DISSERTATION

Titel der Dissertation

The bat baculum:

Histomorphological methods and microCT-based 3D models to distinguish cryptic species of *Pipistrellus* and *Plecotus* and to test functional hypotheses using post-mortem specimens

verfasst von

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"*Nothing in biology makes sense except in the light of evolution*"
(Theodosius Dobzhansky 1973)

"*Nothing in evolution makes sense except in the light of sex*"
(SERG 2013)

Skeleton of *Pipistrellus pipstrellus*
(Volume rendering of microCT images, scale bar: 1cm)

ABSTRACT

With the recent discovery of many cryptic bat species, finding morphological characters discriminating these genetically defined taxa has become a crucial issue. The baculum (os penis) has been assumed to play a role in mate choice and could be a mating barrier between closely related species. Thus, it has been used in species discrimination for a long time. The *Pipistrellus pipistrellus/pygmaeus* species complex is a model system for cryptic diversity in European bats. However, no reliable morphological discriminating characters have yet been found. This dissertation introduces a discriminant function, combining projected length, width, and height of the baculum, which was able to reliably assign 97.9% of the tested specimens (n=48) to their genetically identified species. To establish a baseline for functional anatomy, the baculum was studied histologically and experimentally. The baculum in connection with the surrounding soft tissues of the penis in *Plecotus* and *Pipistrellus* species was evaluated in serial, surface-stained ground sections and semithin sections in combination with microCT imaging. To test hypotheses on baculum function experimentally, the corpora cavernosa of bat penes were inflated post mortem. Baculum positions in relation to the soft tissues were compared in high-resolution microCT virtual sections and 3D models of flaccid and "erect" penes. Histomorphological and experimental results supported both of the hypotheses tested: 1) baculum and corpora cavernosa form a functional unit, supporting both the penile shaft and the glans; and 2) the baculum can protect the distal urethra and urethral opening from compression during erection and copulation.

KURZFASSUNG

Das Finden morphologischer Merkmale zur Bestimmung von Fledermausarten gewinnt mit der Entdeckung weiterer kryptischer Arten zunehmend an Bedeutung. Das Baculum (Os penis), ein oft verwendetes Artbestimmungsmertkmal, spielt vermutlich eine Rolle in der Partnerwahl und könnte als Fortpflanzungsbarriere zwischen nah verwandten Arten fungieren. Für den *Pipistrellus pipistrellus/pygmaeus* Artenkomplex, der ein Modellsystem für die kryptische Diversität europäischer Fledermäuse darstellt, wurde bis jetzt noch kein verlässliches morphologisches Artbestimmungsmerkmal gefunden. Diese Dissertation stellt eine Diskriminanzfunktion aus projizierter Länge, Breite und Höhe des Baculums vor, mit der 97,9% aller getesteten Fledermäuse (n=48) ihrer zuvor molekulargenetisch bestimmten Art zugeordnet werden konnten. Um eine Grundlage für funktionell-anatomische Studien zu schaffen, wurde das Baculum histologisch und experimentell untersucht. Bacula von *Plecotus* und *Pipistrellus* Arten wurden, zusammen mit dem umgebenden Penisgewebe, mit seriellen Dünnschliffen und Semidünnschnitten, in Kombination mit MikroCT Aufnahmen, untersucht. Um Hypothesen zur Baculumfunktion experimentell zu testen, wurden die Corpora cavernosa der Penes toter Fledermäuse mit Formol aufgefüllt. Die Position der Bacula und der Weichgewebe im Verhältnis zueinander wurden, mittels virtueller Schnittebenen und 3D Modellen aus hochauflösenden MikroCT Aufnahmen, zwischen schlaffen und "erigierten" Penes verglichen. Histomorphologische und experimentelle Ergebnisse unterstützen die beiden getesteten Hypothesen: 1) Baculum und Corpora cavernosa bilden eine funktionelle Einheit um gemeinsam sowohl den Penisschaft als auch die Glans penis zu versteifen. 2) Das Baculum kann die Urethra und ihre Öffnung während Erektion und Kopulation davor schützen, komprimiert zu werden.

CONTENTS

1. INTRODUCTION

Reproductive isolation between closely related species is a key factor in speciation [Mayr, 1942]. Occasional breakdown of reproductive isolating mechanisms, leading to hybridization, has been found to occur in most animal groups with well established taxonomies [Mayr, 1963]. The properties whose variability lead to reproductive isolation can be very different in different species. Behavior, vocalization, olfactory cues, and morphology probably all play a role in reproductive isolation. The morphology of reproductive organs could be an important factor [Mayr, 1942]. The baculum is especially interesting, because it is a sexually selected character and often distinctly different in shape and size between closely related species [Hosken et al., 2001]. Although it has been used as a species discriminating character for precisely that reason, especially for Rodentia and Chiroptera, not much is known about its function [Patterson & Thaeler, 1982]. In this dissertation project I quantified individual and interspecific variation of the baculum and studied its probable role in reproductive isolation, and its mechanical functions.

1.1 Cryptic diversity in European bats

Cryptic diversity in European bats poses major challenges to research and conservation efforts. To enable meaningful research on a species' ecology or conservation status, the specimens studied have to be identified reliably [Arlettaz, 1999; Davidson-Watts et al., 2006; Sattler et al., 2007; Ashrafi et al., 2010]. During the last two decades, research on cryptic species has increased dramatically, mostly due to the increasing availability of relatively inexpensive and rapid DNA sequencing. In the Western Palaearctic region alone, 37 morphologically defined vespertilionid bat species were recognized prior to the advent of DNA sequencing in taxonomy. Now a total of 54 species, with new members in every speciose genus, have been identified by the combination of morphological and genetic approaches. Neither method alone could detect all species. Since Western Palaearctic bat species are among the best-studied chiropteran groups in terms of geographic coverage,

number of studied individuals, and applied techniques, an enormous rise in bat species diversity is anticipated in less studied regions such as the tropics [Mayer et al., 2007].

1.2   The study system

After an initial study on functional baculum histomorphology in species of the genus *Plecotus* (Fig. 1.1; Paper I), I switched to the *Pipistrellus pipistrellus/pygmaeus* species complex (Figs 1.2 and 1.4; Papers II-IV), because it is an emerging model system for bat speciation and because it was easier to get sufficient material to study individual varia-tion. Other species used for comparison were *Pipistrellus nathusii* (Paper III) and *Nyctalus noctula* (Fig. 1.3; Paper III), because of their baculum shapes [Hill & Harrison, 1987] and mating systems [McCracken & Wilkinson, 2000], which are similar to those of *Pipistrellus pipistrellus* and *Pipistrellus pygmaeus*.

Fig. 1.1: *Plecotus austriacus* (with kind permission of the photographer, Harald Polt)

Fig. 1.2: *Pipistrellus pipistrellus* (photo: A. N. Herdina)

1.3   *Pipistrellus pipistrellus/pygmaeus* species complex as model system

The *Pipistrellus pipistrellus/pygmaeus* species complex (Figs 1.2 and 1.4) has become a model system for studying cryptic diversity in European bat species, speciation, and reproductive isolation and hybridization between closely related species [Davidson-Watts et al., 2006; Hulva et al., 2010; Sztencel-Jabłonka & Bogdanowicz, 2012]. The discovery of further cryptic bat species, mostly based on molecular genetic data, necessitated the search for morphological species discriminating characters.

The *Pipistrellus pipistrellus/pygmaeus* species complex provides a remarkable system for investigating links between morphology, echolocation call design and resource partitioning [Davidson-Watts et al., 2006; Kaňuch et al., 2007b], and has consequently been the subject of many studies. Most of the studies involving one or both of these cryptic species have focused on the genetic diversity and the discovery of new cryptic species within the genus. However, there has also been a great deal of research on ecology, distribution range and overlap, behavior, physiology, morphology, echolocation, fossil record, and parasite load of *P. pipistrellus* and *P. pygmaeus*.

Fig. 1.3: *Nyctalus noctula* (photo: A. N. Herdina)

1.3.1   The genus *Pipistrellus*

The genus *Pipistrellus* comprises 75 species in 7 subgenera and occurs all over Eurasia, Africa, the Australasian region, and North and Central America [Krapp, 2004]. There are five *Pipistrellus* species in Europe: *P. pipistrellus*, *P. pygmaeus*, *P. hanaki*, *P. kuhlii*, and *P. nathusii* [Krapp, 2004; Evin et al., 2011]. The common pipistrelle (*Pipistrellus pipistrellus* sensu lato (s.l.)) has been considered one of the most abundant and well known bat species in Europe [Jones & Barratt, 1999], until the existence of two separate species, *P. pipistrellus* and *P. pygmaeus*, was discovered by Jones & van Parijs [1993] and confirmed through mitochondrial DNA analysis by Barratt et al. [1995; 1997] and by Mayer & von Helversen [1999; 2001a].

1.3.2   Traits distinguishing the species *Pipistrellus pipistrellus* and *P. pygmaeus*

Since they were assigned to separate species, much research on possible species identification traits has been reported [Barlow & Jones, 1997b; 1999; Häussler et al., 2000; Ziegler et al., 2001; Sendor, 2002; Sendor et al., 2002; von Helversen & Holderied, 2003; Ahlen et al., 2004; Dietz & von Helversen, 2004; Dubourg-Savage, 2006; Dietz et al., 2007; Kaňuch et al., 2007a; Pavlinić et al., 2008; Sztencel-Jabłonka et al., 2009; Evin et al., 2011]. Still, the most useful method for reliable determination of both species of the *P. pipistrellus* complex

Fig. 1.4: left: *Pipistrellus pygmaeus*, right: *Pipistrellus pipistrellus* (upper photo with kind permission of the photographer, Edmund Weiß; lower photo: A. N. Herdina)

(Fig. 1.4) remains genetic analysis along with the analysis of echolocation calls [Mayer & von Helversen, 2001b; Benda et al., 2003b; Kaňuch et al., 2007a; Hulva et al., 2010]. Many attempts to find distinguishing morphological parameters have failed due to the similarity of external characters of the two species [Pavlinić et al., 2008]. Measurable parameters like skull morphology [Barlow & Jones, 1997b; Sztencel-Jabłonka et al., 2009; Evin et al., 2011] and wing morphology [Barlow & Jones, 1999; Häussler et al., 2000] indicate that *Pipistrellus pygmaeus* is on average slightly smaller than *P. pipistrellus*. Unfortunately, the overlap of quantitative parameters between the species is too large, so discrimination of the two sibling species with these features is not feasible when studying single specimens. Qualitative characteristics include the shorter muzzle of *P. pygmaeus*, an internarial ridge (hump between nostrils) in *P. pygmaeus*, and the light to orange colored penis in *P. pygmaeus* as opposed to the dark penis with paler median stripe in *P. pipistrellus* [Häussler et al., 2000;

Ziegler et al., 2001; Sendor et al., 2002; Zöphel et al., 2002; Sattler, 2003; von Helversen &
Holderied, 2003]. Additionally, the musky smell of *P. pygmaeus* can sometimes be useful
to identify live bats [Bartonička et al., 2010]. Those qualitative characters can be useful
if applied by someone with good knowledge of both species. Ziegler et al. [2001] proposed
to use the outer shape of the baculum (Fig. 1.5) as a possible species specific trait, but
also cautioned that to use this trait will first require examining the bacular shape in larger
numbers of individuals.

1.3.3   Molecular genetic studies on *Pipistrellus pipistrellus* and *P. pygmaeus*

Studies about the genetic diversity within the genus *Pipistrellus* have uncovered *Pipistrellus
hanaki* as a further cryptic species and an unexpected number of genetic lineages [Barratt
et al., 1995; 1997; Bullejos et al., 2000; Horáček et al., 2000; Ahlén & Baagøe, 2001; Mayer &
von Helversen, 2001b; Volleth et al., 2001; Jones et al., 2002; Barragan et al., 2003; Hoofer
& Van Den Bussche, 2003; Benda & Ivanova, 2003; Benda et al., 2004b; Hulva et al.,
2004a;b; Karataş et al., 2004; Kruskop & Matveev, 2004; Krystufek & Rezek Donev, 2005;
Ibáñez et al., 2006; Benda et al., 2007; Bryja et al., 2007; Hulva et al., 2007a;b; Kaňuch
et al., 2007b;a; Kruskop, 2007; Mayer et al., 2007; Racey et al., 2007; Benda et al., 2008;
Bryja et al., 2008; Neckářová, 2008; Bryja et al., 2009; Fornůsková, 2009; Hulva et al., 2010;
Sztencel-Jabłonka & Bogdanowicz, 2012]. Thus, it has become important to separate the
new cryptic species and linages morphologically as well as genetically. Sztencel-Jabłonka
& Bogdanowicz [2012] have found bidirectional hybridization and a high probability that
backcrossing and second generation hybrids occur.

1.3.4   Distribution range of *Pipistrellus pipistrellus* and *P. pygmaeus*

Distribution range [e.g. Presetnik et al., 2001; Dietz et al., 2002; Benda et al., 2003a; 2004b;
Řehák et al., 2004; Wermundsen & Siivonen, 2004; Kapfer et al., 2007] and range overlap
[e.g. Hanák et al., 2001; Mayer & von Helversen, 2001b; Benda et al., 2003a; Pētersons,
2003; Agirre-Mendi & Ibáñez, 2004; Benda et al., 2004a] of the two species have been
studied since they were recognized as separate species. They occur sympatrically in most
of their distribution range in central and southeastern Europe and Great Britain [Benda
et al., 2004a; Kapfer et al., 2007]. Allopatric populations of *Pipistrellus pipistrellus* occur
in the Mediterranean region of northern Africa (Morocco, Algeria, and Tunisia) [Benda
et al., 2004b; Hulva et al., 2007a], Afghanistan, [Habibi, 2003], Albania [Prigioni, 1996],

Andorra [Hutson et al., 2008a], China [Smith et al., 2010], Finland [Hulva et al., 2007a], the Jammu-Kashmir region of India [Simmons, 2005], Israel [Mayer & von Helversen, 2001b], Kazakhstan [Hulva et al., 2004a], Lebanon [Hulva et al., 2007a], Liechtenstein [Hoch, 1997], and Pakistan [Mahmood-ul-Hassan & Nameer, 2006]. *Pipistrellus pygmaeus* occurs allopatrically in parts of Denmark [Hulva et al., 2004a] on Malta [Baron, 2007], in Norway [Jones, 1997], and parts of Sweden [Benda et al., 2003a; Hulva et al., 2004a]. For maps of the species' ranges see Hutson et al. [2008a;b].

1.3.5   Reproductive biology

Most studies on reproductive biology were done before *Pipistrellus pipistrellus s.l.* was split into two species. Gerell & Lundberg [1985]; Lundberg & Gerell [1986] found the mating system of *Pipistrellus pipistrellus s.l.* to be resource defense polygyny in Sweden (where both species occur sympatrically; according to Sachteleben & von Helversen [2006], this study most probably describes *P. pygmaeus'* mating system). *P. pipistrellus s.l.* has a mating system with seasonal single-male/multi-female groups, like many other temperate bat species (including *Pipistrellus nathusii* [Heise, 1982] and *Nyctalus noctula* [Sluiter & Van Heerdt, 1966]). After a brief period of males and females roosting in mixed groups in spring after hibernation, single *P. pipistrellus* males establish their mating territories in June and defend them until September [Gerell & Lundberg, 1985]. The females spend the summer in maternity colonies and join the males in their territories in August for mating. During August and September, the composition of single-male/multi-female groups is variable. Males are mating with multiple females in their established territories, but females also move to different mating partners throughout this period [Gerell & Lundberg, 1985; Lundberg & Gerell, 1986; McCracken & Wilkinson, 2000]. Males and females seem to show some fidelity to the same roost sites and some have been found to return in successive yeas [Gerell & Lundberg, 1985; McCracken & Wilkinson, 2000]. In both *P. pipistrellus sensu stricto (s.str.)* and *P. pygmaeus*, males occupy territories and roosts and attract females with songflight displays [Lundberg & Gerell, 1986; Park et al., 1996; Barlow & Jones, 1997a; McCracken & Wilkinson, 2000; Sachteleben & von Helversen, 2006; Jahelková, 2011]. It seems that in *P. pipistrellus s.str.*, sometimes the male territories are too small to contain a defendable resource. Thus, the mating system also shows some similarity with a lek based mating system [Sachteleben & von Helversen, 2006]. The advertisement calls of the two species differ in frequency and in number of components. *P. pygmaeus* songflight calls typically consist of fewer components at higher frequencies than *P. pipistrellus s.str.* calls.

Since advertisement calls and songflight displays play an important role in mate choice, they are very likely to also act as a mating barrier between the species [Barlow & Jones, 1997a; Jahelková, 2011]. Olfactory cues may also play a role in mate choice and mating barriers between *P. pipistrellus* and *P. pygmaeus* [Bartonička et al., 2010].

In bats very little is known about mating behavior, copulation duration, and copulation postures [Glass, 1966; Barclay & Thomas, 1979; Hosken et al., 2001; Dixson et al., 2004; Jahelková, 2011; Dixson, 2012; Liu et al., 2013]. Since, to the best of my knowledge, nothing is known about pipistrelle copulations, I summarize here what is known about copulation in other bat species.

In *Myotis lucifugus*, copulation is initiated by the male immediately after contact with the female, without precopulatory interaction. The male establishes a dorsoventral copulation posture, by positioning himself dorsal to the female. He bites the female on the back of the neck. The male arches his back and secures the female by bracing his feet and thumbs on the substrate. The female often struggles and emits broad-band vocalizations typical of agonistic encounters during the early phase of copulation. The male repeatedly emits a specific copulation call, increasing the intensity and repetition rate as the agitation level of the female increases. When the female ceases to struggle the male decreases the intensity and repetition rate of the copulation call, usually 5 to 10 min into the copulation bout. Copulations also take place in winter. In periods of arousal during hibernation, males indiscriminately attempt to copulate with individuals of either sex. The recipient bats are torpid, so they do not struggle, and no copulation calls are emitted [Barclay & Thomas, 1979].

Rhinolophus ferrumequinum mate hanging from the ceiling. At least in captivity, the male approaches the female, echolocates and touches her with a wing or his muzzle before initiating copulation. The male positions himself dorsal of the female to achieve a dorsoventral copulation posture, arches his back and hugs the female from behind using his feet and thumbs. The female either struggles during the early part of copulation, by flapping her wings at the male, or she remains passive or torpid. The male emits copulation calls with short syllables and long syllables. Usually a male emits more than 10 sequences of copulation calls during each copulation, using short syllables when he tries to insert his penis and long syllables after intromission [Liu et al., 2013].

In *Rhinopoma hardwickei* a courtship display precedes the copulation. The male approaches the female with quivering, half unfolded wings, pulling himself forward by the thumbs. This is repeated until the male covers the female completely. The male gently touches the back of the female with his muzzle and continues his display, until initial vocal protest by the female subsides. Then the male assumes a dorsoventral copulation posture by placing his folded wings between the legs and the forearms of the female, which now stands up on all fours. The female turns her tail to one side, while the male turns his tail up. In some cases a small area surrounding the root of the tail of the male shows movements which are comparable to servicing movements. The male and the female remain in this position for about one to two minutes. The female may show some trembling movements during copulation. Sometimes the female appears to free herself by moving her head to one side and vocalizing. In other cases the male leaves the female after copulation [Khasuria, 1972].

Lasiurus borealis have been observed copulating in flight [Glass, 1966]. Their strong and abundant penile spines are hypothesized to serve as copulatory lock during aerial mating [Cryan et al., 2012].

In the Pteropodidae, more precopulatory interaction seems to take place. A male *Rousettus aegyptiacus* often licks the female's face and genitals prior to copulation [Jahelková et al., 2013]. In *Pteropus scapulatus* copulation is also preceded by orogenital grooming for 20 seconds. Copulation takes place in the dorsoventral position. Both animals hang by their feet in their normal head-down posture, so each animal supports its own weight. The female appears undisturbed by the male's thrusting movements. A single mount lasts 175 seconds, with a single intromission, which includes a mean of 20 seconds of thrusting in most copulations, ejaculation, and copulatory lock for about 155 seconds. Ejaculation, lasting only about 9 seconds, occurs in about 20% of copulations. In 65% of copulations, mating between the same pair resumes, usually after about 35 seconds. Typically each pair undertakes a series of two to three copulations at a time. Mating is still and silent [O'Brien & Nankervis, 1994]. In captive *Cynopterus sphinx*, male and female lick each others faces before copulating in a dorsoventral position (for about 100-300 seconds), and the female sometimes licks her mate's penis shaft, while the glans is inserted in her vagina [Tan et al., 2009].

In many rhinolophid and vespertilionid species, fertilization is delayed for up to seven months after copulation [Racey, 1979]. Sperm is stored in the female reproductive tract, with storage sites varying by species, in the uterus, utero-tubal junction, and/or oviduct [Racey, 1979; Fenton, 1984; Orr & Zuk, 2013]. Males also store sperm, in the ductus deferens and cauda epididymidis [Racey, 1979]. Racey [1979] experimentally found that stored sperm can be viable and fertile for up to 198 days in the uterus, and up to seven months in the cauda epididymidis. This enables hibernating species to mate at a favorable time before (and during) hibernation and to ovulate directly after hibernation. Thus they raise their young when food availability is greatest [Fenton, 1984]. In tropical bat species, sperm is stored for much shorter periods than in bat species which hibernate [Racey, 1979].

Reproductive delays between mating and birth may provide possibilities for postcopulatory sexual selection in bats [Birkhead & Møller, 1993; Orr & Zuk, 2013]. Delayed fertilization could provide more opportunities for sperm competition [Parker, 1970; Wilkinson & McCracken, 2003] by extending the time for ejaculates to interact in the female reproductive tract [Birkhead & Møller, 1993; Wilkinson & McCracken, 2003; Orr & Zuk, 2013]. Multiple mating by females seems to be common in bats. Molecular genetic studies have also found multiple paternity in some bat species, including *Nyctalus noctula* [Mayer, 1995; Wilkinson & McCracken, 2003].

Male testes mass in relation to body size correlates with the size of social groups over a wide range of bat taxa [Hosken, 1997; McCracken & Wilkinson, 2000; Wilkinson & McCracken, 2003]. Testes mass is an indicator for number of produced sperm, and group size an indicator for the risk of sperm competition [Hosken, 1997; McCracken & Wilkinson, 2000]. Thus, it is likely that male reproductive success depends on ejaculate size and the competitive ability of sperm [Parker, 1970; Wilkinson & McCracken, 2003] and that sperm competition is an important factor in the evolution of bat mating systems [Wilkinson & McCracken, 2003].

In *Pipistrellus pipistrellus s.l.*, spermatogenesis takes place in summer and copulations begin in August and September. It is possible that copulations continue during hibernation, like in other temperate bat species. However, most of the females seem to be inseminated by the beginning of hibernation and sperm is stored in the uterus. After hi-

bernation, ovulation occurs in April and May [Racey, 1979].

During their main mating season, from August to October in temperate regions, *Pipistrellus pipistrellus* and *P. pygmaeus* do not share roosts, even in regions where they occur sympatrically [Park et al., 1996]. Mating groups typically consist of one male and up to ten females [Gerell & Lundberg, 1985; Park et al., 1996; McCracken & Wilkinson, 2000].

1.3.6   Roost use in *Pipistrellus pipistrellus* and *P. pygmaeus*

Research on summer roost use and roost switching behavior of *Pipistrellus pipistrellus* and *P. pygmaeus* has found that both species roost in small crevices, e.g. on the outside of buildings behind weather boarding or under roof tiles, in tree holes, or behind loose bark on trees [Feyerabend & Simon, 2000; Cel'uch et al., 2006; Mazurska & Ruczyński, 2008; Kelleher, 2008]. Bats may use buildings in urban areas, but they seem to prefer buildings close to hunting habitats to minimize commuting distance [Mazurska & Ruczyński, 2008]. Preference for roosts in and on buildings or in and on trees seems to vary regionally, probably due to availability of suitable roosts and climate. *P. pipistrellus* was found to have longer commuting distances between roosts and foraging habitats than *P. pygmaeus* [Comber et al., 2006]. Bat boxes can be accepted as alternative summer or autumn roosts by *P. pygmaeus* in habitats where natural roosts are scarce [Bartonička & Řehák, 2007; Michaelsen, 2011]. Generally, *P. pygmaeus* seem to prefer bat boxes that reach higher temperatures in summer, even in the hot Mediterranean climate [Rebelo et al., 2001; Lourenço & Palmeirim, 2004; Flaquer et al., 2006]. In central Europe, *P. pipistrellus* maternity colonies begin to congregate in early May, typically reaching their maximum number of females by the end of May [Feyerabend & Simon, 2000]. Maternity colonies in *P. pipistrellus*, comprising about 25-50 individuals (sometimes up to 200), are typically smaller than in *P. pygmaeus* [Hutson et al., 2008b]. *P. pygmaeus* maternity colonies are typically larger, numbering up to 250 (occasionally up to 3,000) individuals [Hutson et al., 2008b]. Maternity colonies of both *P. pipistrellus* and *P. pygmaeus* use a network of several roosts in an area and switch between them frequently [Feyerabend & Simon, 2000; Bartonička & Gaisler, 2007; Bielik, 2007]. Reasons for roost switching are not clear yet, but they probably include roost temperature, parasite load within the roost, accumulation of feces in the roost, and predation risk [Fenton et al., 1994; Feyerabend & Simon, 2000; Bartonička & Gaisler, 2007].

Winter roosts of *P. pipistrellus* and *P. pygmaeus* have been found in and on build-

ings and in caves, mines, and underground bunkers [Sendor, 2002; Lustrat & Julien, 2003; Horáček, 2004; Kokurewicz, 2004; Smith & Racey, 2009; Murariu & Gheorghiu, 2010]. Large hibernating aggregations of pipistrelle bats, hundreds and up to thousands of individuals, have been found in several European countries (e.g. in Germany, France, Czech Republic, Slovakia and Romania) [Kaňuch et al., 2010]. In some cases *P. pipistrellus* and *P. pygmaeus* have been found in mixed aggregations, consisting of higher numbers of *P. pipistrellus* and some individual *P. pygmaeus*. Most of the larger winter aggregations seem to be just *P. pipistrellus*, even in areas where both species occur sympatrically in summer. Thus, *P. pygmaeus* seems to either prefer more cryptic hibernation roosts in hollow trees and crevices, or to migrate to different regions than *P. pipistrellus* to hibernate [Wojtaszyn et al., 2004; Kaňuch et al., 2010; Murariu & Gheorghiu, 2010].

P. pipistrellus and *P. pygmaeus* hibernate in mixed groups of males and females. In spring, during migration to the summer roosts, males and females can sometimes be found roosting in mixed groups as well. In summer, single males establish mating territories, while the females aggregate in large maternity colonies. During late summer and autumn, seasonal single-male/multi-female groups form for mating, before the migration to the winter roosts begins [Sendor, 2002; Sendor & Simon, 2003; McCracken & Wilkinson, 2000].

1.3.7   Migrations

Despite some reports of migration distances of over 1000km between summer roosts and hibernacula [Strelkov, 1969], *Pipistrellus pipistrellus s.l.* has generally been considered a regional migrant [Hutterer et al., 2005]. However, it seems there are differences in the two species' migration distances [Strelkov, 1969; Gaisler et al., 2003; Hutterer et al., 2005; Racey et al., 2007; Chudárková, 2008; Bryja et al., 2009]. Even in regions with sympatric summer occurrence of *P. pipistrellus* and *P. pygmaeus*, only *P. pipistrellus* was found in invasions of bats in buildings during autumn migration. This supports the hypothesis that the two species might have different migratory patterns [Kaňuch et al., 2010]. Winter records and population genetic results suggest that both species are sedentary in southwestern Europe, with estimated distances of only 10-50km between summer and winter roosts. However, in northern and eastern parts of their distribution range, where winters are more severe, seasonal migrations take place. In these regions, *P. pygmaeus* seems to migrate longer distances than *Pipistrellus pipistrellus* [Hutterer et al., 2005; Sachanowicz et al., 2006; Dietz et al., 2007; Kaňuch et al., 2010; Sztencel-Jabłonka & Bogdanowicz, 2012].

1.3.8   Foraging habitats, prey selection, and niche partitioning of *Pipistrellus pipistrellus* and *P. pygmaeus*

Foraging habitat preferences and prey selection differ between *Pipistrellus pipistrellus* and *P. pygmaeus* [e.g. Glendell & Vaughan, 2002; Russ & Montgomery, 2002; Kusch & Schmitz, 2013]. *P. pipistrellus* seems to be more of a generalist, using a wider variety of foraging habitats than *P. pygmaeus* [Vaughan et al., 1997; Russ & Montgomery, 2002; Kusch & Schmitz, 2013]. However, both species can sometimes be found sharing the same foraging habitat [Bartonička et al., 2008]. *P. pipistrellus* forages mostly in and on the edges of woodlands [Russ & Montgomery, 2002; Džingozovová, 2008], in suburban areas [Bärtschi, 2001; Bartonička & Zukal, 2003; Yiğit et al., 2005], and over lakes and rivers [Warren et al., 2000]. Within the variety of prey items found in *P. pipistrellus* droppings, Diptera of the families Psychodidae, Anisopodidae, and Muscidae are predominant [Barlow, 1997]. *P. pygmaeus* mainly hunts in deciduous woodland [Russ & Montgomery, 2002], and over lakes and rivers [Glendell & Vaughan, 2002; Bielik, 2007; Bartonička et al., 2008]. Diptera of the families Chironomidae and Ceratopogonidae are a predominant part of the diet of *P. pygmaeus* [Barlow, 1997; Bartonička et al., 2008].

Differences in preferred foraging habitat [e.g. Arnold et al., 2002; Sattler, 2003; Davidson-Watts et al., 2006; Kusch & Schmitz, 2013] and spatial segregation within habitats [Nicholls & Racey, 2006a], diet [Barlow, 1997], echolocation calls while foraging [Bartonička & Řehák, 2004; Bartonička et al., 2007], and timing of activity patterns [Nicholls & Racey, 2006a] all contribute to the successful niche partitioning between these morphologically extremely similar species [Nicholls & Racey, 2006b;a].

1.4   The Baculum

The baculum is a heterotopic bone, located in the glans penis, directly above the urethra (Fig. 1.6). A baculum can be found in many species of Rodentia, Chiroptera, Carnivora, Insectivora, and Primates. In most chiropteran families, all of the species have a baculum, but it is completely absent in the families Phyllostomidae and Noctilionidae and absent in some species of the families Mormoopidae, Vespertilionidae, Molossidae, and Pteropodidae [Hosken et al., 2001].

In most species of the vespertilionid genera *Plecotus* and *Pipistrellus*, the distal tip of

the baculum is situated at the urethral opening. The base of the baculum usually consists of two branches, whose proximal ends are linked closely to the corpora cavernosa. The tunica albuginea of both corpora cavernosa continues into the periosteum of the baculum [Matthews, 1937; Topál, 1958; Herdina, 2008; Herdina et al., 2010].

1.4.1   The baculum as taxonomic character

In mammals generally, intraspecific variation of the baculum is very small compared to interspecific variation [Patterson & Thaeler, 1982], making baculum morphology a potentially useful trait for identifying species. Bacula are routinely used as bat species discriminating characters and are usually described when a new species is characterized. Strelkov [1989] identified many different forms of bacula in the plecotine bats and described them as geographical variations. Each of those forms could later be assigned to a separate *Plecotus* species [Spitzenberger et al., 2006]. There are also micromorphological traits, like position and shape of the medullary cavity, that might be useful for species identification in some genera [Herdina, 2008; Herdina et al., 2010].

Bat biodiversity is by no means completely described, as recent new discoveries of species and subspecies demonstrate. It is possible to find new cryptic species based on bacular variation or to support such findings published in studies based solely on molecular biology. The genera *Pipistrellus*, *Plecotus*, *Hypsugo*, and *Eptesicus* were found to be polyphyletic because of their marked differences in baculum shape [Hill & Harrison, 1987]. In this dissertation, we demonstrate how to distinguish *Pipistrellus pygmaeus* and *Pipistrellus pipistrellus* by baculum characters (Fig. 1.5; Paper IV).

