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Morphological diversity of integumentary traits in fowl domestication: insights from disparity analysis and embryonic development

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ABSTRACT

The domestication of the fowl resulted in a large diversity of integumental structures in chicken breeds. Several integumental traits have been investigated from a developmental genetics perspective. However, their distribution among breeds and their developmental morphology remain unexplored. We constructed a discrete trait-breed matrix and conducted a disparity analysis to investigate the variation of these structures in chicken breeds; 20 integumental traits of 72 chicken breeds and the red junglefowl were assessed. The analyses resulted in slight groupings of breed types comparable to standard breed classification based on artificial selection and chicken type use. The red junglefowl groups together with bantams and European breeds. We provide new data on the red junglefowl and four chicken breeds, demonstrating where and when variation arises during embryonic development. We document variation in developmental timing of the egg tooth and feather formation, as well as other kinds of developmental patterning as in the anlagen of different type of combs. Changes in epithelial-mesenchymal signalling interactions may drive the highly diverse integument in chickens. Experimental and comparative work has revealed that the cranial neural crest mesenchyme mediates its interactions with the overlying epithelium and is the likely source of patterning that generates diversity in integumental structures.

1 Introduction

1.1 Chicken breeds

Among birds, chickens (*Gallus gallus domesticus*) are the most important source of protein for many societies all over the world (Crawford, 1990; Al-Nasser et al., 2007; Eda et al., 2016; Bennett et al., 2018). Current ideas on when and how fowl domestication occurred continue to be revised.

Traditionally, domestication of fowl has been hypothesized to have happened around 6000–5000 B.C.E. (Crawford, 1990). A proposed earlier event of domestication around 8000 B.C.E. in northern China, based on mtDNA analysis (Xiang et al., 2014), remains debated (Xiang et al., 2014; Peters et al., 2015; Xiang et al., 2015; Eda et al., 2016). The main ancestor of domestic chicken is the red junglefowl (*Gallus gallus*), but a contribution from at least two other *Gallus* species (Grey junglefowl: *Gallus sonneratii* and Sri Lankan junglefowl: *Gallus lafayetii*) has been postulated (Nishibori et al., 2005; Eriksson et al., 2008; Loog et al., 2017). Several "domestication centres" of chicken have been identified in South and South-East Asia (Tixier-Boichard et al., 2011). The number of chicken breeds is estimated as hundreds, and varieties count in the thousands (Ekarius, 2007), but there is no international commission or standard that regulates the naming and definition of all breeds. Regional poultry breeding societies publish standards where the desired traits for exhibition birds are listed and breeds are grouped into classes. For example, the American Poultry Association (APA) provides periodic descriptions of its recognized breeds in The American Standard of Perfection (ASoP), currently in its 44th edition (American Poultry Association, 2015); the ASoP groups chicken breeds into classes according to geographical origin. In contrast, the British classification system is based on feather hardness, breed rarity and size (e.g., bantam breeds are represented by the small size version of standard breeds, however there are "true bantams" breeds with no large breed equivalent (Scrivener, 2008)). As Darwin (1868) did in his book *The Variation of Animals and Plants under Domestication*, one could trace back the antiquity of certain breeds of chicken following historical records. This was also accomplished by Aldrovandi around 1600 C.E., which has been translated from the Latin and summarized in Lind (1963).

The origins and relationships of breeds are not a straightforward story. Many attempts, mainly from a genetic perspective, have failed to provide a plausible phylogenetic/historical network

framework. Some investigations have found non “genetic distance” between breeds and red junglefowl using microsatellite markers (Kumar et al., 2015) and others, by morphological and genetic evaluation, have surmised that egg-type breeds (mainly European breeds) would be the closest relative to red junglefowl (Niu et al., 2002; Moiseyeva et al., 2003). Genetic analyses and historical records do suggest that the current breeds were established to a large extent by a “second wave” import of chickens from Asia to Europe in the 19th century, therefore a breed framework nowadays would be the result of continuous hybridizations among European and Asiatic breeds (Rubin et al., 2010; Flink et al., 2014). Moreover, the records of some breeds are kept as proprietary information by commercial companies, and interbreeding/crossbreeding experiments are uncommon (Tixier-Boichard et al., 2012). Therefore, historical details on the relationships of breeds, mentioned in the standards and other sources, have been the only way to provide a framework of chicken relationships. Figure 1 depicts such information as a network of the Continental and the English classes taken from the ASoP (Association, 2015), the Rassengeflügel-Standard für Europa (Bund Deutscher Rassegeflügelzüchter, 2016), as well as further literature on poultry breeds (Ekarius, 2007; Scrivener, 2008; Damerow, 2012). This scheme is in concordance with the hypothesis of high hybridization among breeds and with the latter as a mechanism of breed creation, as is well known for domestic dogs (Lord et al., 2017).

1.2 Integumental traits: variation and development

As recognized by Darwin (1868) much diversity in chicken breeds has been generated through domestication. Diversity caused by domestication has been quantified and treated in several other works, but this has been largely restricted to mammals and especially to skulls (Drake & Klingenberg, 2010; Evin et al., 2017; Sánchez-Villagra et al., 2017; Heck et al., 2018; Veitschegger et al., 2018) and/or their constituent parts (Schweizer et al., 2017). Concerning birds, pigeon (Young et al., 2017) and chicken skulls have been recently studied (Stange et al.,

2018). Chicken breeds span a vast amount of morphological (Figure 2) and genetic diversity, however the latter is now decreasing because of a strong focus on selection for commercialization (Muir et al., 2008). An accessible way to investigate the variation of traits among chicken breeds is to focus on the integument, as breed representatives are always depicted, and their integumental features are described meticulously in the exhibition standards (e.g. ASoP). In birds, integumentary structures include the skin, feathers, scales and horns (Lucas & Stettenheim, 1972). Integumentary traits, also, have been paramount to the worldwide distribution and success of chicken breeds. Due to the notably vascularised dermis of the comb and wattles, breeds can become dispersed to suitable climates, as the size and surface area of these integumentary structures presumably relate to climate tolerance. For instance, large combs are prone to frostbite (therefore rare in countries with cold climates), but common in Mediterranean breeds, along with large wattles and earlobes. Furthermore, game birds often have small or absent wattles, which apparently minimises blood loss in cases of cock fight-evoked injuries (Damerow, 2012).

