DEVELOPMENT OF INTEGUMENTARY STRUCTURES IN *ROUSETTUS AMPLEXICAUDATUS* (MAMMALIA: CHIROPTERA: PTEROPODIDAE) DURING LATE-EMBRYONIC AND FETAL STAGES

NORBERTO GIANNINI,* ANJALI GOSWAMI, AND MARCELO R. SÁNCHEZ-VILLAGRA

*American Museum of Natural History, Mammalogy, Central Park West at 79th Street,
New York, NY 10024-5192, USA (NG)*
*Department of Palaeontology, The Natural History Museum, Cromwell Road, London, SW7 5BD,
United Kingdom (AG, MRS-V)*

The pattern of development of integumentary structures and associated tissues in the megachiropteran bat *Rousettus amplexicaudatus* is described on the basis of 18 specimens ranging in crown–rump length (CRL) from 8.5 to 44.4 mm. The ontogenetic sample encompasses specimens that correspond to stages 16 through 22 of the phyllostomid *Carollia perspicillata* (late-embryonic) as well as fetal stages. Although the earlier stages of *R. amplexicaudatus* show little deviations in relative timing or anatomy from *Carollia*, later ones are increasingly different. Integumentary structures show different degrees of developmental penetrance. Derived features in embryonic stages are clearly reflected in adult structures such as a great enlargement of the handplate in relation to the footplate at the moment in which both are fully differentiated. We propose that patagium morphogenesis includes development of dactylopatagium by initial retention and posterior growth of interdigital tissue, coupled with digit elongation; and development of pro-, plagio-, and uropatagium in association with wing muscles unique to bats. Additionally, allometric trends in wingspan, total wing area, arm-wing area, and hand-wing area were estimated with respect to CRL. Changes in the terminology of some *Carollia* stages (16 and 18) are proposed to facilitate comparisons across bat species.

Key words: Chiroptera, development, integument, Megachiroptera, Pteropodidae, *Rousettus amplexicaudatus*, staging, wing development

Bats are remarkable for an extraordinary expression of the integumentary system. The most obvious character of bats are the wings, formed by 4 integumentary tracts collectively known as patagia—the pro-, dactilo-, plagio-, and uropatagium, each derived from different embryonic primordia (Cretekos et al. 2005). But bats also feature extreme variation in the anatomy of ears, rhinarium, and other integumentary structures. Many of these structures appear in poorly known late-embryonic stages, and continue developing throughout the fetal and postnatal period. Detailed descriptions of the development of these integumentary structures are lacking, correlated with the lack of studies of bat ontogeny at these stages.

Most of the recent literature on bat development has focused on implantation and early development (Badwaik and Ras-

weiler 2000). On the opposite end of the developmental process, postnatal development has been documented in a number of bat species (e.g., Isaac and Marimuthu 1996; Jones 1967; Reiter 2004). Comparatively, late-embryonic and fetal development has received little attention.

In this work we study the development of integumentary structures in the Australasian megachiropteran bat *Rousettus amplexicaudatus*. The recent availability of a comprehensive staging system for a bat, the phyllostomid *Carollia perspicillata* (Cretekos et al. 2005), provides a reference framework for the description of development. All mammalian staging systems, including the *Carollia* system, rely heavily on integumentary traits. For this reason, increased knowledge of the development of the bat integument offers the additional reward of providing potential characters for further refinements on staging systems and a broader basis for comparative studies across bat lineages.

The extensive use of integumentary characters in mammalian systematics (Ade 1999; Hutterer 1985; Pocock 1914; Voss and Jansa 2003) and of megabats in particular (Giannini and

* Correspondent: norberto@amnh.org

FIG. 1.—Overview of embryos and fetuses of *Rousettus amplexicaudatus* used in this study. Number indicates the order assigned to each specimen in the growth series, based on crown–rump length (CRL; see Table 1). Specimens are represented at an approximately uniform CRL. Scale bars (on lower right of each specimen): 1 mm.

Simmons 2005) makes the understanding of development of these structures particularly important. Incorporation of ontogenetic data on integumentary structures into phylogenetic analyses will have to wait the accumulation of such data, currently available only for a handful of mammalian species (Ade 1993). However, some significant phylogenetic characters can now be informed with an embryological basis. In turn, particulars of the observed changes in *Rousettus* generate important questions on the development of integumentary structures, especially the wings.

MATERIALS AND METHODS

Rousettus amplexicaudatus (E. Geoffroy, 1810) is a rousettine megachiropteran bat (Pteropodidae: Rousettinae; Bergmans 1997) distributed in the center of the Australasian region from Myanmar to the Solomon Islands (Simmons 2005). A colonial, cave-dwelling species, *R. amplexicaudatus* feeds on nectar, pollen, and fruit of a variety of plants, and exhibits seasonal bimodal polyestry synchronized with plant phenology (Heideman and Utzurrum 2003). Gestation period is about 3.5–4.5 months, followed by 2.5–3 months of lactation; age of 1st pregnancy is between 6 and 9 months (Flannery et al. 1992).

Eighteen ethanol-preserved specimens of *R. amplexicaudatus* in late-embryonic and fetal stages (Fig. 1) were available for examination.