1.4.2   Baculum function in bats

The baculum most likely plays a mechanical role in erection and copulation for bats and other mammals, but the mechanism of its specific function is still not clear [Long & Frank, 1968; Patterson & Thaeler, 1982; Larivière & Ferguson, 2002]. In older studies, it has been suggested that the mammalian baculum arose as a by-product of indirect selection and pleiotropy [Burt, 1936; Mayr, 1963], but the general consensus in the literature is that the baculum probably has adaptive value and is subject to sexual selection [Simmons & Firman, 2013]. The energetic cost for the growth and maintenance of a baculum and the

Fig. 1.5: Bacula of *Pipistrellus pipistrellus*, *Pipistrellus pygmaeus*, *Pipistrellus hanaki*, *Pipistrellus nathusii*, and *Pipistrellus kuhlii* (left to right; 3D surface renderings of microCT images; scaled to the same size; marrow cavity and canals shown in orange) in dorsal view (upper row) and lateral view (lower row)

risks of infection, fracture or breakage [Sanderson, 1950; Bolton et al., 1996; Kierdorf, 1996] strengthen the hypothesis that the baculum is adaptive [Larivière & Ferguson, 2002]. The baculum probably has a precise reproductive function, which exposes it to direct selection and in turn allows bacular variation to function as a reproductive isolating mechanism [Patterson & Thaeler, 1982; Simmons & Firman, 2013]. The mammalian baculum probably has multiple overlapping functions. It could function during intromission indirectly, by providing mechanical support or affecting penile shape. The baculum could also protect the urethra from compression, enable protracted copulations, stimulate the female reproductive tract, provide information about male quality during intromission, or provide reproductive isolation [Dyck et al., 2004; Stockley, 2012]. For a more detailed discussion of baculum function see Paper III.
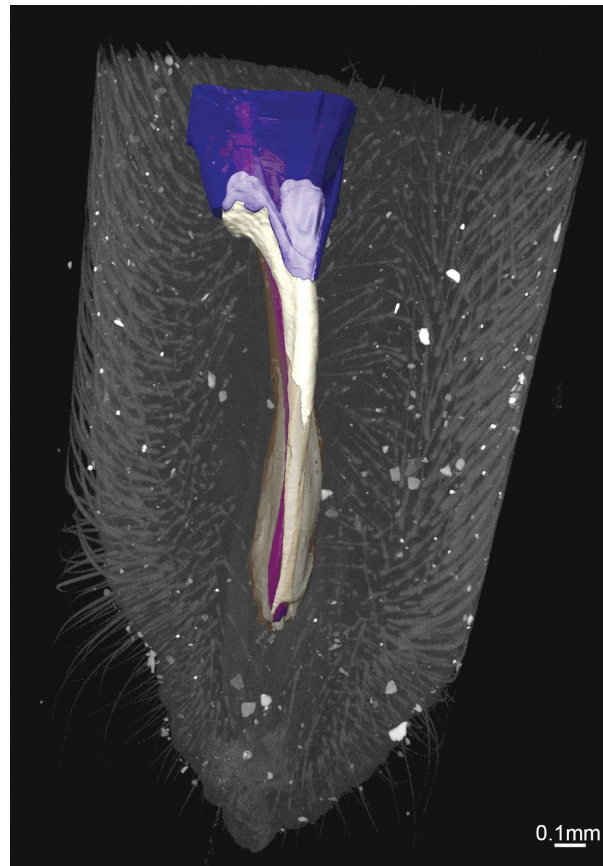
Fig. 1.6: Volume rendering of a *Pipistrellus pipistrellus* penis (glans and distal part of the shaft), overlaid with 3D surface renderings of baculum (white), urethra (purple), corpus spongiosum (brown), and corpora cavernosa (blue)

The most widely accepted theory on the evolution of bacular diversity is that the baculum is subject to sexual selection [Eberhard, 1985; Arnqvist, 1998; Danielsson & Askenmo, 1999; House & Simmons, 2002; Hosken & Stockley, 2004; Lüpold et al., 2004; Simmons & Firman, 2013; Stockley et al., 2013]. This is supported by the specifically differentiated and sometimes elaborate bacula of many mammals and their persistence in various lineages. In house mice, postcopulatory sexual selection on baculum thickness was shown in breeding experiments by Simmons & Firman [2013] and Stockley et al. [2013]. In some primates and carnivores that engage in prolonged intromissions during copulation, the length of the baculum is sexually selected [Dixson, 1987; Dixson & Anderson, 2004; Dixson, 2012]. The existence of variation in bacular morphology within a species allows for cryptic female choice. At the same time, the difference in baculum morphology between closely related species is usually much more pronounced than morphological differences

within a species [Burt, 1960; Patterson & Thaeler, 1982].

1.4.3 Baculum ontogeny

The development of the baculum has so far been studied mainly in species of Rodentia [Retterer, 1887; Ruth, 1934; Friley Jr., 1949; Callery, 1951; Williams-Ashman & Reddi, 1991; Spotorno, 1992; Murakami & Mizuno, 1984; 1986; Murakami, 1987; Edwards, 1997; Izumi et al., 2000; Yonezawa et al., 2011; Weiss et al., 2012] and Carnivora [Petrides, 1950; Scheffer, 1950; Miller et al., 1998; 1999; Albayrak et al., 2008; Schwery et al., 2011].

In *Rattus norvegicus* the adult baculum consists of three distinct parts. The proximal part is derived from a cartilage anlage, the central part is derived from membranous bone, and the distal part is nonlamellar bone [Kelly, 2000]. Rat baculum development starts with a densely packed mass of mesenchymal cells dorsal to the urethral plate in the fetus at 16.5 days after conception. At 18.5 days the mesenchymal mass differentiates into three discernible parts, the corpus cavernosum, and the proximal/central and distal parts of the baculum. The proximal/central part of the baculum differentiates into cartilage and bone until around parturition. Two days after birth a woven membranous bone (without a cartilage precursor) forms in the central part of the baculum [Murakami & Mizuno, 1984]. Three days postpartum a hyaline cartilage forms the proximal part of the baculum. Both cartilage and membranous bone of the proximal/central part of the baculum grow and fuse. Endochondral ossification starts 4-5 days after birth. Within two to three weeks after birth, the central part of the baculum develops lamellar bone in the shaft. Two to three weeks after birth fibrous connective tissue develops in the extracellular matrix of the distal part of the baculum. This fibrous tissue differentiates again into mature chondrocytes. Six weeks after birth calcium deposition starts in the distal part of the baculum. Ten weeks postpartum blood vessels and hematopoietic tissue invade the cartilage of the distal part of the baculum. Endochondral ossification starts around the blood vessels, but the outer area of the distal part of the baculum remains cartilaginous for at least eight months after birth and the bone formed in the distal part of the baculum remain woven bone [Murakami & Mizuno, 1984]. For a synopsis of the influence of hormones on the development of the rat baculum see Yonezawa et al. [2011].

There are a few older works on bat baculum ontogeny. Leydig [1857] describes the baculum as an ossification of the connective tissue septum between the corpora cavernosa

in "*Vespertilio pipistrellus*" (referring either to *Pipistrellus pipistrellus* or *P. pygmaeus*). Gilbert [1892], however, described a cartilage anlage in juvenile specimens of *Vespertilio murinus*.

Baculum ontogeny has been described in developmental stages for *Myotis myotis* [Vlček, 1967], *Nyctalus aviator* ("*Nyctalus lasiopterus aviator*")[Maeda, 1978], *Nyctalus noctula* [Smirnov & Tsytsulina, 2003], and *Vespertilio murinus* [Smirnov & Tsytsulina, 2003]. In *Myotis myotis*, no baculum was found in the first 12 days postpartum [Vlček, 1967]. This could, however, be due to the preparation method used, whereby it might not be possible to extract a cartilage precursor and only an ossified baculum would be found. In *Nyctalus aviator*, *Nyctlus noctula*, and *Vespertilio murinus* a small cartilage anlage of the distal portion of the baculum seems to be present already before birth. Ossification and morphogenesis begin a few days after after birth. In *Myotis myotis*, the baculum was first detected 15 days postpartum as a small oval shaped bone without indentations. It grows fastest between 22 and 51 days after birth. The *Myotis myotis* baculum begins to develop its typical arrow- and saddle-like shape between the 36th and 51st day after birth, the proximal end is still straight at this point. Between 68th and 82nd day postpartum, the protrusions of the proximal end of the baculum develop. In *Nyctalus aviator* ossification of the cartilage anlage starts 10 days after birth. The proximal part of the baculum develops until the 35th day postpartum. The baculum grows in length until 3-9 months of age. In *Nyctalus noctula* the stick-like baculum anlage with a forked distal end remains cartilaginous up to the 2nd or 3rd day postpartum, before it starts to ossify in the distal part of its central axis. Ossification of the *Nyctalus noctula* baculum is completed about 7 days after birth. Then the baculum starts to grow in length. The proximal part of the *Nyctalus noctula* baculum forms between the 15th and the 40th day after birth. In *Vespertilio murinus* the thin, heart shaped baculum anlage ossifies between the 7th and the 15th day after birth. Between the 15th and 25th day postpartum, the proximal part of the baculum develops. The baculum grows for the first two months and the proximal part elongates and thickens. In all the species mentioned, the baculum reaches its typical shape by the time the bats leave the maternity roost in their first autumn, and growth thereafter is minimal but continues at least until the bat reaches sexual maturity [Vlček, 1967; Maeda, 1978; Smirnov & Tsytsulina, 2003].

Baculum ontogeny needs further study on a histomorphological scale in bat species

with different baculum types. In some genera, like *Pipistrellus*, there seems to be plasticity in the development of the medullary cavity and foramina nutritia. While in other genera, like *Plecotus*, the morphology of the medullary cavity seems to be rather species-specific. I plan do research on this topic in the future.

1.5   Aims

The aims of this dissertation work were 1) to quantify inter- and intraspecific variation of baculum micromorphology in cryptic species of the genera *Plecotus* and *Pipistrellus*; 2) to test published hypotheses of baculum function histomorphologically and experimentally; and 3) to test baculum shape and its biometrics as species discriminating characters for *Pipistrellus pipistrellus* and *Pipistrellus pygmaeus*.

1.6   Overview and Author contributions

The first aim of this dissertation is addressed by Paper I (in *Plecotus*) and by Papers II and IV (in *Pipistrellus*), describing inter- and intraspecific variation of baculum histomorphology. The second aim is addressed by Papers I and II, testing published hypotheses of baculum function by our own histomorphologal findings, and by Paper III, testing published hypotheses of baculum function by experimentally inflating and 3D imaging of penes. The third aim is addressed in Paper IV, where it is demonstrated that baculum measurements can be used in a discriminating function to identify *Pipistrellus pipistrellus* and *P. pygmaeus*.

1.6.1   Paper I – Histomorphology of the Penis Bone (Baculum) in the Gray Long-Eared Bat *Plecotus austriacus* (Chiroptera, Vespertilionidae)

Paper I (pages 35-47) is a detailed study of functional baculum histomorphology in *Plecotus austriacus*. Micromorphological evidence of possible mechanical functions of the baculum supports the following hypotheses: 1) That the baculum forms a functional unit with the corpora cavernosa, facilitating force transfer between the tip of the penis to the corpora cavernosa and increasing overall flexural stiffness of the glans and shaft of the penis. 2) That the baculum protects the distal part of the urethra and the urethral opening from compression during copulation. In *Plecotus* the morphology of the medullary cavity of the

baculum seems species specific enough to be useful in species identification, when combined with the external characters and biometrics of the baculum. This paper also demonstrates the possibilities of microCT-derived 3D models for histomorphological studies and for depicting bacula in a more recognizable way than with drawings alone.

This paper was published in *The Anatomical Record* on 13.04.2010.

This part of my dissertation is a continuation from my diploma thesis, but most of the specimens were processed and analyzed after my graduation. The semithin sections, ground sections, and microradiography used were made during my diploma thesis.

Author contributions

Barbara Herzig-Straschil, Brian Metscher, Helge Hilgers, and Hanns Plenk all helped to shape the concept of this paper. Barbara Herzig-Straschil resected the bacula from the penes, measured and photographed the bacula, and preserved them in glycerol. I prepared and evaluated histological semithin sections and ground sections together with Helge Hilgers and Hanns Plenk. Brian Metscher made microCT images of the samples. I wrote the manuscript together with Hanns Plenk and Brian Metscher. All authors added valuable comments and approved of the final version before it was submitted.

1.6.2 Paper II – Correlative 3D imaging of *Pipistrellus* penis micromorphology: validating quantitative microCT images with undecalcified serial ground section histomorphology

The second paper (pages 47-86) presents results of baculum histomorphology in species of the genus *Pipistrellus* and demonstrates the value of correlative imaging combining microCT imaging with ground section light microscopy. The baculum histomorphology of *Pipistrellus pipistrellus* and *P. pygmaeus* extends the results on baculum function of the preceding chapter (Paper I), and it adds the results that the forked tip protects and opens

the dorsal half of the urethral opening even more distinctly than in species of the genus *Plecotus*. It also demonstrates that the marrow cavity of the baculum contains fatty bone marrow. In the studied species of *Pipistrellus* however, the shape and dimensions of the medullary cavity of the baculum show too much individual variability to be useful in species identification.

This paper is in review at the *Journal of Morphology* (submitted 09.07.2014, in review since 30.07.2014).

Herdina Anna Nele, Plenk Jr. Hanns, Benda Petr, Lina Peter H.C., Herzig-Straschil Barbara, Hilgers Helge, Metscher Brian D. Correlative 3D imaging of *Pipistrellus* penis micromorphology: validating quantitative microCT images with undecalcified serial ground section histomorphology.

Author contributions

The project was developed by me, Helge Hilgers, Barbara Herzig-Straschil, Brian D. Metscher, and Hanns Plenk. MicroCT imaging and iodine staining were done by me with help from Brian D. Metscher. I measured and segmented microCT images. Hanns Plenk and I made, stained, and photographed ground sections. Petr P. Benda, Peter H. C. Lina, and Barbara Herzig-Straschil contributed the specimens. All authors contributed ideas and discussed the results. I wrote the manuscript together with Brian D. Metscher and Hanns Plenk.

1.6.3 Paper III – Testing hypotheses of bat baculum function with 3D models derived from microCT

Paper III (pages 87-122) is an experimental study of bat baculum function on post-mortem specimens of *Pipistrellus pipistrellus*, *P. nathusii*, and *Nyctalus noctula*. The corpora cavernosa of penes of freshly dead specimens were inflated with formalin. Comparing microCT images of those "erect" with flaccid penes, differences in the positions of the baculum and the soft tissues of the penes were described. The results of this part of my dissertation further support the functional hypotheses that the baculum can protect the distal urethra and urethral opening from compression during erection and copulation, and that the baculum and corpora cavernosa form a functional unit to support both the penile shaft and the more distal glans tip. This paper reports a novel approach of combining

experimental designs like inflating penes with high-resolution microCT which will help to compare and test the functions of different baculum morphologies, and it forms a foundation for finite element modeling to test biomechanical hypotheses in non-living specimens.

This paper is in review at the *Journal of Anatomy* (submitted 11.09.2013, decision - major revisions - 21.01.2014, revision submitted 05.08.2014).
Herdina Anna Nele, Kelly Diane A., Jahelková Helena, Lina Peter H.C., Horáček Ivan, Metscher Brian D. Testing hypotheses of bat baculum function with 3D models derived from microCT.

Author contributions

After learning the experimental methods of penis inflation (on rodents) from Diane A. Kelly, I designed an experimental study combined with microCT on bat penes. Ivan Horáček and Peter H. C. Lina provided the whole dead bats for this study. I took standard measurements of the bats and inflated the bat penes with formalin together with Diane A. Kelly, Helena Jahelková, and Peter H. C. Lina. Brian D. Metscher and I iodine stained and microCT scanned the penes. I reconstructed and manually segmented the microCT images to create 3D models together with Brian D. Metscher. All authors evaluated and discussed the results. I wrote the manuscript and prepared the figures together with Brian D. Metscher. All authors added valuable comments and corrections and approved the final manuscript.

1.6.4   Paper IV – MicroCT imaging reveals morphometric baculum differences for discriminating the cryptic species *Pipistrellus pipistrellus* and *P. pygmaeus*

The fourth paper (pages 123-136) introduces a discriminant function of simple baculum measurements to identify *Pipistrellus pipistrellus* and *P. pygmaeus*. Projected measurements of baculum length, width, and height used in this discriminant function separate the two cryptic species reliably (97.92%; all but one specimen of this sample were correctly identified). Geometric morphometric analysis was used to find the regions of the baculum showing most inter- and intraspecific variation. Other species identification characters, "wing vein" pattern and penis color and shape, were evaluated on this sample and found to be unreliable in ethanol preserved material.

Author contributions

The idea for this part of the dissertation arose out of discussions between Helge Hilgers and me, but it was shaped and substantiated in discussing the topic with Ivan Horáček, Pavel Hulva, and Petr Benda. Petr Benda and Pavel Hulva made the material for this study available to me from the National Museum in Prague. Together with Brian Metscher, I microCT scanned the bacula within the whole bats. I took all the measurements on the wings and on virtual thick sections of the microCT images of the bacula. For the statistical analyses, I set all the landmarks on virtual thick sections of lateral and dorsal view of the bacula. Christine Mayer taught me geometric morphometric methods and helped me analyze the data statistically. All authors discussed and evaluated the results. I wrote the manuscript and prepared the figures together with Brian Metscher. All authors added valuable comments and approved of the final version before it was submitted.

2. PAPER I (PUBLISHED) *PLECOTUS AUSTRIACUS* BACULAR HISTOMORPHOLOGY

Histomorphology of the Penis Bone (Baculum) in the Gray Long-Eared Bat *Plecotus austriacus* (Chiroptera, Vespertilionidae)

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ABSTRACT

For the first time, the histomorphology of the penis bone of a bat (*Plecotus austriacus*) was examined in detail. From *Plecotus austriacus*, 14 whole penes and 11 isolated bacula were studied and compared to bacula of *Plecotus auritus* and *Plecotus macrobullaris*. The baculum was located on specimen microradiographs and in micro-CT images in the tip of the penis. Using serial semithin sections and surface-stained, undecalcified ground sections, the types of bone and other tissues constituting the baculum were examined by light microscopy. 3D reconstructions were generated from the serial semithin sections and from micro-CT images. The shaft and the proximal branches of the Y-shaped baculum form a tubular bone around a medullary cavity. Since the small diameter of this channel and the main lamellar bone around it resemble a Haversian canal, the baculum is equivalent to a single-osteon bone. Several oblique nutrient canals enter this medullary cavity in the shaft and branches. All ends of the baculum consist predominantly of woven bone. The collagen fiber bundles of the tunica albuginea of both corpora cavernosa insert via fibrocartilage into the woven bone of the branches. Thus, the microscopic structures support the hypothesis that the baculum functions as a stiffening element in the erect penis. In this study, several microscopic imaging techniques were evaluated for displaying the microscopic structures of the baculum. Specimen microradiography, but especially micro-CT proved to be suitable nondestructive methods for accurate and reproducible demonstration and comparison of the three-dimensional structures of the baculum in different bat species. Anat Rec, 293:1248–1258, 2010. © 2010 Wiley-Liss, Inc.

Key words: os penis; bone; osteon; ground sections; microradiography; x-ray microtomography; species identification; function

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INTRODUCTION

A baculum, also called os penis, os glandis or os priapi, occurs in many different mammals, including most species of Chiroptera, Insectivora, Rodentia, Carnivora, and Primates. The name "baculum" was proposed by Thomas (1915), who was the first mammalogist to recognize its value as a taxonomic character (Burt, 1936). The baculum varies greatly among groups, but is commonly invariant within a species (Romer and Parsons, 1977; Patterson and Thaeler, 1982). The penis bone is one of the most highly variable primary sex traits in male vertebrates (Tasikas et al., 2007). In bats the baculum is generally short, situated in the glans dorsal to the urethra, but can reach up to 75% of the penis length in some species (Sinha, 1976). Many hypotheses and speculations have been proposed concerning the function of the baculum.

The macromorphology of the baculum, which has been studied extensively, is a very useful distinctive trait to identify bat species. For a synopsis of the early descriptions and uses of the chiropteran baculum, see Hill and Harrison (1987) and Strelkov (1989a,b). Strelkov (1989a) has identified and hand drawn many different forms of bacula in the plecotine bats (see Fig. 3 in Strelkov, 1989a). At that time, only two species of the genus *Plecotus* were accepted, so he described the different bacula as geographical variations of both species. Each of these bacular forms could later be assigned to a different *Plecotus* species (Spitzenberger et al., 2006).

The microchiropteran genus *Plecotus* is distributed throughout the palearctic region from Ireland to Japan, including North Africa, Macaronesia, and Ethiopia (Horáček et al., 2000). It comprises 19 species, many of which are very similar to each other. *Plecotus austriacus* occurs in most parts of Europe and eastwards to the Ukraine (see Spitzenberger et al., 2006). The characters used most for species discrimination, in order of decreasing frequency, are the skeletal measurements of forearm, thumb and claw, fingers, skull, and feet; fur-color; shape of the baculum; dentition; and pigmentation of the skin on face and ears (Spitzenberger et al., 2002; Tvrtković et al., 2005).

This study was carried out with three aims:

1. To investigate the histomorphology of the baculum in *Plecotus austriacus*, and to correlate microanatomy with macromorphology by using both invasive histological techniques and noninvasive microradiography and X-ray microtomography (micro-CT).
2. To support the most plausible of the many existing theories on bacular function based on its micromorphology.
3. To show that the micromorphology of the baculum includes characters that can potentially be used for differentiating bat species.

While the primary aim is descriptive, the second and third aims above are driven by the following working hypotheses: for the second aim, the general consensus is that sexual selection is influencing bacular morphology, and therefore it must have an identifiable precise mechanical function; and for the third aim microstructural traits of the baculum can be used to distinguish closely related species.

MATERIALS AND METHODS

Specimens

The Mammal Collection at the Natural History Museum Vienna kindly provided all the specimens for this study. In Austria, all species of bats are protected by law. Therefore, the Natural History Museum Vienna only collects bats that are found dead and donated to the museum. Of the species *Plecotus austriacus*, 14 total penis specimens (AM 87/196, KS 08/44, NMW 34241, NMW 36137, NMW 50460, NMW 50464, NMW 50895, NMW 51137, NMW 52193, NMW 52841, NMW 52845, NMW 57250, NMW 64099, and NMW 65273), and 11 isolated bacula specimens (NMW 11490, NMW 11514, NMW 11817, NMW 25151, NMW 34418, NMW 36135, NMW 52197, NMW 52232, ZMB 42943, ZMB 9255, and ZMB 9256) were available. One specimen was available of each of the species *Plecotus auritus* (NMW 50509), and *P. macrobullaris* (NMW 34857) for micro-CT imaging (Abbreviations: Inventory numbers NMW at the Natural History Museum Vienna; AM and KS laboratory abbreviations, mammal collection NMW; ZMB, Zoological Museum Berlin).

Specimen Preservation

The whole bats were usually stored deep-frozen after arrival at the museum, sometimes for several months. Because of the time period between death and fixation, the state of preservation was often suboptimal. When the bats were dissected, the whole penes were stored in 70% ethanol with no additional fixation (Kiernan, 1990; Kryštufek and Hrabě, 1996). Eleven of the penes were cleared in a 6% solution of potassium hydroxide (KOH), calcium-salt stained with Alizarin Red S and transferred to 100% glycerol via ascending glycerol concentrations. Then, the bacula were retrieved by dissection under a stereomicroscope, measured (Fig. 1), and further stored in glycerol solution with thymol crystals added (Kryštufek and Hrabě, 1996). The samples for this study were obtained at several different stages of this process and variously treated to prepare them for the methods detailed below.

Microradiography

The bacula in the intact penes were first located and identified by microradiography, and microradiographs were also taken of the undecalcified ground sections. A high-resolution film (Kodak Professional SO-343™; Eastman Kodak Company, NY) was exposed in a Cabinet X-Ray System (Faxitron Series; HP Company, Palo Alto, CA) for 20 min or 25 min at a voltage of 20 kV or 25 kV and developed under standard conditions.

Micro-CT

X-ray microtomography scans were made of 12 unstained total penes in 70% ethanol, of one iodine-stained (Metscher, 2009) penis, fixed in buffered formalin solution (Lillie and Fullmer, 1954), and of one isolated, decalcified, and iodine-stained baculum in 100% ethanol. A high-resolution micro-CT system (model MicroXCT, Xradia, Inc., Concord, CA, USA) with either a tungsten or a rhodium source was used at a voltage of between 40 kV and 80 kV. Different magnifications gave reconstructed voxel sizes between 4.17 µm and 0.90 µm. Some illustrations in this article are virtual slices taken
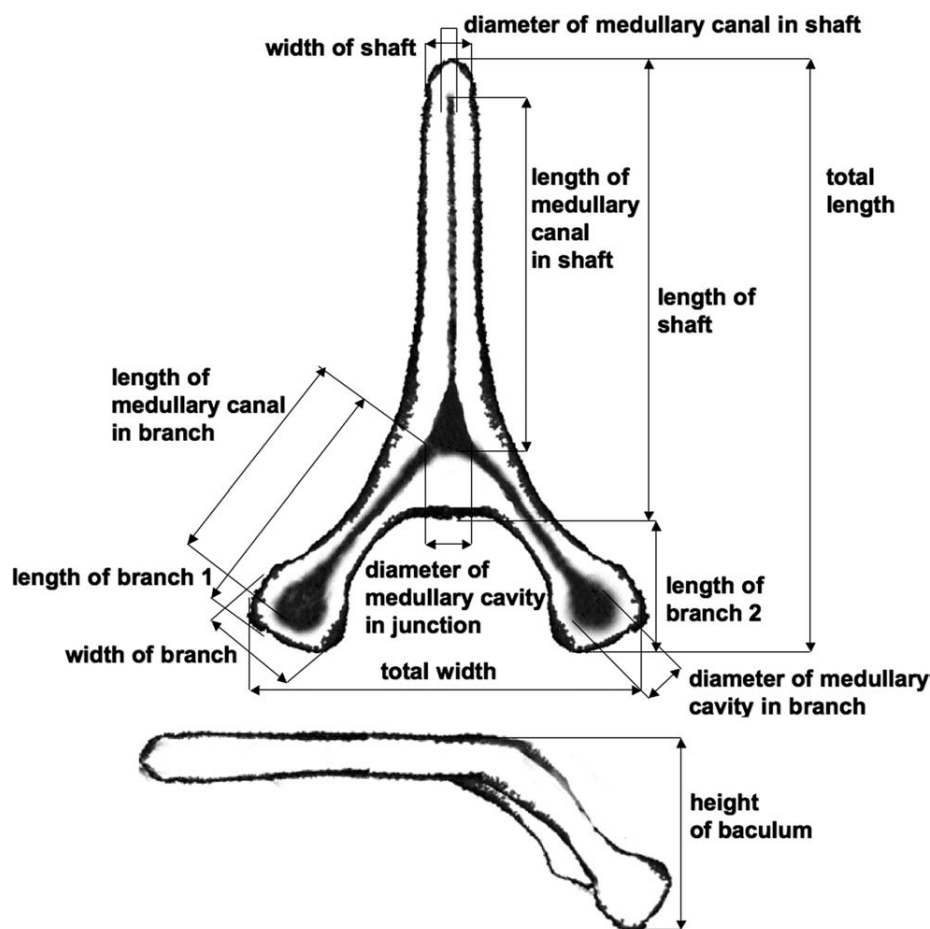
Fig. 1.   Schematic drawing of a baculum with measurements as in Table 1.

from the 3D reconstructions of the micro-CT scans, made using the supplied viewing software.

Histological Techniques

Serial semithin sections. Eleven isolated bacula and one intact penis were embedded in araldite resin according to Romeis (1989). The isolated bacula were prestained red with safranin O (0.5% in 80% ethanol) to make them visible inside the resin. To generate ribbons of serial semithin sections, each resin block was coated with glue (Pattex Compact[TM]; Henkel Central Eastern Europe, Vienna, Austria) mixed with xylol on the side facing the blade. From each isolated baculum or the one whole penis, 300–450 serial sections of 2 μm thickness were cut with a diamond knife on a Reichert Ultracut S microtome (Leica Microsystems, Wetzlar, Germany). The section ribbons were transferred to microscope slides and stained with Richardson's stain (Richardson et al., 1960) or toluidine blue O stain (0.1% in 2.5% sodium (tetra)borate) (modified according to Romeis, 1989). Araldite resin was also used as mounting medium for the glass cover slips.

The stained sections were evaluated by light microscopy using a Nikon Microphot-FXA (Nikon Corp., Tokyo,

Japan). At 25× to 600× magnification, the bone and soft tissues of the penes were examined, dimensions were measured (Fig. 1 and Table 1) with an eyepiece graticule, and photographed with a digital camera (ProgRes C14, Jenoptik, Jena, Germany).

Digital microphotographs of the serial sections were imported into the reconstruction program Amira[TM] 3.11. (Visage Imaging, Berlin, Germany). The pictures were aligned to form an image stack. On each picture, all types of tissue were labeled manually for reconstruction by drawing the contours of the relevant structures. Based on manual image segmentation, a three-dimensional surface model was computed by the software.

Ground sections. One whole penis was embedded in methylmethacrylate resin (Plenk, 1989).

The resin block was cut into rectangular shape on an Emco Swing[TM] band saw (Emco GmbH, Wr. Neudorf, Austria). Using a water-cooled diamond saw (Buehler Isomet[TM] low-speed saw; Evanston, IL) the resin block was cut parallel to the frontal side of the baculum. To the resulting plane, a plexiglas microscope slide was glued with instant adhesive (CA8 Pronto[TM], 3M Comp., St.Paul, MN, USA). A thick section of 3000 μm was cut and then ground (with EXAKT- Type AW 10[TM]) and

TABLE 1. Measurements on bacula (means ± SD in mm)

	Stereomicroscopic Bacula in glycerol	Microscopic		
		Semithin sections	Microradiography	Micro-CT
Isolated bacula $n = 9$	$n = 9$	$n = 7$		
Whole penes $n = 13$		$n = 1$	$n = 1$	$n = 12$
Dimensions				
Total length of baculum	0.81 mm ± 0.05	–	0.85 mm	0.78 mm ± 0.05
Total width of baculum	0.55 mm ± 0.04	0.44 mm ± 0.05	0.43 mm	0.48 mm ± 0.05
Height of baculum	–	0.16 mm ± 0.03	–	0.17 mm ± 0.02
Length of shaft	0.66 mm ± 0.03	–	0.71 mm	0.65 mm ± 0.05
Width of shaft	–	0.16 mm ± 0.05	0.10–0.14 mm	0.16 mm ± 0.02
Height of shaft	–	0.06 mm ± 0.01	–	0.06 mm ± 0.01
Length of branch 1	–	–	0.22 mm	0.30 mm ± 0.03
Length of branch 2	0.16 mm ± 0.04	–	0.13 mm	0.13 mm ± 0.02
Width of branch	–	0.08 mm ± 0.02	0.09–0.12 mm	0.16 mm ± 0.02
Length of medullary canal in shaft	–	–	0.53 mm	0.41 mm ± 0.09
Diameter of medullary canal in shaft	–	0.03 mm ± 0.01	0.02 mm	0.03 mm ± 0.03
Length of medullary canal in branch	–	–	0.30 mm	0.21 mm ± 0.04
Diameter of medullary canal in branch	–	0.06 mm ± 0.01	0.02–0.04 mm	0.05 mm ± 0.01
Diameter of medullary canal in junction	–	0.05 mm ± 0.02	0.04 mm	0.23 mm ± 0.06

polished (Buehler Minimet™ Polisher; Evanston, IL) until the superficial tissue layers of the penis were exposed. Subsequently, the surface of the ground section was ground (1000-grit sandpaper, 4000-grit sandpaper) and polished (alumina polishing powder, particle size 0.3 μm) off in sixteen 100 μm steps until the remaining ground section was 490-μm thick. At each step, the ground section was surface stained with either Giemsa stain (Merck, Darmstadt, Germany; (modified according to Plenk, 1989), or Sanderson's Rapid Bone Stain™ (Surgipath Medical Industries, Richmond, IL, USA) and photographed with the same equipment as above. After every fifth polishing-step, microradiographs were made as described above.

RESULTS

Macromorphology

The baculum is a Y-shaped bone (Fig. 1). Its shaft is straight and almost cylindrical, but flattened dorso-ventrally for most of its length. Distally, the shaft is slightly tapered, ending in a blunt tip. Proximally, the profile of the shaft is slightly convex above and concave below. There is no distinct change toward the base, just a gradual broadening of the shaft. The base (proximal end) is bifurcated into two branches which are shorter than the shaft. The branches of the base are broad and flattened, ending in blunt tips. They are curved toward the base of the penis. In dorsal view, the two branches together form a flattened arch. For macroscopic dimensions of the bacula measured in this study see Table 1.

Microanatomy

Based on micro-CT images made after iodine staining, and on semithin microtome and ground sections, the baculum of *Plecotus austriacus* was found to be situated in the glans penis, directly dorsal to the urethra (Fig. 2). The baculum resembles a bicycle saddle, its proximal base reaching down the sides of the urethra (Figs. 3 and 4). The distal end of the baculum is located near the tip of the glans penis and the opening of the urethra (Fig. 2).

Histomorphology

The shaft and branches of the baculum form a tubular bone with concentric lamellae around a central medullary cavity or canal. In the shaft, this medullary canal is uniformly narrow and runs along the whole length except for the very tip (Figs. 4, 5, and 6). The canal is round to oval in cross section along the shaft and becomes slightly narrower toward the distal end. At the proximal base it widens slightly, and in a distinct crotch it splits into two canals. In the branches of the baculum, the cross section of the medullary cavity is oval and widens steadily toward the proximal tips. For microscopic dimensions of the bacula measured in this study see Table 1. Seven nutrient canals enter the medullary cavity, three of them close to each other along the shaft's axis near the distal tip of the baculum; a fourth canal enters proximally at the crotch. At the base, there are four additional nutrient canals: one in the middle of each branch, and one near the tip of each branch. The respective foramina nutritia of those seven nutrient canals are located ventrally on the shaft, ventrally in the crotch, and on the medial aspects of the branches (Figs. 4, 5, and 6).