Among modern birds, skin outgrowths are variably present (Stettenheim, 2000). In Galliformes, wattles and combs are conspicuous in junglefowls (including chickens) and are also present in other members of Phasianidae such as *Crossoptilon*, *Lophura*, *Phasianus* and *Syrmaticus* (Wang et al., 2013a). Wattles are the most common soft integumentary outgrowths and, apart from junglefowls, they are also present in several species of Casuariidae, Megapodiidae, Numididae, Gruidae, Cathartidae, Meliphagidae, among others (Stettenheim, 2000). Either wattles or combs can be present, or both. For example, in Megapodiidae both structures can be present (Figure 3a), whereas in Cracidae only small wattles occur and instead of a comb, long feathers (crest) are present up to the beak (Figure 3b). At least in Galliformes, these skin outgrowths are typically found in both sexes, but these traits are generally dimorphic, with males often exhibiting larger and more brightly coloured outgrowths (Kimball et al., 2011). Besides, even though these

structures are present at hatching and are traceable in development, at the juvenile-adult transition (maturation) they go through growth and a reorganization process mediated by hormone effects in these tissues (Hamilton, 1952). In addition, it is common in birds to find variable feathering patterns throughout the body: micropatterns and macropatterns (Boer et al., 2017). Micropatterns are variations in the kind of integumental features (as in types of feathers); there are different types in different zones of the body. Macropatterns relate to integumental organization in certain parts of the body, like the absence or presence of feathers in certain areas, as is the case in the “naked neck” chicken breeds (Mou et al., 2011) and ptarmigans (*Lagopus*), in which foot feathers are common even in juveniles (Figure 3c). Foot feathers are present in some chicken breeds as well; Ukokkei (Silkie) chickens show this pattern during embryogenesis; the onset starting around at 11 days of development (Figure 3d), then foot feathers grow (Figure 3e) to become a characteristic feature of the adult phenotype in this breed (Figure 3f).

Chicken integumentary traits have been traditionally studied through a genetic perspective, and the occurrence of specific traits in certain breeds (mainly bred for commercial and exhibition interests) has led the research agenda (Stevens, 1991). Table 1 presents an overview of the genetic basis of the integument traits analysed/discussed in this work. Since the seminal works by Bateson in chickens (Patrick, 2002), the first animal in which Mendelian inheritance was evaluated (comb inheritance), we know that a direct relation “one gene, one trait” is rarely sufficient to explain integumental chicken variation; pleiotropic effects seem to be the rule (Dorshorst et al., 2010; Johnsson et al., 2012; Ng et al., 2012). Similarly, genetic linkage has been well studied in chickens, including sex-linkage scenarios, where integumental traits may segregate with other kinds of characters, as is the case of pea comb and blue eggs (Bitgood, 1985), or the presence of a gene in the sex chromosomes, as is the case of the sex-linked barring (*B*) phenotype in Barred Plymouth Rock chickens (Spillman, 1908).

Table 1 summarizes the interactions and roles of neural crest during development of the integumentary traits studied in the present work. Much of that information derives from experimental embryology, which has demonstrated that epithelial-mesenchymal signalling interactions are the essential basic mechanisms required for the formation and patterning of the vertebrate integument (Schneider, 2005; Schneider, 2018b). Particularly, the tissue layer that induces many of the integumental structures in vertebrates, and consequently in chickens, is the dermis (Dhouailly, 1974). For example, the seminal work of Saunders (Cairns & Saunders, 1954; Saunders & Gasseling, 1957), which involved grafting and assessing the interactions between different body regions of chickens throughout development, provided a foundation for further developmental studies on this matter (Boer et al., 2017). Traits such as combs, wattles, egg teeth, and feathers are appendages that form via outgrowth of the ectodermal epidermis (i.e., epithelium), and are induced and patterned by the prospective dermis (i.e., mesenchyme) (Eames & Schneider, 2005). However, the dermis comes from different origins: neural crest in the head or mesoderm in the limbs and trunk (Schneider, 2005; Noden & Schneider, 2006). For example, the dermis of the limbs originates from the somatopleural component of the lateral plate mesoderm (Mauger, 1972a; 1972b). Conversely, the facial and cranial dermis derive from neural crest mesenchyme (Noden, 1988; Le Douarin et al., 1993), whereas in the otic and occipital regions as well as the posterior cranial vault, the dermis comes from the mesodermal mesenchyme (Couly et al., 1992). The inductive role played by the dermis has been well studied. For instance, the egg tooth is a thickened keratinized structure present around the distal tip of the upper beak surface in birds, mostly recognizable at the stage of hatching (used by hatchlings to tear the egg surface at pipping) (Clark, 1961). The egg tooth is a transitory structure and its presence and shape are variable among birds (Hamburger & Hamilton, 1951; Romanoff, 1960). According to the degree of maturity at hatching (altricial/precocial), the egg tooth can appear at

different developmental stages (Clark, 1961). Neural crest-derived dermis through its interactions with the overlying epidermis, controls the species-specific patterning of the egg tooth, as revealed by transplants of neural crest between quail and duck embryos (Schneider & Helms, 2003). Quail have an egg tooth at the tip of their beak that is a conical protrusion of hard keratin whereas duck have a leathery and flat epidermal nail (Lucas & Stettenheim, 1972). In quail-duck chimeras, which are generated by transplanting cranial neural crest cells that give rise to the dermis, the egg tooth takes on the morphological identity of the donor species despite arising entirely from non-transplanted host epidermis (Schneider & Helms, 2003; Schneider, 2005; Fish & Schneider, 2014; Schneider, 2018b).

In chickens, as in other vertebrates, colours mostly come either from the accumulation of carotenoids or from the activity of melanocytes (eumelanin [black] and phaeomelanin [red]), the latter being neural crest derivatives (Stevens, 1991). Additionally, some contributions to pigmentation come from structural colour and the so-called “uncommon colours”; as pterins, porphyns, psittacofulvins (Li et al., 2005; Hill & McGraw, 2006). Melanocytes affect the colour patterns in chickens during embryogenesis and before sexual maturity. After emigration from the neural tube, neural crest cells arrive in the base of the feather papillae, where the melanoblasts (pre-melanocyte) accumulate. Then, in the collar of the feather, melanocyte cellular projections transfer the pigments to keratinocytes (Wu et al., 2004), and eventually to the protruding feather (Stevens, 1991). Melanocyte pigment production is dependent upon the Golgi apparatus and smooth endoplasmic activities. Perturbations to this delicate process appear to correlate with allelic alterations at certain loci (e.g., “extended black” loci [*E*]) (Davila et al., 2014). For example, the characteristic white feathering present in White Leghorns is the result of a lack of pigment production, likely because of a loss-of-function mutation in the melanocortin 1-receptor (*MC1R*), which is responsible for the accumulation of the eumelanin precursor (Tyrosine) in the cytoplasm

of melanocytes (Kerje et al., 2003). Remarkably, this is not the only way to make a “white feather” chicken, for example Silkies lack melanocytes in their feathers, which makes their plumage white (Kuklenski, 1915).