The specimens belong to the Hubrecht Collection, located at the time of the study in Utrecht, The Netherlands, but currently at the Museum für Naturkunde, Berlin, Germany. The Hubrecht Collection of Comparative Embryology is estimated to represent 600 chordate species in 175 families and is thus considered "the largest and most valuable of its kind in existence" (Richardson and Narraway 1999:591). The specimens of *R. amplexicaudatus* were collected in the former Dutch East Indies, today Indonesia, in the decades of 1890 and 1900 (further information unavailable). The ontogenetic series included adults, which led to the verification of the species as *R. amplexicaudatus*. Our specimen reference number (1–18; Fig. 1; see below) correspond to the following Hubrecht Collection catalogue numbers: 1/1110/1114 (specimen 1), 1/1097/1109 (specimen 2), 1/1178/1192 (specimen 3), 1116/1126k (specimen 4), 1405/1461k (specimen 5), 1116/1126q (specimen 6), 1405/1461m (specimen 7), Dof/1287/1305 (specimen 8), 1115 (specimen 9), 1/1128/1134 (specimen 10), 1/1264/1274 (specimen 11), 1/1309/1368 (specimen 12), 1405/1461q (specimen 13), 1/1275/1279 (specimen 14), 1483/30a (specimen 15), 1/1462/1485b (specimen 16), 1483/45 (specimen 17), and 1/1280/1286 (specimen 18). Specimens ranged in size, using crown–rump length (CRL) as a rough estimator, from 8.5 to 44.4 mm. This 5-fold increase in linear dimensions encompasses the majority of late-embryonic and fetal stages, when most of the development of integumentary structures occurs. Specimens were given a consecutive number in the order of their CRL combined with their developmental stage. In addition, we

TABLE 1.—Events in the development of integumentary structures across the prenatal developmental stages identified in *Rousettus amplexicaudatus*. Specimens are ordered according to crown–rump length (CRL) and assigned either to *Carollia* embryonic stages or to fetal stage (1 category). Characters indicates 1st appearance of integumentary structures across stages. Within each stage, characters are listed in anatomical order (following the *Nomina Anatomica Veterinaria* [World Association of Veterinary Anatomists 1994]). Key structures that allow precise staging are in boldface. Note that stage assignment based on morphology and ordering based on CRL are in good but not perfect agreement. Abbreviations: C, upper canine; DI, 1st digit; DII, 2nd digit; dC, deciduous upper canine; dP3, deciduous upper 3rd premolar; E, early; L, late; M., muscle.

Specimen no.	CRL (mm)	*Carollia* stage	Characters
1	8.5	16	Vibrissal folicles
			Pinna primordium
			Retinal pigment[a]
			Nipple primordia
			Genital tubercle[a]
			Uropatagial primordium
			Propatagial primordium[a]
			Anterior plagiopatagial primordium[a]
			Digital condensations in limb pads
2	8.5	16L	Rhinarium, ala nasi ventralis
			Rhinarium, sulcus medianus
			Antitragus
			Interdigital notches in handplate
			Calcar primordium
3	9.4	17	Dorsal dermal grooves
			Lacrimal commissure
			Posterior plagiopatagial primordium
			Rhinarium, sulcus alaris
			Interdigital notches in footplate
4	13.4	18	Oral papillae
			M. occipitopolicallis
			M. humeropatagialis, fascies
			M. uropatagialis
			Free thumb
			Proximal claw fold, DI−DII
			Proximal claw fold in toes
			Genital tubercle bent
5	16.1	19	Upper lip notch
			Claw primordia
			M. coracocutaneous
6, 7	16.3−16.6	20E	**Eyelids closed**
8, 10, 11	16.8−20.7	20	Widespread bumps of hair follicles
9	17.2	22	Follicles of eyelashes
			Claws achieve adult morphology
			Follicles of eyelashes
			Rhinarium, sulcus lateralis
			Lower lip pads
			Pinna apex attenuated
			Carpal joints distinct
			Genitals begin dimorphic differentiation
12, 14	26.4−28.6	22L	Fold in lower lip for C
			dC and dP3
			Areola mammaria
13	27.3	Fetal	**Vibrissae emerge**
			Pigmentation in palpebral line
			Melanocytes widespread
			Rhinarium, paved epidermis
15	39.1	Fetal	Guard hairs in dorsum
16−17	39.3−42.4	Fetal	Eyelashes emerge
			Short hair widespread on all dorsal parts
18	44.4	Fetal	Hairs in genitalia and uropatagium

[a] Already present in *Carollia* in stages 14 and 15.

examined adult specimens of *R. amplexicaudatus* in the collection of the American Museum of Natural History, New York (AMNH).

We staged the specimens examined here using the proposal by Cretekos et al. (2005), a staging system for *C. perspicillata* of known age. A developmental stage is an arbitrary position along the path of continuous development of an organism that can be easily recognized by the 1st appearance of 1 or more morphological landmarks. Staging is thus the process of identifying the appropriate landmarks in a specimen series in order to define the ontogenetic sequence, or the allocation of a specimen to a particular, previously defined stage based on the presence of morphological landmarks. Staged specimens can be compared within a developmental sequence and across species or lineages.