In the shaft and the branches, particularly the inner surface of the medullary canal, the tubular bone appears lamellar like an osteon. Here, the small, elongated, and oval shaped osteocyte lacunae are arranged between the
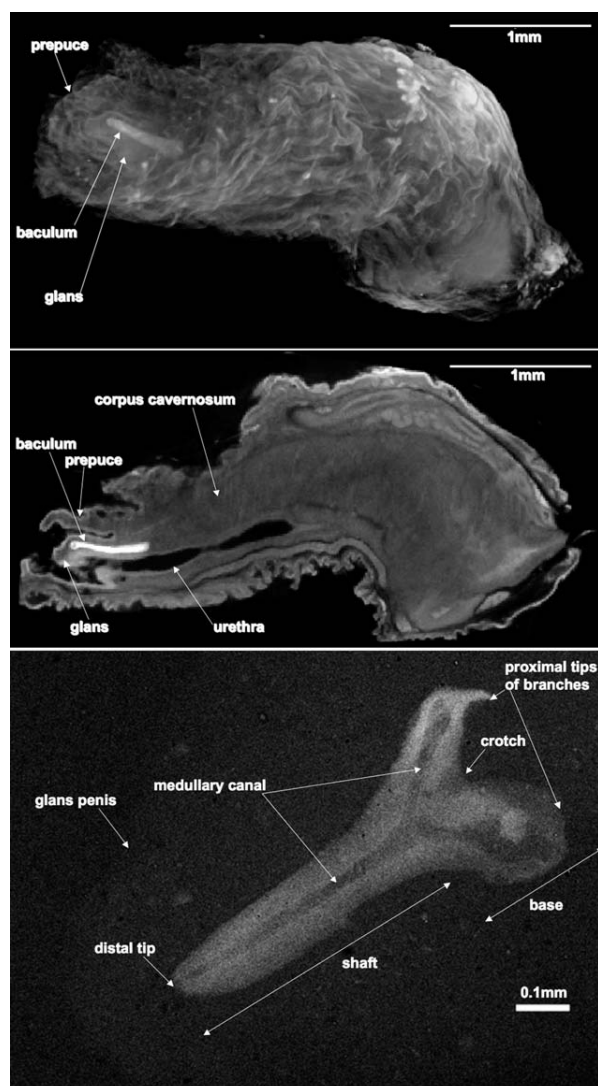
Fig. 2. Top: Volume rendering from micro-CT images of an iodine-stained *Plecotus austriacus* penis (KS 08/44; tungsten source, 79 kV, 43 μA, cubic voxel size: 4.17 μm). Middle: Virtual longitudinal section of the same micro-CT scan, showing the radiodense baculum. Bottom: Film microradiograph of a *Plecotus austriacus* baculum in an unstained total penis specimen (NMW 51137).

lum form a unit enveloped by a continuous fibrous structure. Chondroid cells are scattered between the collagen fiber bundles of the tunica albuginea, where they insert into the woven bone of the branches (Fig. 5). In this fibrocartilaginous area, mineralization fronts can be seen between the bone and the tunica albuginea (Fig. 5).

Bacular Morphology Traits for Species Identification

Comparison of micro-CT volume renderings of bacula from *Plecotus austriacus* with a baculum of *Plecotus auritus* and *Plecotus macrobullaris* (see Fig. 7) revealed a different outer shape and diameter of the canals in all three species. Also, the number and distribution of nutrient canals seems to differ between species. In the bacula from two adult *Plecotus austriacus*, a different canal pattern was found (see Fig. 8). The bacula from juvenile bats of all three species appear to differ according to age in all these aspects.

Problems and Merits of the Applied Methods

The specimens used in this study were originally preserved for only macromorphological examination and measurements. The oldest of them had been preserved in ethanol since 1965. Alcohol fixes and preserves tissues by protein coagulation, dehydration, and antimicrobial action, but is generally acknowledged as a poor substitute for reagents with cross-linking and other fixation properties (Kiernan, 1990). Also, fixation often took place long after the death of the bat. At the Mammal Collection of the Natural History Museum Vienna, bat specimens are kept either in ethanol, or as dry skins and/or skeletons, depending on their state of preservation at acquisition. For the Museum's purposes, deep-freezing the animals until they can be processed is the best solution.

Unfortunately, deep-frozen tissue is less appropriate for histological methods. According to Plenk (1986), immediate postmortem or post-sacrifice dissection is always preferable to cryopreservation. This is because, especially in larger specimens, adequate cellular and soft tissue preservation cannot be achieved even by rapid deep-freezing: subsequent fixation and penetration with the embedding medium is always impaired in previously frozen specimens.

Even though storage in glycerin with thymol is the standard method (Dingerkus and Uhler, 1977; Kryštufek and Hrabě, 1996; Narotsky and Rogers, 2000; Bhudoye et al., 2001), the thymol failed to prevent the growth of mold on the samples. In addition, some of the specimens were found to be decalcified, or partially decalcified at the margins. As thymol is a weak organic acid in solution (Haug, 1999), this could account for decalcification.

The mineralization of the baculum allows for radiographic imaging. Specimen microradiographs are a simple way for depicting and measuring the baculum and the medullary cavity, but radiographic projection images can give an impression of the three-dimensional structure of the mineralized bone only if they are taken in different orientations. Micro-CT proved to be a more complete solution (see also Neues and Epple, 2008), providing not only projection radiographs with down to 0.90 μm pixel size but also data to automatically create virtual sections and 3D-reconstructions. Both the

lamellae. All three ends of the baculum consist primarily of woven bone. Here, the osteocyte lacunae are larger, more rounded, and more numerous than in the shaft, closer together and randomly oriented (Figs. 4 and 5). Zones of bone remodeling, marked by reversal cementing lines, were found between woven bone and the inner lamellar layer in the tubular bone of the branches (Figs. 4 and 5).

The intact penis specimens of *Plecotus austriacus* showed that the tips of the branches of the baculum are linked to the corpora cavernosa (Figs. 2, 4, and 5). While the medial portions of the tunicae albugineae of the two corpora cavernosa merge in the middle, the peripheral portions of the tunicae continue into the periosteum of the baculum. Thus, the corpora cavernosa and the bacu-
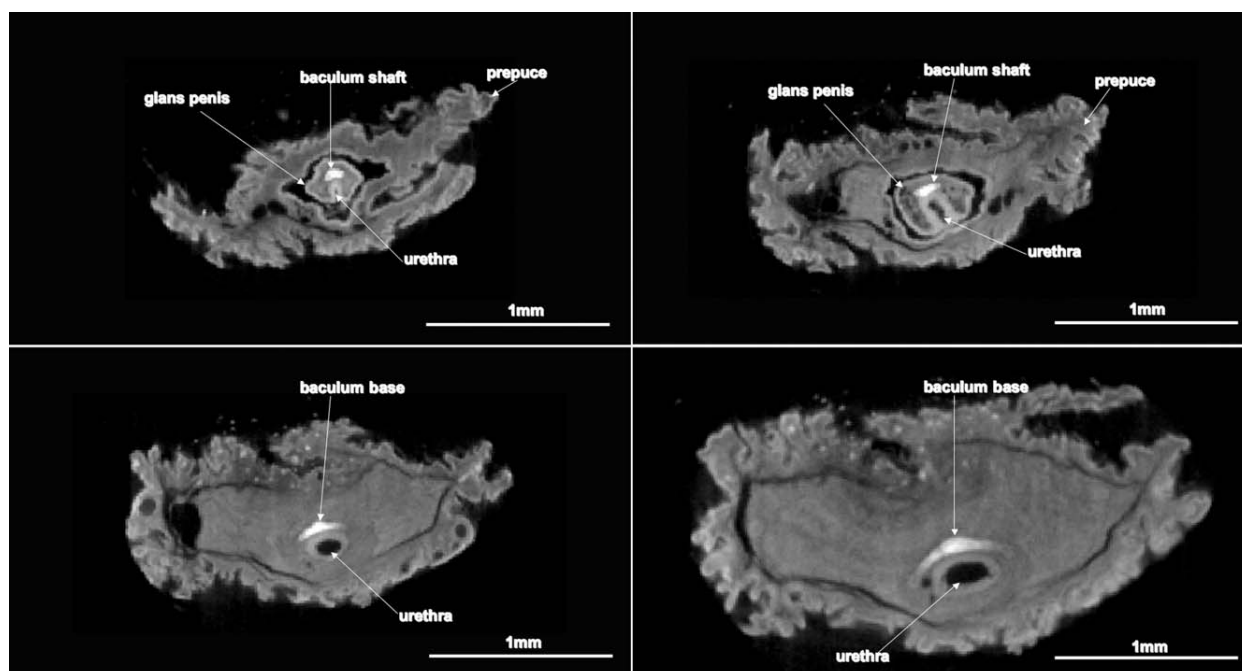
Fig. 3.   Virtual cross sections, from distal (top left) to proximal (bottom right), of the micro-CT scan of an iodine-stained *Plecotus austriacus* penis (KS 08/44; tungsten source, 79 kV, 43 µA, cubic voxel size: 4.17 µm).

radiographic methods are nondestructive and obviously well suited for accurate and reproducible imaging and measurements, which can be used for comparison of the three-dimensional structures of the baculum in different bat species. The drawback of these methods is that they only work with undecalcified samples. A solution to this problem is the whole specimen staining with radiodense contrast media such as iodine solution (Metscher, 2009). 3D reconstruction from semithin serial sections is destructive and much more time consuming but can also use decalcified bone samples, and it can be used to show only selected histological details.

The retrieval of the bacula by dissection under the stereomicroscope also allowed for microscopic measurements. The differences in dimensions measured on isolated bacula in glycerin and those measured on radiographic images and microscopic sections could either be due to intraspecific variation, or to residual tissue attached to the bacula.

DISCUSSION

Microscopic investigation of the baculum in *Plecotus austriacus* showed that the penis bone contains a continuous central medullary cavity or canal, which is entered by several nutrient canals. The shaft and branches of the baculum form a lamellar bone, which we interpret as a single-osteon bone. The typical dimensions of an osteon concur with the measurements of the baculum in *Plecotus austriacus*: According to Knese (1979 and citations therein), the length of an osteon can vary between 3 and 40 mm, the diameter between 154 and 418 µm, and the diameter of its Haversian canal between 20 and 300 µm. Long bones of this single-osteon type are also found in other vertebrates, for example the femur of the Japanese fire-bellied salamander (*Cynops pyrrhogaster*) (Urschitz, 1982). Demeter and Mátyás (1928) describe the whole femur cross section in other amphibians (e.g. *Rana esculenta*) as representing a single large osteon with visible nutrient canals.

The distal end of the bacular shaft and especially the proximal ends of the two branches consist of woven bone with a fibrocartilaginous insertion of the tunica albuginea. In this study of the adult *Plecotus austriacus*, the tunica albuginea of the corpora cavernosa continues into a tubular bone without any remaining evidence of chondral ossification. However, reversal cementing lines between the outer woven bone and the inner lamellar bone point to previous remodeling processes. The occurrence of chondroid cells between inserting fibers in the woven bone appears to be a functional adaptation to biomechanical needs, similar to tendinous insertions at joints. Matthews (1942) already reported that the bacula of some African species of bats are fused with the distal end of the corpora cavernosa. He also found that, in *Hipposideros caffer*, the corpora cavernosa are fused throughout the length of the penis: at their distal end, the tunica albuginea is continuous, through a region of cartilage, with the baculum. Crichton and Krutzsch (2000) reported that the baculum of bats is typically attached to the distal end of the partially fused corpora cavernosa with its two branches. Generally, in the mammalian penis the baculum (when present) is closely associated with the distal portion of the corpora cavernosa (Smith and Madkour, 1980). Wimsatt and Kallen (1952) state that the fetal baculum might be derived from the corpora cavernosa, because the tunica albuginea transforms imperceptibly into the periosteum of the baculum.
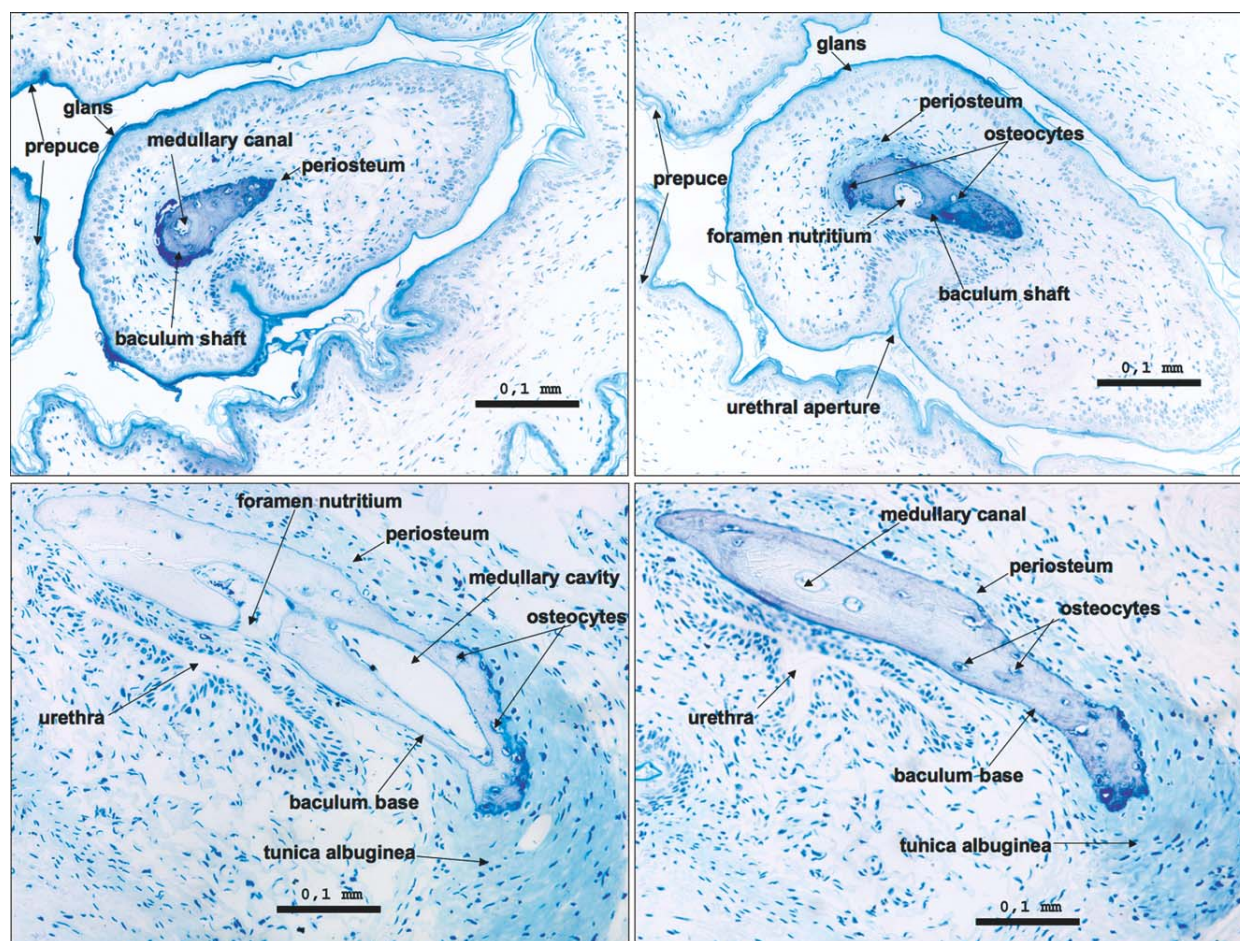
Fig. 4. Semithin (2 μm) oblique cross sections, from distal (top left) to proximal (bottom right), of a *Plecotus austriacus* penis tip, stained with toluidine blue O.

This would imply membranous ossification. On the other hand, according to Kelly (2000), the baculum and the corpus cavernosum originate from the same mesenchymal mass in the embryonic penis in mice (Glucksmann et al., 1976) and rats (Murakami and Mizuno, 1984), but the two structures develop separately, and the fetal baculum has a cartilaginous anlage (Smirnov and Tsytsulina, 2003).

The microanatomy of the plecotine baculum and penis implies a mechanical function of the baculum. Apparently, it forms a functional unit with the corpora cavernosa, enveloped in a common fibrous structure. Even though the microanatomy of the *Rattus norvegicus* penis is different, the interpretation of Kelly (2000) concerning force transfers between baculum and corpus cavernosum seems to be similar. The penis of *Rattus norvegicus* contains only one corpus cavernosum, the distal end of which envelops the flared proximal end of the baculum. She suggests that bending as well as compressive forces on the tip of the penis during intromission are transferred to the tensile wall of the single corpus cavernosum via the baculum in rats. The single-rod baculum is pressed into the proximal end of the corpus cavernosum, with the resulting compression increasing the hydro-static pressure inside the corpus cavernosum. Because of inelasticity of the collagen fibers in the tunica albuginea, this will further increase the stiffness of the corpus cavernosum and thereby the flexural stiffness of the entire penis. The layer of fibrocartilage between the baculum and corpus cavernosum could serve as a flexible joint similar to the joints in the hyoid (Kelly, 2000).

In contrast, the Y-shaped baculum in plecotine bats seems to be the mechanical solution for transferring and distributing the above-mentioned forces equally to and from the two corpora cavernosa. The hydrostatic stiffness of the filled corpora cavernosa can continue into the tip of the glans via the stiffness of the penis bone, as they are enveloped in a common fibrous structure to form a structural unit. The concave surface of the baculum's distal tip in plecotine bats also suggests a function in keeping the distal orifice of the urethra open during copulation. Finally, the fibrocartilage at the insertion of the tunica albuginea into the baculum indicates alternating shearing forces on this joint-like interface during erection and copulation. The function of fibrocartilage at tendon-bone attachments (entheses) seems to be very similar in humans (Benjamin et al., 1986) and other vertebrates (Kelly, 2000).
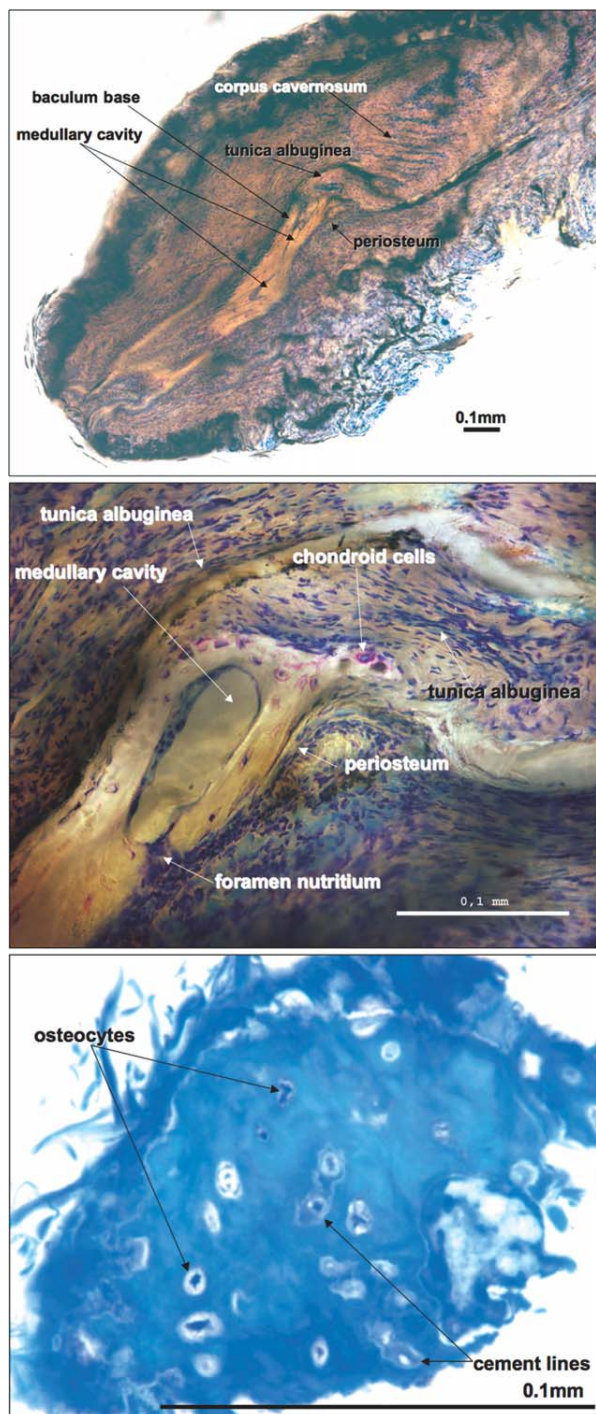
Fig. 5. Top: Undecalcified ground section (longitudinal) of a *Plecotus austriacus* penis tip, Giemsa stained. Middle: Enlarged detail of the same ground section, Giemsa stained. Bottom: Corresponding detail of a semithin (2 μm) cross-section of a *Plecotus austriacus* baculum, stained with toluidine blue O.

The function of the variable shapes of the baculum in various species may be different and the precise mechanical function of the baculum in bats has yet to be

determined. According to Dyck et al. (2004), the mammalian baculum probably has multiple overlapping functions. It functions during intromission indirectly, by affecting penile shape or providing mechanical support. The baculum could also protect the urethra from compression, enable protracted copulations, stimulate the female reproductive tract, provide information about male size or quality during intromission, or facilitate reproductive isolation. For the evolution of the baculum, three hypotheses have been put forward: the vaginal friction hypothesis (Long and Frank, 1968), the prolonged intromission hypothesis (Dixson, 1987, 1995; Dixson and Anderson, 2004) and the induced ovulation hypothesis (Eberhard, 1985). Larivière and Ferguson (2002) give a synopsis of those three hypotheses.

The macroscopic findings of this study are consistent with those of Spitzenberger et al. (2002), who stated that the baculum of the holotype of *Plecotus "microdontus"* (=*P. macrobullaris*) clearly differs in shape from that of *Plecotus auritus* and less clearly from *Plecotus austriacus*. Kiefer and von Helversen (2004) reported that the form of the baculum of *Plecotus macrobullaris* is intermediate between those of *Plecotus auritus* and *Plecotus austriacus*.

In contrast, macroscopic plus microscopic results of the present study—especially 3D micromorphology revealed by micro-CT—show much more similarity between the bacula of *Plecotus macrobullaris* and *Plecotus austriacus* than between either of them and *Plecotus auritus*. In *Plecotus auritus*, the baculum is noticeably more slender than those of *Plecotus macrobullaris* and *Plecotus austriacus* (see Fig. 7). *Plecotus auritus* also has the least saddle-like and least curved baculum in lateral view of the three species. In *Plecotus macrobullaris*, the base is much bulkier than in the other two species. The arch between the branches is more arcuated in *Plecotus macrobullaris* than in *Plecotus austriacus*, almost like an inverted letter U. Light microscopy showed a narrow medullary cavity in the baculum of all three species, which is narrowest in proportion to the baculum cross section in *Plecotus auritus*. In *Plecotus macrobullaris*, the cavity is a little wider than in *Plecotus austriacus* in both the shaft and branches. The difference is especially striking at the junction, where the canals from the branches meet with the shaft's canal. *Plecotus macrobullaris* has a very wide junctional cavity, encased by a narrow bone shell. In *Plecotus auritus*, the medullary canal does not widen at the junction and is thus encased by a thicker bone shell there than in shaft and branches. *Plecotus austriacus* shows an intermediate situation: the medullary canal is slightly widened at the junction.

The genus *Plecotus* (long-eared bats) contains many cryptic species, whose existence was revealed mainly by genetic studies in the last few years. To discriminate all *Plecotus* species morphometrically, many characters and measurements have been successfully used by Spitzenberger et al. (2006). Craniometric variables were used in multivariate analyses (discriminant analyses and UPGMA) to separate the taxa (Spitzenberger et al., 2006). Apart from species discrimination, the mammalian baculum has also been used for age determination (Callery, 1951; Elder, 1951; Patterson and Thaeler, 1982; Smirnov and Tsytsulina, 2003; Dyck et al., 2004) and as phylogenetic trait (for a synopsis see Hill and Harrison,
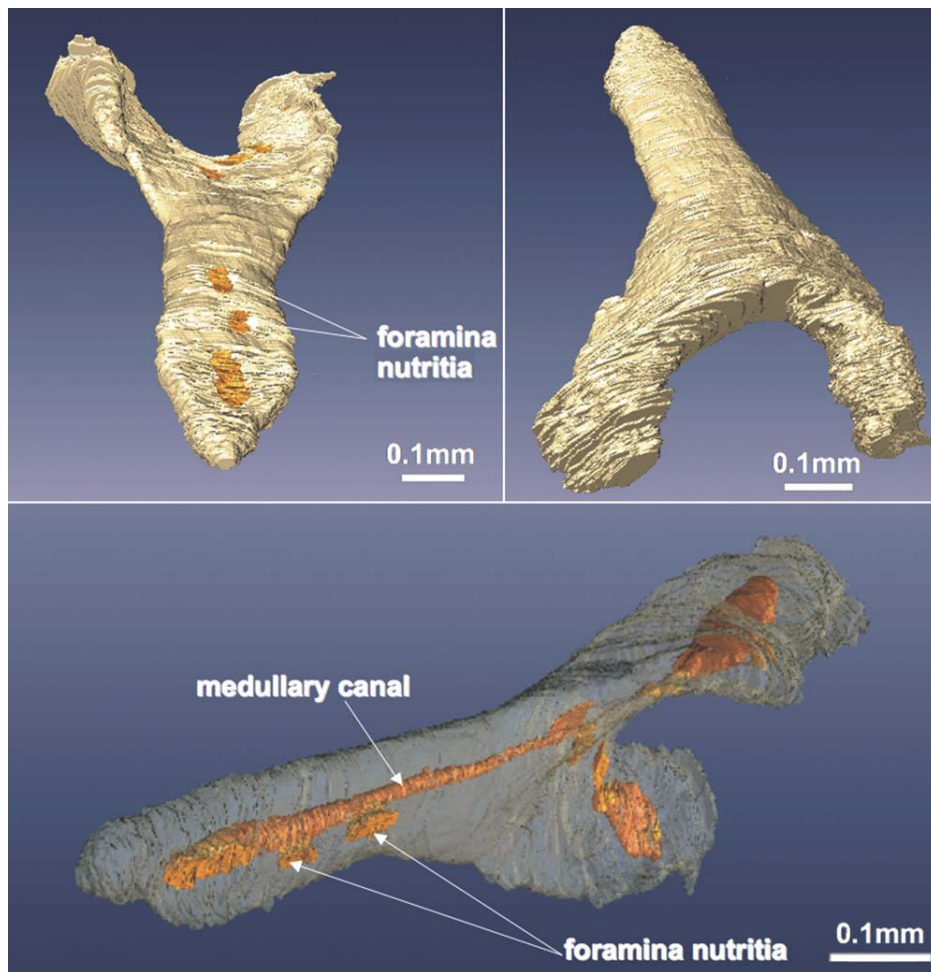
Fig. 6. 3D surface reconstruction from serial semithin (2 μm) cross sections of a *Plecotus austriacus* baculum, showing the medullary canal, foramina, and nutritial canals in orange.
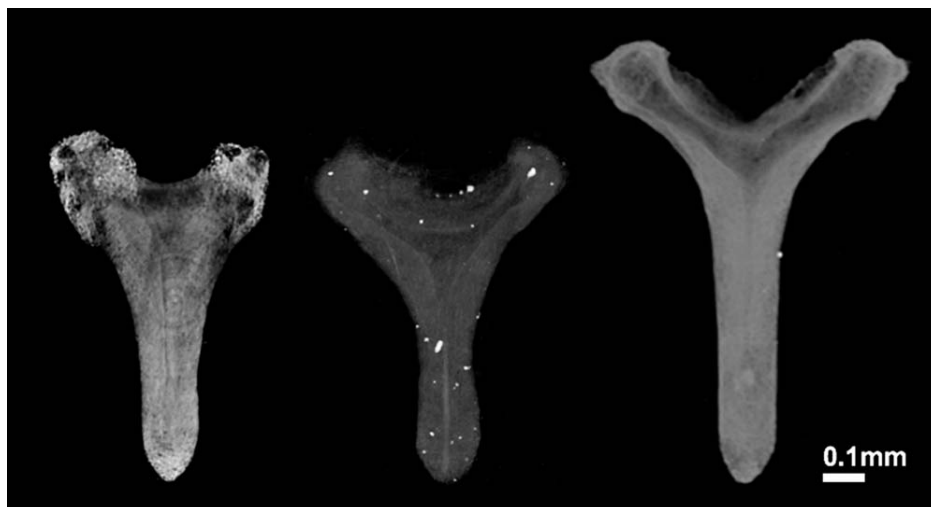
Fig. 7. Volume renderings from micro-CT images of unstained bacula of *Plecotus austriacus* (left) and *Plecotus auritus* (right), and of a decalcified, iodine-stained baculum of *Plecotus macrobullaris* (middle). *P. austriacus* (NMW 34241): tungsten source, 80 kV, 100 μA, cubic voxel size: 0.90 μm. *P. macrobullaris* (NMW 34857) tungsten source, 40 kV, 200 μA, cubic voxel size: 1.0 μm. *P. auritus* (NMW 50509): tungsten source, 60 kV, 66 μA, cubic voxel size: 1.49 μm.

Fig. 8. Volume rendering from micro-CT images of an unstained *Plecotus austriacus* penis (NMW 52193; rhodium source, 77 kV, 47 μA, cubic voxel size: 1.86 μm), showing a baculum with deviating bone canals (outlined in orange).

1987; Frost and Timm, 1992). Newer studies caution that the penis bone could be an unreliable trait for phylogenetic studies (Kearney et al., 2002; Baryshnikov et al., 2003). Clearly, more research on different species is needed, and micro-CT in combination with histomorphology of bones could be valuable tools for studying ontogeny and phylogeny.

In addition to destructive histological evaluation, specimen microradiography, but micro-CT in particular proved to be suitable nondestructive methods for accurate and reproducible demonstration, measurement and comparison of the three-dimensional structures of the baculum in different bat species.

This study confirms that macroscopic details and dimensions along with histomorphology of the baculum can be used for bat species identification. Both these methods have been successfully applied to differentiate *Plecotus austriacus*, *P. auritus* and *P. macrobullaris* (Herdina, 2008).

Future work with these techniques on larger sample sizes in a greater number of species will also provide more insight into potential intraspecific variation in bacular microanatomy. Based on the specimens used in this study, the number and distribution of the bone canals seems to be fairly consistent in adult males. We did, however, find in the micro-CT scans of two bats differing bone canal patterns. These two individuals will be further studied histologically to find possible reasons for

this intraspecific variation. Also, depending on age, the shape of the baculum seems to be quite variable in juvenile bats (Smirnov and Tsytsulina, 2003), which warrants further micromorphological studies.

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3. *PIPISTRELLUS* BACULAR HISTOMORPHOLOGY

Journal of Morphology

Correlative 3D imaging of *Pipistrellus* penis micromorphology: validating quantitative microCT images with undecalcified serial ground section histomorphology

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1 **Correlative 3D imaging of *Pipistrellus* penis micromorphology: validating**

2 **quantitative microCT images with undecalcified serial ground section**

3 **histomorphology**

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19

20 **Correlative bat penis histomorphology**

21

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Herdina   2

26   ABSTRACT

27

28   Detailed knowledge of histomorphology is a prerequisite for studies of function,

29   variation, and development. In bats, as in other mammals, penis and baculum

30   morphology are important in species discrimination and phylogenetic studies. Here,

31   non-destructive 2D and 3D microCT imaging is correlated with light microscopic

32   imaging of surface-stained ground sections to combine advantages of both

33   histomorphological techniques. MicroCT images of bacula and iodine-stained penes of

34   *P. pipistrellus* were correlated with light microscopic images from undecalcified

35   surface-stained ground sections of three penes of *P. pipistrellus* (1 juvenile). These

36   results were compared with microCT images of bacula of *P. pygmaeus*, *P. hanaki*, and

37   *P. nathusii*. The Y-shaped baculum in all studied *Pipistrellus* species has a proximal

38   base with two club-shaped branches, a long slender shaft, and a forked distal tip. The

39   branches contain a medullary cavity of variable size, which tapers into a central canal of

40   variable length in the proximal baculum shaft. Both are surrounded by a lamellar and a

41   woven bone layer and contain fatty marrow and blood vessels. The distal shaft consists

42   of woven bone only, without a vascular canal. The proximal ends of the branches are

43   connected with the tunica albuginea of the corpora cavernosa via entheses. In the penis

44   shaft, the corpus spongiosum-surrounded urethra lies in a ventral grove of the corpora

45   cavernosa, and continues in the glans under the baculum. The glans penis

46   predominantly comprises an enlarged corpus spongiosum, which surrounds urethra and

47   baculum. In the 12 studied juvenile and subadult *P. pipistrellus* specimens the proximal

48   branches of the baculum were shorter and without marrow cavity, while shaft and distal

49   tip appeared already fully developed. The present combination with light microscopic

50   images from one species enabled a more reliable interpretation of histomorphological

51   structures in the microCT images from all four *Pipistrellus* species.

52

53   KEYWORDS: Vespertilionidae, Chiroptera, os penis, x-ray microtomography, iodine

54   staining, histomorphology

55

56

57   INTRODUCTION

58

59   Correlative imaging is a well-established approach in many radiological and light- and

60   electron microscopic studies on diverse topics and materials. Since the invention of

61   radiographic techniques a combination with histomorphological findings is standard for

62   anatomical textbooks. Combining microCT imaging with other 2D or 3D imaging

63   methods adds information from cell and tissue level histomorphology to the overall

64   three dimensional structures. Because of the inherent physical limitations of each

65   method, a correlative approach can yield a significantly broader range of information

66   (Handschuh, Baeumler et al. 2013). Thus, correlative imaging was applied to the further

67   evaluation of bat baculum histomorphology in the present study.

