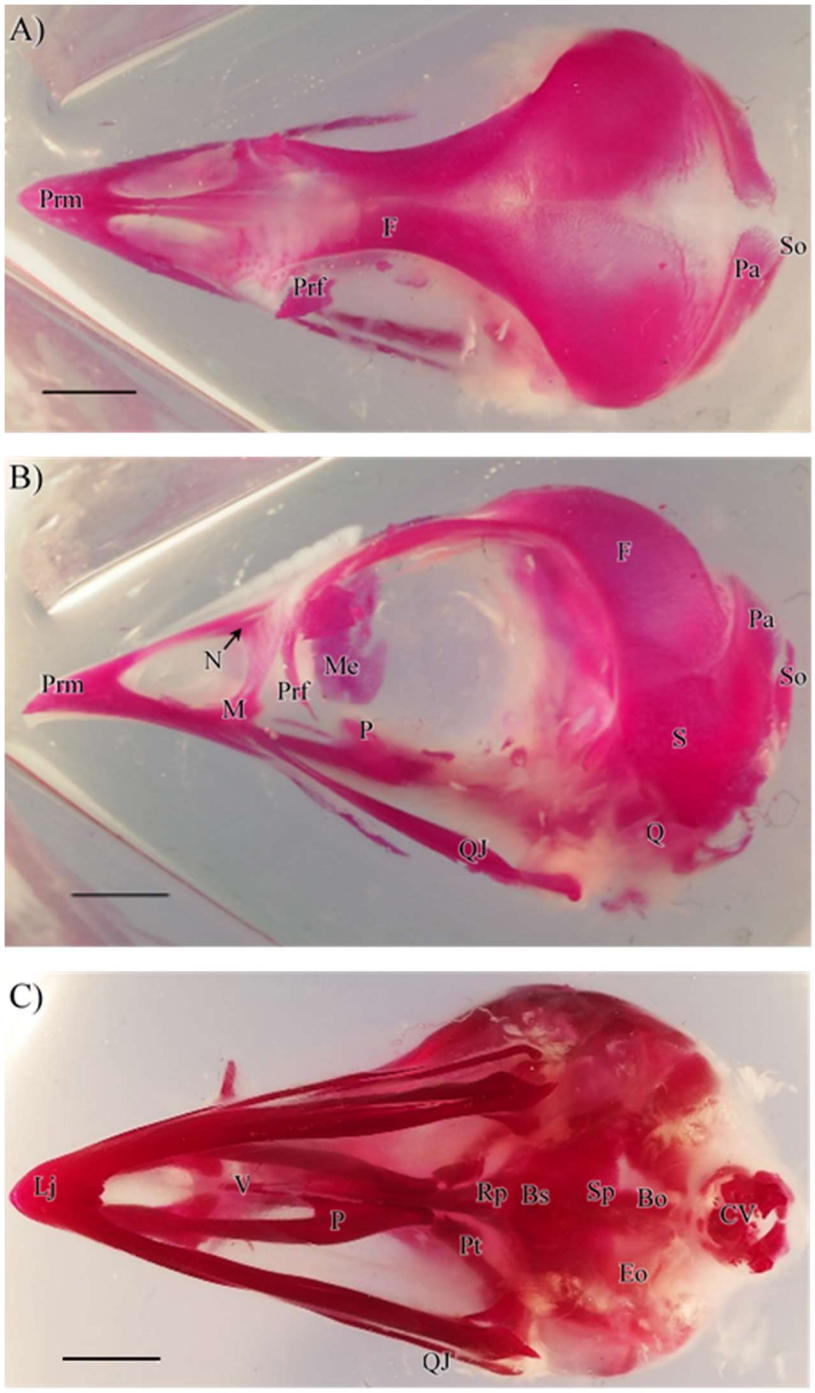

Figure 2.9: Whole-mount cleared and stained chicken skull at stage HH42. The skulls are shown from a dorsal (A), left lateral (B) and ventral (C) views. The rostral side of the skull is on the left. Scale bar is 1 cm. Abbreviations: Bo: Basioccipital, Bs: Basisphenoid, CV: Cervical Vertebrae, Eo: Exoccipital, F: Frontal, LJ: Lower jaw, M: Maxillary, Me: Mesethmoid, N: Nasal, P: Palatine, Pa: Parietal, Prf: Prefrontal, Prm: Premaxillary, Pt: Pterygoid, Q: Quadrate, QJ: Quadratojugal, Rp: Rostroparasphenoid, S: Squamosal, So: Supraoccipital, Sp: Sellaparasphenoid and V: Vomer.

has become more expanded medially along its entire length. The frontals appear to be almost fused to one another for most of their length, except at the caudalmost margin (Figure 2.9A).

2.3.9 Hamburger and Hamilton Stages 43 and 44:

At Hamburger and Hamilton Stages 43 and 44, the skull appears slightly more elongated than the previous stage, with further ossification of the mesethmoid, frontal and parietal (Figures 10 & 11). The mesethmoid extends dorsally to the region between the nasal and frontal, along with the expansion of its dorsal surface laterally. The lateral expansion gives the mesethmoid the shape of a downward facing wedge at stage 43 (Figure 2.10B). At stage 44, the dorsal apex of the mesethmoid reaches the dorsal surface of skull, closing the gap between the frontal and nasal (Figure 2.11B). However, the dorsomedialmost edge of the frontal continues to expand medially (Figure 2.11A). Moreover, the parietals have also expanded medially and rostrally to come into proximity to one another and to the frontals, respectively. The aforementioned expansions of the frontals and parietals further enclose the brain (Figure 2.11B).

2.3.10 Hamburger and Hamilton Stage 45:

At Hamburger and Hamilton Stage 45, the final stage of embryonic development, the skull looks very similar to stage 44, except for the dorsal extension of the mesethmoid (Figures 11 & 12). The dorsal expansion of the mesethmoid forces the medial processes of the premaxillaries very close together. Immediately caudal to the premaxillaries, the

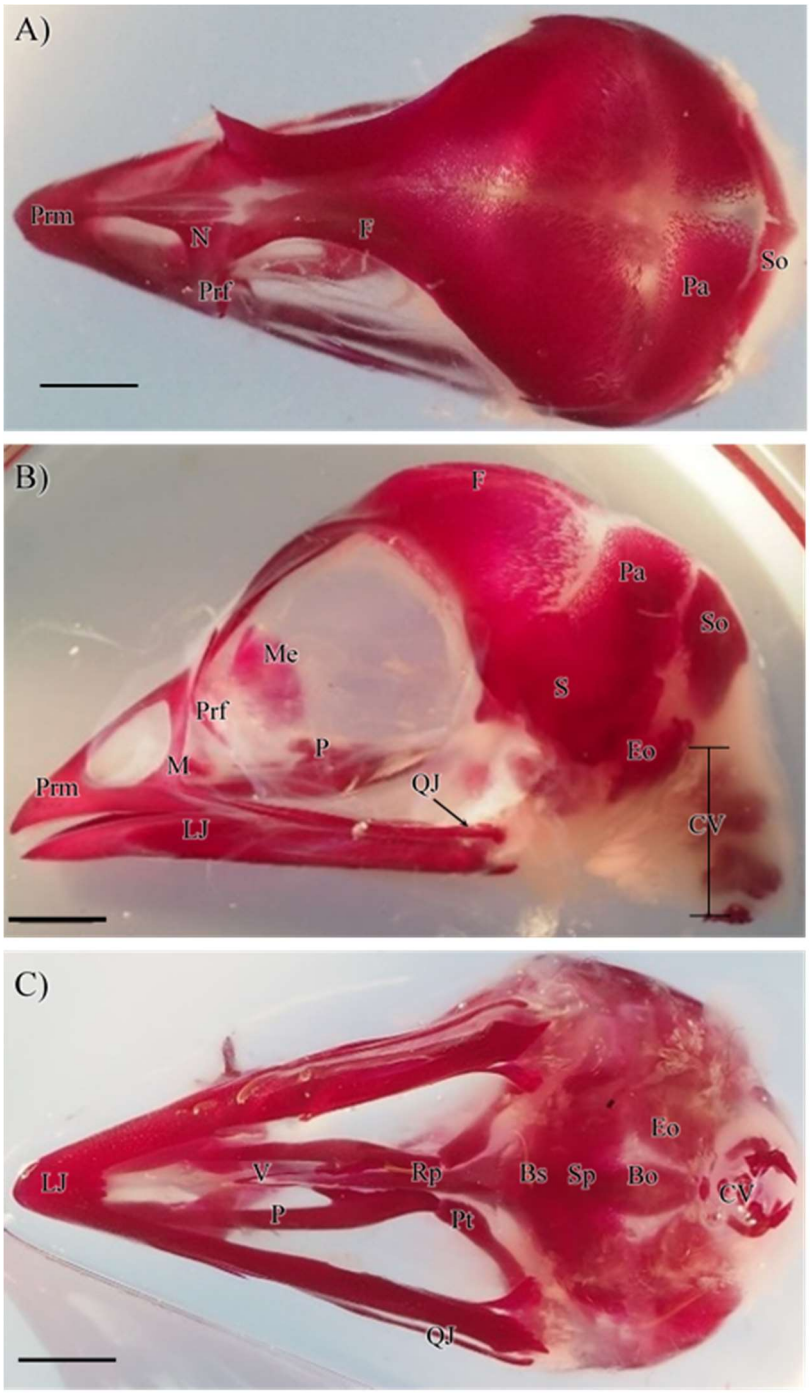

Figure 2.10: Whole-mount cleared and stained chicken skull at stage HH43. The skulls are shown from a dorsal (A), left lateral (B) and ventral (C) views. The rostral side of the skull is on the left. Scale bar is 1 cm. Abbreviations: Bo: Basioccipital, Bs: Basisphenoid, CV: Cervical Vertebrae, Eo: Exoccipital, F: Frontal, LJ: Lower jaw, M: Maxillary, Me: Mesethmoid, N: Nasal, P: Palatine, Pa: Parietal, Prf: Prefrontal, Prm: Premaxillary, Pt: Pterygoid, Q: Quadrate, QJ: Quadratojugal, Rp: Rostroparasphenoid, S: Squamosal, So: Supraoccipital, Sp: Sellaparasphenoid and V: Vomer.