In chickens, the ancestral colour of the skin (i.e., in red junglefowl) is associated with the presence of an enzyme encoded by the dominant *BCDO2* allele, which breaks the carotenoids in the skin and prevents their accumulation (Eriksson et al., 2008). The recessive allele of this gene has been proposed to underlie the grey junglefowl phenotype (yellow skin). When present in modern chickens (homozygosis), this allele affects carotenoid deposition and produces a characteristically yellow skin colour in the epidermis (Rubin et al., 2010). However, in chickens such as those of the Gushi breed, multiple shank colour can be expressed (white, yellow, green and grey). This condition is associated with the sex-linked inhibitor of dermal melanin (Id) (Xu et al., 2017).

Silkie chickens have provided essential information for understanding integumental colour variation within chickens. Their hyperpigmentation, feathered legs, and other traits have been traced genetically, and some of their development has been well studied too (Dorshorst et al., 2010). Hyperpigmentation in this breed (the skin and almost all of the internal organs are black) is due to a particular migratory pattern of neural crest cells, with pigment producing melanocytes populating body zones beyond that observed (e.g., ventral side of the body) in the fowl and in other chicken breeds (e.g., White Leghorn) (Reedy et al., 1999). Lastly, skin colour offers a good example of pleiotropy in chickens. The same factor, agouti signalling peptide (ASIP), which inhibits melanogenesis and regulates skin colour in vertebrates in general, also has been proposed as an appetite stimulant in mammals (Norris & Carr, 2013). Comparing expression of this gene between fowl and White Leghorn reveals that levels are higher in the domesticated form

(Fallahshahroudi et al., 2018). Therefore, this gene could be another factor affecting skin colour, as well as size variation in chickens.

Our goal in the present study is to discuss mechanisms underlying integumentary variation in chicken breeds. We aim to build upon the well documented examples that link genetic variation with epithelial-mesenchyme interactions (Wright et al., 2009; Boije et al., 2012). For this purpose, we clarify when and where integumental traits vary in the red junglefowl and in chickens, as represented by four diverse breeds, as well as define specific “developmental windows” as a means to analyze integument structures in an established comparative morphological context. We propose three developmental morphological backgrounds whereby epithelial-mesenchymal interactions experiments can be conducted. Likewise, we present a discrete trait-breed matrix and a disparity analysis of adult male chicken breeds and red jungle fowl integumental traits, which illustrates the complex nature of the association and distribution of variation in the integument.

2 Results

2.1 Disparity of the integument

To illustrate morphological diversity from easily accessible data, we compiled a dataset of integument traits from the literature. Examples of the traits include comb, earlobe and wattle shape and colour, feathering in head and legs, feather hardness, and the colours of beak, egg shell, eyes, skin and shanks (see Table 1). The first two axes of the Principal Components Analysis (Fig. 4) capture 20.07% of the variation (PC1: 11.62% of variation, PC2: 8.45% of variation). Clusters of All Other Standard Breeds (AOSB, light red) and of the American class (Light brown) were recognised. The Continental (Light blue), English (Purple) and Mediterranean

(Light green) class breeds clustered together. Subsequently this is referred to as the European cluster where applicable. The unclassified breeds (black) were distributed in concordance with the latter as well as with AOSB, but only marginal with the American class breeds. The Asiatic class grouped in the lower half of the plot together with the American class and the European cluster. The red junglefowl (Red) is placed with the European cluster (lower half of the plot). The breed utility was superimposed to the ASoP classification system (Figure 4). By applying the single utility type per breed, no distinctive grouping of breeds could be observed.

The classification according to the British system shows a grouping of the hard-feathered breeds (Light red) in the upper two thirds and heavy soft feathered breeds (Light brown) are grouped in the lower thirds of the scatter plot (Figure 5). The light soft feathered breeds (Light green) occur dispersed, but not in the lower left quarter of the plot. Rare breeds and true bantams (Light blue and magenta) appear ungrouped in the whole range of disparity, however the majority of true bantams (five of seven) cluster close to the red junglefowl.

2.2 Development

Egg tooth

In chickens, the onset of the egg-tooth is observed around 6.5 days of development (HH30), while at one stage prior, this protruding structure is not present (Hamburger & Hamilton, 1951). When recording the development of the egg tooth between 6-7 days of development, we found that this structure develops earlier in red junglefowls (HH29; four specimens of six) than in most chickens (HH30), but there is variation. In the Ukokkei embryos, the egg tooth onset resembles that of the red junglefowls in one specimen of three (Figure 6). At stage HH30, the egg tooth is present in all

the red junglefowl embryos and more conspicuous than in chicken embryos when this structure is present at the same stage (Figure 6). In most chicken embryos, the earliest onset of the egg tooth occurs at stage HH30; in Shamo (one of two specimens) this structure was not present at this stage.

Thigh feathers

Thigh feather tracts are oriented parallel to the body of the embryo, located near the joint between the femur and the tibia (Holmes, 1935). Subsequently, these rows give rise to the characteristic crossing pattern drawing squares present in the middle of the thighs (Lucas & Stettenheim, 1972). Red junglefowl embryos exhibit more complex integumental pigmentation than chicken embryos. For instance, at 11 days of embryonic development (HH37) thigh feathers are larger (or more developed) and show black and reddish pigmentation, whereas the Hubbard and White Leghorn embryos present smaller feathers and almost no pigmentation (Figure 7). The Ukokkei and Shamo embryos present larger feathers, resembling red junglefowl embryos; in contrast, Shamo shows black pigmentation and Ukokkei no pigmentation (Figure 7). Prior to hatching, this distinction is even more conspicuous. Chicken embryos display mainly one-colour pigmentation; red junglefowl embryos exhibit a mixed pattern, with black pigmentation across the eye (present in shamo embryos too [Figure 8,b]), thighs and dorsal axis; while reddish pigmentation is present mostly in the head, shoulders and ventral axis.

Combs

Common throughout modern birds, this integumental outgrowth appears in chickens as a single comb (red junglefowl and some breeds, wild-type) or consists of varieties including pea, rose, walnut, and v-shaped (Lucas & Stettenheim, 1972) (see Table 1). These types of comb have been traditionally described from a genetic perspective. For instance, the single comb comprising one

row of tissue along the dorsal side of the head, differs from the pea comb in showing mainly three longitudinal rows of tissue, due to the presence of the *Pea-comb* dominant (*P*) allele (Stevens, 1991).

The comb is noticeable at 6-7 days of development in both the chicken and red junglefowl. Morphological differences related to each kind of comb can be distinguished at around 16 days of development among fowls and chicken embryos. Red junglefowl embryos show the single serrated anatomy, whereas Shamo embryos show a central tissue row with two main and several lateral parallel smaller outgrowths (Boije et al., 2012). In the case of the Ukokkei embryos, a more complex structure is formed. Transverse rows of tissue are divided in an anterior part, resulting in a lump. The latter is the precursor of the walnut phenotype (Figure 9).