68

69   Combining non-destructive, quantitative microCT imaging with classical

70   histomorphological techniques enables us to take advantage of the possibilities of both

71   methods while avoiding some of the limitations of each. 2D and 3D images derived

72   from microCT scans can contain information on cell and tissue scales, and validating

73   this information with light microscopic evaluation of undecalcified, surface stained

74   ground sections of the same specimen in a corresponding orientation is useful to

75   corroborate findings. Once a structure or tissue type has been calibrated in this way,

76   further conclusions can be drawn from microCT images of other specimens and even

77   specimens of different species. Thus the time consuming and destructive processing of a

78   small number of samples can be used to validate results of a larger number of non-

79   destructive microCT images, allowing us to study specimens which are rare in scientific

80   collections in numbers sufficient to evaluate individual and geographic variation of

81   traits. Additionally, 3D renderings of microCT images contain information that is

82   difficult or impossible to glean even from serial histological sections. In particular,

83   complicated three-dimensional structures are much easier to study in an interactive 3D

84   visualization. Virtual 2D sections from microCT scans of the same specimen can be

85   shown in any orientation and combination, and they can constitute an independent data

86   set that can be useful in further studies.

87

88   The main object of this study, the baculum (os penis, os priapi, os glandis), is an

89   extraskeletal or heterotopic bone present in the glans penis of many species of the

90   orders Carnivora, Chiroptera, Insectivora, Primates, and Rodentia (Patterson and

91   Thaeler 1982). The size of the baculum in relation to the size of the penis varies

92   between species, as does the position of the baculum within the glans penis

93   (Meisenheimer 1921; Patterson 1983). While the shape of the baculum usually does

94   show similarities between the species of a genus, it is often distinctly different between

95   closely related species (Hill and Harrison 1987). Thus, due to its usefulness as a

96   taxonomic character (Thomas 1915; Burt 1936), the macromorphology of bat bacula

97   has been studied extensively. The position of the baculum in context with the

98   surrounding erectile tissues has been studied histologically in several bat species

99   (Ercolani 1868 cited in Matthews 1937; Gilbert 1892; Matthews 1937; Ryan 1991;

100 Ryan 1991) but the histomorphological features of the penis bone have not been

101 described in particular.

102 In a more recent approach, our research group has already applied correlative 2D and

103 3D imaging to describe baculum histomorphology in three species of the bat genus

104 *Plecotus* (Herdina 2008; Herdina, Herzig-Straschil et al. 2010). As suggested at least for

105 the *Plecotus,* the micromorphological traits of shape and position of the medullary

106 cavity in the baculum and the position and number of nutrient foramina may prove

107 useful for species discrimination (Herdina 2008). Research on penis bone

108 histomorphology could also add valuable information about the development of the

109 baculum and its mechanical function. So far, studies on baculum histomorphology and

110 development have been conducted mainly in rodents (Retterer 1887; Ruth 1934; Friley

111 Jr 1949; Callery 1951; Murakami and Mizuno 1984; Murakami and Mizuno 1986;

112 Murakami 1987; Iguchi, Irisawa et al. 1990; Williams-Ashman and Reddi 1991;

113 Spotorno 1992; Murakami, Miyake et al. 1994; Edwards 1997; Izumi, Yamaoka et al.

114 2000; Yonezawa, Higashi et al. 2011; Weiss, Rodriguez Jr. et al. 2012) and in

115 carnivores (Petrides 1950; Scheffer 1950; Miller, Stewart et al. 1998; Miller, Jones et al.

116 1999; Albayrak, Özen et al. 2008; Schwery, Köhnemann et al. 2011).

117

118 We have continued to study the histomorphological traits of penes and bacula not only

119 for their value in bat species discrimination (Herdina, Hulva et al. 2014 in press), but

120 also for determining specific mechanical functions of the penis bone in bats (Herdina,

121 Kelly et al. 2014 submitted). In the scope of these ongoing studies, we have

122   concentrated on the *Pipistrellus pipistrellus* species complex, which is a valuable model

123   system for studying cryptic diversity in European bats (Davidson-Watts, Walls et al.

124   2006; Hulva, Fornůsková et al. 2010). In the present study we have i) refined current

125   correlative 2D and 3D imaging techniques by validating the histomorphology in virtual

126   section images of non-invasive microCT with corresponding images of serial surface-

127   stained undecalcified ground sections; ii) established a histomorphological basis for

128   functional studies of the baculum in the *Pipistrellus pipistrellus* species complex; and

129   iii) collected and interpreted preliminary histomorphological data on *P. pipistrellus*

130   baculum development.

131

132

133   MATERIAL AND METHODS

134

135   The specimens for this study were kindly made available by the National Museum

136   (Natural History) Prague, Czech Republic: *Pipistrellus pipistrellus* (n=30); *P. pygmaeus*

137   (n=24); *P. hanaki* (n=9), all of them genetically identified; by the Naturalis Biodiversity

138   Center, Leiden, The Netherlands: *P. pipistrellus sensu lato (s.l.)* (n=5, two of them

139   subadult, one juvenile); *P. nathusii* (n=3); and by the Natural History Museum Vienna,

140   Austria: *P. pipistrellus s.l.* (n=30, five of them subadult, four juvenile); *P. nathusii*

141   (n=1). The specimens were whole bats or resected penes, preserved in 70% ethanol.

142

143   MicroCT imaging was used for histomorphological and histometrical evaluation of the

144   bacula of all four *Pipistrellus* species (*Pipistrellus pipistrellus*, n=65; *P. pygmaeus*,

145   n=24; *P. hanaki*, n=9; *P. nathusii*, n=4). The penes of whole bats, preserved in ethanol,

146   were scanned unstained. The bats were mounted intact in plastic sample vials in 70%

147   ethanol. Resected bat penes (*P. pipistrellus s.l.*, n=12; *P. nathusii* n=4) were scanned

148   unstained and then scanned again after iodine staining for soft tissues. For contrast

149   staining, they were transferred to 100% ethanol via ascending ethanol concentrations,

150   and stained with 1% (w/v) elemental iodine in 100% ethanol (I2E; Metscher 2009;

151   Herdina, Herzig-Straschil et al. 2010; Metscher 2011) for at least 14 hours, up to several

152   days. Before scanning, the samples were transferred back to 100% ethanol for at least

153   one hour to improve contrast. Resected bat penes were mounted in polypropylene

154   micro-pipette tips (heat-sealed and filled with 70% or 100% ethanol, Metscher 2009)

155   and sealed with Parafilm.

156

157   MicroCT imaging was performed using an Xradia MicroXCT system

158   (www.xradia.com), with a microfocus tungsten source, secondary optical magnification

159   of the scintillator images, and a 2k x 2k cooled CCD camera. Projection images were

160   collected every 0.25° over a rotation of 180° (plus the cone angle Metscher 2009;

161   Metscher 2011) with 2x2 pixel binning, at 4x or 10x optical magnification, exposure

162   times of 4-10 sec, and source voltages of 40-60 kVp at 4-8 W. Tomographic virtual

163   sections were reconstructed using the XMReconstructor software (version 8.1) supplied

164   with the Xradia MicroXCT system, resulting in reconstructed isotropic voxel sizes of

165   2.0-2.5 μm.

166

167   Virtual sections from the reconstructed volume images were evaluated and measured.

168   On virtual thick sections projected length, width, and height were measured of adult *P.*

169   *pipistrellus s.str.* (n=30), *P. pygmaeus* (n=24), *P. hanaki* (n=9), and *P. nathusii* (n=4);

170   and juvenile (n=5) and subadult (n=7) *P. pipistrellus s.l.* (Table 1). Bone and soft tissues

171   were segmented manually using Amira 5.4.5 to create 3D models (Metscher 2009;

172   Metscher 2009; Herdina, Plenk Jr et al. 2010; Metscher 2011; Herdina, Kelly et al. 2014

173   submitted).

174

175   The penes of three *Pipistrellus pipistrellus* (one of them juvenile), which had been

176   iodine stained and microCT scanned, were prepared for light microscopic evaluation of

177   serial surface-stained undecalcified ground sections (Plenk 1986; Herdina, Plenk Jr et

178   al. 2010). The penes were transferred from 100% ethanol to acetone:ethanol (1:1;

179   100%), then 100% ethanol, and infiltrated with pure methylmethacrylate. Subsequently,

180   they were embedded in glass containers in a mixture of 800ml methylmethacrylate,

181   100ml Plastoid N, and 15g benzoyl peroxide in a water bath (28-32°C) for 4 days. The

182   polymerized blocks were hardened at 50°C in a heating cabinet over night. The blocks

183   were trimmed roughly on a band saw (Emco GmbH, 5400 Hallein, Austria) and then

184   sectioned close to the surface of the embedded penis on a low speed saw (Buehler

185   Isomet low-speed saw; Evanston, Illinois, USA). One specimen block each was cut and

186   ground longitudinally, starting from either the dorsal or ventral side of the penis. The

187   specimen block from the juvenile bat was cross-sectioned, starting from the proximal

188   end of the glans penis. The resulting surfaces of the respective specimen blocks were

189   polished and glued (CA8 Pronto instant adhesive, 3M, USA) to a plexiglass slide. An

190   about 3000μm thick section of was cut parallel to this surface. This thick section was

191   ground (EXAKT- Type AW 10, EXAKT Advanced Technologies GmbH, D-22851

192   Norderstedt, Germany; 1000-grit and 4000-grit sandpaper) until the skin of the penis

193   was exposed. This surface and consecutive ground surfaces were polished (Buehler

194   Minimet Polisher; Evanston, Illinois, USA; alumina polishing powder, particle sizes 1.0

195   and 0.3 µm) for surface staining. Before staining they were treated for 2 min in 0.1%

196   formic acid in distilled water (A. dest.), then rinsed in A. dest., and stained 20-40 min in

197   freshly prepared Giemsa-solution (Merck GmbH, Darmstadt, Germany,

198   http://www.merckgroup.com), differentiated in 100 ml A. dest with 5 drops of glacial

199   acetic acid, and rinsed again in A. dest.. Some sections were contrasted with

200   pararosaaniline (Rendl and Haase, in preparation). Dried surfaces were evaluated

201   without cover slips by light microscopy (Nikon E 600) and photographed (Nikon J1,

202   Nikon Ltd. Japan).

203

204

205   RESULTS

206

207   Evaluation of microCT virtual sections and 3D renderings showed that the bacula of

208   *Pipistrellus pipistrellus*, *P. pygmaeus*, *P. hanaki*, and *P. nathusii* are all Y-shaped in

209   dorsal view (Fig. 1). The two branches of the base are slender where they split, but

210   widen considerably towards the proximal ends. The angle of this branching shows inter-

211   and intraspecific variations. In all four *Pipistrellus* species studied the shaft of the

212   baculum is long and slender in proportion to the base. The small distal tip of the

213   baculum is forked and directly encases the upper half of the external urethral orifice

214   within the glans penis.

215

216   **Fig. 1 near here**

217

218   In lateral view (Fig.1), the bacula of *P. pipistrellus*, *P. pygmaeus*, and *P. hanaki* show a

219   pronounced dorsoventral curve, with the broader base tapering towards the narrow tip.

220   The curve shape in those tree species shows considerable individual variation. *P.*

221   *nathusii* bacula are also curved dorsoventrally, but bend back dorsally near the distal

222   end of the shaft. Table 1 contains the mean and range of measurements of the bacula.

223

224   **Table 1 near here**

225

226   MicroCT images also show the position of a medullary cavity and the course of a

227   medullary/vascular canal and various nutrient canals in the bacula (Fig. 1). In most

228   specimens, both widened proximal ends of the branches of the baculum contain marrow

229   cavities, which can be very large and can have several nutrient canals. A narrower

230   marrow canal leads to a central canal in the proximal portion of the baculum shaft. In all

231   four *Pipistrellus* species, considerable individual variation was observed in the length of

232   the canals, the number and position of the foramina nutritia, and further canals

233   sometimes branching off the main canal in the shaft. In three specimens of *P. hanaki*, a

234   medullary cavity was missing completely.

235

236   **Fig. 2 near here**

237   **Fig. 3 near here**

238

239   Light microscopic histomorphology of undecalcified ground sections of *P. pipistrellus*

240   penes showed that the proximal branches (base) of the baculum consist of a layer of

241   woven bone with densely packed irregular osteocytes around an inner layer of lamellar

242   bone (Figs. 2, 3, and 4). In the woven bone layer, the tunica albuginea with

243   fibrocartilage-like cells connects the corpora cavernosa to the baculum (Figs. 3 and 4).

244   Such a connection is identified as an enthesis (Benjamin, Evans et al. 1986). The layer

245   of lamellar bone encases the medullary cavity, filled with fatty bone marrow and blood

246   vessels (Figs. 2, 3, and 4). The proximal part of the baculum-shaft consists of a tubular

247   bone with a central medullary or vascular canal (of variable length and morphology),

248   surrounded by concentric lamellar bone with regularly spaced, oval shaped osteocytes

249   (Figs. 4 and 5). This inner lamellar layer of the proximal shaft is in most places

250   separated from the peripheral subperiosteal woven bone by a distinct cement line (Figs.

251   4 and 5). The distal part of the baculum-shaft with its slender and forked tip consists of

252   woven bone only (Fig.6).

253

254   **Fig. 4 near here**

255   **Fig. 5 near here**

256

257   The thick fibrous tunicae albugineae of the paired corpora cavernosa are merged for

258   most of the length of the penis shaft (Figs. 2 and 3). Where they merge, a thinner

259   continuation of the combined tunicae albugineae forms the incomplete septum

260   pectinatum, allowing blood lacunae to communicate. Between the blood lacunae a

261   network of more fibrous trabeculae can be seen, which connect to the tunica albuginea

262   (Figs. 2 and 3). In microCT virtual cross sections, the corpora cavernosa are shaped like

263   a thick horseshoe, with a ventral groove along their length. At the distal end, the

264   gradually thinning tunica albuginea continues into the fibrous layer of the dorsal

265   periosteum of the baculum (Figs. 2, 3, and 4).

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266

267 **Fig. 6 near here**

268

269 The urethra is lined with urethral mucosa and surrounded by the corpus spongiosum

270 (Figs 2, 3, 4, 5 and especially 6). Proximally, the urethra lies in the ventral grove of the

271 merged corpora cavernosa, and then continues under the baculum towards the forked

272 distal end (Fig. 5). In the glans penis, the corpus spongiosum becomes more voluminous

273 and completely envelops the urethra and the baculum. The corpus spongiosum is also

274 enveloped in a fibrous layer, but thinner than the tunica albuginea of the corpora

275 cavernosa (Fig. 6). Finally, the urethra ends with the external urethral orifice, which is

276 beveled like the tip of a hypodermic needle, its dorsal half being enclosed by the forked

277 tip of the baculum (Figs. 5 and 6). The preputium is thick and vascular (Figs. 2, 5, and

278 6). The inner, visceral surface of the preputium and the skin of the glans consist of a

279 stratified epithelium (Fig. 6). The preputium contains accessory swelling tissue, a

280 subcutaneous layer of fat, and sebaceous glands (Fig. 6). The transition from the normal

281 skin of the penis occurs about where the corpus spongiosum widens. The preputial sac

282 is filled with abundant cellular debris constituting the smegma (Figs. 5 and 6). The

283 outer, parietal surface of the preputium is folded and covered densely with hair (Figs. 2,

284 5, and 6). The epidermis is a stratified squamous keratinized epithelium with a distinct

285 layer of melanocytes (Fig. 6). No keratinized spines or papillary buds were found.

286

287 **Fig. 7 near here**

288

289 Baculum development in *Pipistrellus pipistrellus*

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290

291 In the juvenile and subadult bats, the whole baculum consists of woven bone. While the

292 distal part of the shaft with the forked tip seems to be mostly developed, the proximal

293 base of the baculum is distinctly different from the appearance in adults (Fig. 7). The

294 branches are shorter and not as broad as in adult specimens. The woven bone at the base

295 contains densely packed, single large, round or cubic osteocytes, resembling

296 chondrocytes (Fig. 7A). However, the corresponding microCT images show a

297 mineralized extracellular matrix. In some of the bacula from subadult bats (n=6) and

298 one from a juvenile, a primary medullary cavity was found where the branches of the

299 base merge into the shaft, sometimes with a large opening to the ventral side of the bone

300 (Fig. 7B), sometimes expanding into the distal part of the branches. The other bacula

301 (juvenile n=4, subadult n=1) only had a small primary medullary cavity, where the

302 branches of the base merge into the shaft.

303

304

305 DISCUSSION

306

307 By verifying histomorphological findings on microCT images by light microscopic

308 evaluation of serial surface-stained ground sections, correlative imaging of museum

309 specimens enabled us to study *Pipistrellus* penes in greater detail and in more

310 specimens than would a single technique. Some tissue properties, like discriminating

311 woven from lamellar bone by the shape and size of osteocytes, can readily be studied on

312 virtual sections from microCT images alone (Fig. 4). Soft tissues can be distinguished

313 in microCT images of iodine stained *P. pipistrellus* and *P. nathusii* penes by different

314   grey values – representing different radiodensities – due to the extent to which the

315   tissues take up iodine (Figs. 2-5).

316

317   For other findings, like the identification of fatty marrow or of cement lines, the

318   comparison of microCT images with ground sections of the same individual in the same

319   section plane and orientation was necessary (Figs. 2-4). The ground sections showed

320   more detailed histological traits in the soft tissue, in addition to what we found with

321   microCT. After thus calibrating our microCT images, these structures could be found

322   and confidently interpreted in microCT images of other specimens, even of different

323   species. Additionally, 3D renderings of microCT images provide a better understanding

324   of the overall shape of *Pipistrellus* bacula and facilitate comparisons between species

325   (Figs. 1, 5, and 7).

326

327   We originally evaluated the micromorphology of the baculum to find characters that

328   could potentially be used for differentiating *Pipistrellus* species (Herdina, Hulva et al.

329   2014 in press). Differences in dorsoventral curve shape (Fig. 1), as found between the

330   specimens of *Pipistrellus pipistrellus*, *P. pygmaeus, P. hanaki* seem to represent only

331   individual variation. The location and length of the medullary canal and foramina

332   nutritia in *Pipistrellus pipistrellus*, *P. pygmaeus, P. hanaki,* and *P. nathusii* exhibit

333   much more individual variation than in the *Plecotus* species examined previously

334   (Herdina 2008; Herdina, Herzig-Straschil et al. 2010). This variation ranges from

335   specimens without any medullary canals to specimens with large cavities in the

336   branches of the proximal base, and extremely large foramina nutritia. Therefore these

337   characters do not appear to be usable in *Pipistrellus* species identification.

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338

339   The prevalence of a secondary medullary cavity with fatty marrow in mammalian

340   bacula is unclear. The occurrence of a canal or cavity filled with connective tissue and

341   blood vessels in the baculum has been reported in *Vespertilio murinus* (Gilbert 1892)

342   and in rodents (von Ihering 1885; Retterer 1887; Gilbert 1892; Glucksmann and Cherry

343   1972). Bone marrow, without further description, was reported in the baculum of mice

344   (Iguchi, Irisawa et al. 1990; Iguchi and Ohta 1996; Deveci, Nergiz et al. 2009; Yildiz,

345   Bolat et al. 2010) and rats (Yoshida and Kadota 1980). The occurrence of fatty bone

346   marrow was only mentioned in the baculum of rats (Retterer 1887). The fatty bone

347   marrow found in the medullary cavity of the baculum, together with cement lines as

348   evidence of bone remodeling, identifies the baculum as a mature bone. The baculum can

349   probably alter its thickness as a response to forces applied to it, as was found in

350   comparing sexually active to sexually inexperienced mice (Stockley, Ramm et al.

351   2013).

352

353   The histomorphological structure of the lamellar bone with a central canal and small

354   nutrient canals but no Haversian canals resembles the structure of a single osteon. Thus,

355   it appears that the lamellar portion of the baculum develops like a single secondary

356   osteon in all of the *Plecotus* (Herdina 2008; Herdina, Herzig-Straschil et al. 2010) and

357   *Pipistrellus* species we have studied (Fig. 7). However, the baculum in the studied bat

358   species (Figs. 2-5) is not a single-osteon bone, because of the presence of woven bone

359   around the lamellar portion and because of the occurrence of a secondary medullary

360   cavity filled with fatty marrow, although such bones have been found in other

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361   vertebrates like the femur of the Japanese fire-bellied salamander *Cynops pyrrhogaster*

362   (Urschitz 1982) and other amphibians (e.g. Rana esculenta; Demeter and Mátyás 1928).

363

364   Ground sections of *Pipistrellus pipistrellus* bacula and microCT images of the bacula of

365   all the species studied complement the results of our earlier studies on *Plecotus* baculum

366   histomorphology (Herdina 2008; Herdina, Herzig-Straschil et al. 2010), and results of

367   this study on soft tissue histomorphology corroborate the results of previous studies of

368   bat penes (Matthews 1937; Ryan 1991; Ryan 1991; Herdina 2008; Herdina, Herzig-

369   Straschil et al. 2010). The small glans and the thick preputium, containing accessory

370   swelling tissue, of *P. pipistrellus s.l.* (Figs. 2, 3, 5, and 6) were described by Matthews

371   (1937), who called this swelling tissue the accessory corpus cavernosum (citing

372   Ercolani 1868, who named it in *Vespertilio*). The thick preputium might effect coital

373   locking, as proposed for *Myotis lucifugus* by Wimsatt and Kallen (1952). Contrary to

374   findings in some other bat species (Vamburkar 1958; Ryan 1991; Ryan 1991; Crichton

375   and Krutzsch 2000; Armstrong 2005; Cryan, Jameson et al. 2012), we did not find

376   penile spines on the penes of the species studied.

377

378   Our results provide histomorphological support for two hypotheses of baculum

379   function: 1) The baculum forms a functional unit with the corpora cavernosa (Figs, 2-4),

380   facilitating force transfer from the tip of the penis to the corpora cavernosa and

381   increasing overall flexural stiffness of the glans and shaft of the penis (Kelly 2000;

382   Herdina, Kelly et al. 2014 submitted). 2) The baculum protects the distal part of the

383   urethra and the external urethral orifice (Figs. 5-7) from compression during copulation

384   (Dixson 1995; Herdina, Kelly et al. 2014 submitted). The forked tip of the baculum

385 encloses the upper half of the external urethral orifice in all the *Pipistrellus* species

386 studied.

387

388 Our preliminary results on baculum development in *Pipistrellus pipistrellus* show that

389 the distal part of the baculum reaches its adult shape before the proximal part (Fig. 7).

390 We did not study young enough individuals to confirm if a cartilage precursor to the

391 baculum exists in this species. However, the woven bone tissue in the base of the

392 juvenile and subadult bats, with large round osteocytes resembling chondrocytes (Fig.

393 7A), could be chondroid bone (as described by Schaffer 1930). At least two juvenile

394 specimens were not yet capable of flight, while the subadult bats we studied were

395 already capable of flight and had probably already left the nursery colonies in which

396 they were born. The different states of medullary cavity development we found suggest

397 an invasion of blood vessels from the ventral side of the baculum, where the branches of

398 the base meet the shaft (Fig. 7B).

399

400 Variability in the innervation and the morphology of medullary canals of bone is a well-

401 known phenomenon (Usener 1966). The different degree of variability of the

402 morphology of the medullary canals in the bacula of bat species of the genera

403 *Pipistrellus* and *Plecotus* is interesting because it may imply differences in ontogenetic

404 plasticity. To our knowledge, the ontogeny of the baculum in bats has not been studied

405 histologically, but only on dissected penes or macerated bones (Vlček 1967; Maeda

406 1978; Maeda 1978; Smirnov and Tsytsulina 2003). An interesting follow-up study

407 would be a more thorough investigation of baculum ontogeny to determine whether

408 epigenetic mechanisms are involved in the plasticity of baculum micromorphology.

409

410   Conclusion

411

412   Correlative 2D and 3D imaging can be especially useful for studying valuable museum

413   specimens. High-resolution microCT images can be obtained without modifying or

414   damaging the specimens. Thus a larger number of samples can be studied, even if the

415   study organism is rare in scientific collections. Combining microCT imaging with

416   specialized histomorphological techniques, like surface-stained ground sections, allows

417   accurate identification of histological structures in the microCT images. It also creates

418   an independent data set that can be useful in further studies. Thus correlating a versatile

419   3D image and virtual 2D sections from microCT scans with ground section surfaces of

420   the same specimen in different orientations and combinations becomes possible, even

421   long after the specimen has been returned or processed histologically.

422

423   AUTHOR CONTRIBUTIONS

424   The project was developed by Anna Nele Herdina, Helge Hilgers, Barbara Herzig-

425   Straschil, Brian D. Metscher and Hanns Plenk. MicroCT imaging and iodine staining

426   were done by Brian D. Metscher and Anna Nele Herdina. Anna Nele Herdina measured

427   and segmented microCT images. Hanns Plenk and Anna Nele Herdina made, stained,

428   and photographed ground sections. Petr P. Benda, Peter H. C. Lina, and Barbara Herzig-

429   Straschil contributed the specimens. All authors contributed ideas and discussed the

430   results. The manuscript was written by Anna Nele Herdina, Brian D. Metscher, and

431   Hanns Plenk.

432

448

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623

624

625   Fig. 1: Examples of bacula of *P. pipistrellus*, *P. pygmaeus*, *P.hanaki*, and *P. nathusii*

626   (left to right; 3D surface renderings of microCT images, scaled to the same size to

627   compare shape only; for sizes see Table 1) in dorsal view (upper row) and lateral view

628   (lower row). Medullary cavity and canals shown in orange.

629

630   Fig. 2: Ventral view of a *P. pipistrellus* penis (distal part of the shaft and glans), surface

631   stained ground section (right; Giemsa stain and Pararosaniline; photographs stitched

632   with Adobe Photoshop CS6), comparing the corresponding microCT virtual section

633   (left; iodine stained, overview scan) of the same specimen; ac: accessory swelling

634   tissue, ba: baculum, cc: corpora cavernosa, cs: corpus spongiosum, ha: hair, la: blood

635   lacunae (in the corpora cavernosa), mc: medullary cavity with fatty bone marrow and

636   blood vessels, po: periosteum, pr: preputium, ps: preputial sac, ta: tunica albuginea (of

637   the corpora cavernosa), tr: trabeculae (in the corpora cavernosa), ur: urethra.

638

639   Fig. 3: Corresponding microCT virtual section (left; iodine stained; taken from

640   overview scan) and Giemsa surface-stained ground section (right) of a *P. pipistrellus*

641   penis in ventral view. The visible portion of the baculum (ba) shows woven bone (wb)

642   with densely packed osteocyte lacunae in the periphery, and lamellar bone (lb) with

643   sparse osteocytes around the medullary cavity (mc). ba: baculum, cc: corpora

644   cavernosa, cs: corpus spongiosum, en: entheses, fm: fatty marrow (with blood vessels in

645   between), la: blood lacunae (in the corpora cavernosa), lb: lamellar bone, mc: medullary

646   cavity with fatty bone marrow and blood vessels, po: periosteum, pr: preputium, ps:

647   preputial sac, ta: tunica albuginea (of the corpora cavernosa), tr: trabeculae (in the

648   corpora cavernosa), ur: urethra, wb: woven bone.

649

650   Fig. 4: Corresponding microCT virtual section (left; iodine stained; detail scan of the

651   proximal baculum and surrounding soft tissue) and Giemsa surface-stained ground

652   section (right) of a *P. pipistrellus* penis in dorsal view. The visible portion of the

653   baculum (ba) shows woven bone (wb) with densely packed osteocyte lacunae in the

654   periphery, and lamellar bone (lb) with sparse osteocytes separated by a distinct cement

655   line (cl); around the medullary canal (mc). ba: baculum, bv: blood vessels, cc: corpora

656   cavernosa, cl: cement line, en: entheses, hc: haversian canal, la: blood lacunae (in the

657   corpora cavernosa), lb: lamellar bone, mc: medullary canal with blood vessels, oc:

658   osteocytes, po: periosteum, ta: tunica albuginea (of the corpora cavernosa), ur: urethra,

659   wb: woven bone.

660

661   Fig. 5: MicroCT surface rendering of the baculum (ba), urethra (ur), glans penis (gl),

662   and corpora cavernosa (cc) with volume rendering and virtual section of the penis in

663   lateral view (left; iodine stained; overview scan) and Giemsa surface-stained ground

664   section (right, focus stack of several photographs) of a *P. pipistrellus* penis in ventral

665   view. Showing the position of the distal tip of the baculum (ba) at the external urethral

666   orifice (uo; left) and the bone structure of the baculum-shaft (ba; right). ac: accessory

667   swelling tissue, ba: baculum, cc: corpora cavernosa, cl: cement line, gl: glans penis, ha:

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668 hair, lb: lamellar bone, mc: medullary canal with blood vessels, pr: preputium, ps:

669 preputial sac, uo: external urethral orifice, ur: urethra, wb: woven bone.

670

671 Fig. 6: Giemsa surface-stained ground sections of the distal tip of the penis in two

672 different *P. pipistrellus* specimens. Dorsal view (left) and ventral view (right) show how

673 the distal tip of the baculum encases the dorsal half of the external urethral orifice. ac:

674 accessory swelling tissue, ba: baculum, cl: cement line, cs: corpus spongiosum, ep:

675 epidermis, gl: glans penis, ha: hair, lb: lamellar bone, mc: medullary canal with blood

676 vessels, me: melanocytes, pr: preputium, ps: preputial sac, se: stratified epithelium, sf:

677 subcutaneous fat, sg: sebaceous gland, sm: smegma, um: urethral mucosa, uo: external

678 urethral orifice, ur: urethra, wb: woven bone.

679

680 Fig. 7: Baculum 3D surface renderings, medullary cavity and canals in orange; left:

681 lateral view, middle: dorsal view; projected length: 1.3mm. Slightly oblique ground

682 cross-sections (right, A-D; Giemsa stained) of juvenile *P. pipistrellus.* A: base of the

683 baculum with osteocytes (oc) resembling chondrocytes and a small medullary canal

684 (mc). B: distal part of the baculum-base with large primary medullary cavity (mc). C

685 and D: distal end of the baculum where the forked baculum-tip surrounds the dorsal half

686 of the external urethral orifice. gl: glans penis, mc: medullary cavity/canal, oc:

687 osteocytes, po: periosteum, ur: urethra.