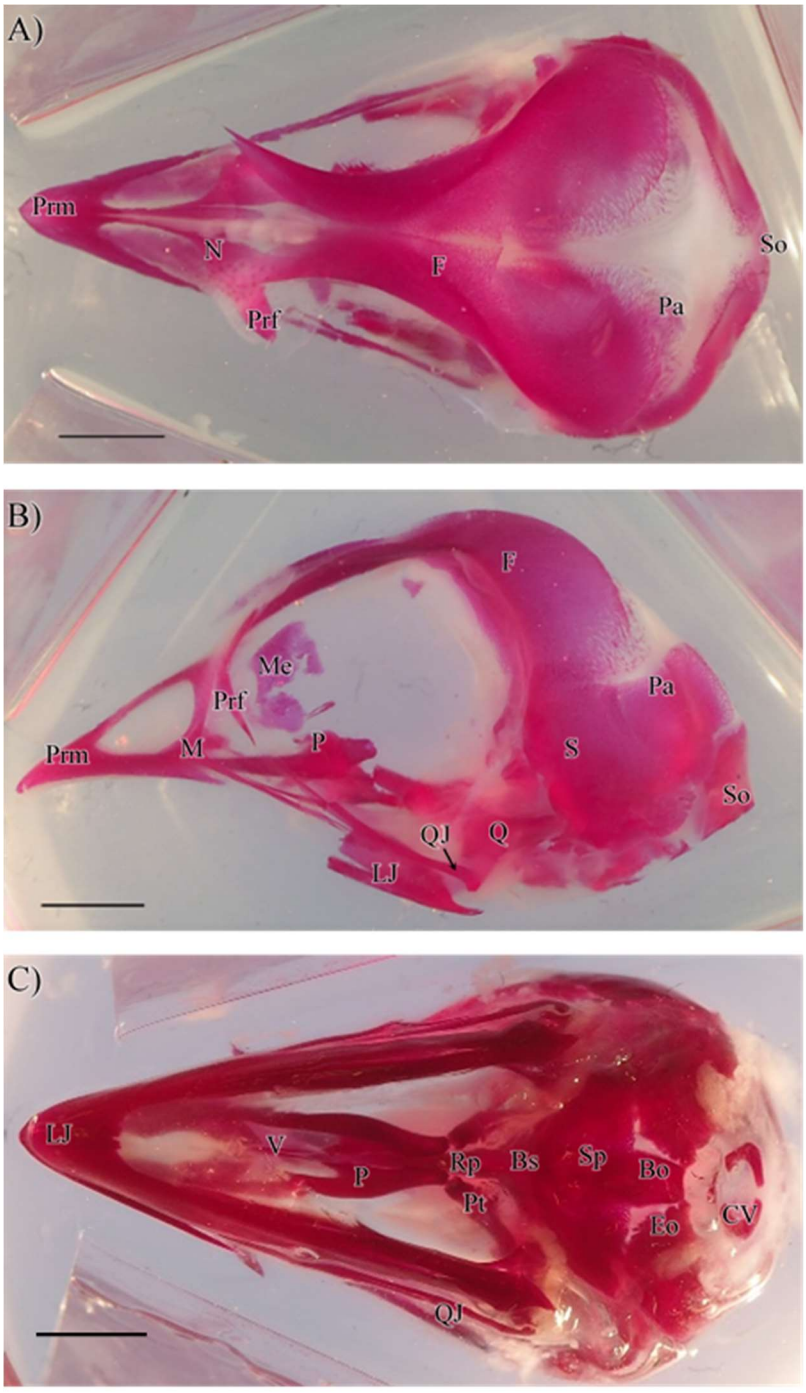

Figure 2.11: Whole-mount cleared and stained chicken skull at stage HH44. The skulls are shown from a dorsal (A), left lateral (B) and ventral (C) views. The rostral side of the skull is on the left. Scale bar is 1 cm. Abbreviations: Bo: Basioccipital, Bs: Basisphenoid, CV: Cervical Vertebrae, Eo: Exoccipital, F: Frontal, LJ: Lower jaw, M: Maxillary, Me: Mesethmoid, N: Nasal, P: Palatine, Pa: Parietal, Prf: Prefrontal, Prm: Premaxillary, Pt: Pterygoid, Q: Quadrate, QJ: Quadratojugal, Rp: Rostroparasphenoid, S: Squamosal, So: Supraoccipital, Sp: Sellaparasphenoid and V: Vomer.

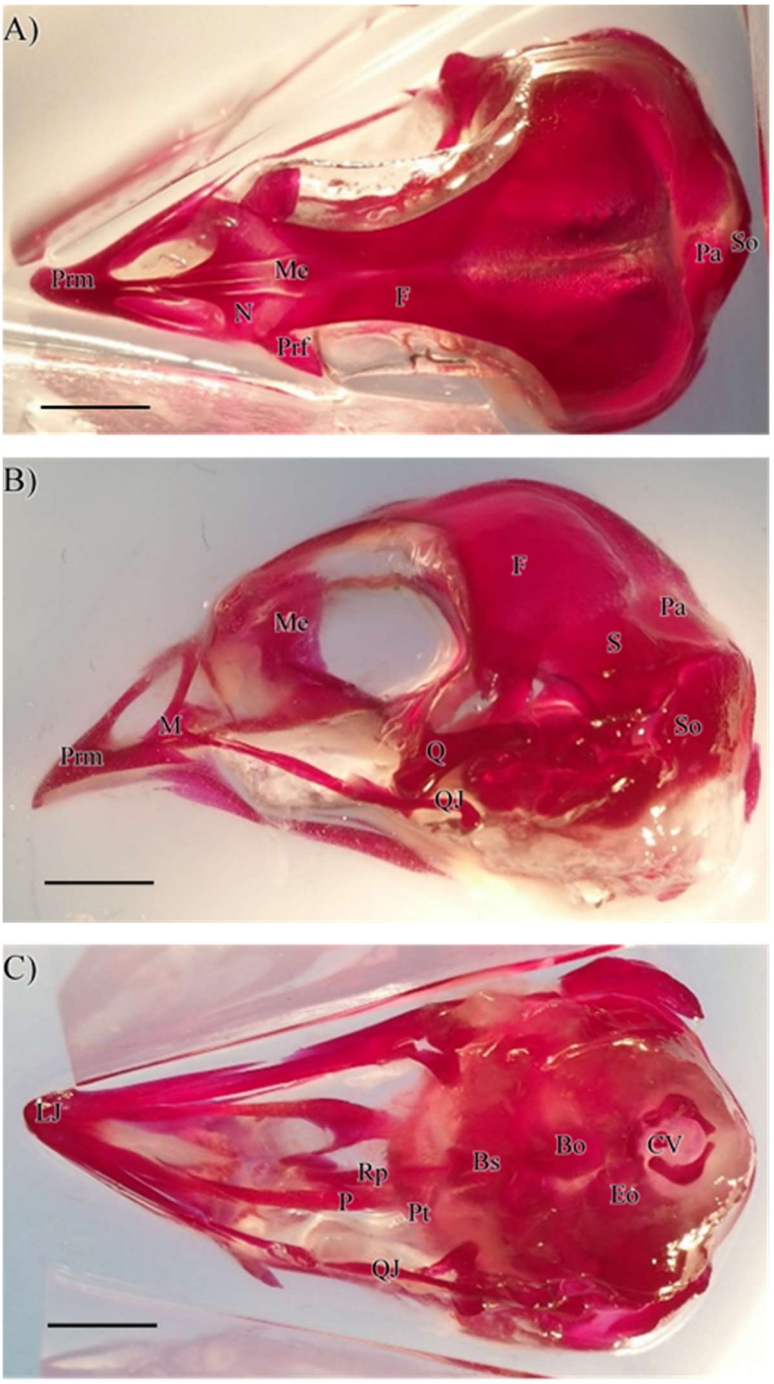

Figure 2.12: Whole-mount cleared and stained chicken skull at stage HH45. The skulls are shown from a dorsal (A), left lateral (B) and ventral (C) views. The rostral side of the skull is on the left. Scale bar is 1 cm. Abbreviations: Bo: Basioccipital, Bs: Basisphenoid, CV: Cervical Vertebrae, Eo: Exoccipital, F: Frontal, LJ: Lower jaw, M: Maxillary, Me: Mesethmoid, N: Nasal, P: Palatine, Pa: Parietal, Prf: Prefrontal, Prm: Premaxillary, Pt: Pterygoid, Q: Quadrate, QJ: Quadratojugal, Rp: Rostroparasphenoid, S: Squamosal and So: Supraoccipital.

dorsal surface of the mesethmoid appears triangular in shape, with a wide rostral margin and a tapering caudal margin that extends into the interfrontal region (Figure 2.11 A & B).

The progression of ossification of the skull from stages HH35 to 44 permits the compilation of an ossification sequence for chicken. Elements of the upper jaw (e.g., the nasal and premaxillary) and the ventral surface of the skull (e.g., pterygoid and palatine) begin ossification before the skull roof elements (e.g., the postocular part of the frontal, parietal and supraoccipital; Figure 2.13). Moreover, about half the cranial elements begin ossifying at stages HH35 and HH36, with the nasal, pterygoid and quadratojugal being the first, and the mesethmoid the last element (Figure 2.14). However, full ossification for most elements took five stages, which is equivalent to five days, to take their final shape. A few elements, such as the prefrontal, took their final shape in three stages, whereas others, such as the frontal, took up to ten stages to form (Figure 2.14).

2.3.11 Variation in the frontal element development

The extensive sampling of embryos at stages HH36 and 37 revealed that the timing and extent of frontal formation varies greatly intraspecifically (Figure 2.15). A single, large ossification spanning the supra- and postocular regions was observed in 70% of the samples from stages HH36 to HH38. On the other hand, eight of the samples did not display any frontal ossification from stage HH36 to very early HH37. Three other samples displayed the supraocular ossification centre alone from stage HH36 to mid-HH37 and only one sample at stage HH37 clearly showed two separate ossification centres (Figure 2.15).

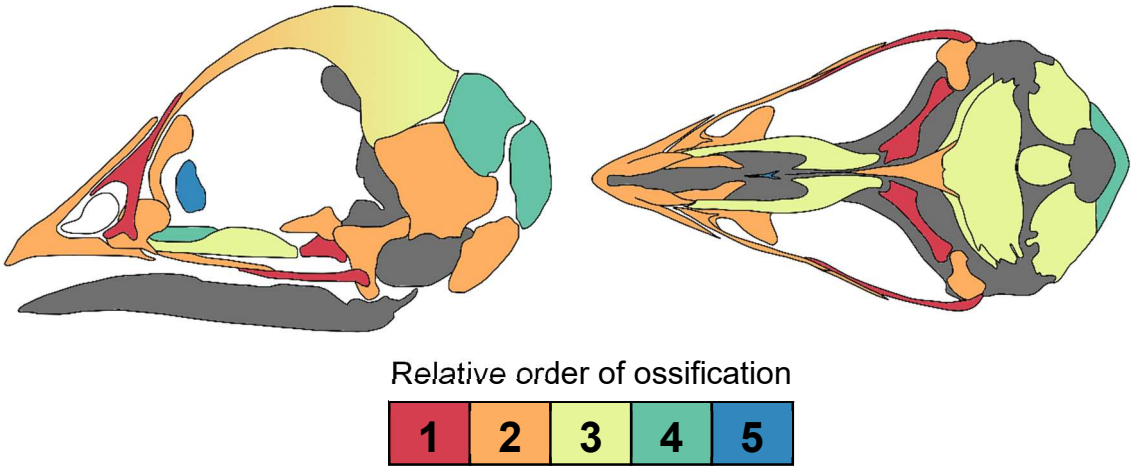

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Figure 2.13: Order of ossification of cranial element of *Gallus domesticus*. The left illustration is modified from Maddin et al. (2016) and the right is modified from Jollie (1957).

Figure 2.14: An ossification sequence of cranial elements in *Gallus domesticus*. The timing of ossification is shown using Hamburger and Hamilton stages (Hamburger and Hamilton, 1992), when the ossification of each element begins and ends, by taking its final shape. The elements are organized in alphabetical order.