Cretekos et al. (2005) used 23 embryonic stages, which were based on the Carnegie system for human development (see Cretekos et al. 2005), but many details were bat specific. We also compared the *Rousettus* series we examined with the series of *Syconycteris australis* (Pteropodidae) studied by Lawrence (1991). She staged embryos and fetuses of the macroglossine pteropodid *S. australis* according to the mammalian system established by Butler and Juurlink (1987), based on the standard Carnegie stages of the human embryo. Lawrence's 42 specimens included Carnegie mammal embryo stage 12 through 23 (last Carnegie stage before fetal period commences), which showed some deviations from the Carnegie mammal embryo stages of Butler and Juurlink (1987). In addition, she developed 6 fetal stages for *Syconycteris*.

Age of the *Rousettus* specimens examined here is unknown, but may be roughly similar to the age of *Carollia* embryos and fetuses of corresponding stage given that gestation period in both species is of comparable length (3.5–4.5 months in *R. amplexicaudatus* [Flannery et al. 1992] and 3.7–4 months in *C. perspicillata* [Cretekos et al. 2005]). *Carollia* stage 16, which occurs 50 days postcoitum in *Carollia*, is the 1st stage recorded in the sample of *R. amplexicaudatus* studied here (see ''Results''); in turn, fetal stage in *Carollia* starts at 90 days postcoitum (Cretekos et al. 2005).

Staged specimens were examined in detail in order to describe the pattern of development of integumentary structures. These included the rhinarium, lips, vibrissae, eyelid, pinna, patagia, claws, and skin and hair cover. Some of these structures are intimately associated with hard mesodermal tissue (cartilage or bone), particularly the rhinarium, pinna, and wing. In this description, we excluded details of the development of underlying hard tissue, which will be presented elsewhere.

Wing shape and area were examined for specimens 5–17. Wing measurements follow Norberg and Rayner (1987), with the exception that wing area does not include the uropatagium, which was too delicate to measure accurately in many specimens. Specimens were digitally photographed with a wing extended as far as possible for measurement. Digital photographs of wings were imported into Photoshop 7.0.1 (Adobe Systems, Inc., San Jose, California), and wing shapes were manually outlined using a brush tool. Resulting outlines were then exported to TPSdig version 2.0.4 (Rohlf 2005) for calculation of wing area and wingspan.

RESULTS

Staging

The available sample of embryos of *R. amplexicaudatus* encompasses *Carollia* stages 16 through 22. The younger specimens of *R. amplexicaudatus* correspond well to the *Carollia* staging system, and the older ones less so (Table 1). The least-developed specimen of *R. amplexicaudatus* (1097/1109,

CRL 8.5 mm; see Fig. 2) can be safely allocated to *Carollia* stage 16, named "nose-leaf primordium" by Cretekos et al. (2005). This specimen shows several features that appear at earlier stages of *Carollia* (retinal pigment, and propatagium and plagiopatagium primordia) but it exhibits 2 traits, appearance of pinna primordium and uropatagium primordium, that are typical of stage 16. Specimen 2, having approximately the same CRL, is in a slightly more advanced stage, 16L, because it presents clear interdigital notches in the handplate (Fig. 2). In turn, specimen 3 shows interdigital notches in the footplate and a lacrimal notch, therefore belonging to stage 17 (designated as "tongue out" by Cretekos et al. [2005]). In specimen 4 a large number of changes had occurred (Table 1), most notably the "free thumb" that gives its name to stage 18. The appearance of claw primordia places specimen 5 in stage 19 (Cretekos et al. 2005:731, figure 4o), named "peek-a-boo" by Cretekos et al. (2005) because the developing wings cover the face of the embryo partially hiding the eyes. Specimens 6 and 7 are slightly larger than the preceding one and their eyes are closed (stage 20E). Stage 21 is not defined by Cretekos et al. (2005). In specimen 9, the claws in both the forelimb and hind limb are ventrally excavated and appear essentially identical to those of adults. Therefore we assigned specimen 9 to stage 22. Specimen 13 exhibits characters that appear in several different *Syconycteris* fetal stages of Lawrence (1991): pigmentation in the palpebral line (*Syconycteris* fetal stage 2), melanocytes widespread in the body (fetal stage 4), and emerging vibrissae (fetal stage 6). That is, this specimen unambiguously is in the fetal stage, but it does not fit in specific *Syconycteris* categories. As a consequence, we assigned specimens 13 and older to an unspecified fetal category.

Integumentary Structures

Rhinarium and lips.—In stages 16 through 20, the rhinarium is not sharply defined against the surrounding epidermis, but the pars internarica is relatively wide by stage 16, as in the adult stage (Figs. 2 and 3). In stage 16L the ala nasi ventralis, the plica alaris, and an indistinct sulcus medianus appear. With the addition, in stage 17, of the sulcus alaris, the narial opening is shaped into a fully recognizable therian morphotype (as termed and figured by Ade [1999:207, plate X]). This morphology is completed later in the last embryonic stage (stage 22; specimen 9) with the appearance of the sulcus lateralis, which separates the pars supralabialis from the muzzle skin laterally. The typical reticulated epidermis of the rhinarium (a system of polygonal plaquets separated by furrows or surface grooves, called rhinoglyphics [Hill 1948] or nose prints [Evans 1993]) becomes evident in specimen 13 (fetal stage).