688

TABLE 1. Measurements on the bacula of juvenile and subadult *P. pipistrellus s.l.*, and adult *P. pipistrellus s.str.*, *P. pygmaeus*, *P. hanaki*, and *P. nathusii*. Arithmetic mean ($\bar{x}$) and range of projected length, width, and height.

	projected length $\bar{x}$ (range)	projected width $\bar{x}$ (range)	projected height $\bar{x}$ (range)
P. pipistrellus (n=5) juvenile	1267.3μm (1185.9-1377.6)	210.1μm (182.7-237.9)	230.4μm (205.2-267.8)
P. pipistrellus (n=4) younger subadult	1364.5μm (1253.5-1422.4)	210.2μm (166.2-230.8)	276.6μm (205.2-322.3)
P. pipistrellus (n=3) older subadult	1570.3μm (1541.8-1589.1)	287.9μm (236.9-265.6)	333.2μm (317.8-354.3)
P. pipistrellus (n=30) adult	1687.7μm (1531.5-1835.4)	371.7μm (285.8-417.9)	366.9μm (309.9-458.6)
P. pygmaeus (n=24) adult	1559.3μm (1420.6-1767.8)	325.9μm (281.5-397.3)	351.9μm (296.0-510.7)
P. hanaki (n=9) adult	1478.7μm (1319.1-1762.2)	315.1μm (272,0-393.5)	294.5μm (243.0-387.9)
P. nathusii (n=4) adult	1375.5μm (1292.0-1447.5)	399.4μm (367.9-430.8)	246.4μm (241.1-259.6)

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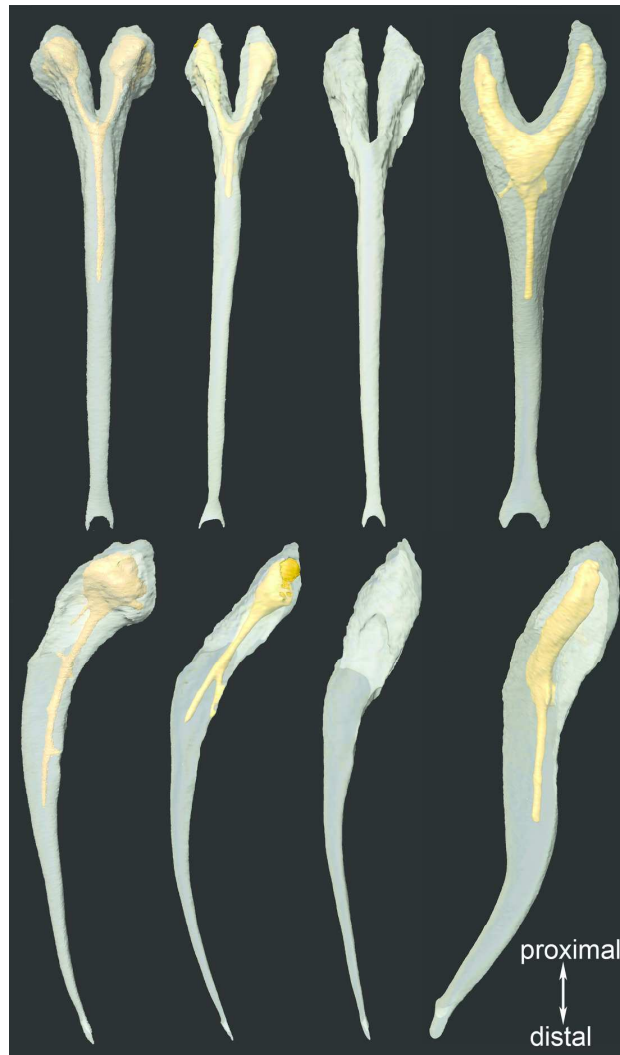
Fig. 1: Examples of bacula of *P. pipistrellus*, *P. pygmaeus*, *P.hanaki*, and *P. nathusii* (left to right; 3D surface renderings of microCT images, scaled to the same size to compare shape only; for sizes see Table 1) in dorsal view (upper row) and lateral view (lower row). Medullary cavity and canals shown in orange.
135x230mm (300 x 300 DPI)

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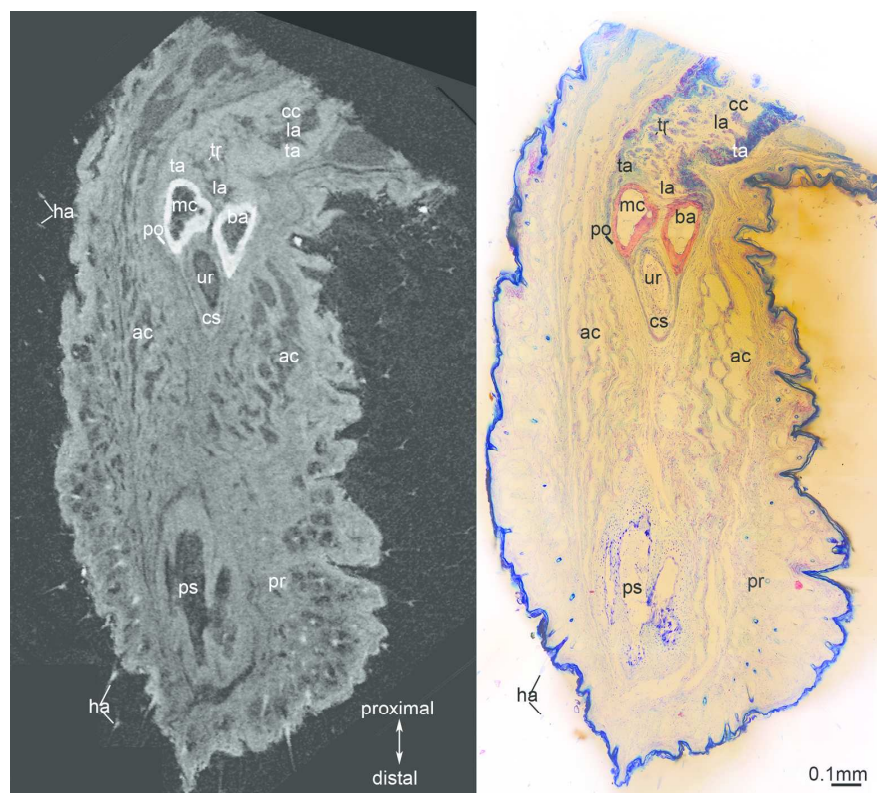
Fig. 2: Ventral view of a *P. pipistrellus* penis (distal part of the shaft and glans), surface stained ground section (right; Giemsa stain and Pararosaniline; photographs stitched with Adobe Photoshop CS6), comparing the corresponding microCT virtual section (left; iodine stained, overview scan) of the same specimen; ac: accessory swelling tissue, ba: baculum, cc: corpora cavernosa, cs: corpus spongiosum, ha: hair, la: blood lacunae (in the corpora cavernosa), mc: medullary cavity with fatty bone marrow and blood vessels, po: periosteum, pr: preputium, ps: preputial sac, ta: tunica albuginea (of the corpora cavernosa), tr: trabeculae (in the corpora cavernosa), ur: urethra.
170x153mm (300 x 300 DPI)

Fig. 3: Corresponding microCT virtual section (left; iodine stained; taken from overview scan) and Giemsa surface-stained ground section (right) of a *P. pipistrellus* penis in ventral view. The visible portion of the baculum (ba) shows woven bone (wb) with densely packed osteocyte lacunae in the periphery, and lamellar bone (lb) with sparse osteocytes around the medullary cavity (mc). ba: baculum, cc: corpora cavernosa, cs: corpus spongiosum, en: entheses, fm: fatty marrow (with blood vessels in between), la: blood lacunae (in the corpora cavernosa), lb: lamellar bone, mc: medullary cavity with fatty bone marrow and blood vessels, po: periosteum, pr: preputium, ps: preputial sac, ta: tunica albuginea (of the corpora cavernosa), tr: trabeculae (in the corpora cavernosa), ur: urethra, wb: woven bone.
170x126mm (300 x 300 DPI)
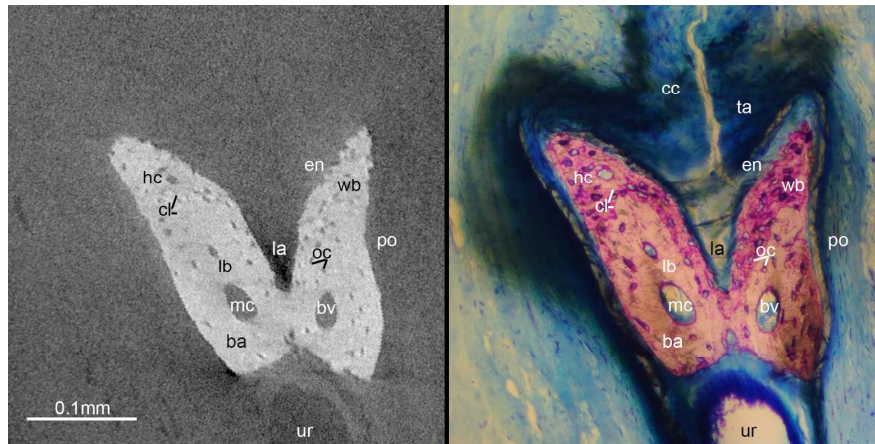
Fig. 4: Corresponding microCT virtual section (left; iodine stained; detail scan of the proximal baculum and surrounding soft tissue) and Giemsa surface-stained ground section (right) of a *P. pipistrellus* penis in dorsal view. The visible portion of the baculum (ba) shows woven bone (wb) with densely packed osteocyte lacunae in the periphery, and lamellar bone (lb) with sparse osteocytes separated by a distinct cement line (cl); around the medullary canal (mc). ba: baculum, bv: blood vessels, cc: corpora cavernosa, cl: cement line, en: entheses, hc: haversian canal, la: blood lacunae (in the corpora cavernosa), lb: lamellar bone, mc: medullary canal with blood vessels, oc: osteocytes, po: periosteum, ta: tunica albuginea (of the corpora cavernosa), ur: urethra, wb: woven bone.
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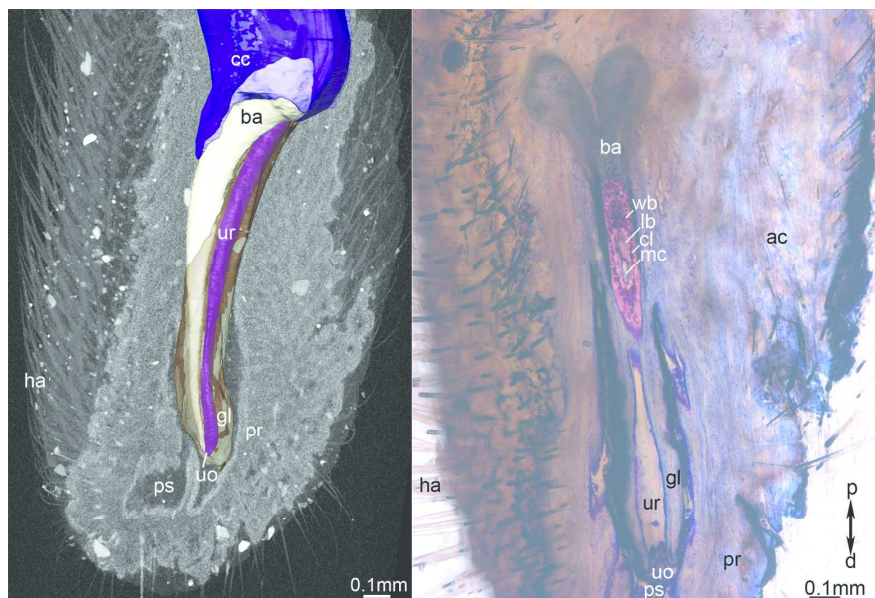
Fig. 5: MicroCT surface rendering of the baculum (ba), urethra (ur), glans penis (gl), and corpora cavernosa (cc) with volume rendering and virtual section of the penis in lateral view (left; iodine stained; overview scan) and Giemsa surface-stained ground section (right, focus stack of several photographs) of a *P. pipistrellus* penis in ventral view. Showing the position of the distal tip of the baculum (ba) at the external urethral orifice (uo; left) and the bone structure of the baculum-shaft (ba; right). ac: accessory swelling tissue, ba: baculum, cc: corpora cavernosa, cl: cement line, gl: glans penis, ha: hair, lb: lamellar bone, mc: medullary canal with blood vessels, pr: preputium, ps: preputial sac, uo: external urethral orifice, ur: urethra, wb: woven bone.
170x115mm (300 x 300 DPI)
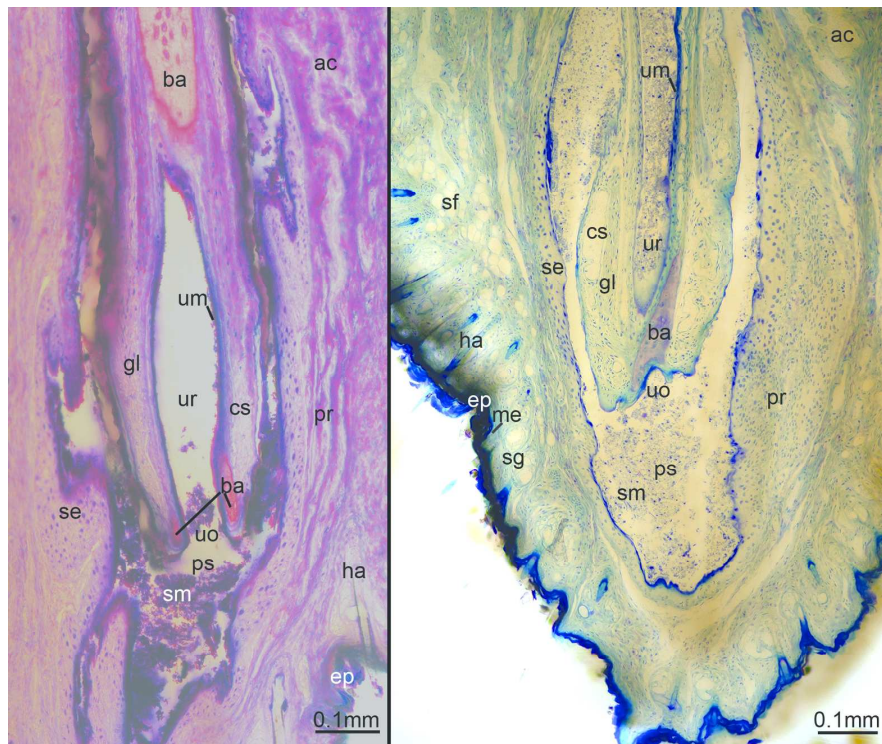
170x143mm (300 x 300 DPI)
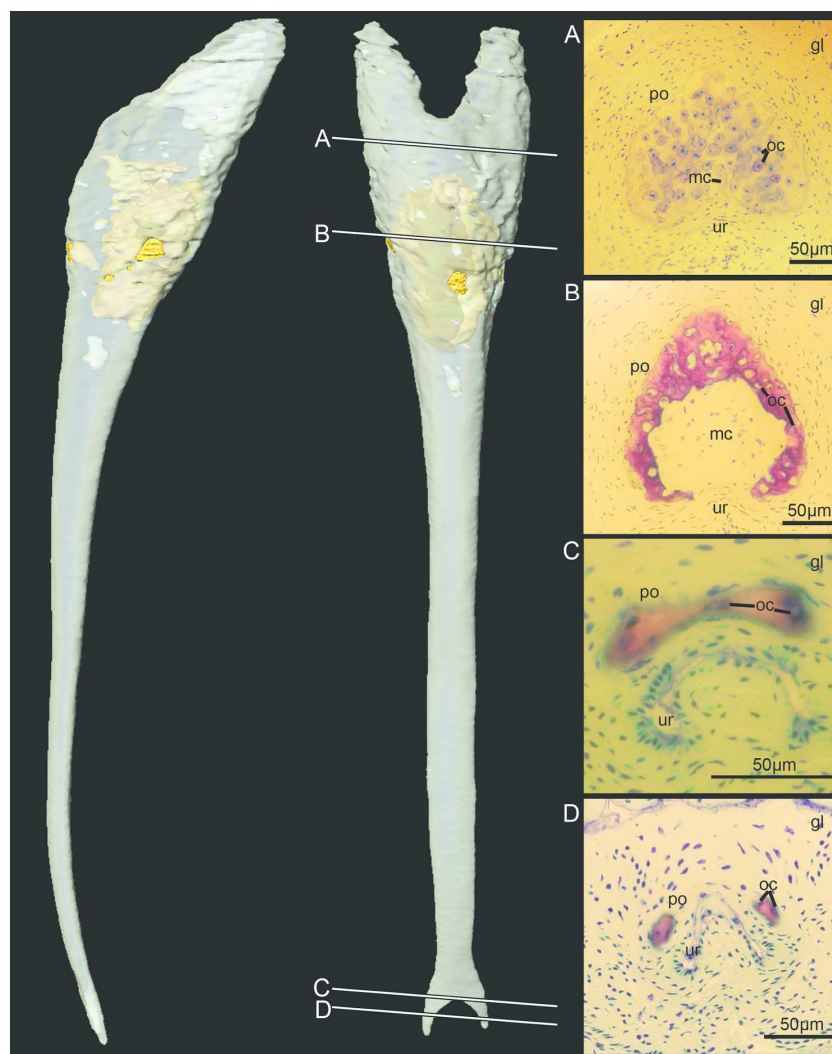
Fig. 7: Baculum 3D surface renderings, medullary cavity and canals in orange; left: lateral view, middle: dorsal view; projected length: 1.3mm. Slightly oblique ground cross-sections (right, A-D; Giemsa stained) of juvenile *P. pipistrellus*. A: base of the baculum with osteocytes (oc) resembling chondrocytes and a small medullary canal (mc). B: distal part of the baculum-base with large primary medullary cavity (mc). C and D: distal end of the baculum where the forked baculum-tip surrounds the dorsal half of the external urethral orifice. gl: glans penis, mc: medullary cavity/canal, oc: osteocytes, po: periosteum, ur: urethra.
170x214mm (300 x 300 DPI)

4. PAPER III (IN REVISION) BACULUM FUNCTION IN BATS

1   **Baculum function in bats**

2

3   **Testing hypotheses of bat baculum function with 3D models**

4   **derived from microCT**

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25   **Abstract**

26   The baculum (os penis) has been extensively studied as a taxon-specific character in bats and

27   other mammals but its mechanical function is still unclear. There is a wide consensus in the

28   literature that the baculum is probably a sexually selected character. Using a novel approach

29   combining postmortem manipulation and 3D imaging, we tested two functional hypotheses in

30   the common noctule bat *Nyctalus noctula*, the common pipistrelle *Pipistrellus pipistrellus,*

31   and Nathusius' pipistrelle *P. nathusii*: 1) whether the baculum can protect the distal urethra

32   and urethral opening from compression during erection and copulation; and 2) whether the

33   baculum and corpora cavernosa form a functional unit to support both the penile shaft and the

34   more distal glans tip. In freshly dead or frozen and thawed bats, we compared flaccid penises

35   with artificially "erect" penises, which were inflated with 10% formalin. Penises were stained

36   with alcoholic iodine and imaged with a lab-based high-resolution x-ray microtomography

37   system. Analysis of the 3D images enabled us to compare the changes in relative positions of

38   the baculum, corpora cavernosa, urethra, and corpus spongiosum to one another between

39   flaccid and "erect" penises. Our results support both functional hypotheses, indicating that the

40   baculum probably performs two different roles during erection. Our approach should prove

41   valuable for comparing and testing the functions of different baculum morphologies in bats

42   and other mammals. Moreover, we have validated an essential component of the groundwork

43   necessary to extend this approach with finite element analysis for quantitative 3D

44   biomechanical modeling of penis function.

45

46   **Keywords**: penis bone, micro-CT, x-ray microtomography, Chiroptera, functional

47   morphology, 3D model, iodine stain

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49

50   **Introduction**

51

52   Many different mechanical and behavioral hypotheses have been proposed for baculum

53   function in bats, and in mammals more generally. However, the only studies on baculum

54   function based on experimental data were done in rats (*Rattus norvegicus*) by Kelly (2000)

55   and in mice (*Mus musculus domesticus*) by Simmons and Firman (2013) and Stockley et al.

56   (2013).

57

58   A single mechanical function for all the different baculum shapes is very unlikely (Ruth,

59   1934, Dixson, 2012), so there are probably various and overlapping mechanical functions

60   (Dyck et al., 2004). The baculum and corpora cavernosa could form a functional unit due to

61   their tight connection and shared envelope of fibrous connective tissue. This functional unit

62   could increase the flexural stiffness of the penis by transferring bending forces from the distal

63   end of the glans penis to the corpora cavernosa during copulation (Long and Frank, 1968,

64   Kelly, 2000). The baculum could increase the stiffness of the corpora cavernosa by being

65   pressed into their proximal ends and thus increasing the hydrostatic pressure within the

66   corpora cavernosa during copulation (Long and Frank, 1968, Kelly, 2000). The baculum

67   could affect penile shape during erection, and it could protect the distal end of the urethra

68   from compression, especially in species in which coital locking occurs (Dyck et al., 2004,

69   Dixson, 2012). The baculum may allow males to gain intromission if males are larger then

70   females or if the penis is inserted before it is fully erect (Long and Frank, 1968, Dixson,

71   2012). It might also serve to expand the female cervix to optimize sperm deposition (Long

72   and Frank, 1968).

73

74   In bats of the genera *Pipistrellus* (Herdina et al., 2014b) and *Plecotus* (Herdina et al., 2010)

75 our histological data also supports the hypotheses that the baculum protects the urethra from

76 compression during copulation (Dyck et al., 2004), and that it forms a functional unit with the

77 corpora cavernosa. During copulation this functional unit can transfer bending forces from the

78 distal end of the glans penis to the corpora cavernosa, thereby increasing the flexural stiffness

79 of the whole erect penis (Long and Frank, 1968, Kelly, 2000, Herdina et al., 2010, Herdina et

80 al., 2014b).

81

82 In bats, mating systems and songflight behavior have been described for several species,

83 including *Nyctalus noctula* (McCracken and Wilkinson, 2000), *Pipistrellus pipistrellus*

84 (Sachteleben and von Helversen, 2006), and *P. nathusii* (Jahelková and Horáček, 2011). In all

85 three species the mating systems have been described as resource defense polygyny with

86 seasonal single-male multi-female groups, but it seems to be more complicated and might

87 share traits with a lek based mating system (Sachteleben and von Helversen, 2006, Jahelková

88 and Horáček, 2011). However, in bats very little is known about mating behavior, copulation

89 duration, and copulation postures (Glass, 1966, Barclay and Thomas, 1979, O'Brien and

90 Nankervis, 1994, Hosken et al., 2001, Dixson et al., 2004, Sachteleben and von Helversen,

91 2006, Jahelková and Horáček, 2011, Dixson, 2012, Liu et al., 2013). For mammals in general,

92 behavioral functions of the baculum have also been proposed. The baculum could enable

93 protracted copulations (Dixson, 1987, Dixson, 2012) and could stimulate the female

94 reproductive tract (Dyck et al., 2004, Dixson, 2012). It could play a role in eliciting

95 neuroendocrine responses necessary for induced mating postures and ovulation, or the

96 stimulation of corpus luteum function, in species that depend on mating to trigger these events

97 (Patterson and Thaeler Jr, 1982, Dixson, 2012). The baculum could also provide information

98 about male size or quality during intromission (Dyck et al., 2004). This could enable the

99 baculum to function as a reproductive isolating mechanism (Patterson and Thaeler Jr, 1982).

100   Further studies on baculum function, mating systems, and copulation behavior in bats can also

101   contribute to answering questions on the evolution of bacular diversity and the mechanism of

102   sexual selection on baculum shape and size (Patterson and Thaeler Jr, 1982, Eberhard, 1993,

103   Dixson et al., 2004, Hosken and Stockley, 2004, Lüpold et al., 2004).

104

105   Aims

106   In this study we tested two functional hypotheses in three bat species with similar baculum

107   shapes, the common noctule bat *Nyctalus noctula*, the common pipistrelle *Pipistrellus*

108   *pipistrellus,* and Nathusius' pipistrelle *P. nathusii*: 1) whether the baculum can protect the

109   distal urethra and urethral opening from compression during erection and copulation (Dixson,

110   1995); and 2) whether the baculum and corpora cavernosa form a functional unit to support

111   both the penile shaft and the more distal glans tip (Kelly, 2000).

112

113

114   **Material and methods**

115

116   The specimens for this study were made available by the Department of Zoology, Charles

117   University, Prague (*Nyctalus noctula*, n=15), the Naturalis Biodiversity Center, Leiden

118   (*Pipistrellus pipistrellus*, n=9; *Pipistrellus nathusii*, n=10) and the Natural History Museum

119   Vienna (*Pipistrellus nathusii*, n=1). We chose these three species for this study, because of

120   general similarities in their overall baculum morphology (Hill and Harrison, 1987) and mating

121   systems (McCracken and Wilkinson, 2000). The *Nyctalus noctula* specimens were collected

122   approximately 40 hours after they died in construction work at their hibernaculum; they were

123   not preserved or frozen during that time. The *Pipistrellus* specimens were found dead or died

124   in rescue stations and were frozen shortly after death (between 2009 and 2013). We thawed

125   them directly before preparation. All the specimens used in this study will be vouchered in the

126   mammal collection of the Natural History Museum Vienna.

127

128   We follow the definition of the glans penis of Meisenheimer (1921), where the corpora

129   cavernosa end in the shaft of the penis and do not extend into the glans, whereas the baculum

130   is always found completely within the glans.

131

132   The corpora cavernosa in the penes of 26 bats were inflated with formalin (see Fig. 1 and

133   Kelly, 2000). The penis was carefully resected close to the pubic bone. Sewing thread was

134   tied loosely around the penis near its proximal end. The needle (30G, diameter 0.30mm) of a

135   syringe filled with 10% formalin was inserted into a corpus cavernosum at the proximal (cut

136   off) end. The thread was pulled taut around the penis and needle. The formalin was slowly

137   pushed into the corpora cavernosa, until it started to leak out at the tied-off end. The needle

138   was removed and the thread tied tightly. The penis was then fixed in 10% formalin for at least

139   7 days. Additionally, the flaccid penes of 9 bats were fixed in 10% formalin.

140

141   After fixation the penes were transferred to 100% ethanol via ascending ethanol

142   concentrations. They were stained with 1% (w/v) elemental iodine in 100% ethanol (I2E;

143   Metscher, 2009a, Herdina et al., 2010) for at least 14 hours to several days. Before scanning,

144   the samples were transferred back to 100% ethanol for at least one hour to improve contrast.

145   Samples were mounted in polypropylene micro-pipette tips (heat-sealed and filled with 100%

146   ethanol; Metscher, 2009b) and sealed with parafilm.

147

148   High resolution microCT images were made with the Xradia MicroXCT system in the

149   Theoretical Biology Department at the University of Vienna, using a microfocus tungsten

150　source, secondary optical magnification of the scintillator images, and a 2k x 2k cooled CCD

151　camera (www.xradia.com). Projection images were collected every 0.25° over a rotation of

152　180° (plus the cone angle; Metscher, 2011) with 2x2 pixel binning, 4x optical magnification,

153　exposure times of 4-10 sec, and source voltages of 40-60 kVp at 4-8 W.

154

155　Tomographic sections were reconstructed using the XMReconstructor software (version 8.1)

156　supplied with the Xradia system. Reconstructed voxel sizes were 4.0-8.0μm for the

157　*Pipistrellus* samples, and 3.4-5.0μm for the *Nyctalus* samples. Samples too long to fit within

158　one field of view at the desired magnification were scanned in two overlapping segments

159　along the rotation axis, and the reconstructions were concatenated with the Xradia

160　XMContoller program's stitching function.

161

162　The reconstructed images were converted to image stacks of virtual sections and exported to

163　Amira 5.4.5 (http://www.vsg3d.com/amira/overview). In Amira, two penes per species (one

164　flaccid and one inflated) were visualized as volume renderings and the following elements

165　were manually segmented: the baculum, the lacunae of the corpora cavernosa, the tunica

166　albuginea of the corpora cavernosa, the urethral lumen, and the corpus spongiosum (with the

167　urethra). Surface renderings of the penes in their flaccid and "erect" state were compared

168　among species.

169

170

171　**Results**

172

173　The three species studied were chosen to investigate the function of this general baculum

174　shape, with a long slender shaft and bifurcated ends, but we also found certain differences

175   among them. The largest of the species, *Nyctalus noctula*, has the largest baculum absolutely,

176   but also in relation to penis length. In *N. noctula*, only the distal-most part of the baculum is

177   enveloped by the corpus spongiosum (Fig. 3, gray arrows), while in both pipistrelle species

178   more than half of the baculum shaft is enveloped (Figs 4 and 5, gray arrows). Baculum shape

179   is very similar in *N. noctula* and *Pipistrellus pipistrellus* with a dorsoventral curve in lateral

180   view. The baculum of P. nathusii is bent back towards the dorsal surface of the penis in mid-

181   shaft, while curved proximally like in the other species. Soft tissue morphology of the penis is

182   similar all three species, except for the shape of the prepuce and the portion of the baculum

183   covered by corpus spongiosum tissue in the glans penis. Also, in the "erect" penis of *P.*

184   *nathusii* the corpora cavernosa seem to show greater expansion than in both other species.

185

186   When comparing flaccid penis anatomy with the anatomy of penes with erect corpora

187   cavernosa (Fig. 2), a few changes in the position of tissues to one another are immediately

188   visible in all three species (Figs 3-5, supplementary material). In the "erect" penes, the

189   lacunae of the corpora cavernosa expand and stretch out the tunica albuginea. Stretching the

190   collagenous tunica albuginea changes its position within the penis and turns it from a thick,

191   folded layer in the flaccid penis to a straight, taut, thin one. The corpora cavernosa expand in

192   length and diameter, keeping their general U-shaped cross section owing to the network of

193   trabeculae crosslinking the inner walls of the tunica albuginea. In the "erect" penis, the skin is

194   drawn into a taut surface, indicating that the corpora cavernosa take up most of the available

195   space within the shaft of the penis. With their U-shaped cross section, they surround the

196   urethra and the corpus spongiosum on the ventral side.

197

198   When the corpora cavernosa expand, they straighten both the urethra and the corpus

199   spongiosum. In the flaccid penis, the urethra, surrounded for its whole length by the corpus

200   spongiosum, follows the curve of the flaccid penis. When the corpora cavernosa are erect, the

201   urethra and corpus spongiosum are straight, embedded in the ventral surface of the corpora

202   cavernosa in the shaft, and lying directly underneath and partly around the baculum within the

203   glans. The proximal, laterally divided end of the baculum surrounds the urethra laterally and

204   dorsally (Figs 3-6).

205

206   The distal end of the baculum is directly dorsal to the urethra, and both are surrounded by the

207   corpus spongiosum. A distal broadening of the corpus spongiosum around the bone can be

208   seen in the distal part of the glans, where the praeputium is distinguishable from the glans

209   (Figures 3-5, gray arrows). The dorsal half of the urethral opening in the glans is directly

210   surrounded by the forked distal tip of the baculum (Figure 6; Figures 3-5, black arrow).

211

212   From the 3D images of this study, we can confirm that the proximal end of the baculum is

213   linked to the corpora cavernosa over a much larger area than we previously thought (Herdina

214   et al., 2010): not only the very end is connected, but also a broad area of the dorsal surface all

215   along the bifurcated part of the proximal base of the baculum (see Herdina, 2008, Herdina et

216   al., 2014b).

217

218   From our previous histological studies, we know the baculum is tightly linked to the corpora

219   cavernosa via entheses. The tunica albuginea merges into the periosteum of the baculum

220   without a histologically distinguishable border between the tissues (Herdina, 2008, Herdina et

221   al., 2010, Herdina et al., 2014b). Thus when the corpora cavernosa expand and stiffen, the

222   baculum is pulled into a straight line with the corpora cavernosa in *Nyctalus noctula* and *P.*

223   *pipistrellus*, or into an angle with the tip pointing dorsally in *P. nathusii*, moving the distal tip

224   up dorsally and pulling the proximal end down (Figs 3-5, white arrows).

225

226

227 **Discussion**

228 Shape and size of the baculum show a great variety in bats. Nevertheless, its basic designs are

229 clade-specific and provide key diagnostic criteria often used in taxonomy, particularly in the

230 speciose groups rich in convergent phenotypes. The largest family of bats, Vespertilionidae, is

231 a typical example (compare Hill and Harrison, 1987, Horáček and Hanák, 1985). However,

232 despite numerous biometrical and descriptive comparisons, little is known about functional

233 integration of these structures into penial morphology. Here we have presented a new

234 approach to analyzing function using post-mortem samples. Detailed 3D models based on

235 high-resolution microCT proved valuable for comparing flaccid penes with experimentally

236 inflated "erect" ones on a micromorphological scale, and functional anatomical analysis of the

237 3D models provided support for two different hypotheses on the mechanical functions of the

238 baculum.

239

240 The most widely accepted theory on the evolution of bacular diversity is that the baculum is

241 subject to sexual selection (Eberhard, 1985, Arnqvist, 1998, Danielsson and Askenmo, 1999,

242 House and Simmons, 2003, Hosken and Stockley, 2004, Lüpold et al., 2004, Simmons and

243 Firman, 2013, Stockley et al., 2013). This is supported by the specifically differentiated and

244 sometimes elaborate bacula of many mammals and their persistence in various lineages, and

245 baculum morphology is variable enough within a species to allow sexual selection to take

246 place. At the same time, the difference in baculum morphology between closely related

247 species is usually much more pronounced than differences in baculum morphology within a

248 species (Burt, 1960, Patterson and Thaeler Jr, 1982). In house mice, postcopulatory sexual

249 selection on baculum thickness, through female choice, was shown in breeding experiments

250   by Simmons and Firman (2013) and Stockley et al. (2013). In some primates and carnivores

251   that engage in prolonged intromissions during copulation, the length of the baculum is

252   sexually selected (Dixson, 1987, Dixson and Anderson, 2004, Dixson, 2012).

253

254   In general, the form of the baculum results from integration of three structural units differing

255   in morphology, development, and function: a proximal part interfacing with the corpora

256   cavernosa, a distal part related to the urethral opening, and a medial part responding to

257   prolongation of the structure (Kelly, 2000). Compared to ancestral arrangements, the derived

258   baculum type of the studied taxa shows striking divergences in the morphology of particular

259   parts: a robust and broad proximal unit, an early-developing distal bifurcation (Maeda, 1978),

260   and a quite prolonged stick-like medial unit.

261

262   The baculum is directly dorsal to the part of the urethra that goes through the glans, and the

263   distal forked tip of the baculum surrounds the dorsal half of the urethral opening (Fig. 6;

264   Herdina, 2008, Herdina et al., 2010, Herdina et al., 2014b). One hypothesized function of the

265   baculum is that it protects the distal urethra and urethral opening from compression during

266   erection and copulation (Dixson, 1995). The distal part of the baculum is surrounded by the

267   corpus spongiosum, which anchors the urethra to the bone. The reconstructed and segmented

268   3D images show that this arrangement is likely to help straighten out the urethra and corpus

269   spongiosum during erection, when the bone is pushed up and forms a straight line with the

270   corpora cavernosa. If we could inflate the corpus spongiosum as well, we might see

271   morphological changes in the shape of the distal tip of the glans. Similar to some rodent

272   species, the glans might have a species-specific bell shape when fully erect (Holmes et al.,

273   1991). Inflating the corpus spongiosum might also help to test other hypotheses on baculum

274   function; e.g. if the baculum could stimulate the female reproductive tract during copulation,

275   or serve as an indicator for male quality (Dyck et al., 2004).

276

277   The baculum and the corpora cavernosa are enveloped in a common fibrous structure to form

278   a contiguous mechanical unit. Since there is no histologically distinct separation of the tunica

279   albuginea and the baculum's periosteum (Herdina, 2008, Herdina et al., 2010, Herdina et al.,

280   2014b), the baculum seems to be a stiffening element in the glans. This suggested the

281   hypothesis that the baculum and corpus cavernosum form a functional unit to support both the

282   penile shaft and the more distal glans tip (Kelly, 2000). Here, we observed that the baculum is

283   connected to the corpora cavernosa over a large area along the proximal end and dorsal

284   surface of its bifurcated base (Figs 3-5, gray arrows), and the bone even changes position in

285   relation to the other penis tissues when the corpora cavernosa are inflated. This supports the

286   conclusion that the baculum and corpora cavernosa form a functional unit to stiffen the penis

287   through to the glans during erection.

288

289   Of course, many mammalian species without a baculum to take on these functions have penes

290   that function adequately for copulation. In humans, the corpus spongiosum seems to be

291   responsible for protecting the urethral opening during copulation (Hatzichristou et al., 2003).

292   The corpus spongiosum is a low-pressure system (Purohit and Beckett, 1976, Kelly, 2000) but

293   it expands the glans significantly in many species, so in species lacking a baculum the glans

294   could take over the function of the baculum as a stiffening element (Holmes et al., 1991).