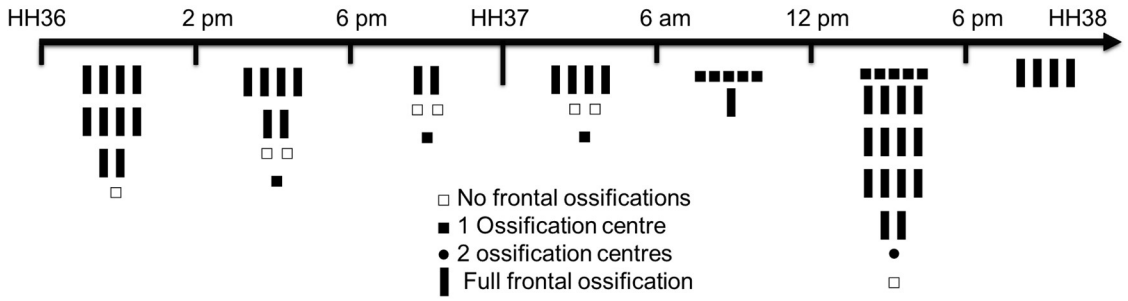

Figure 2.15: Intraspecific variation in the timing of the formation of the frontal in *Gallus domesticus*. The frontal forms from two different ossification centres that seem to appear at different times between stages 36 and 37.

2.4 Discussion:

The staging table obtained revealed that most chicken cranial elements form between stages HH35–45 and over 5 stages (approximately 5 days). Moreover, the elements of the upper jaw and the ventral side of the skull form before the skull roof elements. However, some elements, such as the prefrontal, took only approximately 3 days to reach their final shape, while the frontal took approximately 10 days. Furthermore, the formation of the frontal appears to vary greatly intraspecifically between stages HH36 and 37 with most skulls having a large singular frontal ossification centre.

The ossification sequence of chicken that has been examined by Jollie (1957) using a similar methodology, was largely in agreement with the current results. Although Jollie (1957) reported the timing of ossification in days of incubation, specimens in that study were translated here to HH stages in order to compare that previous sequence with the current one. It was found that most of the elements ossify in the same relative order in both sequences, given an error margin of one HH stage difference (Table1). However, the timing of the first ossification of the nasals and pterygoids appeared here at stage HH35, 8-9 days of incubation, whereas Jollie (1957) reported their appearance after 11 days of incubation. Moreover, the first ossification of the mesethmoid appeared here at stage HH41, after 15 days of incubation, whereas Jollie (1957) reported it to appear after 17 days.

Table 1: A comparison of the ossification sequences for the chicken skull showing the differences in the relative order of bone ossification. Large discrepancies between the ossification sequences are indicated in red.

Element	Jollie (1957)	This study	Jollie (1957)	This study
	Order of ossification		*Stage of initiation of ossification*	
Basioccipital	4	5	39	39
Basisphenoid	2	3	37	37
Exoccipital	3	3	38	37
Frontal	2	2	37	36
Jugal	2	2	37	36
Maxillary	2	2	37	36
Mesethmoid	8	7	43	41
Nasal	2	1	37	35
Orbitospheno-id	5	5	40	37
Palatine	2	3	37	37
Parasphenoid	2	4	37	38
Parietal	3	5	38	39
Prefrontal	2	2	37	36

Premaxillary	2	2	37	36
Pterygoids	2	1	37	35
Quadrates	2	2	37	36
Quadratojugal	1	1	36	35
Rostropara-sphenoid	2	2	37	36
Sellapara-shenoid	2	3	37	37
Squamosal	1	2	36	36
Supraoccipital	5	5	40	39
Vomer	4	5	39	39

The discrepancies between the results of Jollie (1957) and mine are likely due to differences in the rate of growth and/or differences in alizarin red uptake by the specimens. The latter probable reason could also explain the observed bilateral asymmetries in the ossification of an element (Mitgutsch et al., 2011). For example, in one specimen at stage HH37 the frontal postorbital ossification was seen on the left side but not the right side of the same specimen (Figure 2.4B). Similarly, Mitgutsch et al. (2011) reported the occurrence of the same phenomenon in the development of the parietal and opisthotic bones in ducks. Mitgutsch et al. (2011) attributed ossification asymmetries to variation in the speed of mineralization, which in turn is due to minute differences in incubation condition in one egg. However, the effect of these differences would need to be examined more closely in future studies.

Similarly, the observed variation in the initiation of frontal bone formation requires detailed examination in future studies. Intraspecific variation in the ossification sequence obtained here was only observed in the frontal bone, which could be due to several reasons. One of these reasons could be differences in the speed of migration of neural crest and mesodermal cells across individuals. This possibility can be examined using real-time tracking of cell migration between embryos. However, another possible explanation is the variation in cell proliferation, osteogenesis and/or mineralization. This second possibility can be examined using BrdU labelling for cell proliferation (Li et al., 2008), osterix (Cao et al., 2005) and osteocalcin (Hauschka and Reid, 1978) for osteogenesis and mineralization, respectively. Each of these possibilities requires detailed studies to explain the observed variation in frontal bone ossification (Figure 2.15).

The staging table produced in this study lacks an examination of lower jaw elements and the ear bones. The reason for the shortcoming is the falling off of the lower jaw during the clearing process, which prevented its examination across all 11 stages. Moreover, the location of the ear bone is medial to the squamosal, making it difficult to observe externally, and so was not included in this study. Another limitation of this study is the use of a small number of replicates for most stages of development. Although, replicates for most stages did not observably vary, the use of a larger sample size per stage will provide a more thorough examination of the variation in chicken skull development.

However, one of the novel aspects of this study is its attempt to follow the development of a cranial element until it takes its final shape (i.e. shape at hatching). The justification for such an attempt is the visually clear difference between the time it took for some elements to fully form (e.g. frontal) as compared to others (e.g. prefrontal). However, the difficulty with such an attempt is to accurately define what taking on its final shape means. The definition used here is the absence of a clear visual change in the shape of an element beyond a particular stage. However, the senescence of shape change would be better determined using geometric morphometric methods in a future analysis.

2.4.1 The chicken ossification sequence

The early formation of the beak elements and the ventral elements makes a few predictions about the migration of neural crest cells (CNCCs) and skull bone ossification. The ossification of the derivatives of the mandibular and preotic streams at the same stages, HH35-36, has two possible implications. The first implication is that the

mandibular and preotic streams arrive at their respective destinations at the same time in chicken, then begin the processes of acquiring an osteogenic fate. The second implication is that the mandibular stream could arrive earlier than the preotic stream, as has been shown in mammals (Serbedzija et al., 1992), and the initiation of ossification is delayed in the mandibular arch. Similarly, the early appearance of both rostral and caudal ossifications in the upper jaw, e.g. premaxillary and quadratojugal, implies that CNCCs might arrive at the ventral side of the mandibular arch before it arrives at the dorsal side. However, the initiation of ossification is delayed on the ventral side of the mandibular arch compared to that on the dorsal side. Finally, the rate of migration or timing of initiation of ossification in paraxial mesodermal cells compared to CNCCs remains an open question. My results did not indicate any significant difference in the timing of ossification of the mesodermally derived elements compared to CNCC derived ones. An exception to the former observation, is the ossification of the frontal bone.

2.4.2 Element ossification period and implications for intracranial ontogenetic allometry

The supraocular ossification centre of the frontal bone ossified before the postorbital one. This could be due to delayed relative migration or initiation of ossification in mesodermal cells compared to CNCCs. However, the frontal bone took the longest amount of time to develop – from stages HH 36 to 45, equivalent to 10 days – which leads to three possible explanations. The first explanation is that the size of the frontal bone region is seemingly so large that requires a lot of time to fully ossify. The second explanation is that the mesodermally-derived portion of the frontal develops more slowly than the CNCC-derived portion. The third possibility is that the mesodermally-

derived portion develops at the same rate as the CNCCs, but the braincase region it covers is larger than the supraocular region.

The premaxillary, also required a relative long period of time to fully form (fusion of the paired elements at HH39 and full ossification of the rostral tip at HH 44; approximately 5 days). The slow ossification of the beak tip might be due to the decrease in the rate of cell proliferation that is known to occur in beak tips of zebra finch (Fritz et al., 2014). Cell proliferation is one of the early steps in intramembranous ossification that precedes differentiation and osteogenesis (Ornitz and Marie, 2002). In zebra finch, the tip of the primordial beak is known to contain a region of locally high cellular proliferation, whose rate of proliferation decreases as the beak grows (Fritz et al., 2014). Therefore, it is possible that because cellular proliferation begins to decrease in rate, consequently bone ossification also slows down. Contrary to the development of the premaxillary, the prefrontal forms over three stages, approximately 3 days, which is the shortest amount of time that any element took to develop. The reason why it takes such a comparatively short time remains an open question. Similarly, the timing of the development of the mesethmoid is also puzzling, for it is the last element to begin ossification in the chicken skull.

The relative period that each element took to ossify could assist future research in explaining the known negative allometric relationship between bird beaks and braincases (Bright et al., 2016; Linde-Medina, 2016). Linde-Medina (2016) inferred that the beak develops more slowly than the braincase in Galliformes. The relatively slow rate of development of the beak and the observed early onset of its ossifications leads to a possible scenario. The mesenchyme of the beak begins proliferating at an earlier stage

than the mesenchyme of the braincase. Afterwards, it proceeds with differentiation and ossification earlier than the braincase elements, but at a slower rate. This scenario can be tested by checking for the appearance of condensed mesenchymal cells in the beak region at stages HH34–35, and absence of these cells in the braincase region. Moreover, the rate of the ossification of each beak element can be measured at stages HH35 and onwards, and the rate of the ossification for braincase elements can be measured from stages HH37 onwards. The rates of ossification of the beak elements can be averaged and compared with the averaged braincase element to verify the proposed scenario.

2.4.3 The search for homologs using sutures

The staging table indicated that the search for a suture between the supraocular and postorbital ossification centres of the frontal would be best conducted by examining stages HH36 and 37. At these stages the two ossification centres of the frontal are visible, and thus a suture, if present, might be found between them at this time. If such a suture was found, then it would support the hypothesis that the frontal is a composite bone (Evans and Noden, 2006; Maddin et al., 2016; Smith-Paredes et al., 2018).

2.4.4 Summary

In summary, questions of the timing, the number of ossification centres, and the length of time an element takes to reach its final shape rely on the use of a staging table. Similarly, investigations into the timing of formation, orientation and location of cranial sutures depend on the use of a staging table. The chicken staging table has shown most cranial elements form between stages HH35–45 (i.e. over ten days). The table has also shown that elements of the upper jaw and the ventral side of the skull form before the

skull roof elements. Moreover, the two ossification centres of the frontal were revealed to appear separately between stages HH36 and 37. This timing is crucial for the search for a suture between the two ossification centres, which would assist in resolving the controversy concerning the homology of the avian frontal.

Chapter 3: Suture histology in the chicken skull: implications for the homology of the avian frontal bone

3.1 Abstract:

The study of vertebrate evolution relies heavily on the identification of homologs in different lineages. A variety of definitions have been used to identify homologs, including the morphological and developmental definitions, which have subsequently led to controversies about the homology of traits. An example of such a conflict concerning the homology of bone elements is the avian frontal. According to the morphological definition, the supra- and postorbital ossifications in the avian skull comprise what is called a frontal, but embryologically these ossifications have been proposed to be a frontal and parietal, respectively; if correct, the element is then, instead, a frontoparietal. Since sutures are known to occur between discrete cranial bone elements, this hypothesis was tested by histologically examining the gap between the two ossification centres of the frontal for an ontogenetic series of *Gallus domesticus* and comparing these with the four known sutures in the skull of the chicken. It was determined that, based to the absence of the middle vascular layer within it, the histology of the gap did not resemble that of any of these four sutures. Instead, the histology of the gap more closely resembled the histology of the presumptive region of a single bone (frontal), thus, rejecting the hypothesis that an ancestral frontal and parietal fused to form a frontoparietal. This project represents the first histological examination of embryonic cranial sutures in Aves.