Intimately associated with the rhinarium is the morphology of the upper lip. The pars supralabialis of the rhinarium reaches ventrally, and fuses to, the upper lip. By stage 22, both the upper and lower lips are finely parted by minute incisions, but these subdivisions remain inconspicuous, never leading to the formation of separate odontoid papillae as in other megabats (see Andersen 1912; Giannini and Simmons 2005). Also in specimen 9, bulges related to the upper and lower deciduous

Fig. 2.—*Rousettus amplexicaudatus*, scanning electron microscope image, caudolateral view of one of the smallest embryos (specimen 2, 8.5 mm in crown–rump length) in stage 16L of the growth series examined. Scale bar: 1 mm.

canines and 3rd premolars become apparent. The apices of the corresponding teeth are visible in specimens 12 to 14. In specimens 12 and 14, other papillary structures appear. These include a large, single papillae on the vestibular side of the lower lip immediately caudal to the lower canine, and an associated fold that in the adult receives the upper canine when the mouth is closed. In specimens of stage 22L, a thinning in the upper lip caudal to the upper canine appears. This inconspicuous notch, and the set of upper and lower lip papillae, remain in subsequent developmental stages and also are found in adults. Finally a large, single papilla is present in the vestibular side of the upper lip of specimen 13, the 1st assigned to the fetal stage.

As is the case for Glires (Ade 1999), the development of the rhinarium is closely tied to that of the upper lip. Future studies of heterochrony (Smith 2001) could reveal the presence of a developmental module formed by these 2 structures.

Rousettus (as other megabats) exhibits externally in the lower lip 2 large epidermal pads diffusely divided by a median sulcus. These pads are present in stage 22, and specimen 13
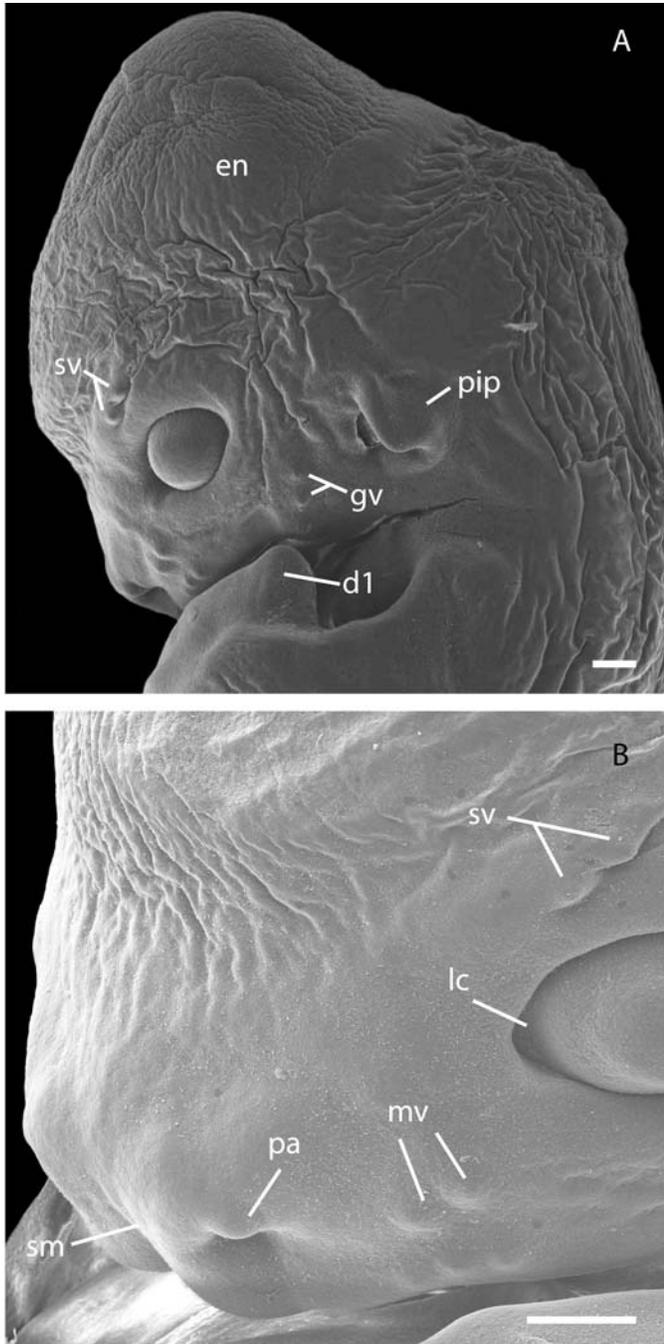
FIG. 3.—*Rousettus amplexicaudatus*, scanning electron microscope image (specimen 1) of A) lateral view of head, and B) close-up (rostrolateral view) of rostrum. Abbreviations: en, encephalic region; d1, 1st digit; gv, bumps for genal vibrissae; lc, lacrimal commissure; mv, bumps for mystacial vibrissae; pa, rhinarium, plica alaris; pip, pinna primordium; sm, rhinarium, sulcus medianus; sv, bumps for supercilliary vibrissae. Scale bars: 0.25 mm.