295

296   In this first application of our new approach to three species of vespertilionid bats, it was not

297   possible to draw conclusions about intraspecific variation from our rather small sample. In

298   related studies, we have described intraspecific variation in baculum shape in *Pipistrellus*

299   *pipistrellus* (Herdina et al., 2014a) and compared *P. pipistrellus* and *P. nathusii* baculum

300   histomorphology and size (Herdina et al., 2014b). There is little interspecific variation, as we

301   specifically chose the species for the similarities they show in overall baculum morphology

302   and mating systems. The shared features of the bacula in all three species are the bifurcated,

303   sturdy base, the long, slender shaft with its forked tip, and the dorsoventral curve in lateral

304   view. The main difference in baculum shape is the way the baculum of *P. nathusii* bends back

305   towards the dorsal surface of the penis in mid-shaft, while curved proximally like in the other

306   species. The larger species *N. noctula* has a larger baculum than both of the pipistrelle

307   species. Soft tissue morphology is very similar, except for the shape of the prepuce and the

308   portion of the baculum covered by corpus spongiosum tissue in the glans penis. Also, in the

309   "erect" penis the corpora cavernosa seem to show greater expansion in *P. nathusii* than in

310   both other species.

311

312   In this study only the corpora cavernosa were inflated, but not the corpus spongiosum, nor the

313   accessory swelling tissue within the praeputium. This limited the functional hypotheses we

314   were able to test with this experiment. Even with morphological structures this small, it

315   should be possible to inflate all of the swelling tissues in the penis, possibly using a

316   chiropteran species larger than a pipistrelle. All the accessory swelling tissue within the large

317   praeputium, together with the tiny distal end of the glans also brings up the question of

318   whether the preaputium can even be pulled back during copulation. According to Matthews

319   (1937), in vespertilionids the minute glans can be exposed by retracting the prepuce in

320   specimens examined immediately after death. Inflating all the swelling tissues would also be

321   an important step towards an even more anatomically complete 3D model of an important

322   reproductive organ and its function.

323

324   This new methodological approach to an old problem helps to accurately follow the changes

325   of tissue positions relative to one another, even in post-mortem samples. The efficacy of

326   generating and analyzing anatomically accurate 3D models also opens up the possibility of

327   using microCT-based models to create finite element models for quantitative testing of more

328   complex functional hypotheses, such as the effects the increasing hydrostatic pressure within

329   the penis during copulation (Kelly, 2000). To test hypotheses on baculum function like

330   stimulating the female reproductive tract (Dyck et al., 2004), an extended approach involving

331   female reproductive anatomy and behavioral studies would be necessary.

332

333   **Conclusion**

334

335   Both functional hypotheses tested – that the baculum protects the urethral opening and that it

336   forms a functional unit with the corpora cavernosa to provide stiffness within the glans – are

337   supported by our data. The novel approach of combining experimental designs like inflating

338   penes with high resolution microCT will help to compare and test the functions of different

339   baculum morphologies, and it paves the way for finite element modeling to test

340   biomechanical hypotheses in non-living specimens.

341

342   **Conflict of interests**

343   The authors have no conflicts of interests.

344

358

359   **Authors' contributions**

360   IH and PHCL provided the samples for this study. ANH inflated the bat penes with formalin

361   and took standard measurements of the bats together with DAK, HJ, and PHCL. BDM stained

362   and microCT scanned the samples. ANH and BDM reconstructed and manually segmented

363   the microCT images to create 3D models. All authors evaluated and discussed the results.

364   ANH and BDM wrote the manuscript and prepared the figures, all authors added valuable

365   comments and corrections and approved the final manuscript.

366

367

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478

479

480

481   **Supplementary material**

482

483   **Supplementary material 1** Movie of an inflated *Nyctalus noctula* penis

484   **Supplementary material 2-7** Pictures of 3D surface rendering of inflated *Nyctalus noctula*

485   penis, with the tissues added step by step. Orange: outer shape of the penis (volume

486   rendering), white: baculum, red: blood lacunae in the corpora cavernosa, green: tunica

487   albuginea of the corpora cavernosa, blue: urethra, pink: corpus spongiosum.

488   **Supplementary material 8** 3D PDF of a *Pipistrellus pipistrellus* baculum

489

490

491   **Figures**

492

493   **Fig. 1** Photos of inflating the penis of a male *Pipistrellus pipistrellus* with formalin

494   left: the bat with its label and a syringe with 30-gauge needle; top right: the same needle

495   inserted in the resected penis of the same bat with the base tied shut before inflation; bottom

496   right: penis after inflation with formalin leaking out at the base.

497

498   **Fig. 2** Schematic drawing of an inflated bat penis

499   The distal tip of the penis is pointing to the left, the proximal base of the penis to the right.

500   Gray arrows mark where the corpus spongiosum is enveloping the baculum. White arrows

501   mark the area where the baculum and corpora cavernosa are linked. Black arrow shows the

502   urethral opening.

503

504   **Fig. 3** 3D models of *Nyctalus noctula* penes

505   3D surface renderings of manually segmented tissues overlaid on volume renderings of a

506   flaccid (top) and an inflated (bottom) *N. noctula* penis. Gray arrows mark where the corpus

507   spongiosum is enveloping the baculum. White arrows mark the area where the baculum and

508   corpora cavernosa are linked. Black arrow shows the urethral opening. Orange: outer shape of

509   the penis (volume rendering), white: baculum, red: blood lacunae in the corpora cavernosa,

510   green: tunica albuginea of the corpora cavernosa, blue: urethra, pink: corpus spongiosum.

511

512   **Fig. 4** 3D models of *Pipistrellus pipistrellus* penes

513   3D surface renderings of manually segmented tissues overlaid on volume renderings of a

514   flaccid (top) and an inflated (bottom) *P. pipistrellus* penis. Gray arrows mark where the

515   corpus spongiosum is enveloping the baculum. White arrows mark the area where the

516   baculum and corpora cavernosa are linked. Black arrow shows the urethral opening. Orange:

517   outer shape of the penis (volume rendering), white: baculum, red: blood lacunae in the

518   corpora cavernosa, green: tunica albuginea of the corpora cavernosa, blue: urethra, pink:

519   corpus spongiosum.

520

521   **Fig. 5** 3D models of *Pipistrellus nathusii* penes

522   3D surface renderings of manually segmented tissues overlaid on volume renderings of a

523   flaccid (top) and an inflated (bottom) *P. nathusii* penis. Gray arrows mark where the corpus

524   spongiosum is enveloping the baculum. White arrows mark the area where the baculum and

525   corpora cavernosa are linked. Black arrow shows the urethral opening. Orange: outer shape of

526   the penis (volume rendering), white: baculum, red: blood lacunae in the corpora cavernosa,

527   green: tunica albuginea of the corpora cavernosa, blue: urethra, pink: corpus spongiosum.

528

529   **Fig. 6** Detail of the penis tip and urethral opening of *Pipistrellus pipistrellus*

530   left: 2D virtual lateral section, right: 3Dvolume rendering; both depicting the distal part of the

531   glans penis with the distal part of the urethra, the distal tip of the baculum and parts of the

532   praeputium of a *P. pipistrellus* penis.

Fig. 1 Photos of inflating the penis of a *Pipistrellus pipistrellus* with formalin.
left: the bat with its label and a syringe with 30-gauge needle; top right: the same needle inserted in the resected penis of the same bat with the base tied shut before inflation; bottom right: penis after inflation with formalin leaking out at the base.
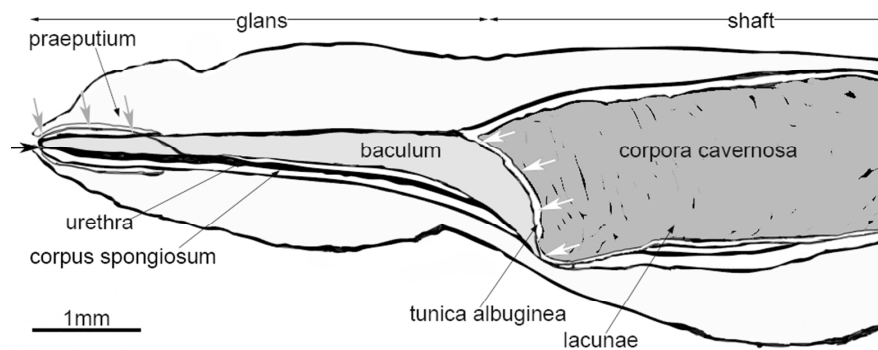207x126mm (180 x 180 DPI)

Fig. 2 Schematic drawing of an inflated bat penis.
The distal tip of the penis is pointing to the left, the proximal base of the penis to the right. Gray arrows mark where the corpus spongiosum is enveloping the baculum. White arrows mark the area where the baculum and corpora cavernosa are linked. Black arrow shows the urethral opening.
344x150mm (72 x 72 DPI)

Fig.3 3D models of *Nyctalus noctula* penes.
3D surface renderings of manually segmented tissues overlaid on a volume renderings of a flaccid (top) and an inflated (bottom) *N. noctula* penis. Gray arrows mark where the corpus spongiosum is enveloping the baculum. White arrows mark the area where the baculum and corpora cavernosa are linked. Black arrow shows the urethral opening. Orange: outer shape of the penis (volume rendering), white: baculum, red: blood lacunae in the corpora cavernosa, green: tunica albuginea of the corpora cavernosa, blue: urethra, pink: corpus spongiosum.

299x193mm (300 x 300 DPI)
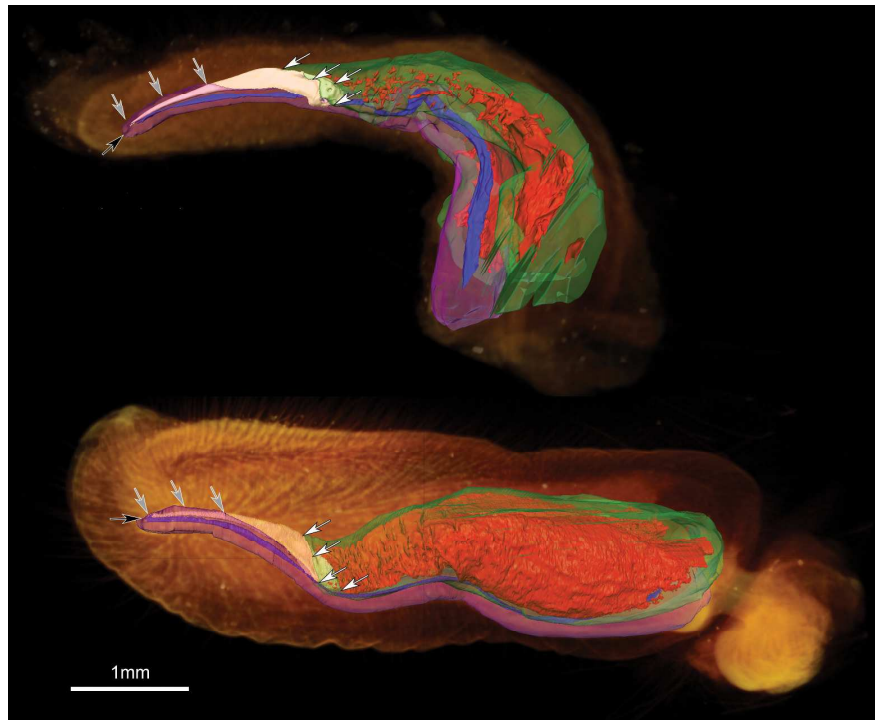
Fig. 4 3D models of *Pipistrellus pipistrellus* penes.
3D surface renderings of manually segmented tissues overlaid on volume renderings of a flaccid (top) and an inflated (bottom) *P. pipistrellus* penis. Gray arrows mark where the corpus spongiosum is enveloping the baculum. White arrows mark the area where the baculum and corpora cavernosa are linked. Black arrow shows the urethral opening. Orange: outer shape of the penis (volume rendering), white: baculum, red: blood lacunae in the corpora cavernosa, green: tunica albuginea of the corpora cavernosa, blue: urethra, pink: corpus spongiosum.
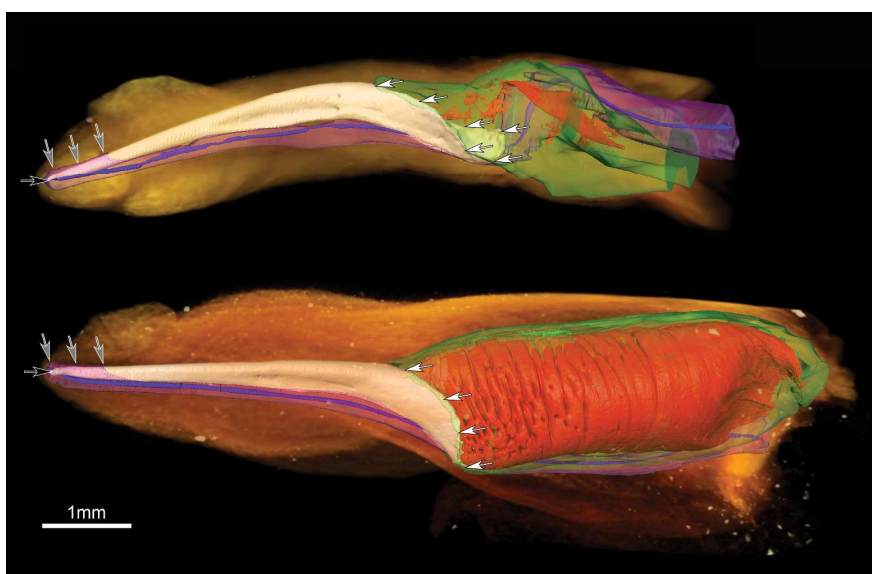297x242mm (300 x 300 DPI)

Fig. 5 3D models of *Pipistrellus nathusii* penes.
3D surface renderings of manually segmented tissues overlaid on volume renderings of a flaccid (top) and an inflated (bottom) *P. nathusii* penis. Gray arrows mark where the corpus spongiosum is enveloping the baculum. White arrows mark the area where the baculum and corpora cavernosa are linked. Black arrow shows the urethral opening. Orange: outer shape of the penis (volume rendering), white: baculum, red: blood lacunae in the corpora cavernosa, green: tunica albuginea of the corpora cavernosa, blue: urethra, pink: corpus spongiosum.

326x303mm (300 x 300 DPI)
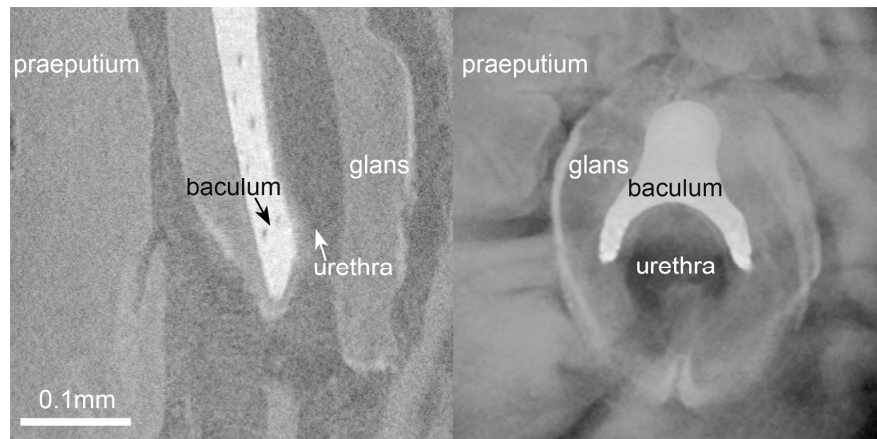
Fig. 6 Detail of the penis tip and urethral opening of *Pipistrellus pipistrellus*.
left: 2D virtual lateral section, right: 3Dvolume rendering; both depicting the distal part of the glans penis
with the distal part of the urethra, the distal tip of the baculum and parts of the praeputium of a *P.
pipistrellus* penis.
220x109mm (300 x 300 DPI)

Supplementary material 2-7 Pictures of a 3D surface rendering, overlaid on a volume rendering, of an inflated *Nyctalus noctula* penis, with the tissues added step by step. Orange: outer shape of the penis (volume rendering), white: baculum, red: blood lacunae in the corpora cavernosa, green: tunica albuginea of the corpora cavernosa, blue: urethra, pink: corpus spongiosum.

Supplementary material 2 3D volume rendering, of an inflated *Nyctalus noctula* penis.

1381x906mm (72 x 72 DPI)

Supplementary material 3 3D surface rendering of the baculum (off-white), overlaid on a volume rendering,
of an inflated *Nyctalus noctula* penis.
1381x906mm (72 x 72 DPI)

Supplementary material 4 3D surface rendering of the baculum (off-white) and the filled lacunae of the corpora cavernosa (red), overlaid on a volume rendering, of an inflated *Nyctalus noctula* penis.

1381x906mm (72 x 72 DPI)
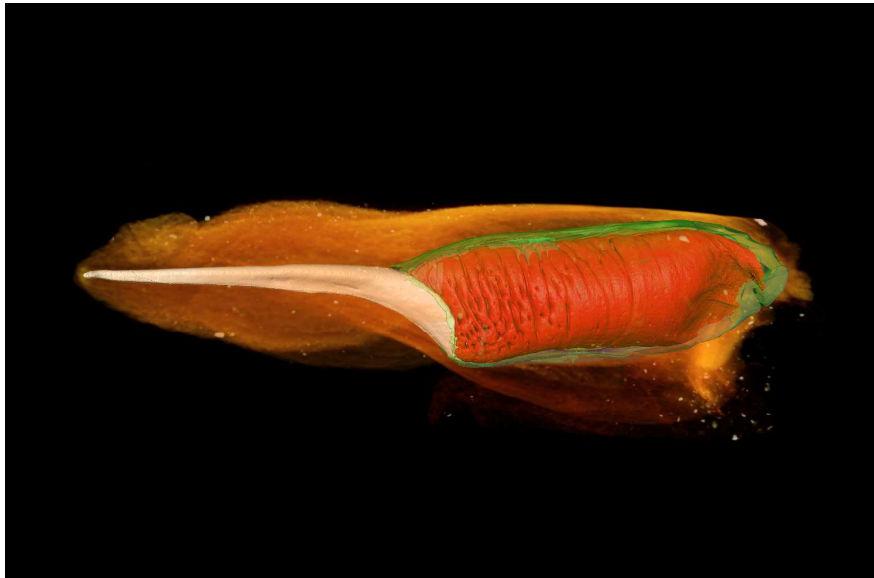
Supplementary material 5 3D surface rendering of the baculum (off-white), the filled lacunae of the corpora cavernosa (red), and the tunica albuginea of the corpora cavernosa (green), overlaid on a volume rendering, of an inflated *Nyctalus noctula* penis.
1381x906mm (72 x 72 DPI)
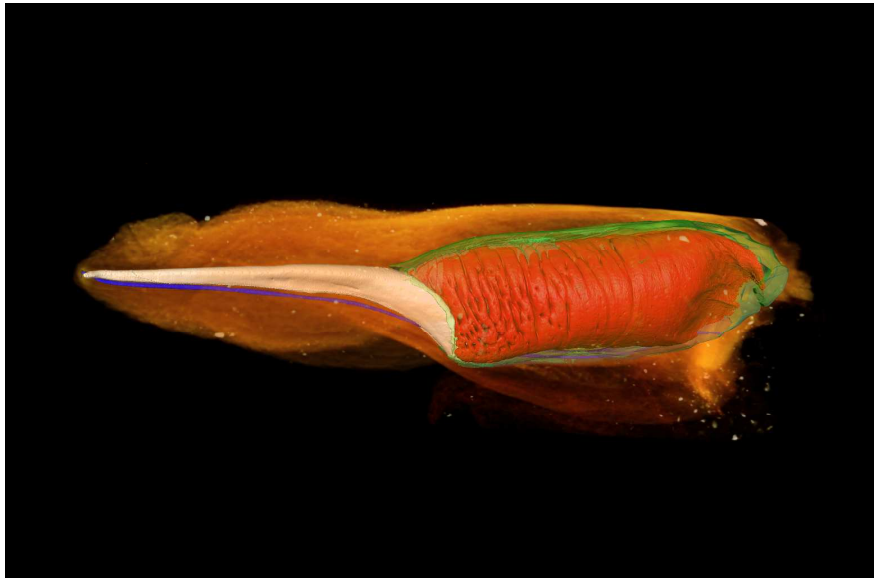
Supplementary material 6 3D surface rendering of the baculum (off-white), the filled lacunae of the corpora cavernosa (red), the tunica albuginea of the corpora cavernosa (green), and the lumen of the urethra (blue), overlaid on a volume rendering, of an inflated *Nyctalus noctula* penis.
1381x906mm (72 x 72 DPI)
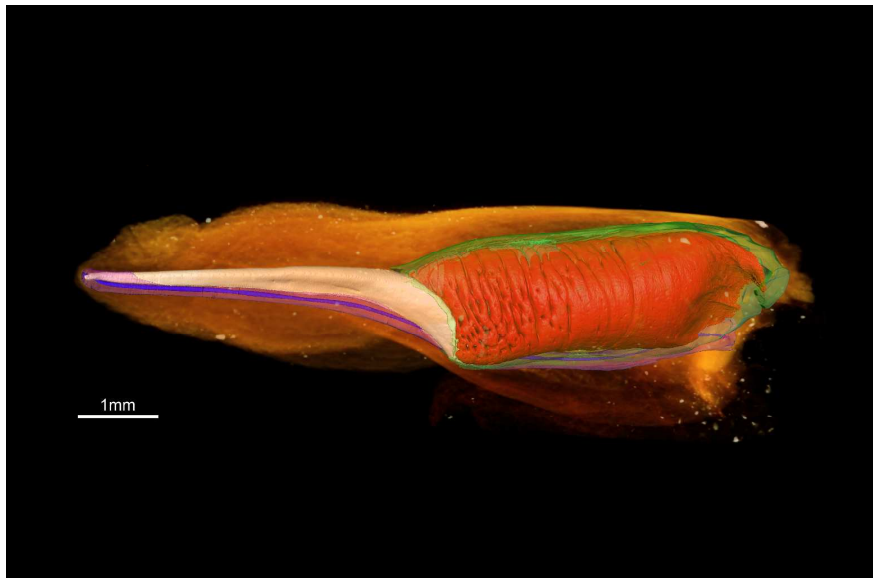
Supplementary material 7 3D surface rendering of the baculum (off-white), the filled lacunae of the corpora cavernosa (red), the tunica albuginea of the corpora cavernosa (green), the lumen of the urethra (blue), and the corpus spongiosum and urethra (pink), overlaid on a volume rendering, of an inflated *Nyctalus noctula* penis.
1381x906mm (72 x 72 DPI)

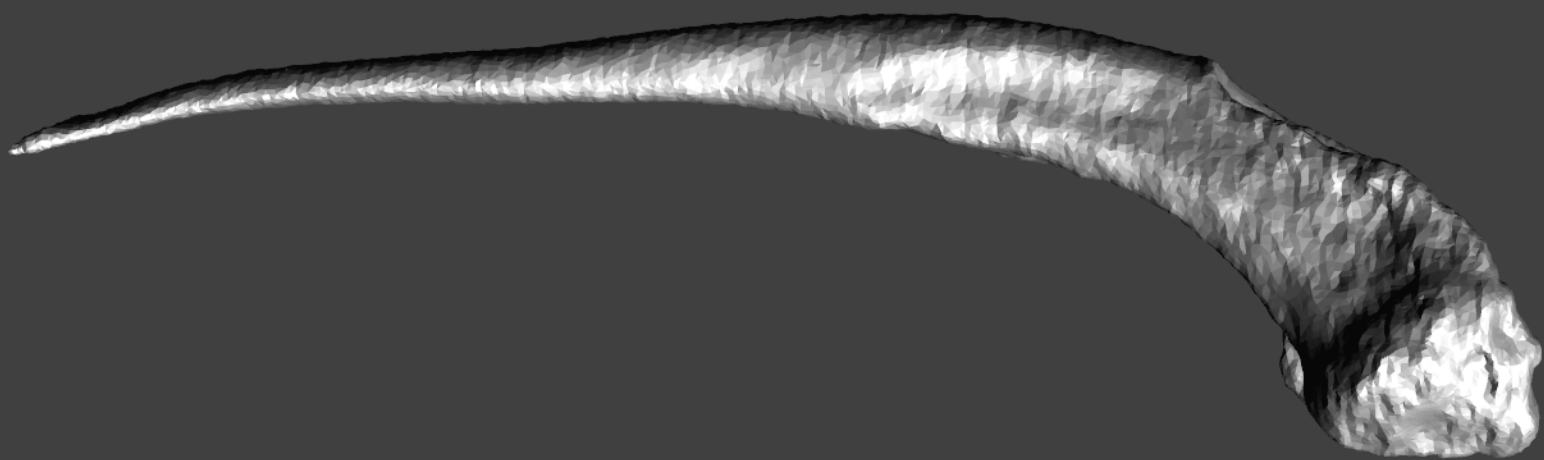# 5. PAPER IV (PUBLISHED) DISCRIMINATION OF *P. PIPISTRELLUS* AND *P. PYGMAEUS*

MicroCT imaging reveals morphometric baculum differences for discriminating the cryptic species *Pipistrellus pipistrellus* and *P. pygmaeus*

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With the recent and continuing discovery of further cryptic bat species, it is essential to find morphological species discriminating characters. *Pipistrellus pipistrellus* (common pipistrelle) and *Pipistrellus pygmaeus* (soprano pipistrelle) have been recognized as separate species since 1997, but no reliable morphological species discriminating trait has yet been found. The most commonly used morphological species discrimination traits are 'wing vein' pattern and shape and color of the penis, but these have not been validated on sets of genetically identified specimens. The baculum (os penis) has long been used successfully in species discrimination in bats and other mammals. In this study, we tested the reliability of the established traits and demonstrated how to reliably separate the common pipistrelle and the soprano pipistrelle by simple baculum measurements. The bacula of museum specimens of these two species and of *Pipistrellus hanaki* were imaged with high-resolution microCT. Several measurements were taken on the size-calibrated volume images, and their value for species discrimination was tested by discriminant analysis with leave-one-out cross validation. We showed that *P. pipistrellus* and *P. pygmaeus* specimens can be discriminated by measuring the projected length, height, and width of the baculum (*n* = 48; all but one classified correctly). Geometric morphometrics was used to analyze and locate variations in baculum shape. Principal component analysis of baculum variation was not sufficient to separate these species. Most of the interspecific variation in baculum shape can be found in the proximal third (the base) of the baculum, and most individual variation can be observed in lateral view, especially in the dorsoventral curve. Quantitative details of morphology are becoming more important to distinguish cryptic species and understand their phylogeographic distributions. The simple baculum measurements can be used to classify single specimens and could be taken without microCT, on a resected baculum.

*Key word*s: os penis, morphometrics, micro-CT, x-ray microtomography, Vespertilionidae

INTRODUCTION

Finding morphological characters to properly describe and discriminate newly discovered cryptic species is becoming an important challenge, with the growing number of species discovered due to the rise of molecular techniques in taxonomy. The problem posed by cryptic species to the traditional morphospecies concept has long been recognized (Mayr, 1942), and the widespread application of genetic tools has revealed that morphologically similar population groups can consist of several genetically distinct species (Baker *et al.*, 1995; Bruna *et al.*, 1996). In Palearctic bats, cryptic diversity has emerged as a major subject of research in the last decade.

Cryptic species or species complexes in the Western Palearctic have been discovered in the genera *Eptesicus*, *Hypsugo*, *Myotis*, *Pipistrellus*, *Plecotus*, and *Rhinolophus* (Jones and Barlow, 2001; Mayer and von Helversen, 2001*a*, 2001*b*; von Helversen *et al.*, 2001; Hulva *et al.*, 2004*a*; Ibáñez *et al.*, 2006; Spitzenberger *et al.*, 2006; Mayer *et al.*, 2007; Benda and Vallo, 2012; Salicini *et al.*, 2013). Cryptic species frequently go undetected because their diagnostic features are in sensory modalities very different from our own, such as olfactory cues or the ultrasonic calls of microchiropteran bats (Davidson-Watts *et al.*, 2006).

The *Pipistrellus pipistrellus*/*Pipistrellus pygmaeus* species complex is an iconic example of this

problem. The common pipistrelle, one of the most widespread European bat species and previously considered a monotypic taxon, actually comprises two cryptic species, *Pipistrellus pipistrellus* (Schreber, 1774) and *Pipistrellus pygmaeus* (Leach, 1825) (Jones and van Parijs, 1993; Barratt *et al.*, 1995, 1997). Recently, further cryptic variation was discovered within the Mediterranean basin (Hulva *et al.*, 2004*a*, 2007, 2010). The structure of the species complex comprises many lineages with small ranges in statu nascendi on Mediterranean islands (e.g., *Pipistrellus hanaki* on Crete) and peninsulas, and two sympatric continental sibling species. Thus the *P. pipistrellus* species complex is a remarkable model system for investigating links between morphology, echolocation calls, and niche partitioning (Davidson-Watts *et al.*, 2006). In small Mediterranean demes (e.g., on Cyprus), hybridization has probably occurred in the past (Hulva *et al.*, 2010). There is even some genetic evidence for hybridization still occurring in Central Europe (Sztencel-Jabłonka and Bogdanowicz, 2012).

Most of the recent studies on the genus *Pipistrellus* have focused on its genetic diversity, including the discovery of new cryptic species (e.g., Benda *et al.*, 2004; Hulva *et al.*, 2004*a*, 2007, 2010; Mayer *et al.*, 2007; Racey *et al.*, 2007; Bryja *et al.*, 2009; Sztencel-Jabłonka and Bogdanowicz, 2012). Subsequently, the requirement has arisen to separate the new cryptic species morphologically as well as genetically. In the last decade a great number of studies have been published on one or both of the two cryptic species *P. pipistrellus* and *P. pygmaeus*, referring to differences in many respects and marking them as truly separate species. These have included studies on distribution (e.g., Mayer and von Helversen, 2001*b*; Benda *et al.*, 2003; Řehák *et al.*, 2004; Isaksen and Landsgard, 2007; Derouiche *et al.*, 2009), habitat requirements (e.g., Davidson-Watts *et al.*, 2006; Bartonička *et al.*, 2008; Smith and Racey, 2009; Kaňuch *et al.*, 2010; Kusch and Schmitz, 2013), behavior (e.g., Russ *et al.*, 2005; Bartonička, 2008; Petrželková *et al.*, 2009), physiology (e.g., Azzali *et al.*, 2003; Abramochkin *et al.*, 2006; Waters and Wong, 2007; Becker *et al.*, 2013), and breeding biology (e.g., Aşan-Bayedmir and Albayrak, 2006; Bielik, 2007).

Even though much effort has been devoted to finding reliable morphologic species discriminating characters, so far none have been established. Still, the most useful methods to date for reliable determination of both species of the *P. pipistrellus/*

P. pygmaeus complex remain genetic sequence comparison and the analysis of echolocation calls (Mayer and von Helversen, 2001*b*; Benda *et al.*, 2003; Kaňuch *et al.*, 2007). Because the external characters of the two species are quite similar, many attempts to find distinguishing morphological parameters have failed (Pavlinić *et al.*, 2008). Measurable parameters like wing morphology (Barlow and Jones, 1999; Häussler *et al.*, 2000) and skull morphology (Barlow *et al.,* 1997; Sztencel-Jabłonka *et al.*, 2009) indicate that *P. pygmaeus* is slightly smaller than *P. pipistrellus*. However, these findings are limited by the fact that there is significant overlap in these characters between the two species (Barlow *et al.*, 1997).

Of the qualitative characters described, the 'wing vein' pattern, referring to the patterns of elastic bands and fibers in the bat wing (von Helversen and Holderied, 2003), and shape and color of the penis (Häussler *et al.*, 2000) are used most frequently. Bartonička *et al.* (2010) also found that the distinctive musky smell of *P. pygmaeus* can be used to identify the species in live animals; however, this character can vary depending on age and physiological condition of the individual bat (Benda, personal observation). Qualitative characters can be useful if applied by someone with good knowledge of both species, for example the shorter muzzle of *P. pygmaeus,* an internarial ridge (hump between nostrils) in *P. pygmaeus* and different coloration of the face (Häussler *et al.*, 2000; Ziegler *et al.*, 2001; Sendor *et al.*, 2002; Zöphel *et al.*, 2002; Sattler, 2003; von Helversen and Holderied, 2003).

The use of baculum shape (penis bone, os penis, os glandis, or os priapi) as a possible species specific trait has been proposed by Ziegler *et al*. (2001), who also cautioned that to use this trait will first require examining the bacular shape in statistically relevant numbers of individuals. Bats show a great variety in shape and size of the baculum. Nevertheless, its basic forms are clade-specific. Thus they provide key diagnostic criteria, which have been used successfully as taxonomic characters in bats and other mammals, particularly in the speciose groups rich in convergent phenotype evolution. The largest family of bats, Vespertilionidae, can serve as a typical example (cf., Horáček and Hanák, 1985; Hill and Harrison, 1987). Phylogenies were based on baculum shape even before molecular techniques were developed. The polyphyly of the genera *Pipistrellus*, *Plecotus*, *Hypsugo*, and *Eptesicus* was discovered because of their marked differences in baculum shape, e.g. the reason for the separation of

Pipistrellus subflavus was its distinctive baculum (Hill and Harrison, 1987).

In this study we aimed to (1) critically evaluate the reliability of the taxonomic characters penis color and shape, and 'wing vein' pattern, which also are used in field identification, using genetically identified specimens; and (2) demonstrate that baculum shape and size can be used as discriminating characters to consistently separate *P. pipistrellus* and *P. pygmaeus*.