3 Discussion

3.1 Disparity analysis

Morphological diversity of chicken breeds, as illustrated by the integument in the present study, reveals groupings that are in line with geographical and particular functional classifications. We realize that the use of discrete character data may not be the optimal approach to understand morphological diversity using a disparity analysis, as discussed in (Gerber, 2018), but the method integrates information that is easy to gather, provides a way of documenting and comparing traits in an exploratory way, and can be useful for generating hypotheses on underlying developmental mechanisms.

The American class breeds form a group that is distinguishable from the European cluster and AOSB. There is an indication that the skin colouration, which is mostly yellow in American class

breeds, is a main driver of this cluster under the premise that pale pink is the ordinary skin colour (Ancestral condition) (Stettenheim, 2000; American Poultry Association, 2015). If only integument characters are considered, then the AOSB group in the upper half of the disparity plot depicts a clear separation from the other classes, with only the Phoenix breed clustering with the European group and red junglefowl. Furthermore, the wattles represented in this group are mostly small or absent. Only Yokohama, Shamo, Aseel and Malay do not group together with the other AOSB (see Figure 4). The Yokohama breed has a distinguished long tail and no information on feather hardness was found. These conditions explain the respective location of the Yokohama in the context of the AOSB. Nevertheless, this clustering together in the upper third of the scatter plot could be related with the “Game” sub-classification that is given to these breeds (breeds that were originally created for cockfighting (Ekarius, 2007)).

The low resolution of the European cluster may result from a lack of information regarding the analysed traits or be due to a strong genetic relatedness of the respective breeds, as depicted in Figure 1. This result supports the prevailing notion of high levels of hybridization in the origin of modern chicken breeds. The assignment of breeds into classes in the British system is based on different aspects of (i) three feather types, (ii) rare breeds and (iii) true bantams, where the respective feather type used in this work (hard feathered) do not consistently cluster together in our analysis, rather only partially in the disparity space (Figure 5). The basis of its classification does not correspond with integumentary characters as studied here. Only the majority of the breeds corresponding to the true bantams (five of seven) cluster together close to the red junglefowl (Figure 5). In terms of utility of breeds, neither ornamental, laying nor meat breeds are grouped with respect to integumentary characters (Figure 4). This notably applies to multi-purpose breeds as well, as Shamo which often is considered meat, game or ornamental breed (Ekarius, 2007; Scrivener, 2008; Damerow, 2012).

The results of the disparity analysis are in concordance with the hypothesis of high hybridization among chicken breeds and fit with the large amount of supporting data from genetic analyses (Rubin et al., 2010; Flink et al., 2014). Remarkably, we found that the red junglefowl is grouping in the European cluster close to Leghorns and bantam breeds, in concordance with previous works based on morphological and genetic data (Moiseyeva et al., 2003; Kumar et al., 2015). Other interesting findings are the grouping of Araucana with AOSB and other Asiatic breeds, supporting the Polynesian origin hypothesis (Storey et al., 2007), and the position of Cornish close to the “game” breeds far away from English breeds. Perhaps exposing its origin, which is mainly the cross of Aseel and Malay with other British game breeds (see Figure 1).

From a methodological aspect, we conclude that PCA-based disparity analysis using the Claddis package is useful for investigation of intra-species variation. Past studies that have attempted to resolve the ancestral relationships of domesticated fowl also included non-integumentary characters in their morphological analyses (Stevens, 1991; Moiseyeva et al., 2003). These characters included flightiness, weight, stance, body measurements, beetle brow (prominent forehead projecting over the eyes), and the position of the occipital foramen. Expanding our trait-breed matrix to include non-integumentary characters is another strategy that might be useful for subsequent diversity analysis of chicken breeds.

3.2 Development

We hypothesize that variations in timing of egg tooth formation in fowls and chickens results from changes to the developmental interactions within the cranial neural crest. On the cellular level, such changes could include an initially smaller-sized population of inducing neural crest

mesenchyme that results from differences in rates of proliferation, cell cycle dynamics, or migratory behaviour (Schneider, 2005; Schneider, 2018a). On the molecular level, these changes could involve regulatory changes to the timing, location, or amounts of expression of genes known to mediate epithelial-mesenchymal interactions in the integument such as members and targets of the Sonic Hedgehog (*SHH*), Bone Morphogenetic Protein (*BMP*), Fibroblast Growth Factor (*FGF*), and Wingless (*WNT*) signalling pathways (Schneider, 2005; Fish et al., 2014). This would be similar to what has been observed to underlie variation in other cranial integumental structures like feather buds in quail and duck (Eames & Schneider, 2005) and in the transition from a single comb to a pea comb development in chickens (Wright et al., 2009).

Regarding hatching time, chickens hatch after 21 days of incubation, however, differences in the rate of development among chicken breeds have been reported (Hamburger & Hamilton, 1951). In the case of red junglefowl, hatching occurs 12 to 24 hours earlier than in White Leghorn (data not shown). Variation in the timing of egg tooth development and different developmental rates could be related to the level of activity (or degree of precociousness) between red junglefowl and chickens, but to establish this relationship more samples and statistical tests will be needed. Red junglefowls, in comparison with their domesticated counterparts, are more active and show higher fear response (Agnvall et al., 2018). Possibly, this behavioural difference related to the domestication process, is coupled with morphological variation and early hatching.

In the same manner, our data on feather development illustrate how the delicate network of mesenchymal-epithelial interactions may vary between the wild ancestor and the domesticated forms. Not only do we find changes in the neural crest-mediated patterns of cranial feather pigmentation, but also we expect that mesodermal mesenchyme likely plays an equivalent inductive role in the trunk, and underlies the shift in the timing of feather outgrowth. The

"developmental window" identified in this work, could be a key aspect to help understand the developmental bases of adult morphological variation that results from domestication.

Although comb variation has been mapped using genetics as a tool and data exist on how this trait can change among chickens, more comparative developmental morphology work is needed in order to understand the variation in this structure during domestication. Comb morphology can be clearly observed before hatching and offers an excellent system for investigating mechanisms that link development and evolution. For example, mutations in certain regulatory domains can lead to ectopic expression of genes either in the mesenchyme or epithelium, which in turn can alter comb morphology. This is the case for the pea comb phenotype, which occurs when there is tandem duplication in the regulatory domain of the transcription factor Sox5 (Wright et al., 2009). Moreover, reduced expression of Patched (*Ptch1*) and Smoothened (*Smo*), which are SHH receptors, has been observed in pea comb mesenchyme. When SHH signalling is attenuated in wild-type embryos, a pea comb-like phenotype is produced (Boije et al., 2012). Similarly, the "buttercup" and "v-shaped" phenotypes are the result of the ectopic expression of the gene Eomesodermin (*EOMES*) at the epithelium by duplication at the *CMC1* gene (Dorshorst et al., 2015).