(the smaller fetal specimen) has acquired a reticulate epidermis (by the same time the rhinarium does).

Specialized face hairs.—The highly modified hairs of the face, vibrissae, and eyelashes show a very different pattern of development. Vibrissal follicles are already present in stage 16. In specimens 1 and 2, mystacial, supercilliary, and genal vibrissal follicles are already present (Fig. 3). The bumps of 3 mystacial follicles are arranged such that 2 follicles appear in a dorsal row and 1 is the 1st member of a ventral row of follicles. A supercilliary ridge is present, with 2 vibrissal follicles. Two genal follicles are distinct in stage 16, as well as a 3rd follicle located near the mouth commissure. Although the number of supercilliary and genal vibrissae remains the same until adulthood, a steady increase in the number of primordial mystacial vibrissae is observed. In stage 16L, follicles are more prominent in general, and the mystacial bumps have increased in number to form a dorsal row of 2 follicles and a less-developed ventral row of 3 (Fig. 3). Number 3 has 3 rows of supralabial vibrissae, formed by 3, 2, and 3 bumps from dorsal to ventral row. Infralabial vibrissae also appear in this stage. In stage 18, 4 rows of large mystacial follicles are present, formed by 3, 2, 3, and 5 bumps from dorsal to ventral row, accompanied by 1 row of some 6 smaller bumps immediately dorsal to the lip. A 2nd row of even smaller bumps appears in stage 19 immediately dorsal to the lip. Number 4 also exhibits the 1st mental bumps. This arrangement is likely final, and it is remarkable that interramal vibrissae, absent in adult megabats, never form. It is not until the fetal stage (specimen 13) that the vibrissae emerge, and are already >3 mm in length in specimen 15. By contrast, the 1st eyelashes appear much later (specimen 16), and are associated with pigmentation (see details below).

Eye.—Retinal pigment is present in the round open eye of specimen 1 (stage 16). The eyelids (palpebrae) are thickened, and a small lacrimal commissure appears in specimen 2 (Fig. 3). In stage 17, the eyes start to close, taking an almond shape, and a lacrimal notch becomes evident. Eyelids are closed in specimens 6 and 7, marking stage 20E. In the closely related *Rousettus aegyptiacus*, eyes remain closed until about 10 days postpartum (Kulzer 1958); we assume that this is also the case in *R. amplexicaudatus*. This represents an important developmental difference with the comparatively precocial neonates of *C. perspicillata*, which are born with eyes open (and have mobile ears—Cloutier and Thomas 1992).

First follicles of eyelashes become apparent in specimen 9, and pigmentation is distributed along the palpebral line in specimen 13 (fetal stage). Finally, eyelashes sprout in later fetal specimens (16 and 17).

Pinna.—The primordium of the pinna is a distinct bump dorsal to the external acoustic meatus in stage 16. In stage 16L, the growing pinna is directed caudoventrally, and the primordium of the antitragus forms a low arch in the ventral side of the external acoustic meatus (Fig. 2). A rounded antitragus is present in stage 17. In this stage, the pinna takes a triangular shape and folds down; it continues to grow in length through the successive stages. In stage 20E, the antitragus is comparatively large and oval. The apex of the pinna is slightly attenuated in stage 22. Hair follicles appear on the pinna surface in the beginning of the fetal stage. Based on *R. aegyptiacus*, the pinnae remain folded until approximately 10 days postpartum, when they become erect and mobile (Kulzer 1958).

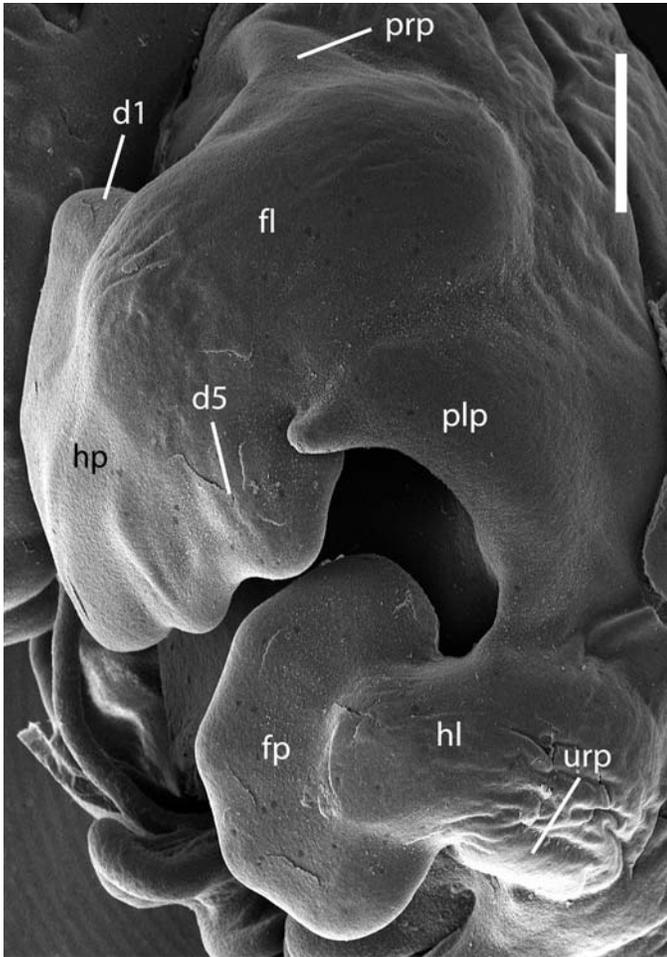*Patagia*.—The primordia of the propatagium, plagiopatagium, and uropatagium are present in the 1st specimen of the