MATERIAL AND METHODS

The material for this study was kindly made available to us by the National Museum (Natural History) in Prague. The animals had been preserved and stored in 70% ethanol and all of them had been genetically identified to the species level. In total, we analyzed 119 specimens of the genus *Pipistrellus*: *P. pipistrellus*: Continental Europe (Czech Republic, Slovakia): 3 ♂♂, 7 ♀♀; Middle East (Iran, Lebanon, Syria): 29 ♂♂, 20 ♀♀; North Africa (Morocco): 4 ♂♂, 3 ♀♀. *P. pygmaeus pygmeaus*: Continental Europe (Czech Republic, Greece): 6 ♂♂, 6 ♀♀; Middle East (Turkey, Iran): 12 ♂♂, 5 ♀♀. *P. pygmaeus cyprius*: Cyprus: 6 ♂♂. *P. hanaki*: Greece (Crete): 7 ♂♂; North Africa (Libya): 2 ♂♂, 9 ♀♀. For museum inventory numbers and more information on the specimens' collection data see Appendix and Fig. 1.

Specimens of *P. pipistrellus* and *P. pygmaeus pygmaeus* were used to test the reliability of species discriminating characters, while the other specimens were described only for comparison. 'Wing vein' pattern was studied on all bats. Penis morphology was studied on all but five (four *P. pipistrellus*, one *P. hanaki*) of the male bats (these *P. pipistrellus* were added to sample later and only the 'wing vein' pattern was examined; the baculum of the *P. hanaki* holotype had been resected, thus there was no penis to study). Baculum characters were studied on all but six (*P. pipistrellus*) of the male specimens (which were added to the sample after the microCT scanning had been done).

Age Determination

Specimen age was estimated by the ossification of the metacarpophalangeal joint of the third finger (Baagøe, 1977).

Evaluation of External Characters

'Wing vein' pattern

To evaluate this character, patterns of the elastic bands between the forearm and the metacarpal of the fifth finger (Fig. 2) were drawn, photographed, and described. For evaluating the 'wing vein' pattern as discriminating character, we followed the description by von Helversen and Holderied (2003). The pattern typical for *P. pipistrellus* only contains one uninterrupted 'cell', while the typical pattern for *P. pygmaeus* contains a second uninterrupted cell right above the first one (Fig. 2).
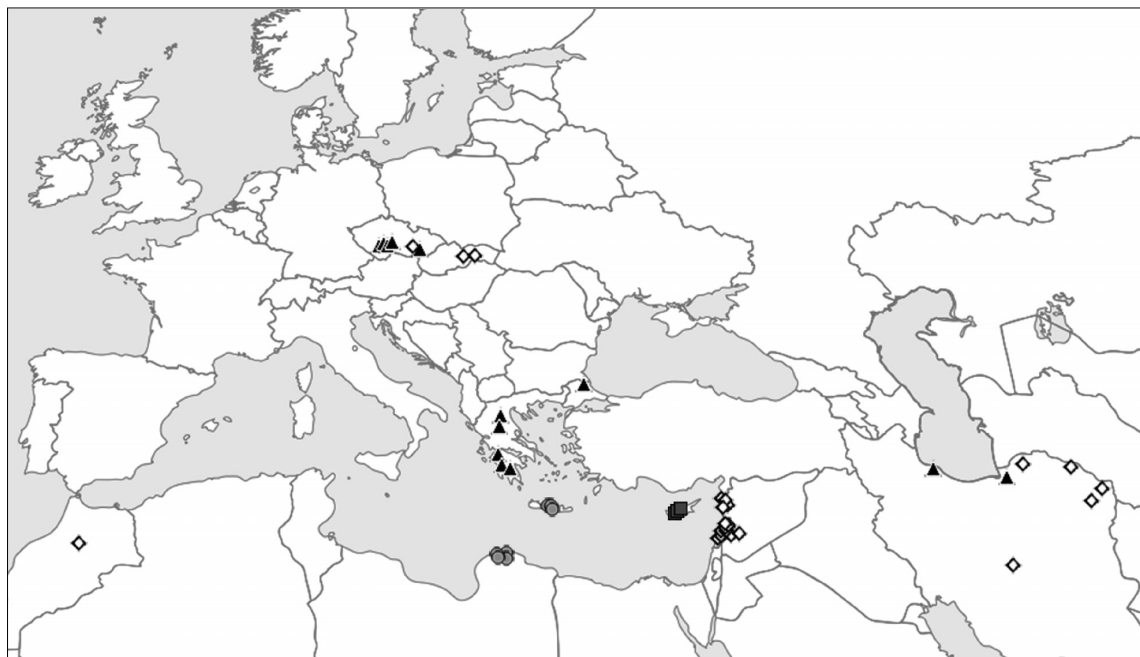

FIG. 1. Map of collecting localities of specimens of *P. pipistrellus* (◇), *P. pygmaeus* (▲), *P. pygmaeus cyprius* (■), and *P. hanaki* (●). For location names and collection dates see Appendix (map taken from www.weltkarte.com/welt/weltatlas/weltkarte-blank-vektorgrafik.htm, image in the public domain)
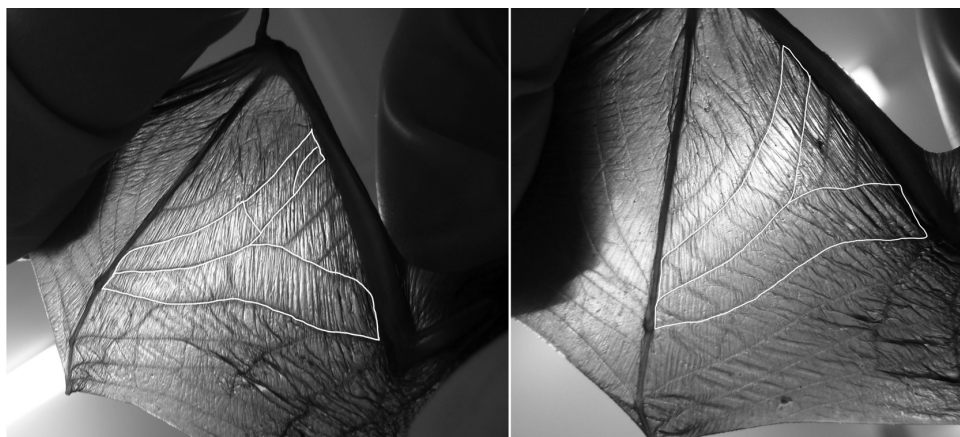
Fig. 2. Typical patterns of elastic bands and fibers in the wing ('wing vein' pattern), in *P. pipistrellus* (left) and *P. pygmaeus* (right). Diagnostic features are marked with white lines. While both species have an uninterrupted 'wing cell' between forearm (at elbow-height) and metacarpal of the 5th finger, differences can be seen in the 'wing cell' directly above. In the pattern typical for *P. pipistrellus* (left), this 'cell' is intersected by smaller elastic bands. In the pattern typical for *P. pygmaeus* (right) this 'cell' is uninterrupted

Penis morphology

The penes of the male specimens were studied under a stereomicroscope. Shape, color, and presence or absence of a median stripe were described. Drawings and photographs were made. The penes were categorized as typical for *P. pipistrellus*, or typical for *P. pygmaeus* according to Dietz and von Helversen (2004) and Dietz *et al.* (2007), or as intermediate (e.g. with the shape typical for one species, but the color typical for the other species).

The typical *P. pipistrellus* penis is shaped like a dorsoventrally flattened club. Its color usually is (dark-) gray with a lighter, whitish median stripe on the dorsal side of the glans (Dietz and von Helversen, 2004). Compared to *P. pipistrellus*, the typical penis shape of *P. pygmaeus* is more uniform in diameter, but it also widens towards the glans. Depending on reproductive state, penis color of *P. pygmaeus* can be orange, skin-colored, or yellowish. It usually does not have a lighter median stripe (Dietz *et al.*, 2007).

Evaluation of Baculum Characters

MicroCT imaging

High resolution X-ray microtomographic (microCT) imaging was used to visualize and measure the bacula. Unstained whole bats in 70% ethanol were imaged without further treatment or dissection. The bats where mounted in 70% ethanol in sealed plastic test tubes. The scans were made with a high-resolution lab-based Xradia MicroXCT system (Zeiss X-Ray Microscopy, Pleasanton, CA, USA; www.xradia.com), which is equipped with a microfocus tungsten source, secondary optical magnification of the scintillator images, and a 2k × 2k cooled CCD camera. Projection images were taken every 0.25° over a rotation of 180° (plus the cone angle — Metscher, 2011) with 60 sec exposure, 4× optical magnification, and a source voltage of 40 kV at 4–8 W. Tomographic sections were reconstructed with the XMReconstructor software (version 8.1) supplied with the Xradia system, resulting in reconstructed isotropic voxel sizes of 2.0–2.5 μm.

Measurements

In the male specimens the following projected measurements were taken on thick sections of the microCT volume images of the baculum with the Xradia TXM3DViewer software (version 1.1.6) supplied with the Xradia system: length, width, height, width of the forked tip, width of each of the branches of the base, space between the branches of the base in the middle of their length, and length of the indentation in the base (Fig. 3). All measurements were taken by the same person (ANH).
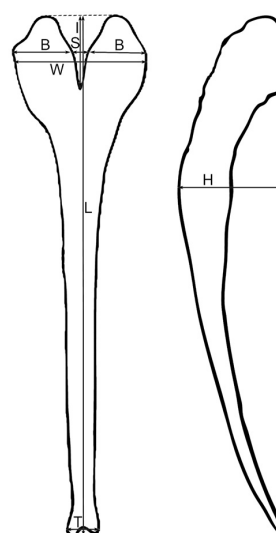

Fig. 3. Schematic drawing of the baculum measurements; dorsal view (left) and lateral view (right). L — projected length, W — projected width, H — projected height, T — projected width of the forked tip, B — projected width of each of the branches of the base, S — space between the branches of the base in the middle of their length, and I — length of the indentation in the base (arrows)

Statistical analyses

A discriminant analysis with leave-one-out cross validation between *P. pipistrellus* and *P. pygmaeus* was used on all the baculum measurements and on just the projected length, width, and height using PAST (http://nhm2.uio.no/norlex/past/download.html; Hammer *et al.*, 2001). Summary statistics of penis and 'wing vein' pattern characters were used to determine a percentage of individuals correctly identified by these traits.

Geometric morphometrics enables the statistical analysis of the shape of morphological structures based on Cartesian landmark coordinates, rather than linear, areal, or volumetric variables (Weber *et al.*, 2001; Bogdanowicz, 2009; Mitteröcker and Gunz, 2009). Images of virtual thick sections of the bacula from microCT volume images were taken in lateral and in dorsal view, with the Xradia software package. All landmarks were set by the same person (ANH) on all of the images using tpsDig (http://life.bio.sunysb.edu/morph/; Adams *et al.*, 2004; Mitteröcker and Gunz, 2009). On each dorsal image, 3 landmarks and 60 semi-landmarks were set. On each lateral image, one landmark and 50 semi-landmarks were set. The landmark sets were analyzed with tpsRelw (http://life.bio.sunysb.edu/morph/; http://www.wolfram.com/mathematica/new-in-9/?fp=middle; Adams *et al.*, 2004; Mitteröcker and Gunz, 2009) and Mathematica (http://www.morpho-tools.net/index.html; Mitteröcker and Gunz, 2009; Madsen, 2013).

The used landmark configurations were superimposed by generalized Procrustes analysis (Rohlf and Slice, 1990) to standardize orientation, location and size. Semi-landmarks were allowed to slide along their corresponding curve to minimize bending energy. Bending energy is a measure of local shape differences between individuals and the average shape (Bookstein, 1997). We visualized average shape for all three species and average differences in shape between the species. We performed between-group principal component analyses (Mitteröcker and Bookstein, 2011) for dorsal and lateral shape.

Results

'Wing Vein' Pattern

We were able to correctly classify 78.9% of the bats (*n* = 95; *P. pipistrellus* and *P. pygmaeus*) by the 'wing vein' pattern trait (Fig. 2 and Table 1) as proposed by von Helversen and Holderied (2003). Of the 66 *P. pipistrellus* specimens 58 were correctly identified, and two were classified as *P. pygmaeus*. Of the 29 *P. pygmaeus pygmaeus* 17 were correctly identified, and four were classified as *P. pipistrellus*.

Of the six *P. pygmaeus cyprius* only one showed a modified *P. pygmaeus* 'wing vein' pattern. This bat had three uninterrupted 'wing cells'. Two bats of this population showed a *P. pipistrellus*-like 'wing vein' pattern. *Pipistrellus hanaki* specimens exhibited a *P. pipistrellus*-like pattern most often. A *P. pygmaeus* like pattern was observed on two specimens. Different patterns on the left and right wing of the same bat where observed in six *P. pipistrellus*, eight *P. pygmaeus*, one *P. pygmaeus cyprius*, and five *P. hanaki*.

Penis Shape and Color

We were able to classify correctly 72.0% of the bats (*n* = 50, *P. pipistrellus* and *P. pygmaeus*) by penis shape and color (Table 2). Of the six *P. pygmaeus cyprius*, three had a *P. pygmaeus*-like penis, while the other three showed an intermediate penis color and shape. In *P. hanaki,* penis color and shape were most often intermediate between *P. pipistrellus* and *P. pygmaeus*.

Baculum Biometrics

A discriminant analysis was performed to determine whether baculum measurements can be used to discriminate *P. pipistrellus* and *P. pygmaeus*. The discriminant analysis between *P. pipistrellus* (*n* = 30) and *P. pygmaeus pygmaeus* (*n* = 18; excluding the presumably hybrid population from Cyprus; Hulva *et al.*, 2010) using projected length (L), width (W), and height (H) of the baculum (Fig. 3) yielded the following discriminant function:

$$x = (L*0.030194 + W*0.06156 + H*0.024837) - 78.2545$$

New specimens can be classified by multiplying the measurements in μm with the discriminant function factors for length (0.030194) width (0.06156) and height (0.024827) and subtracting the offset value (78.2545) from the resulting sum (Hammer *et al.*, 2001; Hammer, 2012). Specimens with a resulting value of *x* greater than 0 should be classified as *P. pipistrellus* and specimens with *x* less than 0 as *P. pygmaeus*. This function correctly assigned all but one of the bats in our sample (97.92%) to its genetically determined species (Fig. 4). This was verified by leave-one-out cross validation with each of the specimens.

TABLE 1. Percentage of specimens exhibiting typical 'wing vein' pattern for *P. pipistrellus* and *P. pygmaeus* (classified after von Helversen and Holderied, 2003)

Character	*P. pipistrellus* (*n* = 66)	*P. pygmaeus* (*n* = 29)	*P. hanaki* (*n* = 18)
P. pipistrellus pattern (%)	87.9	13.8	61.1
P. pygmaeus pattern (%)	3.0	58.6	11.1
Left and right wing different (%)	9.1	27.6	27.8

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TABLE 2. Percentage of specimens exhibiting typical penis shape and color for *P. pipistrellus* and *P. pygmaeus* (classified after Dietz and von Helversen, 2007)

Character	*P. pipistrellus* (*n* = 32)	*P. pygmaeus* (*n* = 18)	*P. hanaki* (*n* = 8)
P. pipistrellus shape & color (%)	68.8	22.2	0
P. pygmaeus shape & color (%)	31.2	77.8	25.0
Intermediate (%)	0	0	75.0

Geometric Morphometrics of the Baculum

The variation of baculum shape within and between the three *Pipistrellus* species was visualized (Figs. 5 and 6) and quantified with geometric morphometrics, but the species could not be classified reliably by between-group principal component analyses of 2D shape variation, applied laterally or dorsally (Fig. 7). However, this analysis showed that most of the interspecific variation in baculum shape can be found in the proximal third (the base) of the baculum (Figs. 5 and 6). Generally, in the baculum of *P. pipistrellus* the two branches of the proximal bifurcated base are broader, shorter, and more club-shaped than in *P. pygmaeus* and in most *P. hanaki*. The branches of the base in the *P. pipistrellus* baculum are closer together, with a smaller angle than in *P. pygmaeus* and in most *P. hanaki*. The distal tip of the baculum also shows differences between the species: the baculum of *P. pipistrellus* tapers uniformly towards the forked tip gradually, while the baculum of *P. pygmaeus* typically narrows into

a slender neck just proximal to the forked tip, where it widens again to a width similar to that of the *P. pipistrellus* baculum tip. *P. hanaki* displays an intermediate shape. Most of the individual variation can be observed in lateral view, in the shape of the curve (Fig. 5). In some of the bats, the baculum is curved evenly, while in others the base is rather straight and stands at an angle to the rest of the bone, which can have different degrees of curvature as well.

DISCUSSION

Pipistrellus pipistrellus and *P. pygmaeus* show a broad overlap in variation ranges of almost all phenotypic characters, including those for which certain discrimination value was suggested (Häussler *et al.*, 2000; von Helversen and Holderied, 2003). We reexamined this matter using a set of genetically identified specimens and found that only baculum measurements can be used to reliably distinguish males of these cryptic bat species.

Our study indicates that the 'wing vein' pattern species discriminating character should not be relied upon. Even if most of the *P. pipistrellus* specimens exhibit the pattern von Helversen and Holderied (2003) proposed, too many *P. pygmaeus* exhibit this pattern also. External shape and color of the penis might be slightly more reliable than the 'wing vein' pattern as a field character, but still should not be relied upon alone. They can be very useful in combination with other characters, such as face length and color, internarial ridge, and the smell of the bat, if used by someone with good knowledge of both species. In ethanol preserved or dried material, the colors fade and the shape is often hardly recognizable, so in this sample we were only able to classify 72% of the specimens correctly (fewer than with the 'wing vein' pattern trait).

A correct species identification can be achieved with the aid of a discriminant function calculated as a weighted sum of measurements of the projected length, width, and height of the baculum. The discriminant function was determined only for the two continental sibling species, which inhabit most of
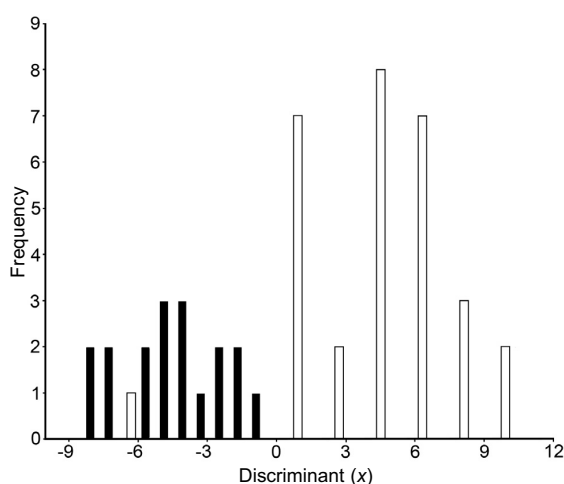

FIG. 4. Discriminant analysis, classifying *P. pipistrellus* (white, *n* = 30) and *P. pygmaeus* (black, *n* = 18) by projected measurements of baculum length, width, and height. One specimen of *P. pipistrellus* (NMP 94542) was misclassified as *P. pygmaeus*
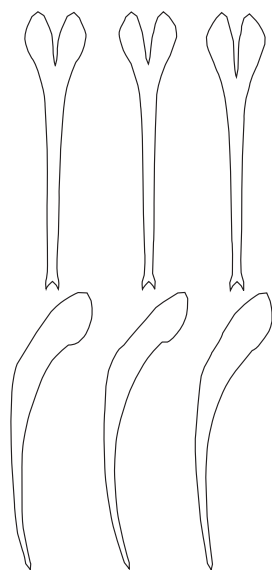
FIG. 5. Average shape of the bacula of *P. pipistrellus* (left), *P. pygmaeus* (middle), and *P. hanaki* (right), in dorsal view (upper row) and in lateral view (lower row), calculated using geometric morphometrics

the Western Palearctic (the common pipistrelle *P. pipistrellus* sensu stricto and the soprano pipistrelle *P. pygmaeus* sensu stricto). The *P. pipistrellus* species complex includes several (mostly allopatric) populations inhabiting peninsulas and islands of the Mediterranean basin. These small Mediterranean populations show higher diversification in morphology and the situation is more complicated (Evin *et al.*, 2011). In the Mediterranean basin, reticulate evolution presumably occurred, and some populations are of hybrid origin (Cyprus, Maghreb).

Applying discrimination criteria to hybrid population is problematic, owing to many phenomena affecting their phenotypes, including higher variability of hybrids and occurrence of transgressive phenotypes. Therefore, for testing species discriminating characters, we did not include the six *P. pygmaeus cyprius*. These bats are from the morphologically and genetically distinct population on Cyprus and show different baculum characteristics (Fig. 7). They were originally classified as a subspecies of *P. pygmaeus* with mitochondrial DNA analysis (Benda *et al.*, 2007). Later analysis showed their nuclear DNA to be closer to *P. pipistrellus* and there is evidence for hybridization between the two species in their distant past on Cyprus (Hulva *et al.*, 2010).

Similarly the misclassification of one specimen in the discriminant function might also be due to hybridization. The one *P. pipistrellus* specimen (NMP 94542) that was misclassified as *P. pygmaeus* with the discriminant analysis of baculum measurements stems from the Maghrebian population in Morocco. The misclassified specimen shows intermediate character states, possibly owing to a hybrid origin. Phylogenetic distance of the Maghrebian *P. pipistrellus* from the continental form may be related to divergent evolutionary pathways of both lineages (Hulva *et al.*, 2004*b*). A distant position of this lineage was observed also in the morphospace derived from geometric morphometric analysis of skulls (Evin *et al.*, 2011). Moreover, cryptic diversity in mtDNA combined with homogenous nuclear pool indicates past reticulate evolution (Hulva *et al.*, 2010). Hybridization may lead to peculiar phenotypic evolution, including transgressive phenotypes (Renaud *et al.*, 2009). While the Maghrebian population is presumably sedentary and perhaps it was never in contact with the *P. pygmaeus* lineage, the opposite is true for *P. pipistrellus* sensu stricto, in which character displacement could not be excluded (Hulva *et al.*, 2010). Considering the hypothesized
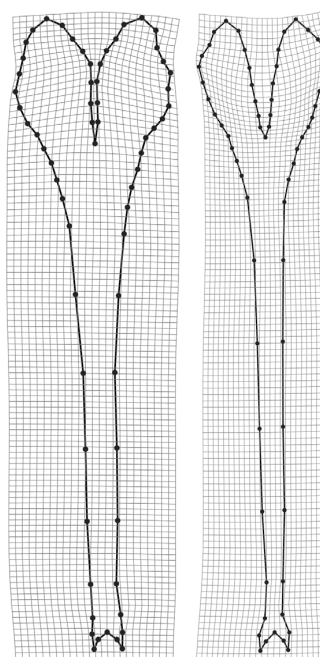

FIG. 6. Deformation grids showing exaggerated shape differences (1.5 times standard deviation) between the average shapes of *P. pipistrellus* (left) and *P. pygmaeus* (right), visualized using geometric morphometrics
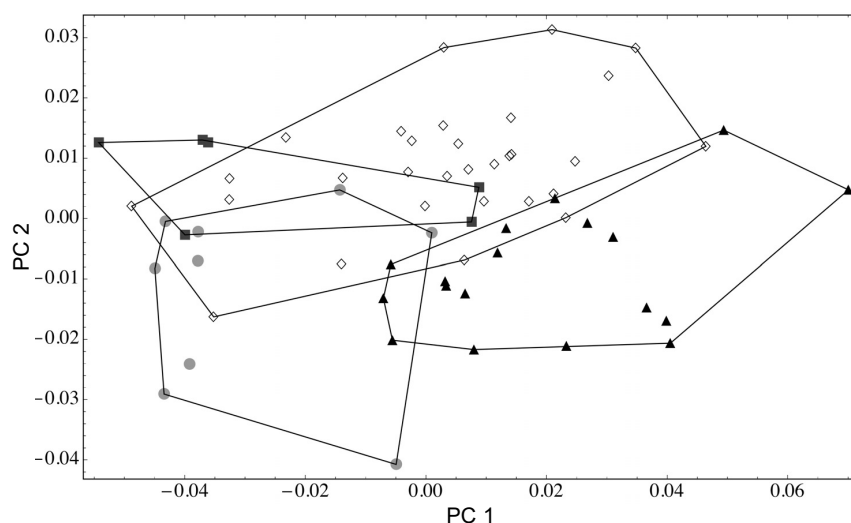
Fig. 7. Between-group principal component analysis of dorsal baculum shape of *P. pipistrellus* (◇, $n = 32$), *P. pygmaeus* (▲, $n = 18$), *P. hanaki* (●, $n = 9$), and *P. pygmaeus cyprius* (■, $n = 6$)

role of the baculum in sexual selection (Eberhard, 1985; Arnqvist, 1998; Danielsson and Askenmo, 1999; House and Simmons, 2002; Hosken and Stockley, 2004; Lüpold *et al.*, 2004) and the possibility of interspecific hybridization of both forms (Hulva *et al.*, 2010; Sztencel-Jabłonka and Bogdanowicz, 2012), divergent selection may also act on penile and bacular morphology.

Owing to the lack of anatomical landmarks on the baculum, we applied 2D geometric morphometrics to virtual thick-sections of 3D microCT imaging data. In geometric morphometric analyses it is possible to use a large number of semi-landmarks, which are allowed to slide along a curve between neighboring landmarks, with only one fixed anatomical landmark. We were not able to classify the three species *P. pipistrellus*, *P. pygmaeus*, and *P. hanaki* with between-group principal component analysis (Fig. 7). However, it was very useful for visualizing and finding areas on the penis bone with maximal variation. In lateral view, the dorsoventral curve of the baculum outline shows large and overlapping individual variation, so it is not useful as a species discrimination character (Fig. 5). In dorsal view, most of the between-species variation could be observed in the base (the proximal third) of the baculum (Figs. 5 and 6).

The baculum measurements we introduced as a new reliable species discriminating character can be taken most precisely and non-destructively on museum material with microCT imaging. However, researchers who do not have access to a microCT

system can still use this discriminating character to identify specimens by resecting the baculum and measuring it under a standard light microscope. High definition 2D X-ray imaging could also be used, if special care is taken to take radiographs at the correct angles. This, and the fact that a single specimen can now be identified by using the discriminant function we introduced here, makes the baculum a very good trait for discriminating dead *P. pipistrellus* and *P. pygmaeus*, especially for museum specimens. Unfortunately the equivalent of the baculum in females, the os clitoridis or baubellum, has seldom been studied in bats (Brown, 1967; Long and Frank, 1968) and has not been found in the pipistrelle species under study. Thus there is no comparable trait to use for species identification in females. The search for a reliable species identification character to be used on live bats in the field will also have to continue.

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APPENDIX

List of the material examined

Pipistrellus hanaki Hulva et Benda, 2004 — **Libya**: 1 ♂, 2 ♀♀ (NMP 49890–49892 [S+A]), 'Arqub Ash Shafshaf, 18 May 2002, leg. M. Andreas, P. Benda, V. Hanák, A. Reiter and M. Uhrin; – 4 ♀♀ (NMP 49894–49897 [S+A]), Wadi Al Kuf, Al Bayda, 19 May 2002, leg. M. Andreas, P. Benda, V. Hanák, A. Reiter and M. Uhrin; – 1 ♂, 2 ♀♀ (NMP 49903, 49904 [S+A], 49902 [A]), Wadi Al Kuf, Massah, 20 May 2002, leg. M. Andreas, P. Benda, V. Hanák, A. Reiter and M. Uhrin; – 1 ♀ (NMP 49884 [S+A]), Wadi Al Minshiyah, 17 May 2002, leg. M. Andreas, P. Benda, V. Hanák, A. Reiter and M. Uhrin. **Greece, Crete**: 1 ♂ (NMP 92344 [S+A]), Monastiraki, 2 June 2008, leg. P. Benda, P. Georgiakakis and V. Hanák; – 5 ♂♂ (NMP 92349–92351, 92353 [S+A], 92352 [A]), Rouva forest, 4 June 2008, leg. P. Benda, P. Georgiakakis and V. Hanák; – 1 ♂ (NMP 92323 [S+A]), Zaros, 25 May 2008, leg. P. Benda and V. Hanák.

Pipistrellus pipistrellus (Schreber, 1774) — **Czech Republic**: 1 ♀ (NMP 90013 [S+A]), Brno, 19 August 2000, leg. Z. Řehák. **Iran**: 1 ♂ (NMP 90832 [S+A]), Emam Qoli, 24 May 2006, leg. P. Benda and A. Reiter; – 1 ♀ (NMP 90776 [S+A]), Karizbalagh, 17 May 2006, leg. P. Benda and A. Reiter; – 1 ♂

(NMP 90791 [S+A]), Shurlaq, 18 May 2006, leg. P. Benda and A. Reiter; – 2 ♂♂, 2 ♀♀ (NMP 90838, 90840, 90841 [S+A], 90839 [A]), Tunel-e Golestan, 26 May 2006, leg. P. Benda and A. Reiter; – 1 ♀ (NMP 48113 [S+A]), Yazd, 4 May 1997, leg. P. Benda. **Lebanon**: 3 ♀♀ (NMP 93534–93536 [A]), Adonis, Nahr Ibrahim, 1 June 2010, leg. P. Benda and M. Uhrin; – 4 ♂♂, 1 ♀ (NMP 91895, 91896. le102, le103, le105 [S+A]), Afqa Cave, 26 June 2006, 17 January 2008, leg. P. Benda, I. Horáček, P. Hulva, R. Lučan and M. Uhrin; – 2 ♀♀ (NMP 93531, 93532 [A]), Balaa, 31 May 2010, leg. P. Benda and M. Uhrin; – 1 ♂ (NMP le101 [S+A]), Er Rouais Cave, 26 June 2006, leg. I. Horáček, P. Hulva and R. Lučan; – 2 ♀♀ (NMP 93547, 93548 [A]), Faraya El Mzar, 2 July 2010, leg. P. Benda and M. Uhrin; – 1 ♂, 1 ♀ (NMP 91902, 91903 [S+A]), Haqel El Aazime, 18 January 2008, leg. P. Benda, I. Horáček, R. Lučan and M. Uhrin; – 1 ♀ (NMP 93572 [A]), Jenta, 8 June 2010, leg. P. Benda and M. Uhrin; – 6 ♂♂ (NMP le19–le22, le24 [S+A], le26 [A]), Jezzine, 23 June 2006, leg. I. Horáček, P. Hulva and R. Lučan; – 2 ♂♂ (NMP le12, le13 [S+A]), Nahr Es Safa, 22 June 2006, leg. I. Horáček, P. Hulva and R. Lučan; – 1 ♂ (NMP le118 [S+A]), Qadicha Cave, 27 June 2006, leg. I. Horáček, P. Hulva

A. N. Herdina, P. Hulva, I. Horáček, P. Benda, C. Mayer, *et al.*

APPENDIX. Continued

and R. Lučan; – 2 ♂♂, 1 ♀ (NMP le169, le193, le212 [S+A]), Ras El Assi, 29 June 2006, leg. I. Horáček, P. Hulva and R. Lučan. **Morocco**: 4 ♂♂, 3 ♀♀ (NMP 94540–94545, 94546 [S+A]), Bekrite, 28 April 2008, leg. P. Benda, J. Červený, A. Konečný and P. Vallo. **Slovakia**: 2 ♂♂, 5 ♀♀ (NMP 90016–90022 [S+A]), Ernya Cave, 20 April 2003, leg. P. Benda, V. Hanák and M. Uhrin. – 1 ♂, 1 ♀ (NMP 90014, 90015 [S+A]), Tisovec, 24 July 2002, leg. P. Benda, E. Hapl and M. Uhrin. **Syria**: 2 ♀♀ (NMP 48084, 48085 [S+A]), Ar Rawda, 1 July 1998, leg. M. Andreas, P. Benda & M. Uhrin; – 1 ♀ (NMP 48902 [S+A]), Baniyas, 31 May 2001, leg. M. Andreas, P. Benda, A. Reiter and D. Weinfurtová; – 4 ♂♂ (NMP 48981– 48984 [S+A]), Maalula, 30 April 2001, leg. R. Lučan; – 1 ♀ (NMP 49986 [S+A]), Ras Al Bassit, 29 April 2001, leg. R. Lučan; – 1 ♂, 1 ♀ (NMP 48871, 48872 [S+A]), Sarghaya, 28 May 2001, leg. M. Andreas, P. Benda, A. Reiter and D. Weinfurtová; – 3 ♂♂ (NMP 48060–48062 [S+A]), Slinfeh, 29 June 1998, leg. M. Andreas, P. Benda and M. Uhrin.

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6. DISCUSSION

6.1 *Pipistrellus pipistrellus* and *Pipistrellus pygmaeus* as model species complex for cryptic diversity in European bats

Cryptic diversity in European bats poses major challenges to studies on ecology or conservation status of European bat species [Arlettaz, 1999; Davidson-Watts et al., 2006; Sattler et al., 2007; Ashrafi et al., 2010]. Molecular genetic methods have boosted the discoveries of new cryptic species, often splitting a well studied species into two or more new species [Mayer et al., 2007]. These methods are also used in species identification, but morphological species discrimination characters are still necessary, especially in field studies and when using old museum material. These new cryptic species complexes also present a unique opportunity for researching niche partitioning, reproductive isolation, phylogeographic character variation, and morphological differentiation [Davidson-Watts et al., 2006; Hulva et al., 2010]. The *Pipistrellus pipistrellus/pygmaeus* species complex is an exceptionally good model system for this research, because of the species' distribution ranges with sympatric as well as allopatric populations, and because a great deal of research has been done before and after the separation of the cryptic species. [Davidson-Watts et al., 2006; Hulva et al., 2010]. However, until this study, no reliable morphological species identification characters have been found for this species complex, despite considerable effort [Barlow & Jones, 1997b; 1999; Häussler et al., 2000; Ziegler et al., 2001; Sendor, 2002; von Helversen & Holderied, 2003; Ahlen et al., 2004; Dietz & von Helversen, 2004; Dubourg-Savage, 2006; Dietz et al., 2007; Kaňuch et al., 2007a; Pavlinić et al., 2008; Sztencel-Jabłonka et al., 2009]. This motivated my research for Aim 3 of this dissertation.