3.2 Introduction:

The approximately 500 million years of vertebrate evolution have led to a vast diversity of vertebrates (Benton, 2015). Understanding vertebrate diversity has relied on examining two different facets of evolution: innovation and homology (Tschopp and Tabin, 2017). Evolutionary innovations are novel traits that uniquely appear in a particular lineage, such as feathers (Brusatte et al., 2015), and are not known to occur in any ancestral lineages (Rabosky, 2017). Homologs are traits that appear in multiple lineages, but that are considered to be the same based on a definition of homology (Tschopp and Tabin, 2017). Multiple definitions of homology currently exist, each with its own set of criteria. For example, the phylogenetic definition of homology would posit two traits as homologous if their common ancestor possessed that trait (Rieppel, 1994). In contrast, the older, historical morphological definition of homology posits two traits as homologous based on similarity of their shape and topology (Panchen, 1994; Rieppel, 1994), e.g., the humerus can be recognized across vertebrate lineages based on its shape and proximal location in the forelimb relative to the body (Panchen, 1994). An evolutionary developmental biology (evo-devo) definition of homology is based on the similarity in the developmental pathways of two traits (Panchen, 1994). If two traits develop in a similar way in two different lineages, they are said to be homologous regardless of their morphology and, sometimes, even regardless of whether or not the trait is present in a common ancestor (e.g. deep homology). For example, the human and *Drosophila* eyes develop in a similar manner using similar regulatory genes, which would make them homologs of each other (Tschopp and Tabin, 2017), yet their common ancestor lacks eyes and they are morphologically quite different. The application of

different definitions of homology to the same part of an organism has led to controversies regarding its homology. One such example is the avian frontal, where the morphological definition of homology has led to naming the avian frontal as a frontal and the developmental definition has led to it being named a frontoparietal (i.e., a fused frontal and parietal) (Evans and Noden, 2005; Maddin et al., 2016).

The avian frontal is a skull roof bone that occurs within the supraocular and postorbital regions of the skull, where it forms a roof over the forebrain (Romer, 1956; Fabbri et al., 2017). Caudal to the frontal, the parietal occurs dorsal to the midbrain (Fabbri et al., 2017). In mouse and axolotl, the frontal develops from a population of embryonic cells called cranial neural crest cells, whereas the parietal develops from cranial mesodermal cells (Jiang et al., 2002; Maddin et al., 2016). In chickens, however, the frontal develops from both types of embryonic cells (Evans and Noden, 2006). There, neural crest cells migrate from the dorsal side of the embryonic fore- or midbrain to the frontal region, forming a population of mesenchymal cells that differentiates into bone cells, which form a supraocular ossification centre (Evans and Noden, 2006). Caudal to this, cranial mesodermal cells migrate from the ventrolateral sides of the mid-and hindbrain to the postorbital region of the skull and form a population of mesenchymal cells that differentiates into bone cells, which form a postorbital ossification centre (Evans and Noden, 2006). These two ossification centres then fuse with one another to form the frontal (see Chapter 2 for developmental timing and variation therein). Posterior to the frontal, the avian parietal develops entirely from mesodermal cells (Evans and Noden, 2006), as in the other model species (Jiang et al., 2002; Maddin et al., 2016). The dual origin of the avian frontal has led several authors to suggest that the avian frontal is

instead a frontoparietal that formed from a fusion event between an ancestral frontal and parietal during the evolution of birds (Evans and Noden, 2006; Maddin et al., 2016).

An aspect that may help in settling the controversy surrounding the identity of the avian frontal is whether or not a suture forms between its two ossification centres. Cranial sutures form during skull development in vertebrates and play a variety of roles in skull development (Slater et al., 2008). Many bones of the skull form through a process called intramembranous ossification in which disconnected populations of mesenchymal cells condense and differentiate into osteoblasts (Berendsen and Olsen, 2015). Osteoblasts secrete an organic bone matrix precursor called osteoid that contains collagen type I (Ben Shoham et al., 2016). Subsequently, a calcium matrix is deposited inside the osteoid in the form of plates and rods (trabeculae) that then traps osteoblasts within it, turning them into osteocytes. The resulting structure is then referred to as an ossification centre (Saladin and McFarland, 2018). An ossification centre grows via osteoblasts at the osteogenic front to form a bone (Jin et al. 2016). A bone can be defined as a discrete unit of bone tissue that is, mostly, separated from other bone tissues by a suture or a joint. Some elements, such as the human frontal, occipital and temporal, form from the fusion of multiple ossification centres that do not appear to be separated by sutures or joints (Rice, 2008).

The precise definition of a suture varies depending on the context in which they are examined (Opperman, 2000; Curtis et al., 2013). Embryologically, a suture is a fibrous joint that connects skull bones together, coordinates bone growth and provides flexibility for accommodating other features, such as enlarged brains (Maloul et al., 2014). Structurally, the definition of a suture depends on its state; open, closed or fused.

There are additional functional and paleontological definitions of sutures (Maloul et al., 2014; Bailleul and Horner, 2016), but these are, unfortunately, not typically applicable to developmental studies. However, the structural features of a suture are histologically observable and are, therefore, most relevant to the issue of frontal homology, as they would permit the identification of a developing suture, if present.

Regardless of how sutures are defined, they form when the osteogenic fronts of two bone elements come close together. The part of the osteogenic front that contributes to a suture is called sutural bone (Bailleul and Horner, 2016). The space between two sutural bones remains open in response to signals (such as fgf2 (Rice et al., 2000)) from the dura mater located ventral to the suture (Opperman, 2000). The dura mater is one of the layers of the meninges surrounding the brain and spinal cords (Adeeb et al., 2012) and the signal from it diminishes during development, the sutural bone of each element approaches the other, and in combination they form one of three possible suture types: a butt-joint suture, a beveled suture, or a tongue-and-groove suture (Rice, 2008). The butt-joint suture is one in which the two sutural bones are separated by a thin vertical gap. A beveled suture is an overlapping joint, where one of the elements extends on top of the other (Rice, 2008). The tongue-and-groove suture occurs when the sutural bone of one element forms a concave recess to accept its convex counterpart (Rice, 2008).

These types of sutures can occur in one of three states: open, closed, or fused. Histologically, an open suture contains sutural bone, a cambial layer, a middle vascular layer and a uniting layer (Figure 3.1) (Bailleul and Horner, 2016). The cambial layer is a

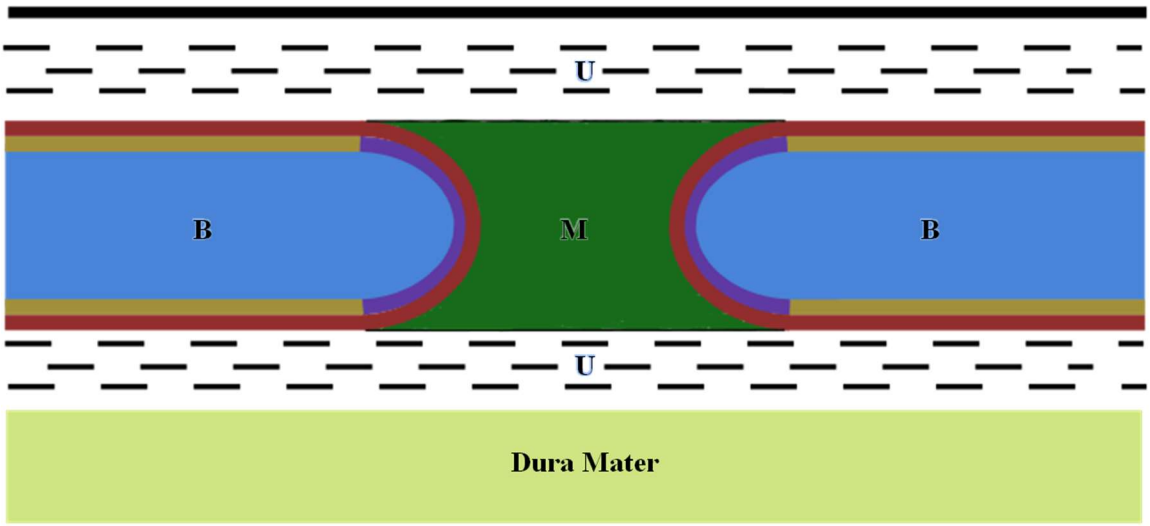

Figure 3.1: A schematic illustration of some of the components of an open suture. The sutural bone is shown in purple, the osteogenic front is shown in yellow and the cambial layer is shown in red. The thick black line at the top is the outer skin. B: Bone, M: Middle vascular layer, U: Uniting layer. Illustration is based on Jones et al. (2011) and Teven et al. (2014).

dense layer of osteoprogenitors, fibroblasts and collagen fibres that surround the bone element's matrix (Bailleul and Horner, 2016). The middle vascular layer is composed of collagen fibres, mesenchymal cells, and fibroblasts (Bailleul and Horner, 2016). The uniting layer is composed of collagen fibres that extend above and below both elements matrices and connects them together (Bailleul and Horner, 2016).

Histologically, a closed suture is composed of sutural bone, a dense fibrous layer and a connecting layer (Bailleul et al., 2016; Bailleul and Horner, 2016). The dense fibrous layer appears parallel to the sutural bone and composed of collagen fibers and a few fibroblasts (Bailleul and Horner, 2016). The connecting layer is composed of bundles of collagen fibers that connect the bone matrices to the dense fibrous layer (Bailleul and Horner, 2016). Closed sutures often have an interdigitating contact between bone elements, where the sutural bone of one element has a series of alternating concavities that accept processes of the other element, resulting in a sinuous contact between elements. A fused suture is one where the two bone matrices of the elements are continuous with each other and there are no structural signs of a suture are visible (Bailleul and Horner, 2016).

The aim of the current study was to examine the developing chicken frontal element to determine if a suture forms within it. Specifically, whether the gap between the two ossification centres of the frontal resembles any known sutures in the chicken skull. A resemblance was assessed based on the presence or absence of known histological components of a suture. The presence of such histological features would imply that the chicken frontal element is a fused frontoparietal, whereas their absence would support the current nomenclature as a singular bone called a frontal.

3.3 Materials and Methods:

3.3.1 Experimental design

In order to determine the presence of a suture within the frontal of a chicken, the staging table of the skull developed in Chapter 2 was consulted to establish the timing of formation of the two ossification centres and, thus, the gap in between them. Interpretation of data from the staging table reveals that the anterior ossification centre appears prior to the posterior one, at approximately stage HH36, and that the posterior ossification appears at stages HH37. The location, orientation and approximate time of formation of the four comparative sutures were also determined using the staging table.

Four sutures were targeted for comparative histological examination: the nasal-frontal, frontal-parietal, parietal-supraoccipital, and interpremaxillary suture. The first three sutures are transversely-oriented, which is similar to the orientation of the gap within the frontal, and show what histological features appear in a transversely-oriented suture. The nasal-frontal suture was sectioned at stages HH37. The frontal-parietal and parietal-supraoccipital sutures were sectioned at stages HH42, 44 and 45 in order to observe any structural changes through development. These sutures will eventually fuse in the mature skull, but that fusion is not observable in the time frame examined here (Konig et al., 2016). The interpremaxillary suture was chosen as it allows for examination of the structure of a suture during fusion. Fusion of the two premaxillaries in the rostral region of their suture begins at approximately stage HH40 (Chapter 2). This suture remains unfused in the caudal region until a posthatching stage. Finally, a region of presumptive bone that does not contain a suture was also examined. This was the caudal portion of the postorbital ossification centre of the frontal, sectioned at stage HH36,

where the bone is in the process of growing posteriorly towards the parietal. This would permit histological comparison of the gap between the two frontal ossifications with that of a region that does not produce a suture.