Overall, chickens are a great model system for studying the evolutionary developmental biology of domestication. However, the hegemony of genetics and genomic explanations of the way morphological structures might change in the context of domestication, leave a gap in understanding how morphological structures change via modifications to molecular and cellular programs. Although comparing the genomes of any two given breeds that show variation in the integument will undoubtedly reveal genetic differences, these genetic differences do not explain how morphological variation is generated (Alberch, 1991). The broad phenotypic diversity among

chickens and the fact that the embryos of these birds are available and experimentally tractable in the lab, gives us exceptional opportunities to investigate how morphological structures are built during ontogeny and how they change through phylogeny (Burt, 2007; Stern, 2018). Artificial selection and hybridization have clearly been the main mechanisms that drive the origin of new chicken breeds. Yet, what has been missing from this discussion, for instance, is how selection for specific morphological traits or behaviours leads to the concordant transformation of many other characters. Neural crest cells have been proposed as a key developmental mechanism linking the phenotypic co-variation that is observed in domesticated species of mammals due primarily to their role in patterning a diverse array of derivatives (Wilkins et al., 2014; Sánchez-Villagra et al., 2016), and they may be playing a similar role in birds as evidenced by the integrated changes that occur in the integumentary system. Examining further into the developmental mechanisms of phenotypic change in the context of domestication, would undoubtedly shed light on one of the central topics in this special kind of evolution.

Conclusion

The results of disparity analysis suggest that (1) the visualization of artificial selection is not comparable to standard breed classification by the ASoP and furthermore, the assignment of types like egg-type, meat-type and ornamental, is not (2) in concordance with chicken integumental traits and only perhaps restricted to some ornamental breeds (Game). The position of the red junglefowl in our analysis corresponds with previous works based in both morphological and genetic data. We further conclude that the assessment of non-discrete characters such as shank colour raises a non-trivial issue with our methodology. This is due to subjective aspects in data collection and insufficient metrical standardization in the breeding literature. The

development of a character assessment protocol is intended to normalise data acquisition and enhance comparability between datasets generated by different researchers.

Our results comparing embryological stages of the red junglefowl and four breeds reveal variation in the timing of onset of formation in certain structures (e.g., thigh feathers). Over the course of many decades, much progress has been made in finding the genetic bases of phenotypic traits in chickens. The developmental perspective can be further examined in order to shed light on the morphological evolution driven by domestication. The study of the neural crest, which is involved in generating many diverse integumentary traits in chicken like skin colour and epidermal appendages such as the egg tooth and comb (Table 1), should be a rich subject. Intraspecific chimeric grafting experiments (between fowl and chicken embryos) like the neural crest transplants performed in quail and duck embryos previously (Schneider & Helms, 2003), would untangle how epithelium-mesenchyme interactions lead to variation during the domestication of chickens.

Materials and methods

Disparity of integument

As a measure of disparity (variation) of the integument traits, we compiled information on 20 features (Table 1) for males of 72 chicken breeds and the red junglefowl. The integument characters were extracted from illustrations and descriptions in the American Standard of Perfection (American Poultry Association, 2015) and complemented with other sources (Ekarius, 2007; Damerow, 2012; Bund Deutscher Rassegeflügelzüchter, 2016). The trait/breed matrix was compiled using the software Mesquite V 3.40 (Maddison & Maddison, 2018). The disparity analysis was performed using the Claddis package in R version 1.1.383. This analysis calculates

morphological diversity and rate implementing a minimum distance or change approach (Lloyd, 2016).

The assignment of utility to the respective breeds was performed by means of literature-based information and by assigning one type of use to the respective breed. The attribution is based on information derived from SBL (Standard Breed Literature). This specific classification is not represented in the underlying matrix. The results of the disparity analysis were then subjected to interpretation of applicable groupings according to (1) classification as applied by the ASoP and (2) the type of use (Ekarius, 2007; Damerow, 2012).

Development

We adhered to generally accepted guidelines for the humane treatment of avian embryos as described in S3.4.4 of the *AVMA Guidelines for the Euthanasia of Animals: 2013 Edition*. Embryos were not allowed to hatch and were euthanized through rapid and humane methods. Both fowl and chicken eggs were incubated at 37,8 °C and 60-70% of humidity. Fowl and White Leghorn embryos were collected at University of Linköping (Sweden); Hubbard embryos at University of Zurich (Switzerland); Ukokkei and Shamo embryos at Niigata University (Japan). Embryos were fixed in 4% PFA, and then stored at 4°C for further anatomical descriptions, comparisons and photographing. Embryos were staged (HH) following the normal table for chicken embryos (Hamburger & Hamilton, 1951).

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No.	Name	Character	Genes/Origin/Developmental basis
1	Comb types	single|rose|pea|cushion|buttercup|carnation|strawberry|V|walnut	Pea; misexpression of *SOX5* (Wright et al., 2009). Rose; misexpression of *MNR2* (Imsland et al., 2012). Walnut; combination of Pea and Rose effects (Imsland et al., 2012). Buttercup/V-shaped; ectopic expression *EOMES* by *CMC1* duplication (Dorshorst et al., 2015).
2	Ear lobe size	small, absent|medium|large	?
3	Ear lobe colour	red|white|red with white spot|gypsy|turquoise	Red/White; *SLCO1B3* (Nie et al., 2016), *PIK3CB*, *B4GALT1* and *TP63* (Luo et al., 2018). Black; *EDN3* (Dharmayanthi et al., 2017)
4	Wattle size	small, absent|medium|large	Misexpression of *SOX5* (Wright et al., 2009)
5	Wattle colour	red|black, mulberry|gypsy	Black; *EDN3* (Dharmayanthi et al., 2017)
6	Egg shell colour	white|tinted|brown|dark brown|blue	Blue; *SLCO1B3* (Wang et al.,2013b; Wragg et al., 2013)
7	Beard and muffs	absent|present	Change expression of *HOXB8* (Guo et al., 2016)
8	Crest	absent|present	Ectopic expression of *HOXC8* (Wang et al., 2012)
9	Neck feathers	absent|present	Cis-regulation of *BMP12/GDF7* (Mou et al., 2011)
10	Ear tufts	absent|present	*TBX1* (Noorai et al., 2012)
11	Vulture hooks	absent|present	*Pti* (Dorshorst et al., 2010)
12	Leg feathering	clean|moderate|feathered	*Pti* (Dorshorst et al., 2010)
13	Tail length	short|medium|long	*LOC431648* (Wang et al. 2017)
14	Rumpless	no|yes	*IRX1* and *IRX2* (Noorai et al., 2012)
15	Hard feathered	no|yes	Hypothetic Ha locus (Crawford, 1990)
16	Shank colour	slate|black|yellow|willow|blue|white|varieties	Yellow; *BCDO2* (Eriksson et al., 2008). Black; *EDN3* (Dharmayanthi et al., 2017). Several colours; *GRAMD3* expression (Xu et al, 2017)
17	Feet-bottom colour	slate|black|yellow|willow|blue|white|varieties	Yellow; *BCDO2* (Eriksson et al., 2008). Black; *EDN3* (Dharmayanthi et al., 2017). Several colours; *GRAMD3* expression (Xu et al, 2017)
18	Eye colour	reddish bay|brown|varieties|red yellow iris|black honey iris|pearl|black	Combination of pigments (Hill and McGraw, 2006)
19	Beak colour	horn|yellow|black|white|blue|varieties	Combination of pigments (Ralph, 1969)
20	Skin colour	white|yellow|pinkish white|black	Yellow; *BCDO2* (Eriksson et al., 2008). Black; *EDN3* (Dharmayanthi et al., 2017). Several colours; *GRAMD3* expression (Xu et al, 2017)
21	Egg tooth	-	Neural crest cells induction (Schneider and Helms, 2003)