FIG. 4.—*Rousettus amplexicaudatus*, scanning electron microscope image, lateral view of specimen 1 showing autopod plates and patagial primordia. Note degree of digital condensation (finger rays), anteroposterior patterning, and absolute size of handplate as compared to footplate. Abbreviations: d1, 1st digit; d5, 5th digit; fl, forelimb; fp, footplate; hl, hind limb; hp, handplate; plp, plagiopatagium primordium; prp, propatagium primordium; urp, uropatagium primordium. Scale bar: 0.5 mm.

FIG. 5.—*Rousettus amplexicaudatus*, scanning electron microscope image, caudolateral view of specimen 1 showing rump, hind limbs, tail, and genital tubercle. Abbreviations: cap, calcar primordium; fp, foot plate; gg, genital groove; gt, genital tubercle; hl, hind limb; ru, rump; ta, tail; urp, uropatagium primordium. Scale bar: 0.5 mm.

Rousettus series examined here, stage 16 (see development of dactilopatagium in next section). The propatagium primordium consists of a thickening of the skin in the cranial border of the forelimb, and can be seen in a similar stage in specimen 2 (Fig. 4). The growing propatagium reaches the thumb in stage 18. Also in this stage, the m. occipitopolicallis is clearly visible on the cranial (leading) edge of the propatagium.

The plagiopatagium primordium is a digitiform integumentary projection that partially overlaps the caudal border of the handplate. It is continued caudally by a wide and thick sheet of skin on the side of the trunk, which connects to the hind limb above the level of the footplate via an extremely small caudal primordium. The plagiopatagium grows by expanding and thinning its surface, so in subsequent stages the cranial plagiopatagial primordium appears to decrease its relative size until no trace of such projection is found in stage 19. Before it disappears, the plagiopatagi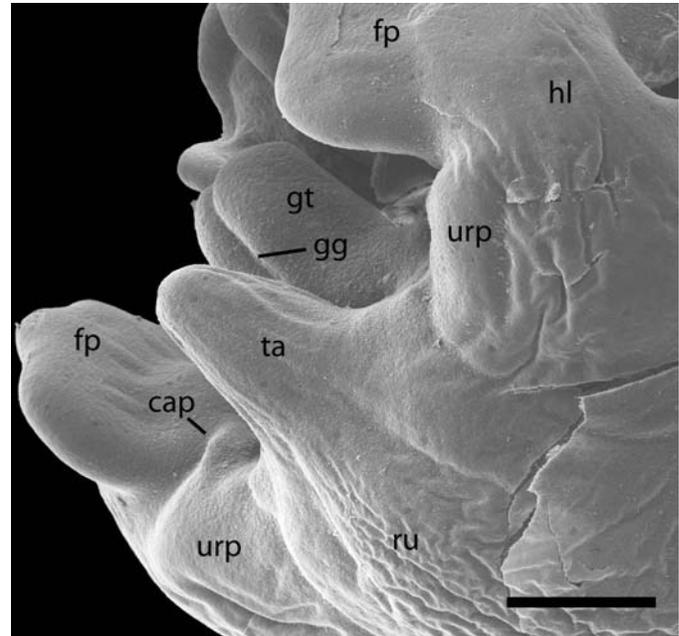al primordium reaches the tip of digit V. At stage 18, the m. humeropatagialis and the 1st wing reticulations are visible. The former originates in the humerus dorsal to the elbow joint, crosses the entire plagiopatagium, and ends in the cranial plagiopatagial primordium. The wing reticulations ("long fascie" of Andersen [1912]) consist of elastic fibers provided with striated muscle arranged transversally with respect to the m. humeropatagialis and associated fibers. Some 6 such fascie are present in stage 18. The number of long fascie increases to 8 in specimen 18. The number, 8, is about the adult number, considering that individual variation certainly exists: 2 adult specimens of *R. amplexicaudatus* (AMNH 27390 and AMNH 31778) exhibited 8 and 9 long fascie, respectively. Extensive reticulation of wings can be observed in specimen 13 (fetal stage). The posterior part of the plagiopatagium, which appeared as a minute projection near the contact area with the hind limb in stage 16, continues growing toward the foot, and in fetal specimens it is already inserted in the dorsal surface of the 1st toe (the adult condition). Finally, the m. coracutaneous, which originates from the proximal humerus and inserts into the axillary part of the plagiopatagium (Vaughan 1970), is visible in stage 19.