3.3.2 Embryo collection and slide preparation

Nine white leghorn chicken eggs were obtained from Canadian Food Inspection Agency. Eggs were incubated at 38°C at 50% humidity in a styrofoam incubator (Hova-Bator Genesis 1588, Incubator Warehouse) in accordance with Carleton University's animal care policies. Eggs were maintained under these conditions for eight days, at which time sampling of embryos began. Sampled embryos were staged according to Hamburger and Hamilton stages (HH; Hamburger and Hamilton 1992).

A sample that included stages HH35-37, 40, 42, 44, and 45 (one sample per stage) was isolated and their eyes and skin were removed to improve embedding and sectioning. Subsequently, specimens were placed in 10% phosphate-buffered formalin solution (Fisher Scientific #SF1004) overnight at room temperature. The next morning, specimens were moved through a sucrose in phosphate-buffered saline (PBS) solution series to prepare them for embedding, following the procedure of Piekarski et al. (2014). First, specimens were placed in a solution of 15% sucrose (Fisher Chemical, #S5500) in PBS and left overnight. The next morning specimens were placed in a solution of 30% sucrose in PBS and left for 24 hours. Following this, the next morning specimens were placed in a solution of equal parts 30% sucrose in PBS and optimal cutting temperature (OCT) solution (Fisher Scientific, #4585) and left for a further 24 hours. The next morning specimens were placed in a solution of 100% OCT solution for 24 hour. Afterwards, the

heads were placed in a mounting mould filled with OCT in either transverse or parasagittal orientation. Blocks were then quickly frozen by submerging the mould in bath of 100% ethanol chilled with dry ice.

Resulting blocks were adhered to a metal mounting stub and sectioned on a Thermo Scientific HM525 NX cryostat. Sections were cut at a thickness of 5 to 12 μm and then left to adhere to the glass slides overnight. For each specimen, approximately 50 sections were cut and most slides were stained with Masson trichrome stain following the method of Sheehan and Hrapchak (1980). Masson's trichrome stain was used because it stains collagen blue and cell cytoplasm red (Sheenan & Hrapchak, 1980). Collagen is present in bone matrix (Carneiro and Leblond, 1959) and in the cambial and middle vascular layers of a suture (Bailleul and Horner, 2016). Combined with the red cell dye, Masson's trichrome stain permits distinguishing between extracellular elements, such as collagen fibres and bone matrix, and cells of different shapes. Thus, the major structural features of developing sutures would be observable, if present.

3.4 Results:

3.4.1 General histological observations:

The Masson's trichrome stain stained the trabeculae of the bone as well as the thread-like collagen fibres blue (Figure 3.3B). Furthermore, the cytoplasm of all different types of cells were stained red and they can only be distinguished from each other by their shape or location. Osteocytes occur within the bone matrix (Klein-Nulend et al., 2003) and were observed as red cells in the sections (Figure 3.4B). Osteoblasts and

osteoprogenitor cells usually surround the bone matrix during ossification. Irregular shaped cells on the bone matrix surface are interpreted here as these cells (Bailleul and Horner, 2016). Fibroblasts are known to have an elongated wavy shape (Ha et al., 2014) and were seen as stained red structures in different regions of the sutures (Figure 3.2). The distribution of the collagen fibers, bone cells and fibroblasts allowed for the identification of the two major components of a suture: the cambial layer and the middle vascular layer (Figure 3.2) (Bailleul and Horner, 2016). The cambial layer, that surrounds the bone matrix, appeared as a layer of densely packed cells with a few collagen fibres running in between them (Figure 3.2). Adjacent to the cambial layer, another layer with many collagen fibres and some fibroblasts was observed. This is the middle vascular layer (Figure 3.2) (Bailleul and Horner, 2016).

3.4.2 The nasal-frontal suture:

The parasagittal sections of the nasal-frontal suture at stage HH37 show that the caudal end of the nasal and the rostral end of the frontal are thin, caudally-tapering blue layers of bone matrix (Figure 3.2). The nasal-frontal suture in chicken is an open, beveled suture at this stage, with the nasal overlapping the frontal. The bone matrix of each element is surrounded by a cambial layer. Between the sutural bone portion of each element is a middle vascular layer that contains collagen fibres and fibroblasts oriented in a direction parallel to the plane of the bone matrices (Figure 3.2).

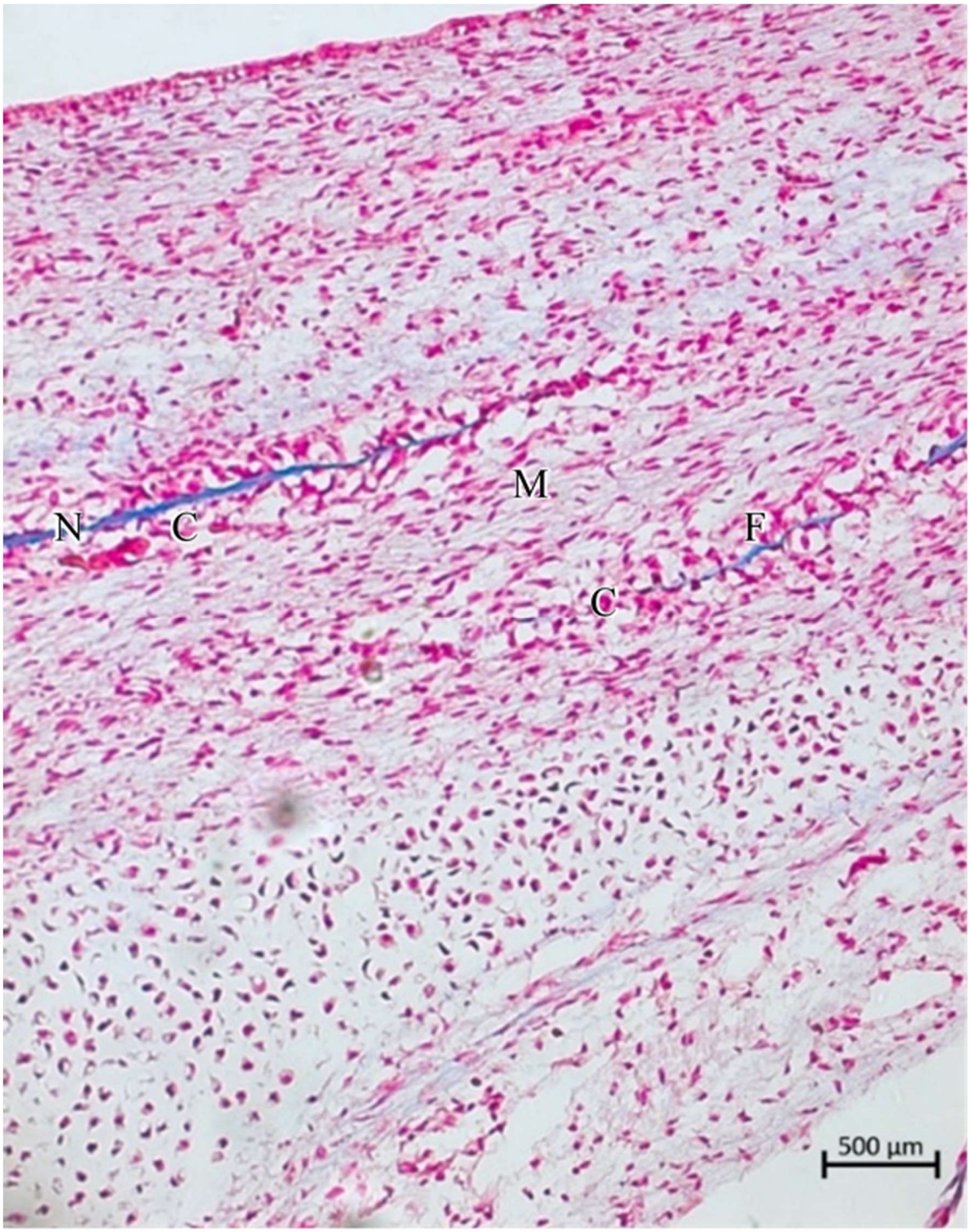

Figure 3.2: A parasagittal section of the nasal-frontal suture at stage HH37 in the developing skull of *Gallus domesticus*. The section shows the bone matrices of the nasal (N) and frontal (F) in blue, whereas the cambial layer (C) is seen surrounding the bone matrices in red. The middle vascular layer (M) is seen running parallel to the bone matrices with an abundance of fibroblasts, that appear as elongated wavy red cells.

3.4.3 The frontal-parietal suture:

The parasagittal sections of the frontal-parietal suture at stage HH42 show that the caudal end of the frontal and the rostral end of the parietal are thin, tapering layers of bone matrix, each of which is surrounded by a cambial layer. The suture is an open, beveled suture at this stage, with the frontal overlapping the parietal. In the space between the two bones elements is the middle vascular layer that contains collagen fibres that are oriented in a direction almost perpendicular to the plane of the two bones (Figure 3.3A). The overall structure of the suture at stage HH44 is very similar to that at stage HH42. However, at stage HH44 the overlap between the frontal and parietal matrices has increased, and the collagen fibres of the middle vascular layer are more disorganized within a thick vascular layer (Figure 3.3B). This is similar to what is seen at stage HH45 (Figure 3.3C).

3.4.4 The parietal-supraoccipital suture:

The parasagittal sections of the parietal-supraoccipital suture at stage HH42 show that caudal end of the parietal and rostral end of the supraoccipital appear as thick, tapering bone elements with developed trabecular structure within the bone (Figure 3.4A). The caudal end of the parietal curves ventrorostrally while the rostral end of the supraoccipital curves dorsocaudally, paralleling one another and giving the suture an open, beveled appearance at this stage. Surrounding each element is a thin cambial layer, and between the two elements is a middle vascular layer with collagen fibres oriented parallel to the long axis of this layer (Figure 3.4A). The same histological features seen at stage HH42 were also seen at stages HH44 and 45 (Figure 3.4B&C).