Table 1: List of integumentary traits, character states for the disparity analysis and their genetic background and/or developmental basis/origin. Colours correspond to the developmental origin of each trait. Light blue: secondarily neural crest derivative; Light orange: neural crest derivate; Light green: no neural crest derivate. Egg tooth is not part of the disparity analysis. ? = unknown

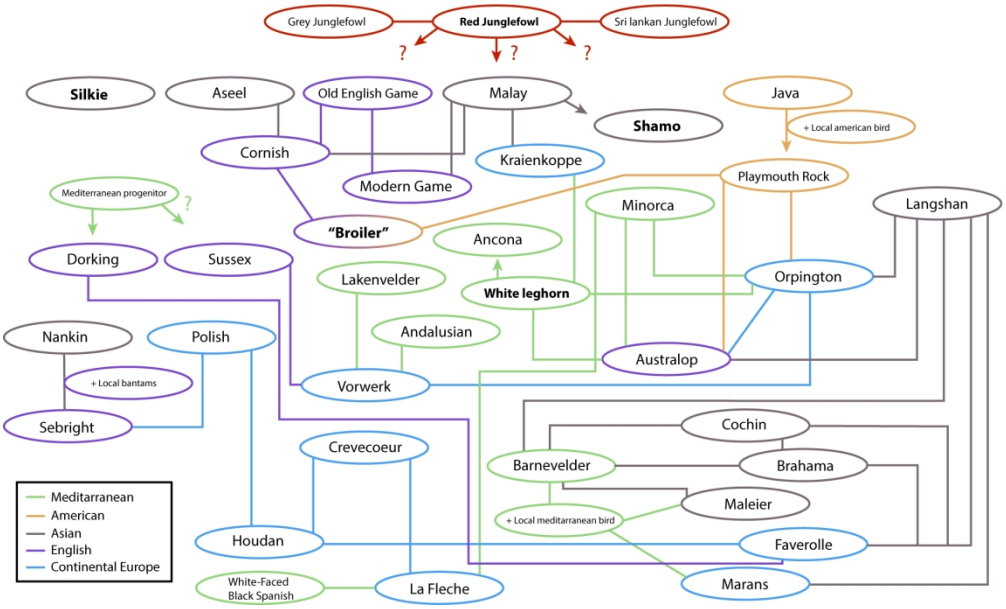

Figure 1: Chicken breeds framework within the English and Continental Europe clusters. Relationships based on historical reports of crosses and hybridizations. In bold are the breeds of embryos used in this work (Ukokkei is the given name for Silkies in Japan; Hubbard is a broiler breed).

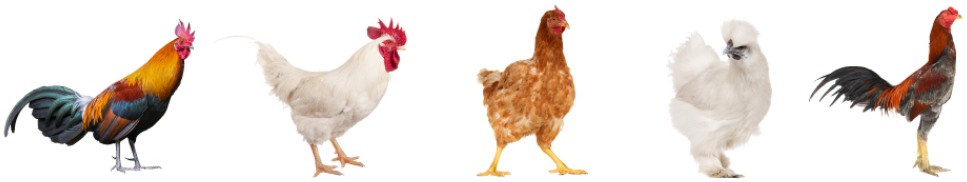

Figure 2: Morphological diversity through domestication in the chicken: Adult specimens of the fowl and chicken breeds included in the embryological work mentioned here. Left to right: Red junglefowl (the wild form), White Leghorn, Hubbard, Ukokkei and Shamo.

Figure 3: Integument diversity within Galliformes and Ukokkei foot feathering development (ptilosis). (a) Wattled brushturkey (Aepypodius arfakianus). Arrows mark wattles and comb. (b) Rufous-bellied chachalaca (Ortalis wagleri). Arrows mark wattles and crest. (c) Adult and fledgling rock ptarmigan (Lagopus muta). Arrows mark foot fethering. (d) Ukokkei chicken embryo 11 days old. Arrow marks feather tracks. (e) Ukokkei chicken embryo 15 days old. Arrow marks foot feathering growing. (f) Ukokkei chicken adult. Arrow shows foot feathering.

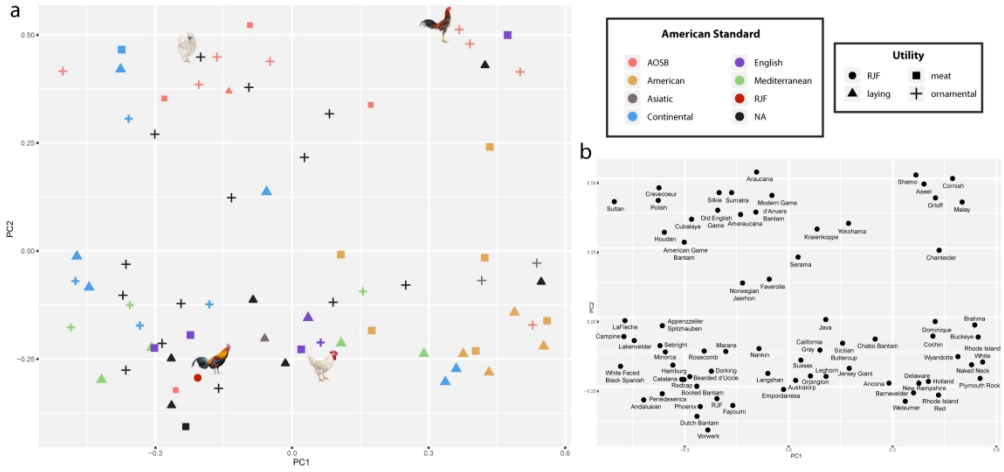

Figure 4: PCA of 20 integumentary characters in chicken breeds and red junglefowl (scatter plot). (a) Colours are assigned to classes according to the ASoP by the APA. A grouping based on breed utility type has been overlaid (best estimation based on information of Standard Breed Literature). (b) Position in the scatter plot of the 72 breeds and red junglefowl. AOSB = All other standard breed.