The uropatagium primordium is barely differentiated as a thick caudal border of the hind limb in stage 16. A tiny primordium of a calcar attaches to the uropatagium primordium in the next stage, 16L (specimen 2; Fig. 5). In stage 18, the m. uropatagialis is differentiated, the uropatagium reaches the tail, and a fully differentiated calcar is present.

The variables wingspan, total wing area, arm-wing area (plagiopatagium and propatagium), and hand-wing area (dactylopatagium), measured across this growth series, are

Table 2.—Wing measurements for specimens of the series of *Rousettus amplexicaudatus* embryos and fetuses. Specimens 1–4 do not yet exhibit measurable areas. For specimen 5, the plagiopatagium was torn and only the dactylopatagium could be measured. For specimens 11 and 14, the dactylopatagium and plagiopatagium were torn and could not be adequately measured. Specimens are shown in Fig. 1.

Specimen no.	Wing area (mm^2)	Hand-wing area (mm^2)	Arm-wing area (mm^2)	Wingspan (mm)
5	(Torn)	16.9	(Torn)	22.45
6	66.68	37.25	29.43	31.02
7	46.53	20.56	25.97	26.11
8	67.98	32.54	35.44	32.45
9	110.93	64.1	46.83	42.98
12	360.83	142.17	218.66	71.62
13	484.8	201.13	283.67	78.44
15	1,265.22	626.41	638.81	129.96
16	1,690.18	649.84	1,040.34	138.94
17	1,532.55	637.21	895.34	130.46

listed in Table 2. The square root of both arm- and hand-wing area grow in an approximately linear fashion with respect to CRL. The square root of arm-wing area grows isometrically with CRL (slope = 0.98; expected slope under isometry = 1.00), whereas the square root of the hand-wing apparently exhibits a slower growth rate (slope = 0.80). However, the small sample size prevented statistical tests being conducted on this series.

Digits and claws.—The developmental stages of forelimbs and hind limbs already differ significantly by the 1st specimen of the *Rousettus* series. The handplates of stage 16 specimens (1 and 2) differ dramatically in size from the footplates, with the handplate being about twice as large as the footplate (Fig. 4). In stages 16 and 16L, the handplate evinces finger rays and interdigital notches (Fig. 4), whereas the footplate shows only slight toe condensations only in the plantar side and no sign of recessing interdigital tissue (Fig. 5). In the next stage (stage 17; specimen 3), the first 2 digits of the hand are clearly differentiated, and the foot exhibits deep interdigital notches and conical toes. In stage 18 (specimen 4), claws are beginning the process of differentiation, as the proximal claw fold (after Hamrick 2001a) of thumb and index finger become apparent. In turn, a constriction marks the joint of the ungual phalanx in the toes. Faint proximal claw folds are present. In this stage the thumb is free from the handplate. All claws are fully distinct from the surrounding epidermis by stage 19 (specimen 5). In stage 22 (specimen 9) the claws approach adult morphology, showing a lateral flange, and a ventral side excavated and sharply distinct from the toe pad. In stage 22L (specimens 12 and 14) the dorsal aspect of claws becomes translucent as the keratinization process continues.

Genitalia.—A relatively large and elongate genital tubercle (Figs. 2 and 5) is present in the smallest embryos of this series, which is consistent with our interpretation of specimen 1 as equivalent to *Carollia* stage 16 (the genital tubercle appears in *Carollia* stage 15). A well-developed urogenital groove is present in all specimens up to stage 22. The genital tubercle is

bent from stage 18 to 20, when external genitalia probably begin dimorphic differentiation. Because of relatively poor preservation, we were unable to document such differentiation in detail, but specimens in the late fetal stage show essentially young adult genital morphology except for hairs being sparse and short. Specimen 18, a male, exhibits a well-developed penis tightly enclosed in a praeputium, with the scrotum being inconspicuous.

Skin and hair cover.—The aspect of the skin in early embryos of this series is generally smooth in ventral parts and limbs and wrinkled in the crown and dorsum. In stage 17, a pair of skin ridges appears along the dorsum, and fades out by stage 20. By this stage a noticeable thickening of the skin is evident. In late fetal specimens, the dorsum is longitudinally crossed by a number of grooves representing skin folds. It is likely that both the ridges and these grooves represent stages of skin growth in which the skin surface is larger than the body surface, so that foldings appear.

In stage 20, most of the previously naked skin shows widespread bumps that correspond to developing hair follicles, except on wings and pinnae. These primordial hair follicles contain 5–15 pigmented bodies (likely clusters of melanocytes), but this composition becomes evident only in 1st fetal-stage specimens (e.g., specimen 13). Later in fetal development (specimen 15), isolated guard hairs emerge and are distributed on all dorsal body parts. These hairs appear on average longer on the dorsum. In specimens 16 and 17 a 2nd layer of shorter, more numerous hairs grouped in small clusters of some 5 units cover the dorsum and also the crown and forearm. Crown hairs are even wooly in texture, whereas forearm hairs are very short. In the rump, all hairs appear thicker and appressed to the back, as typical of adult *Rousettus*. In specimen 18, sparse hairs are distributed on the genitals and some hair appears on the trailing edge of the uropatagium. The dorsa of the feet also acquire sparse hair. Up to now, all ventral parts lacked hairs; a few isolated hairs appear in the throat of specimen 18.