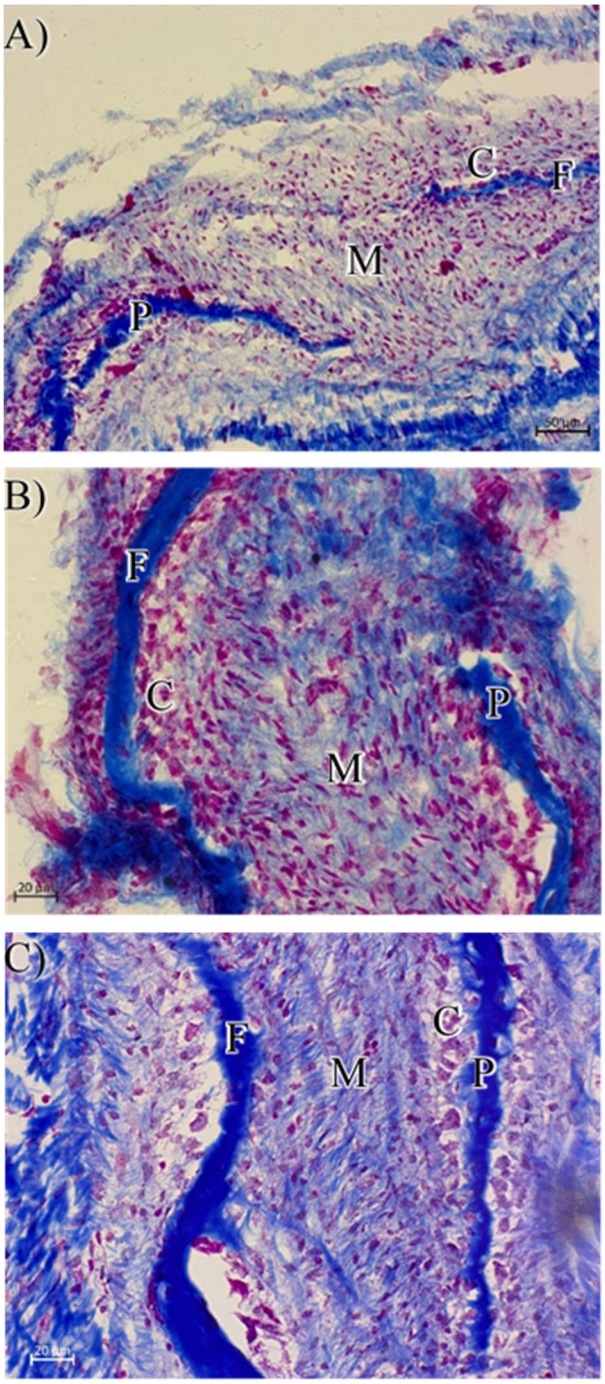

Figure 3.3: A parasagittal section of the frontal-parietal suture at three different stages in the developing *Gallus domesticus* skull. The sections show the bone matrices of the frontal (f) and parietal (P) in blue while the cambial layers (C) surrounding each of them is stained in red. The middle vascular layer (M) is seen with an abundance of fibroblasts and collagen fibers in it. The histology of the frontoparietal suture does not change markedly between stages 42 (a), 44 (b), and 45 (c).

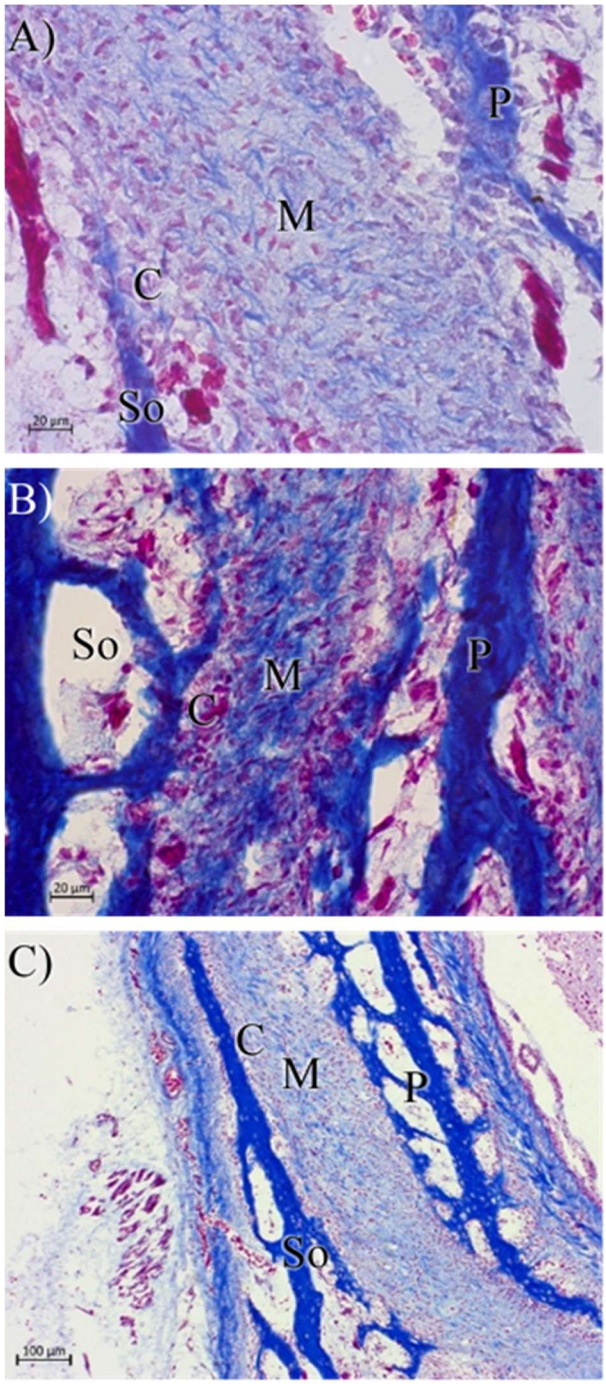

Figure 3.4: A parasagittal section of the parietal-suprapccipital suture at three different stages in the developing *Gallus domesticus* skull. The sections show the bone matrices of the parietal (P) and supraoccipital (So) in blue while the cambial layers (C) surrounding each of them is stained in red. The middle vascular layer (M) is seen with an abundance of fibroblasts and collagen fibers in it. The histology of the frontoparietal suture does not change markedly between stages 42 (A), 44 (B), and 45 (C).

3.4.5 The interpremaxillary suture:

The rostral transverse sections of the interpremaxillary suture at stage HH40 showed left and right bones as inverted V-shaped elements with a vertical, butt-joint suture between them (Figure 3.5A&C). A cambial layer surrounds the bone matrix of each premaxilla. Between the cambial layers of each premaxilla is the middle vascular layer, which contains collagen fibres and fibroblasts oriented vertically. In the rostral region of the interpremaxillary suture, the middle vascular layer is very thin, and two cambial layers are in very close proximity. The sutural bone of each element almost touches the other in places along the suture (Figure 3.5A&B). In the caudal region, the middle vascular layer remains thick, and widely separates the cambial layers of left and right premaxillaries (Figure 3.5C&D).

3.4.6 The region of presumptive bone formation:

The parasagittal sections of the caudal region of the frontal at stage HH36 allowed for the observation of a region of presumptive bone formation with no suture present. At the caudal end of the frontal, a thin layer of closely packed cells extends posteriorly beyond the limit of the bone matrix portion of the element, on the same level as the matrix (Figure 3.6). The density of the cells in the thin layer seems to decrease in the caudal direction (Figure 3.6). Above and below the dense layer of cells is a relatively thick layer of collagen fibers and fibroblasts that run parallel to it.

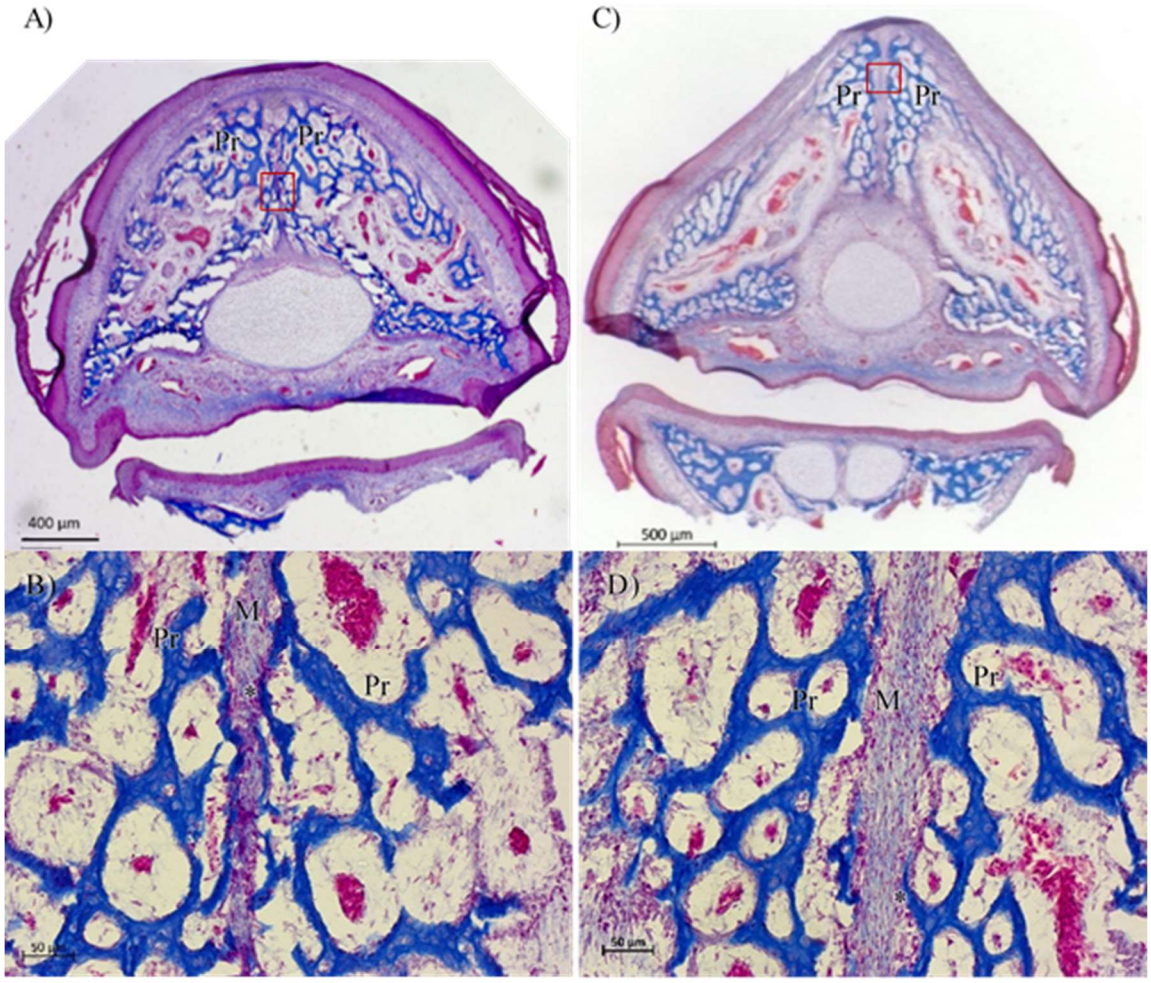

Figure 3.5: A transverse section of the interpremaxillary suture at stage HH40 in the developing *Gallus domesticus* skull. The sections show the bone matrices of the two premaxillaries (Pr) in blue while the cambial layers (*) surrounding each of them is stained in red. The middle vascular layer (M) is seen running parallel to the bone matrices with an abundance of fibroblasts and collagen fibers in it. a) rostral transverse section of the fusing premaxillaries. b) A close up of the region in the red box, showing the reduction in the thickness of the middle layer of the suture during fusing. c) caudal transverse section of the unfused premaxillaries. d) A close up of the region in the red box, showing the thickness of the middle layer in the unfused suture.