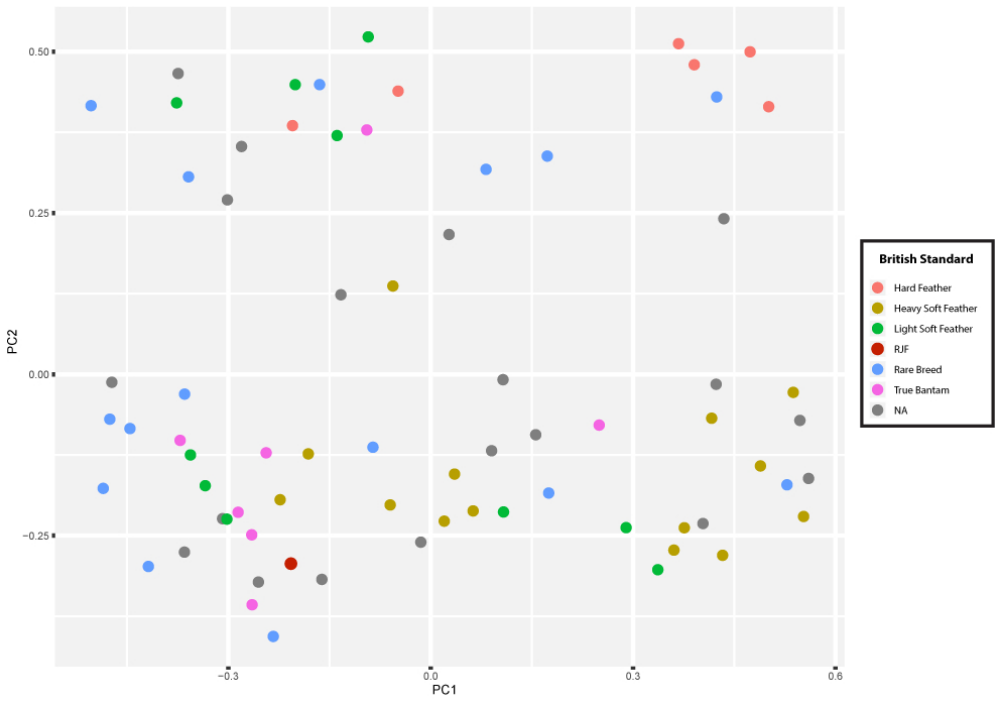

Figure 5: PCA of 20 integumentary characters in chicken breeds and red junglefowl according to the British system (Scrivener, 2008). The colour assignment is based on different aspects of (i) three feather types, (ii) rare breeds and (iii) true bantams.

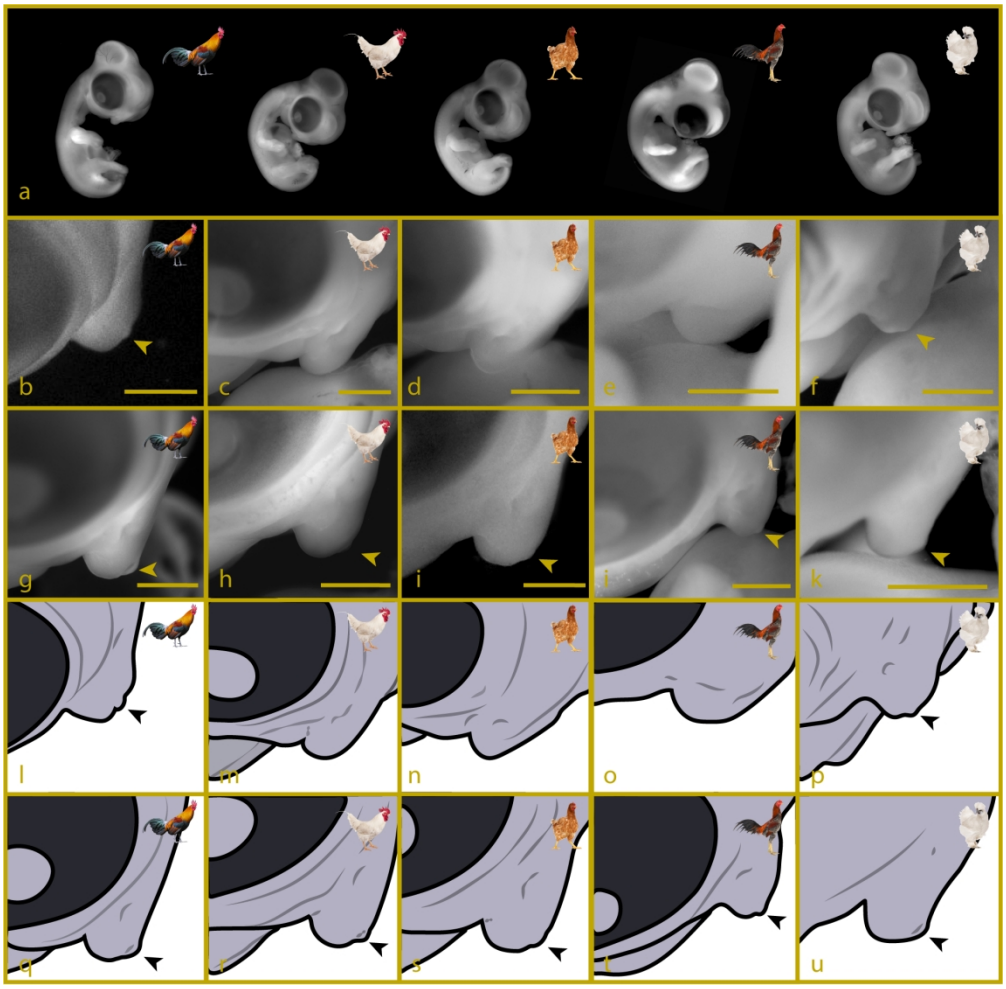

Figure 6: Egg tooth development between 6-7 days of development in red junglefowl and chicken embryos. (a) Red junglefowl, White Leghorn, Hubbard, Shamo and Ukkokei embryos at HH29, images to scale. (b,l) Red junglefowl embryo and corresponding drawing at HH29. (g,q) Red junglefowl embryo and corresponding drawing at HH30. (c,m) White Leghorn embryo and corresponding drawing at HH29. (h,r) White Leghorn embryo and corresponding drawing at HH30. (d,n) Hubbard embryo and corresponding drawing at HH29. (i,s) Hubbard embryo and corresponding drawing at HH30. (e,o) Shamo embryo and corresponding drawing at HH29. (j,t) Shamo embryo and corresponding drawing at HH30. (f,p) Ukokkei embryo and corresponding drawing at HH29. (k,u) Ukokkei embryo and corresponding drawing at HH30. Arrows mark egg-tooth presence. Bars = 1 mm.