Mammary buds are already present in stage 16. They consist of a bulge and an indistinct slitlike transversal depression. This morphology remains during the entire prenatal period. In specimen 12 (stage 22L) an areola mammaria is evident.

DISCUSSION

The *C. perspicillata* staging system introduced by Cretekos et al. (2005) represented a useful framework for the study of prenatal development in another bat, the remarkably different megachiropteran *R. amplexicaudatus*, described here. Our study demonstrates that a staging system can be used as a general reference (Dettlaff and Dettlaff 1961), but its usefulness in comparative studies may be limited, because older specimens showed more deviations from the *Carollia* staging system than did younger ones. Comparison with staging in another megachiropteran, *S. australis* (Lawrence 1991), further supports this contention. As development progresses, the adult structures characteristic of *Rousettus* differentiate. As shown recently (Bininda-Emonds et al. 2003; Jeffery et al. 2002), sequence heterochronies can alter the set of morphological

characters, usually used in staging systems, appearing at any one time. With that in mind, the data presented in this paper could be used in the future together with those for other bat species, in studies of heterocrony using methods based on event-pairing (e.g., Jeffery et al. 2005).

For comparative purposes, terminology of the *Carollia* staging system proposed by Cretekos et al. (2005) requires adjustment. First, the term "nose-leaf primordium," applied to the equivalent of mammal stage 16, is not applicable to most bats; we propose its replacement by "pinna primordium." Second, the term "peek-a-boo," applied to the equivalent of mammal stage 19, does not refer in itself to any specific anatomic structure; our proposal is to replace it by "claw primordia." In our view, our modifications facilitate the comparisons across bat species (all of which have pinnae and claws) on the basis of structures with widely accepted anatomical names.

Examination of the ontogeny of integumentary structures in the megabat *R. amplexicaudatus* reveals how several remarkable specializations are generated during prenatal growth. The 1st stages in which structures with significant integumentary contribution are clearly recognized show features that are derived if compared to other mammalian embryos with less extreme integumentary structures. For instance, the handplate of *Rousettus* (as in *Carollia*) is double the size of the footplate at the moment in which both are fully differentiated (stage 16; in most mammals, handplate and footplate are of comparable size). On the other hand, extreme specializations of bats, such as the patagia, do not appear fully formed (with similar connections and proportions as in the adult) when complete limbs are present. Comparative analyses of development in mammals show that adult variation in limb proportions has "high developmental penetrance," that is, adult differences are observed at early ontogenetic stages (Hamrick 2001b; Richardson 1999). The alternative condition, "low developmental penetrance," involves a gradual change from an ancestral condition to a derived one. Integumentary structures in *Rousettus* cannot be placed at either end of the spectrum.

Based on our own observations and in accordance with expectations derived from other studies and from the existing knowledge on limb morphogenesis, bat wing membrane morphogenesis includes 2 different aspects. One concerns the tissue between digits 2 and 5 in the forelimb, the other the tissue between the shoulder, wrist, forelimb, and hind limb. The formation of the dactilopatagium results from persistence in adulthood of interdigital tissue present earlier in development. Most mammalian species show interdigital cell death during growth (Tickle 2002); lack thereof is under simple mutagenic control (Muragaki et al. 1996). In bats, the interdigital tissue not only is retained, but it is further developed into an extensive dactilopatagium with all the elements typical of wing membranes. The hand skeleton that supports the dactilopatagium further exhibits remarkable elongation and anteroposterior patterning (Cretekos et al. 2005—more properly, mediolateral patterning, by which the development of finger structure strongly differs from digit I to V). That is, the retention of interdigital tissue and its posterior full development, coupled

with an enlarged and anteroposterior patterned handplate, ultimately lead to the formation of the characteristic chiropteran hand wing. Concerning the development of the skeleton of the bat wing, some of the genetic changes associated with its evolution are beginning to be studied. Chen et al. (2005) reported expression patterns of the *Hoxd13* gene in the phyllostomid *C. perspicillata* different from those in the mouse model, differences that are likely associated with development of the wing.

Development of the propatagium, plagiopatagium, and uropatagium involves a different mechanism. As shown by Cretekos et al. (2005) in species of Phyllostomidae and in this work in *Rousettus*, these portions of the bat wing grow from the proximal regions of the limbs and expand as development progresses. It is significant that the 3 patagial primordia are integumentary tracts that contain specific muscles (occipitopolicallis, humeropatagialis, and uropatagialis), all of which are unique to bats (Vaughan 1970). We suggest that the development of the extensive wing membranes that form the pro-, plagio-, and uropatagium is intimately linked to the origin and development of the corresponding muscles and their associated nerves and vessels. Particularly in the case of the plagiopatagium, the digitiform shape of the primordium and its evident distal progression seems to lead the formation of wing membrane as it grows toward the tip of digit V. If the development of wing membrane is indeed linked to the presence and growth of such muscles, as we postulate here, then the development of the entire bat wing, and perhaps its evolution, depends on the concurrence of 3 factors: retention of interdigital tissue; enlarged, anteroposterior patterning in the handplate; and muscular novelties capable of channeling extensive dermal growth, each specific of a patagial region.

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