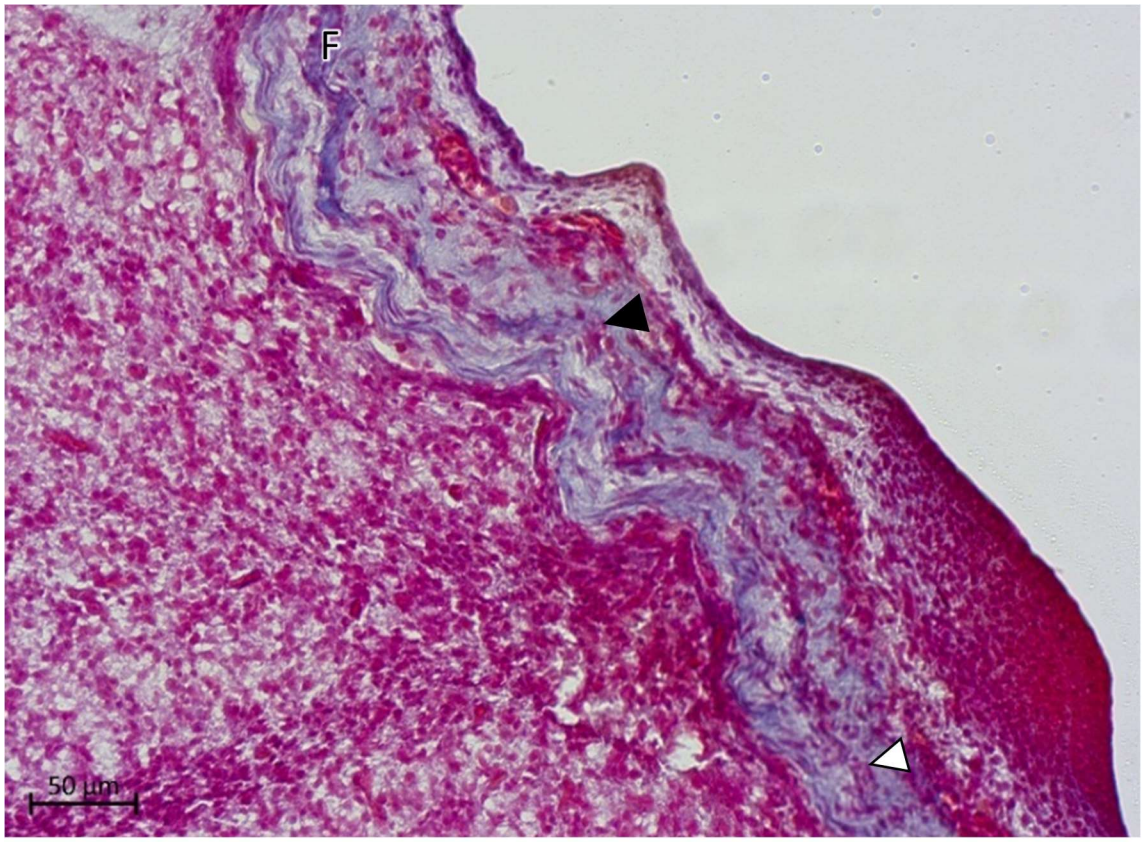

Figure 3.6: A parasagittal section of the developing caudal end of the frontal bone element at stage HH36 *Gallus domesticus* skull. The frontal bone is stain in blue (F) and proximal to it a layer of condensed cells appears stained red (black arrowhead) that gradually becomes less condense (white arrowhead) ventrally. Rostral direction is to the left.

3.4.7 The histology of the gap in the frontal:

The parasagittal sections of the developing frontal at stage HH35 yielded two separate ossification centres. The sections showed the two ossification centres of the frontal as thin blue layers of bone matrix located in the supraocular and postorbital regions. There is a thick layer of very tightly packed cells, similar to those seen in the caudal region of the frontal (Figure 3.6), which extends rostro-caudally from one ossification centre to the other, on the same level as the ossifications. A few dispersed collagen fibres were seen within the thick layer of packed cells and many collagen fibres can be seen above and below the thick layer (Figure 3.7).

3.5 Discussion:

Four sutures – frontal-nasal, frontal-parietal, parietal-supraoccipital and interpremaxillary – were observed in the developing chicken skull. As sutures in an open state, the first three sutures all contained two sutural bones (stained blue), cambial layers (stained red) and middle vascular layers (stained mostly blue). As for the fusing interpremaxillary, it contained similar histological components as these open sutures, except with only a very thin middle vascular layer and very close contact of the cambial and bone matrix surfaces as fusion was commencing. On the other hand, the gap between the two ossification centres had only one thick layer of cells connecting the two ossification centres and lacked the cambial and middle vascular layers. The thick cellular layer was sandwiched between two fibrous layers. This histological structure resembled that of the region of presumptive frontal bone formation, which also contained a thick

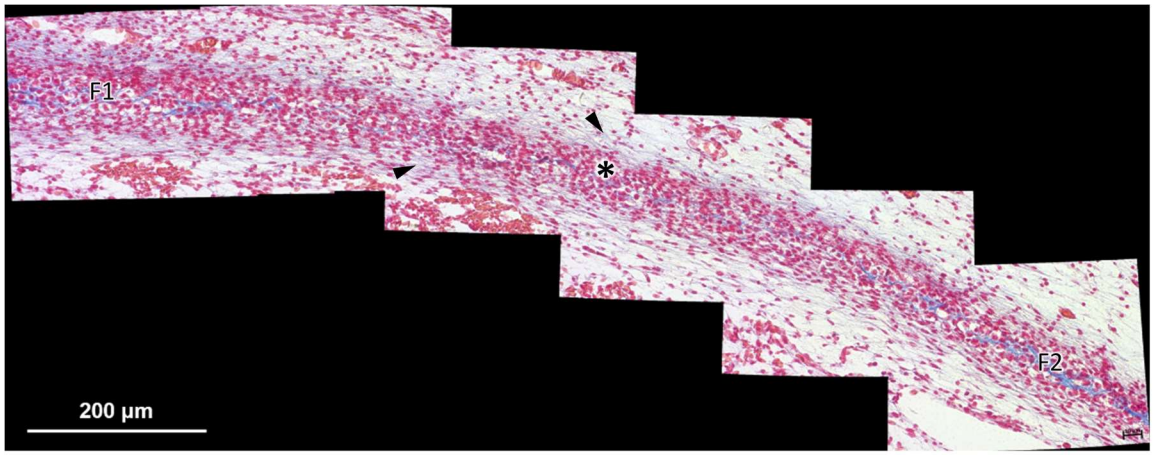

Figure 3.7: A parasagittal section of the gap between the two ossification centres of the frontal bone element at stage HH35 in the developing *Gallus domesticus* skull. The supraocular (F1) and postocular (F2) ossification centres are stained blue and between them is a layer of condensed cells (asterisk), both of which is surrounded by a layer of collagen fibers and fibroblast cells (arrow heads).

cellular layer sandwiched between two thick fibrous layers and lacked cambial and middle vascular layers. Together these observations suggest the gap between frontal ossification centres does not likely represent an open or fusing suture.

These observed morphology and histological features of the sutures examined here in chicken are overall very similar to their counterparts in other species, such as the rat (*Rattus norvegicus domestica*; Adab et al., 2002). Five days after birth, the nasal-frontal suture in rats is composed of two overlapping sutural bones (Adab et al., 2002), giving it a beveled suture morphology, like in chicken. Moreover, A layer of osteoblasts are seen surrounding the sutural bones (Adab et al., 2002), which resembles the cambial layer seen surrounding the sutural bones in the chicken skull. However, the middle vascular layer was not observed in rat, but instead a layer of mesenchymal cells, referred to as sutural mesenchyme, is present in the same location (Adab et al., 2002). The frontal-parietal suture (i.e. the coronal suture) and parietal-supraoccipital suture (i.e. the lambdoid suture) are also known to be of a beveled type in mice (Slater et al., 2008; Behr et al., 2011), which are similar to the avian ones. The morphology of the interpremaxillary suture in rats resembles the unfused portion of the chicken interpremaxillary suture in that they are both of the butt-joint type (Katsaros et al., 2006). However, the histological components of the coronal and lambdoidal sutures have not been described in mice or rats.

The histology of the gap between ossification centres has not been previously explicitly documented in any taxa. A comparison between the histology of the four selected sutures and that of the gap revealed the absence of any features that would suggest a suture is present. The absence of a middle vascular layer separating two

cambial layers and the appearance of a continuous cellular layer connecting the two ossifications, indicate that the gap is not an open suture. Moreover, the gap lacks the dense fibrous and connecting layers of a closed suture (Bailleul and Horner, 2016). The gap also did not appear to be of any of the sutural types known to occur in amniotes, which leads to the conclusion that the gap does not resemble any of the known types or states of sutures. Moreover, even the fusing portion of the interpremaxillary suture contained a middle vascular layer, albeit a very thin one, and cambial layers surrounding each premaxillary. The absence of a middle vascular and cambial layers in the gap implies that it is not a fusing suture either. In contrast, the observed similarity between the gap and the presumptive frontal bone formation leads to the conclusion that the gap is probably a region of active bone formation. Furthermore, it strongly suggests that the avian frontal bone is not a frontoparietal as it does not appear to be the product of fusion of two separate bone elements.

3.5.1 The development of the avian frontal

Whether the avian frontal is frontoparietal or simply a frontal, the timing its formation seems to be at odds with earlier studies. Smith-Paredes et al. (2018) have shown that two separate ossification centres appear at stage HH36, which is similar to the results I have obtained (Chapter 2). At the histological level, however, the two separate ossification centres were visible at stage HH35, which is a day earlier than HH36. The timing of ossification of the frontal has so far been determined using alizarin red staining, which stains calcium crystals (Puchtler et al., 1969) but the use of aniline blue dye in the Masson's trichrome stain used here might be capturing an earlier stage of ossification, that of osteoid formation. Aniline blue stains collagen fibers blue (Sheenan and

Hrapchak, 1980), which is a major component of the osteoid that forms before calcification of presumptive bone tissue (Scott-Savage and Hall, 1979). If the aniline blue dye is staining osteoid, then frontal bone formation begins at or earlier than stage HH35 and that the frontal bone element forms from two separate regions of osteoid. The two regions of osteoid, the supraocular and postorbital ones, then become calcified at stage HH36 forming two ossification centres, as indicated by alizarin staining, and the two ossification centres become linked to one another during stage HH36 or HH37. This developmental scenario needs to be confirmed by further examination by future studies.

Despite the lack of knowledge about ossification centre linkage, the occurrence of multiple ossification centres in the frontal bone region has been documented in other taxa besides avians. In zebrafish, the frontal is comprised of two ossification centres (Kague et al., 2012), similar to that of chicken. Interestingly, the rostral ossification centre of the frontal is derived from neural crest cells and the caudal centre is derived from mesodermal cells (Kague et al., 2012), which is highly similar to the derivation of the chicken frontal. Frogs possess a frontoparietal, which is considered a fusion of an ancestral frontal and parietal (Rocek, 1988). In *Xenopus laevis* the frontoparietal develops from a pair of rostrocaudally-elongate ossification centres that eventually fuse into a single bone element (Slater et al., 2009), thereby obscuring signs of discrete ossification centres homologous with a frontal or parietal. However, in experiments on the yellow-bellied toad (*Bombina variegata*), the inhibition of thyroid hormone (TH) by thiourea led to the appearance of three ossification centres in the frontoparietal region, as opposed to the single ossification centre seen in normal development of that species (Smirnov and Vassilieva, 2009). The first two ossifications were interpreted as corresponding to the

frontal portion and the third is interpreted as corresponding to the parietal portion of the compound frontoparietal (Smirnov and Vassilieva, 2009). In the common frog (*Rana temporaria*), TH inhibition led to the appearance of only two ossification centres, one interpreted as corresponding to the frontal and the other to the parietal in that species (Smirnov and Vassilieva, 2009). Detailed histological analysis of frog frontoparietal bone development would make for useful comparisons with that of the avian frontal; however, the histology of these structures has not been examined to date. Furthermore, the Smirnov and Vassilieva (2009) study begs the question as to what the effect of TH is on the number of ossification centres in birds, for it is known that some taxa, such as members of the palaeognathae clade, only have only one frontal ossification centre (Maxwell, 2009).