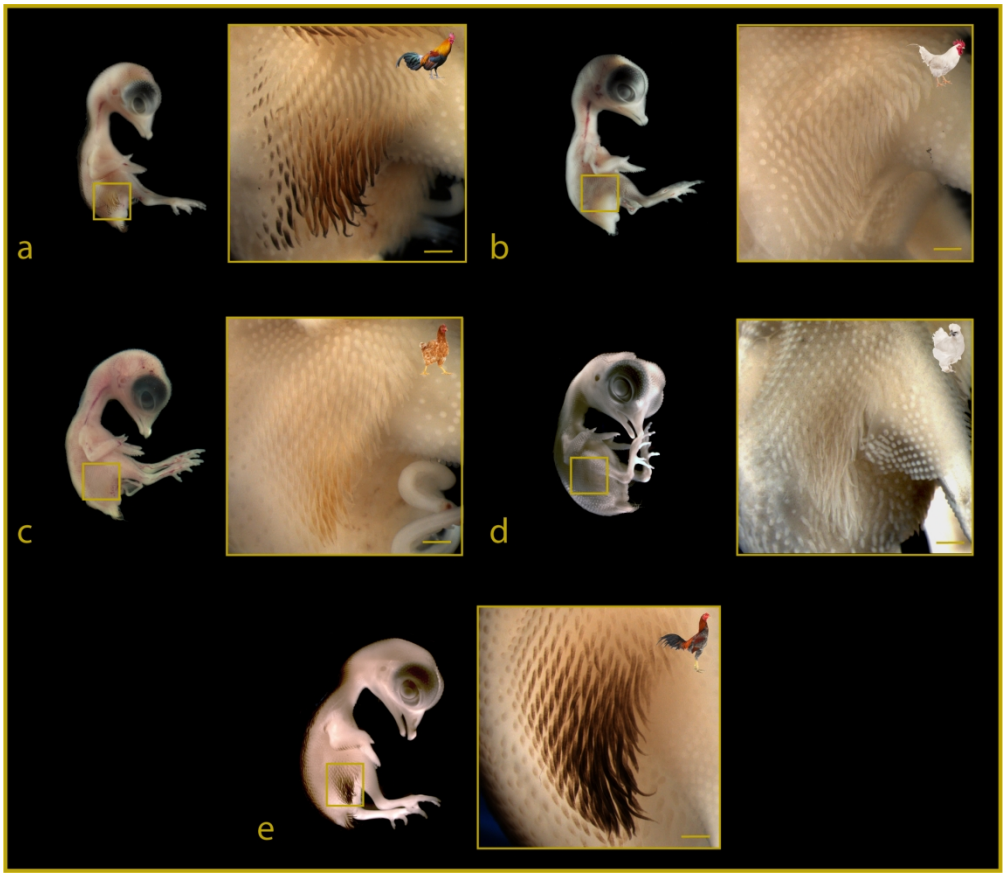

Figure 7: Thigh feathers development between 11-12 days of development in red junglefowl and chicken embryos (HH37). (a) Red junglefowl embryo. (b) White Leghorn embryo. (c) Hubbard embryo. (d) Ukokkei embryo. (e) Shamo embryo. Whole-mount embryos to scale. Bars = 1mm.

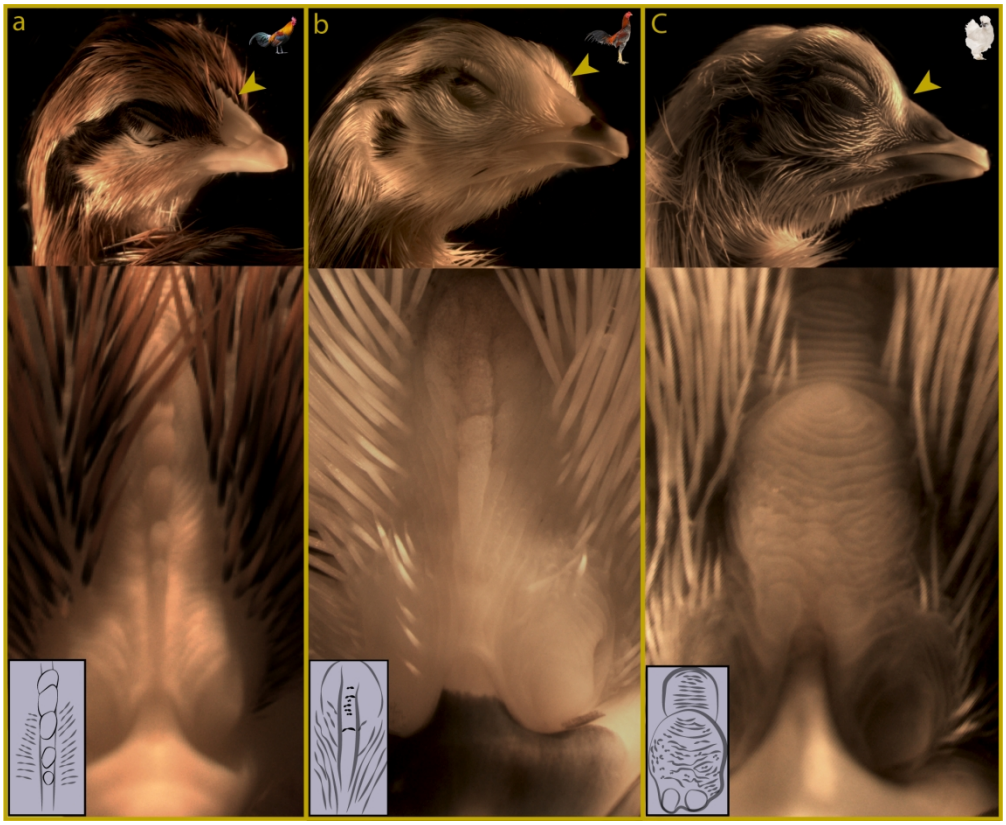

Figure 8: Comb anatomy in fowl and chicken embryos at 16 days of development. (a) Red junglefowl embryo. (b) Shamo embryo. (c) Ukokkei embryo. Arrows mark comb position in the head. Corresponding drawings depict the anatomy of the combs in frontal view.