3.5.2 The homology of the avian frontal bone element

Regardless of the causes of the variation in the number of frontal ossification centres, the naming of the avian frontal bone element as a frontal is now supported by a combination of topological and developmental features. The position of the element caudal to the nasal, dorsal and dorsocaudal to the orbits, and dorsal to the forebrain indicates that the element is mostly likely homologous with the similarly-named bone element of other tetrapod taxa (Romer, 1956; Fabbri et al., 2017). Moreover, the conserved position of the frontoparietal suture dorsal to the boundary between the fore- and midbrains across reptilia (Fabbri et al., 2017), along with the absence of a suture between the two frontal ossification centres, indicate that it is not a fused frontoparietal. However, one aspect of the development of the avian frontal that remains at odds with this interpretation – the mesodermal origin of the postorbital ossification. This might be

explained as a form of developmental system drift, in which a homologous structure in two different lineages develop in different ways (Piekarski et al., 2014). Further investigation is needed to verify this inference.

However, the use of a multitude of approaches in establishing homology might be a better approach than the use of singular criterion. For example, absence of a suture between the two ossification centres of the frontal bone sheds doubt on the singular use of ossification centres for questions of bone homology as done by Smith-Paredes et al. (2018). However, the results obtained in this Chapter agree with the conclusions of the study by Smith-Paredes et al. (2018) in that the avian frontal is not a frontoparietal. However, there, Smith-Paredes et al. (2018) used whole-mount cleared and stained chicken and alligator skulls to argue that the avian postorbital ossification is instead homologous to the postorbital bone element in alligators. The argument was based on comparing the location and shape of the postorbital bone element in alligators with the postorbital ossification of the frontal in chicken (Smith-Paredes et al., 2018). The authors acknowledge that the postorbital ossification in chicken does not end up forming a separate bone element, as might be predicted if it were the postorbital bone, but a criterion was not discussed that can be used to define what a separate bone element is. Thus, under their argumentation, the postorbital bone element fuses with the frontal. Interestingly, they then argued that the lacrimal bone in both alligators and most avians develops from two ossification centres that then fuse with one another (Smith-Paredes et al., 2018). This interpretation assumes that a complete understanding of the homology of lacrimal (including its developmental history) exists for alligator. In a sense, these authors use the same observation to make vastly different interpretations of bone

homology. The results of my study suggest that care must be taken when interpreting observations of developmental ossification that may, at first glance, appear to be intuitive. Additionally, these interpretations could be further complicated by the possibility that ossification centres might form (or not) for reasons that are not related to homology, such as TH levels in the embryo (Smirnov and Vassilieva, 2009). The use of suture presence/absence, and investigation of their presence under different TH levels, might complement the use of ossification centres in answering questions of homology. The presence of a suture between the two ossification centres of the lacrimal may shed light on whether or not it is a fused element.

The singular use of sutures to distinguish between a fused bone or a singular bone element is one of the limitations of this study. For example, bone matrices may partially fuse with each other, which could lead to the two partially-fused elements receiving different names. An example of partial fusion is the cranial dome of pachycephalosaurids, in which the outermost portion of the frontal-parietal suture is fused (no evidence on the surface), whereas internally a suture remains visible (Evans et al., 2018). However, the degree to which the two elements are fused that warrants naming the dome a frontoparietal is difficult to define. Moreover, if the frontal and parietal elements are entirely fused, as in *Xenopus* (Piekarski et al., 2014), then referring to portions of the frontoparietal as frontal or parietal is difficult to do with certainty without embryological data. If the embryological data has shown two separate ossification centres with a suture between them, then that would support the name given to the amphibian frontoparietal (Piekarski et al., 2014).

Another limitation of this study is the use of a single replicate per stage, which assumes the absence of intraspecific variation in suture histology. Moreover, the ontogeny of each suture was not examined from presumptive suture formation until fusion, which makes the comparison with the gap between the frontal ossification centres incomplete. These limitations indicate the need for detailed histological examination of the ontogeny of the sutures in chickens to be carried out in future studies.

3.5.3 Summary

In summary, comparative fate mapping studies have hypothesized that the avian frontal is a frontoparietal. However, since the frontal and parietal are two separate elements, then a suture should appear between them. The comparison between the histology of the four known sutures in the avian skull with that of the gap between the two ossification centres of the frontal has indicated that the gap does not resemble any of those sutures. Moreover, the interpremaxillary suture provided a histological examination of a closing suture, which also did not resemble the frontal gap. On the other hand, the frontal gap most closely resembled the caudal region of the forming frontal, where no suture is present. These observations suggest that the avian frontal is not likely a fused frontoparietal element. The limitations of the singular use of ossification centres, sutures or developmental origins indicate that multiple lines of evidence need to be considered together for the homology of a cranial element to be determined. These include gene regulatory networks, ossification centres comparisons, suture examinations, topological relations, and function. If most of these lines of evidence support a particular homological relationship, then the homology of a cranial element can be established.

Chapter 4: Conclusion

The debate regarding the homology of the avian frontal began with comparative fate mapping of the chicken, mouse and axolotl skulls (Evans & Noden, 2006; Maddin et al., 2016). The mouse and axolotl fate maps revealed that the frontal element is derived from neural crest cells, while the parietal element is derived from mesodermal cells (Jiang et al., 2002; Maddin et al., 2016). In chicken the frontal is derived from both neural crest and mesodermal cells (Evans & Noden, 2006). This developmental comparison led to the hypothesis that the avian frontal is a fused frontoparietal element (Maddin et al., 2016). The frontoparietal hypothesis contradicts the traditional nomenclature of the frontal element as a frontal. The traditional nomenclature is based on the location of the frontal in the skull and its relations to other elements in the skull (Romer, 1956). Moreover, the traditional avian nomenclature has recently been supported by geometric morphometric and comparative archosaurian developmental data (Fabbri et al., 2017; Smith-Paredes et al., 2018). With the aim of contributing novel data to this issue, an investigation based on the hypothesis that a suture would be present if the frontal was indeed a fusion of the frontal and parietal, was conducted.

In Chapter 2, the search for a suture in the gap between the two ossification centres of the frontal element began with the production of a staging table for the skull of the chicken. The staging table revealed that most of the elements of the skull form between stages HH35 and HH45, and that most of them reach their final shape in roughly five days. The staging table showed that elements of the upper jaw and the ventral side of the skull begin to ossify first, before the skull roof elements. Moreover, the prefrontal was found to take a period of three stages to take its final shape, whereas the frontal

spanned nine stages. Crucially, this staging table revealed that the gap between ossification centres of the frontal might be found between stages HH36-37. However, the heavy sampling during these stages revealed a great deal of variation in frontal formation, where most skulls contained a large singular frontal ossification that spanned both the supraocular and postorbital regions, but some others had either a single or no ossifications. The large amount of variation in the timing of the formation of the frontal hindered the identification of the exact stage when the gap could be found. However, the observed variation could be due differences in staining and/or reflect copious amount of natural intraspecific variation in frontal ossification.

In Chapter 3, a specimen at stage HH35 was found with two separate ossification centres present in histological sections. There, the gap between the two ossification centres contained a thick layer of condensing cells that connects the two ossification centres and two (dorsal and ventral) layers of collagen fibers. These histological features are different from those observed in the four known sutures examined. All of the four sutures contained two cambial layers that surround the bone matrix and possessed a middle vascular layer containing collagen fibres. In contrast, the presumptive region of bone formation contained a layer of condensed cells and two (dorsal and ventral) layers of collagen fibers. These results imply that the gap does not contain a suture as expected if the two ossifications centres are two separate bone elements, frontal and parietal, that fused with each other. This interpretation is further supported by the presence of a middle vascular layer in the fusing interpremaxillary suture, which was not seen between the ossification centres in the frontal.

The conclusion that can be derived from all these observations is that the frontal of chicken develops from the fusion of two ossification centres (and not bone elements) at stages HH36-37, and it is not likely to be a frontoparietal. The conclusion supports the hypothesis of the loss of the postparietal in archosaurs (Fabbri et al., 2017), and the current interpretation of the elements of the archosaurian cranial roof as frontal, parietal and supraoccipital (Fabbri et al., 2017). Moreover, the conclusion leads to a possible scenario of the evolution of the theropodian frontal. The frontal in basal theropods (such as *Tyrannosaurus* rex) is likely to have been derived from neural crest cells, and the parietal from mesodermal cells. This hypothesis is based on the relative conservation of the vertebrate skull fate map, and it requires verification through an examination of alligator cranial development. However, as the cerebrum increased in size during the evolution of birds (Balanoff et al., 2013), the frontal bone increased the use of mesodermal cells to accommodate the expanding cerebrum. This latter hypothesis could be verified by examining the co-development of the brain and skull roof in different species of birds. Bird species with relatively small cerebrum might have a small mesodermal contribution to their developing frontal, compared to bird species with larger cerebrums.

Appendices

Appendix A : Staining protocols and recipes

A.1 Staining protocols:

A modified version of Masson Trichrome stain for connective tissue was used (Sheehan and Hrapchak, 1980):

1- Slides were placed in dehydration ethanol series (25%,50%,75%,100%) 5 minutes each

2- Incubation in Bouin's Fixative solution (#HT10132, Sigma) overnight

3- Placed in distilled water until the yellow color makes faint for approximately 6 hours.

4- Placed in Mayer's Hematoxylin Solution (#MHS1, Sigma) for 5 minutes

5- Rinsed in distilled water

6- Placed in Xylidine ponceau and Acid fuchsin solution for 15 minutes.

7- Rinsed in distilled water

8- Placed in 1% Phosphomolybdic acid solution for 15 minutes.

9- Stained with Aniline Blue Solution for 15 minutes

10- Rinsed in distilled water

11- Placed in 1% Acetic acid for 5 minutes

12- Dehydrated in 95% ethanol and 100% ethanol twice. Then placed in Xylene twice for a minute each.

13- Mount the slide using a permount solution (#SP15500, Fisher Scientific) and place a coverslip on top of them.

A.2 Recipes:

Xylidine ponceau and Acid Fuchsin solution (100 mL):

100 µl glacial acetic acid

25 mg xylidine ponceau (#190260250, Fisher Scientific).

25 mg Acid fushin (#400210250, Fisher Scientific).

Fill up to 100 mL with water

Phosphomolybdic acid solution (100 mL):

100 mg phosphomolybdic acid (#A237100, Fisher Scientific)

Fill up to 100 mL with water

Anline Blue solution (100 mL):

0.2 g Anline Blue (#A96725, Fisher Scientific)

200 µL glacial acetic acid

Fill up to 100 mL with water

Appendix B : Replicates table

Table 2: The number of replicates per stage for establishing the chicken skull staging table.

Hamburger and Hamilton Stage	Number of replicates
HH35	2
HH36	31
HH37	32
HH38	3
HH39	2

HH40	3
HH41	3
HH42	1
HH43	4
HH44	2
HH45	2

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