6.2   Discriminating characters for *Pipistrellus pipistrellus* and *Pipistrellus pygmaeus*

6.2.1   The baculum's use in discriminating *Pipistrellus pipistrellus* and *Pipistrellus pygmaeus*

In this dissertation, I tested baculum shape and its biometrics as species discriminating characters for *Pipistrellus pipistrellus* and *Pipistrellus pygmaeus* (Aim 3; Paper IV). Baculum shape and projected length are often used as taxonomic characters. Ziegler et al. [2001] proposed using the baculum, especially the shape of the dorsoventral curve in lateral view, as a species discrimination character for this cryptic species pair. Together with my coauthors on Paper IV, I used 2D geometric morphometrics to locate the areas of the baculum with the largest inter- and intraspecific variation and to quantify this variation (Aim 1; Paper IV). We also tried to use the shape differences in dorsal view for species discrimination. The most promising baculum shape variation between the two species could be observed in dorsal view (Figs 3, p.127; 5, p.130; and 6, p.130 of Paper IV), in the proximal base of the baculum. A between-group principal component analysis showed differences between the species, but no complete separation (Fig. 7, p.131 of Paper IV).

In lateral view (Figs 3, p.127 and 5, p.130 of Paper IV), the shape of the curve and the degree of curvature show the most intraspecific variation. A between-group principal component analysis showed no discernible difference in lateral shape between the species. Thus, the shape of the baculum in lateral view is not useful in species discrimination for *Pipistrellus pipistrellus* and *P. pygmaeus*, refuting this hypothesis of Ziegler et al. [2001].

Originally, I planned to describe the typical shape of the medullary canal, and the number and location of foramina nutritia, in the bacula of *Pipistrellus pipistrellus* and *Pipistrellus pygmaeus*, hoping to find useful species discrimination characters there. However, the individual variation turned out to be so large that there were hardly any similar features, and no one feature was found in all of the specimens of any of the species researched. I found specimens with extensive medullary cavities and many foramina nutritia, especially in the proximal third of the baculum (the base). The medullary canal sometimes extended up to the middle of the baculum, but never into the distal third of the baculum's length. There were two specimens without any medullary cavity or canal (*Pipistrellus*

hanaki NMP pb3977, NMP pb3979). The foramina nutritia varied from several large holes in the base of the baculum to only one small opening (or none in case of the two specimens without medullary cavity), most frequently on the ventral side of the baculum (Paper IV). In contrast, the morphology of the medullary canal in the *Plecotus* species studied seems to be quite species specific with only little individual variation. Occasionally, there are specimens with a totally different morphology of the canal (see Paper I) [Herdina, 2008; Herdina et al., 2010]. This variability in itself is interesting, as it suggests plasticity in ontogenetic development. It might be epigenetic plasticity, but research would be necessary on embryologic and postpartum developmental stages of bat species showing variability compared with species showing more conserved morphology.

I found that with baculum biometrics *Pipistrellus pipistrellus* and *P. pygmaeus* can be classified reliably. I measured the bacula on virtual thick sections of microCT images, oriented laterally or dorsoventrally. Of all the measurements taken (Fig. 3 of Paper IV, p.127), projected length, projected width, and projected height were most useful for classifying the specimens. With a discriminant analysis of those three measurements, all but one bat were identified correctly (Fig. 4 of Paper IV, p.129). The resulting discriminant function can now be used to classify even single specimens. This is even possible with old museum specimens or long-dead found specimens. More accurate measurements are possible on virtual sections, but researchers without access to a microCT facility can also use this species identification character by measuring resected bacula.

6.2.2   Other discriminating characters tested

Additionally, I critically evaluated the reliability of "wing vein" pattern and penis color and shape for species identification (Fig. 2 of Paper IV, p.127). The "wing vein" pattern character is unreliable for identifying specimens, because too many *Pipistrellus pygmaeus* show the pattern typical for *P. pipistrellus*. Also, some specimens were found to have different patterns on the left and right wing. Penis color and shape might be slightly more reliable in the field, but on ethanol preserved specimens the percentage of correctly identified specimens was even lower than with the "wing vein" character, due to changes in color and deformation. I concluded that the "wing vein" pattern character should not be used, and penis color and shape should be used for species identification only in the field, and only in combination with other characters like fur color and face shape.

6.3   The baculum for species discrimination more generally

Because many mammalian species have distinct bacula, baculum shape and length have often been used as taxonomic characters successfully. The baculum has been used not only for species discrimination, but also on higher taxonomic levels. The marked differences in the species' baculum shapes led to the discovery of the polyphyly of the bat genera *Pipistrellus*, *Plecotus*, *Hypsugo*, *Eptesicus*, *Myotis*, and *Rhinolophus* [Jones, 2001; Mayer & von Helversen, 2001a;b; von Helversen et al., 2001; Hulva et al., 2004a; Ibáñez et al., 2006; Spitzenberger et al., 2006; Mayer et al., 2007; Benda & Vallo, 2012; Salicini et al., 2013]. Even though the baculum is very useful in species discrimination, individual variation can be a problem. Thus it is necessary to study a larger number of specimens from throughout the range of the species before describing typical baculum shape. Measurements like baculum length can also be useful for species identification, but there is often an overlap between the species. This can make the application of a discriminant analysis more useful than simply comparing measurements, especially since it is only slightly more complicated.

6.4   Baculum function

Understanding the mechanical and behavioral mechanisms of mammalian reproduction is an important prerequisite for studying reproductive isolation and speciation processes. The baculum probably plays a mechanical role in copulation for bats and other mammals, but its specific function is still unknown. There are probably various and overlapping mechanical functions [Dyck et al., 2004], as a single function for all the different baculum shapes and sizes seems very unlikely [Ruth, 1934].

To shed more light on baculum function in bats, I combined two different approaches: histomorphological and experimental techniques, both in combination with 3D models derived from microCT images. Most of the species used in this study are cryptic species of vespertilionid bats with a Palearctic distribution range. In both *Pipistrellus* (Papers II-IV) and *Plecotus* (Paper I), different patterns of sympatric and allopatric distribution occur. Studying baculum morphology and function in the context of reproductive isolation seemed an especially promising contribution to understanding different speciation processes in the evolution of those species.

6.4.1   The baculum-corpora cavernosa interface

Our histomorphological (Papers I and II) and experimental (Paper III) results strongly support the hypothesis that the baculum and corpora cavernosa together form a functional unit, which increases the flexural stiffness of the whole length of the erect penis by transferring bending forces from the distal end of the glans penis to the corpora cavernosa during copulation [Long & Frank, 1968; Kelly, 2000]. The importance of the connection of the baculum within the glans penis and the paired corpora cavernosa in the shaft of the penis can be supported histologically by the tight connection of these tissues (Figs 4, p.42 and 5, p.43 of Paper I; and Figs 2, p.80; 3, p.81; and 4, p.82 of Paper II). The occurrence of cartilaginous cells (Fig. 5 of Paper I, p.43) and of entheses (Fig. 3 of Paper II, p.81) at the interface of those tissues strongly suggest bending forces are occurring in this part of the penis [Benjamin et al., 1986; Kelly, 2000]. That this is a functional unit is further corroborated by the shared fibrous collagen envelope of the tissues, consisting of the periosteum of the baculum and the tunica albuginea of the corpora cavernosa (Figs 4, p.42 and 5, p.43 of Paper I; and Figs 2, p.80; 3, p.81; and 4, p.82 of Paper II). No distinct border was found between those tissues.

The mechanical function of the tightly knit connection of the baculum and corpora cavernosa was demonstrated in the experimental part of this study (Paper III). When the corpora cavernosa are inflated, the baculum changes position relative to the other penis tissues (Figs 3, p.111; 4, p.112; and 5, p.113 of Paper III). The corpora cavernosa stiffen and form a straight line together with the baculum. The baculum's distal tip is pushed upwards, caused by the change in position. This movement also straightens the urethra and its surrounding corpus spongiosum. This additionally supports the hypothesis that the baculum could affect penile shape during erection [Dyck et al., 2004].

So far, we could neither support nor refute the hypothesis that the baculum could increase the stiffness of the corpora cavernosa by being pressed into their proximal ends and thus increasing the hydrostatic pressure within the corpora cavernosa during copulation [Long & Frank, 1968; Kelly, 2000]. However, in the species we studied it seems unlikely. Histological sections showed only a part of the area where the baculum and corpora cavernosa are connected. Experimental inflation of the corpora cavernosa revealed that the area where the tissues are connected spans most of the dorsal side of the base of the baculum. Thus it seems difficult for such a large portion of the bone to be pressed into

the corpora cavernosa, especially with the shared fibrous envelop of tunica albuginea and periosteum, when the tunica albuginea is expanded fully already.

6.4.2   The baculum protecting the urethra

The hypothesis that the baculum protects the distal end of the urethra from compression during copulation [Dyck et al., 2004] is also supported by both our histomorphological and our experimental results. In the bat species we studied, the baculum is located directly dorsal of the urethra. The distal tip of the baculum is positioned right at the urethral opening (Figs 2, p.40 and 3, p.41 of Paper I; Figs 5, p.83; 6, p.84; and 7, p.85 of Paper II; and Figs 3, p.111; 4, p.112; 5, p.113; and 6, p.114 of Paper III). This could be shown especially clearly in the 3D images (Fig. 5, p.83 of Paper II; Figs 3, p.111; 4, p.112; and 5, p.113 of Paper III; and Fig. 1.6 in the Introduction). In the *Pipistrellus* species the forked tip surrounds the upper half of the urethral opening, which is slightly beveled, like the opening of a hypodermic needle. Here the tissues are positioned next to each other. They do not seem as tightly connected as the proximal end is connected to the corpora cavernosa, but still connected enough to change the shape of the urethra and corpus spongiosum when the baculum changes position. This might be necessary to allow the baculum to change position between flaccid and erect state.

6.5   Discussion of the methods used in this dissertation

I used a number of different classical and modern methods, sometimes in novel combinations. The correlative application of these methods to a different field of research shows their usefulness for systematic research as well as for functional morphology.

6.5.1   MicroCT imaging

The use of x-ray microtomography in the biological sciences, especially for mineralized tissue research, is well established [Neues & Epple, 2008]. However, using microCT for quantitative studies including soft tissue micromorphology is a relatively new approach [Zainon, 2012; Metscher, 2013]. The techniques for soft tissue iodine staining (Papers I-IV) have been developed in our lab by one of my advisors, Brian Metscher [see Metscher, 2009a;b]. The combination of histological techniques like ground sections of few specimens with microCT imaging of a larger number of specimens has proved to be especially success-

ful (Papers I and II). Thus, a statistically relevant number of specimens could be studied non-destructively and quantitatively with micromorphological resolution. The histological details were verified (destructively) with serial semithin sections and surface-stained ground sections, while microCT images provided the 3D spatial context. Tomographic images also allow for segmentation of specific tissues, which was especially important for studying functional anatomy (Paper III).

6.5.2 Geometric morphometrics

We used geometric morphometrics (Paper IV) to determine which areas of the baculum show the highest inter- and intraspecific variation. I was also hoping to separate *Pipistrellus pipistrellus* and *P. pygmaeus* by baculum shape. Although a between-group PCA (Fig. 7 of Paper IV, p.131) showed differences in baculum shape between the species, it was not enough to reliably separate them.

6.5.3 Baculum biometrics

MicroCT images offer a highly accurate way to repeatably take even small measurements on the baculum. More accurate measurements are possible with the Xradia TXM3D Viewer software on virtual thick sections of microCT images than on a resected baculum using a measuring eyepiece. Because of the tight connection of the baculum to the corpora cavernosa, it is hardly ever possible to separate these tissues physically without either having residues of soft tissue on the bone or damaging or decalcifying the bone. Measurements other than projected length and width on resected bacula are further complicated by difficulties mounting the bacula, due to their small size in most species of bats and rodents.

6.5.4 Discriminant analysis

Two discriminant analyses with leave-one-out cross validation were calculated using PAST [Hammer et al., 2001] to find discriminating characters for *Pipistrellus pipistrellus* and *P. pygmaeus*. The first analysis used all of the measurements taken on the bacula of *Pipistrellus pipistrellus* and *P. pygmaeus* (Fig. 3 of Paper IV, p.127). The second one used only the projected measurements of length, width, and height of the bacula. The second discriminant analysis, of just the simple measurements, separated the groups much better: only one *Pipistrellus pipistrellus* specimen was misclassified (Fig. 4 of Paper IV, p.129).

6.5.5   Microradiography

Microradiography (Paper I) was used to determine position and orientation of the baculum within the methylmetacrylate embedded penis, before the ground sections were made (Fig. 2 of Paper I, p.40). This way, we were able to choose the exact angle for cutting and grinding to get dorsoventral sections or cross sections of the baculum.

6.5.6   Histomorphology

Undecalcified, serial, surface-stained ground sections (Papers I and II) and serial semithin cross sections (Paper I) were used to describe baculum histomorphology and to test hypotheses on baculum function (Figs 4, p.42 and 5, p.43 of Paper I; and Figs 2-7, pp. 80-85 of Paper II) . The ground sections turned out to be the more useful technique in functional bone histomorphology, because the bone is processed without the decalcification required for microtome sections. The surface staining of ground sections makes the consecutive use of different staining agents possible, as they are ground away in the next step and thus do not interfere with the next stain. Of course, the downside of continually grinding away the ground section's surface is that in the end only the last section is preserved. Serial semithin sections are much more prone to artifacts and missing sections [Streicher et al., 1997; Ourselin et al., 2000; Handschuh et al., 2010], but all of the sample is preserved as sections. It is possible to do 3D reconstructions from photos of sections using both methods, but the z-axis resolution is higher and more constant using semithin sections (Fig. 6 of Paper I, p.44).

6.5.7   Inflation of penes

Diane Kelly established this method combined with histological sections on rats [Kelly, 2000], armadillos [Kelly, 1997; 1999], and turtles [Kelly, 2002; 2004] to test functional hypotheses of the different penis tissues in post-mortem specimens. I combined her method (Fig. 1 of Paper III, p.109) with microCT imaging to get a better understanding of the three-dimensional penis micromorphology in *Pipistrellus* (Figs 4, p.112 and 5, p.113 of Paper III) and *Nyctalus* (Fig. 3 of Paper III, p.111). Additionally, I found out that penis inflation also works on specimens frozen shortly after death and thawed prior to the penis inflation. My future work with Diane Kelly and Betsy Dumont will be based on microCT images of inflated bat penes, in a project to study baculum function with finite element analysis.

6.6   Conclusion and outlook

The application of new 3D methods has revived an old field of morphological research. The value of the baculum as a taxonomic character has been recognized since 1885 [von Ihering, 1885; Thomas, 1915; Strelkov, 1989], but more accurate measurements can be taken now [Herdina, 2008; Herdina et al., 2010]. Identifying the specimens used in any scientific study is prerequisite to drawing meaningful conclusions on a species' range, habitat, conservation status, population biology, behavior, or physiology [Arlettaz, 1999; Davidson-Watts et al., 2006; Sattler et al., 2007; Ashrafi et al., 2010]. The importance of finding reliable morphological species discriminating characters has not diminished with the advent of molecular genetic methods. On the contrary, with the continuing discovery of further cryptic species, it is necessary to establish reliable criteria for the identification of specimens in the field and in old museum collections. While the search for field identification characters and characters to identify females continues, we have found such a species discriminating character for male museum specimens of *Pipistrellus pipistrellus* and *P. pygmaeus*.

To research reproductive isolation and speciation processes, it is important to understand the mechanical and behavioral mechanisms of mammalian reproduction. With the results of this work, we are one step closer to understanding the function of the baculum in the mechanical process of copulating and in reproductive isolation of closely related species. This longstanding question has been the subject of many hypotheses since the discovery of penis bones [Stockley, 2012]. However, there have been hardly any experimental studies on the topic [Kelly, 2000; Sharir et al., 2011; Stockley et al., 2013; Simmons & Firman, 2013]. Very recently other researchers have approached the question with mating experiments in mice [Ramm et al., 2010; Stockley et al., 2013]. The novel approach of combining experimental designs like inflating penes with high resolution microCT will help to compare and test the functions of different baculum morphologies, and it paves the way for finite element modeling to test biomechanical hypotheses in dead specimens. This dissertation has built the foundation for a finite element study of baculum function and sexual selection mechanisms in bats, which I have started in collaboration with Elisabeth R. Dumont, Diane Kelly, Patricia L.R. Brennan, and Teri Orr.

In the longer term, basic research on the mechanical functions of penis tissues in the process of mammalian copulation could also lead to insights useful for the treatment of medical conditions, such as Peronie's disease or erectile dysfunction.

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8. ZUSAMMENFASSUNG (SUMMARY IN GERMAN)

Reproduktive Isolation spielt eine entscheidende Rolle im Artbildungsprozess [Mayr, 1942]. Gelegentliche Ausfälle dieser Barriere, die zu Hybridisierung führen, sind in allen Tiergruppen mit gut erforschter Systematik beschrieben worden [Mayr, 1963]. Eigenschaften, deren Variabilität zu reproduktiver Isolation führen, können in verschiedenen Tiergruppen unterschiedlich sein. Dabei kann die Morphologie der Geschlechtsorgane ein wichtiger Faktor sein [Mayr, 1942]. Das Baculum ist deshalb interessant, weil es ein Merkmal ist, das unter sexueller Selektion steht und bei nah verwandten Arten oft deutliche Unterschiede in Form und Größe aufweist [Hosken et al., 2001]. Obwohl das Baculum, genau aus diesem Grund, schon lange erfolgreich zur Artbestimmung eingesetzt wird, ist nicht viel über seine Funktion bekannt [Patterson & Thaeler, 1982]. In dieser Dissertation habe ich inner- und zwischenartliche Variation des Baculums quantifiziert, und seine wahrscheinliche Rolle bei der reproduktiven Isolation und seine mechanischen Funktionen untersucht.

Die kryptische Diversität innerhalb der europäischen Fledermausarten stellt eine große Herausforderung für Forschung und Artenschutz dar. Eine zuverlässige Bestimmung der untersuchten Tiere ist die Voraussetzung, um sinnvolle Aussagen über z.B. Verhalten, Morphologie oder Erhaltungszustand einer Art tätigen zu können [Arlettaz, 1999; Davidson-Watts et al., 2006; Sattler et al., 2007; Ashrafi et al., 2010]. Nach meiner erste Studie über die funktionelle Morphologie des Baculums bei *Plecotus* habe ich zu dem kryptischen Artkomplex *Pipistrellus pipistrellus/pygmaeus* gewechselt. Dieser Artkomplex entwickelt sich immer mehr zu einem Modellsystem für kryptische Diversität europäischer Fledermäuse. Bis jetzt wurde kein verlässliches morphologisches Merkmal gefunden, um diese beiden Arten zu unterscheiden. Zu Vergleichszwecken wurden außerdem *Pipistrellus nathusii* und *Nyctalus noctula* untersucht, weil ihre Bacula denen von *Pipistrellus pipistrellus* und *Pipistrellus pygmaeus* ähneln.

Die Ziele dieser Dissertation waren 1) inner- und zwischenartliche Variation der Baculum-Mikromorphologie kryptischer Fledermausarten der Genera *Plecotus* und *Pipistrellus* zu quantifizieren; 2) bekannte Hypothesen über Baculumfunktionen histomorphologisch und experimentell zu testen; und 3) Baculum Form und Biometrie als Merkmale zur Bestimmung von *Pipistrellus pipistrellus* und *Pipistrellus pygmaeus* zu testen.

Diese Dissertation stellt eine Diskriminanzfunktion aus projizierter Länge, Breite und Höhe des Baculums vor, mit deren Hilfe alle untersuchten Tiere, außer einem, verlässlich der vorher molekulargenetisch bestimmten Art zugeordnet werden konnten. Die funktionelle Anatomie des Baculums wurde histologisch und experimentell untersucht. Serielle Dünnschliffe mit Oberflächenfärbung und Semidünnschnitte, in Kombination mit MikroCT Aufnahmen, wurden von den Bacula und dem jeweils umgebenden Penisgewebe von *Plecotus* und *Pipistrellus* Arten gemacht. Um Hypothesen zur Baculumfunktion experimentell zu testen, wurden die Corpora cavernosa der Penes toter Fledermäuse mit Formol aufgefüllt. Die Position des Baculums und der Weichgewebe im Verhältnis zueinander wurden, mittels virtueller Schnittebenen und 3D Modellen aus hochauflösenden MikroCT Aufnahmen, zwischen schlaffen und "erigierten" Penes verglichen. Beide getesteten Hypothesen konnten sowohl mit den histologischen als auch mit den experimentellen Ergebnissen gestützt werden: Das Baculum kann die Urethra und ihre Öffnung während Erektion und Kopulation davor schützen, komprimiert zu werden. Das Baculum und die Corpora cavernosa bilden eine funktionelle Einheit um gemeinsam sowohl den Penisschaft als auch die distale Glans penis zu versteifen.

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APPENDIX

A. CURRICULUM VITAE

Personal Information

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I am a zoologist with a research focus on bat biology. My research interests include
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Education

03/2009 to present	Dissertation (PhD thesis) The bat baculum: histomorphological methods and microCT-based 3D models to distinguish cryptic species of *Pipistrellus* and *Plecotus* and to test functional hypotheses using post-mortem specimens Advisors: Ao.Univ.-Prof.Dr. Helge Hilgers and Dr. Barbara Herzig
07/2008 to present	Doktorats-Studium der Naturwissenschaften (equivalent to PhD program) Department of Theoretical Biology Faculty of Life Sciences, University of Vienna
14/07/2008	Diploma examination, graduation with honors (equivalent to Master of Science)

10/2005 - 07/2008	Diploma thesis Light microscopy of the penis bone (baculum) in the *Plecotus* species (Chiroptera) in Austria Advisors: Ao.Univ.-Prof.Dr. Helge Hilgers and Dr. Barbara Herzig
10/1997 - 07/2008	Diplomstudium Biologie, Zoologie at University of Vienna
03/06/1997	Matura (final secondary-school graduation diploma qualifying for university entrance), graduation with honors
1989 - 1997	Realgymnasium Rahlgasse 4, 1060 Wien (Austrian secondary school with focus on natural sciences and mathematics)

Present Position

10/2011 to present	Lecturer at University of Vienna, Department of Theoretical Biology, Spring semester 2011-2014: Seminar, Scientific Literature and Communication for Biologists, with Brian D. Metscher. Autumn semester 2011-2013: Seminar, Writing Theses and Articles in English, with Brian D. Metscher
05/2009 to present	Sub-group coordinator within the Intersessional Work Group on Wind Turbines and Bat Populations of the Advisory Committee, EUROBATS, The Agreement on the Conservation of Populations of European Bats, United Nations Environment Programme
12/2005 to present	Founding member of BatLife Austria – bat research and conservation, secretary and coordinator of public awareness programs in schools and at bat events

Scientific Research Experience

03/2014 - 06/2014   Research assistant in microCT study of cyprinid fish pharyngeal jaws for Harald Ahnelt, Dept. of Theoretical Biology, University of Vienna – operation of Xradia and Skyscan microCTs, data evaluation in Amira

05/2013   Field assistant in radio-tracking study of *Myotis emarginatus* foraging habitats for Friederike Spitzenberger and Bernd Ohlendorf in Burgenland

12/2011 - 07/2012   Histological lab research assistant for Hanns Plenk, Bone and Biomaterials Research, Institute for Histology and Embryology, Medical University of Vienna – histological staining, ground sections, electron microscopy (SEM) with Edax element analysis

01/2011 - 07/2011   Analysis of common kestrel (*Falco tinnunculus*) pellets in the project "City Slickers – Erfolgsstrategien des Turmfalken als Großstadtjäger", project coordinator: Anita Gamauf, the Natural History Museum Vienna

04/2006 - 06/2006   Holding practical courses for bat workers on bat biology, ecology, and species identification for BatLife Austria

01/2005 - 06/2006   Taxidermist at the Natural History Museum Vienna – scientific preparation of small mammals (skeletons, study skins and stuffed specimens, and specimens preserved in ethanol); maintenance and use of the dermestid beetle colony

06/2003 - 12/2008   Bat research and public awareness projects for the speleology club TFC Hannibal

10/1999 - 06/2002   Research assistant for Alexander Erlach at the Konrad Lorenz Institute for Evolution and Cognition Research in Altenberg – behavioral experiments on mudskippers (*Periophthalmus sp.*)

International Experience

08/2013	Field assistant in radio-tracking study of *Uroderma bilobatum* foraging habitats and roost visiting behavior for Frank Bonaccorso and David Villalobos-Chaves
02/2012 - 07/2012	Marietta Blau Fellowship awarded by the Austrian Federal Ministry of Science and Research and the Austrian agency for international mobility: 4 months at Charles University in Prague, Czech Republic; 2 months at University of Massachusetts in Amherst, USA – Data collection for my PhD project and learning new techniques: molecular genetic methods and Finite Element Analysis
09/2010	Training in mist-netting of birds in the scope of a bird banding project at Passo Brocon, Italy with Stefano Noselli and Francesca Rossi of the Museo Tridentino di Scienze
05/2006 - 5/2011	Representation of BatLife Austria at meetings of the Advisory Committee, EUROBATS, UNEP (AC11 Luxembourg, AC14 Cyprus, AC16 Georgia)
10/2003 - 12/2003	Field assistant on Barro Colorado Island in Panama for Christoph Meyer – bat mist-netting, identification, banding, subcutaneous transponder implantation and recording of echolocation calls
10/1999	Field course on marine ecology in Jordan – commensalism in Red Sea goatfish (*Parupeneus forsskali*), scuba diving for data collection, species identification and statistical analysis
07/1999 - 08/1999	Internship at Coastal Field Research Center ORES in Quebec, Canada – photo-identification of whales, ethological data collection, hydrophone recording of whale calls, analysis of krill samples, supervision of course participants

Affiliations   BatLife Austria

North American Society for Bat Research

The Society for Integrative & Comparative Biology

Deutsche Zoologische Gesellschaft

DAN Europe

Awards and Fellowships

10/2013   PhD Completion Grant, awarded by the Dean's Office of
the Faculty of Life Sciences, University of Vienna

12/2012   Dissemination – support for attending conferences for
young scientists of the University of Vienna, for attending the
42nd North American Symposium on Bat Research

11/2012   Luis F. Bacardi Bat Conservation Award, sponsored by the
Lubee BatConservancy, for my talk at the 42nd North American
Symposium on Bat Research

02/2012 - 07/2012   Marietta Blau Fellowship, awarded by the Austrian Federal
Ministry of Science and Research and the Austrian agency
for international mobility 4 months at Charles University in
Prague, Czech Republic; 2 months at University of Massachusetts
in Amherst, USA

11/2010   Invited speaker for two talks on penis morphology at the fall
conference 2010 of Arbeitskreis Fledermäuse Sachsen-Anhalt
e.V. and Landesreferenzstelle für Fledermausschutz
Sachsen-Anhalt in Germany

08/2010   Student Award for the Best Poster Presentation at the 15th
International Bat Research Conference in Prague, Czech Republic

Publications

Herdina Anna Nele, Plenk Jr. Hanns, Benda Petr, Lina Peter H.C., Herzig-Straschil Barbara, Hilgers Helge, Metscher Brian D. In review at the *Journal of Morphology* (submitted 09.07.2014, in review since 30.07.2014). Correlative 3D imaging of *Pipistrellus* penis micromorphology: validating quantitative microCT images with undecalcified serial ground section histomorphology

Herdina Anna Nele, Kelly Diane A., Jahelková Helena, Lina Peter H.C., Horáček Ivan, Metscher Brian D. In review at the *Journal of Anatomy* (submitted 11.09.2013, decision - major revisions - 21.01.2014, revision submitted 05.08.2014). Testing hypotheses of bat baculum function with 3D models derived from microCT

Herdina Anna Nele, Hulva Pavel, Horáček Ivan, Benda Petr, Mayer Christine, Hilgers Helge, Metscher Brian D. 2014. MicroCT imaging reveals morphometric baculum differences for discriminating the cryptic species *Pipistrellus pipistrellus* and *P. pygmaeus*. *Acta Chiropterologica* 16(1): 157 - 168

Herdina Anna Nele, Herzig-Straschil Barbara, Hilgers Helge, Metscher Brian D., Plenk Jr. Hanns. 2010. Histomorphology of the penis bone (baculum) in the gray long-eared bat *Plecotus austriacus* (Chiroptera, Vespertilionidae). *The Anatomical Record* 293: 1248 - 1258. http://www3.interscience.wiley.com/journal/123345780/abstract

Herdina Anna Nele. 2008. Light microscopy of the penis bone (baculum) in the *Plecotus* species (Chiroptera) in Austria. Diploma thesis: Department of Theoretical Biology, Morphology Section, Faculty of Life Sciences, University of Vienna. 74 pages

Herdina Anna Nele, Schlögl Gerhard, Spitzenberger Friederike. 2006. Fledermäuse brauchen Freunde. (Bats need friends – brochure about bat biology and the species in Naturpark Landseer Berge, Austria) BatLife Österreich and Naturpark Landseer Berge. 27 pages

Publications in preparation

Herdina Anna Nele, Kelly Diane A., Brennan Patricia, Pulaski Dan, Grosse Ian, Purna Nugraha Taufiq, Metscher Brian D., Dumont Elizabeth R. in preparation. Finite Element Analysis reveals probable mechanisms for sexual selection on the penis bone in *Cynopterus* (Chiroptera, Mammalia)

Herdina Anna Nele, Hulva Pavel, Horáček Ivan, Benda Petr, Mayer Christine, Hilgers Helge, and Metscher Brian D. in preparation. Individual and phylogeographic variation of the *Pipistrellus* baculum

Harald Ahnelt, Herdina Anna Nele and Metscher Brian D. in preparation. Diversity and variability of pharyngeal dentition and tooth rows of African chedrin fishes (Cyprinidae: Danioninae)

Conference Presentations

Herdina Anna Nele, Plenk Jr. Hanns, Benda Petr, Lina Peter H.C., Herzig-Straschil Barbara, Hilgers Helge, Metscher Brian D. 2014. Poster: *Pipistrellus pipistrellus* (Chiroptera, Vespertilionidae) postnatal baculum development. In: Sarto-Jackson I, Callebaut W, editors. Vienna, Austria 22nd - 25th July 2014: p358

Herdina Anna Nele, Hulva Pavel, Horáček Ivan, Benda Petr, Mayer Christine, Hilgers Helge, and Metscher Brian D. 2014. Poster: Individual and phylogeographic variation in the *Pipistrellus* baculum. In: Society for Integrative and Comparative Biology 2014 Annual Meeting Abstract Book. Austin, Texas, USA, 3rd - 7th January 2014: p145

Herdina Anna Nele, Hulva Pavel, Horáček Ivan, Benda Petr, Mayer Christine, Hilgers Helge, and Metscher Brian D. 2013. Talk: The *Pipistrellus* baculum: Individual and phylogeographic variation and cryptic species discrimination. In: Bonaccorso F, Kwiecinski G, Mistry S, editors. Abstracts of the 16th International Bat Research Conference and the 43rd North American Symposium on Bat Research. San José, Costa Rica 11th - 15th August 2013: p 68

Herdina Anna Nele, Jahelková Helena, Kelly Diane A., Pulaski Dan, Brennan Patricia L.R., Orr Teri J., Dumont Elizabeth R., Horáček Ivan, Metscher Brian D. 2013. Talk: A micromorphological-experimental approach to baculum function in bats. The Anatomical Record, Volume 296, Special Feature ICVM 2013. 10th International Congress of Vertebrate Morphology. Barcelona, Spain, 8th - 12th July 2013: p C-033

Herdina Anna Nele, Kelly Diane A., Brennan Patricia, Pulaski Dan, Grosse Ian, Purna Nugraha Taufiq, Metscher Brian D., Dumont Elizabeth R. 2012. Talk: The function of the baculum – a longstanding question (partly) answered. In: Bonaccorso F, Kwiecinski

G, Mistry S, editors. Abstracts – 42nd Annual Symposium of the North American Society for Bat Research. San Juan, Puerto Rico, 24th - 27th October 2012: p 23 Luis F. Bacardi Bat Conservation Award, sponsored by the Lubee Bat Conservancy and Dissemination – support for attending conferences for young scientists of the University of Vienna

Herdina Anna Nele, Metscher Brian D., Gruber Daniela, Hahn David, Lederer Johann, Plenk Jr. Hanns. 2012. Poster: Combined microCT, LM and BSE-SEM evaluation of a trephine bone biopsy after human extraction-socket filling with Bio-Oss-Collagen®. In: Richards RG, Roomans G, Roughley PJ, editors. European Cells and Materials XIII: Bone Fixation, Repair & Regeneration (Focus CMF, Spine, Trauma, Vet), 24th - 26th June 2012. eCM 24, Supplement 1: p 48

Herdina Anna Nele, Plenk Jr. Hanns, Pokorny Petra, Hilgers Helge, Herzig-Straschil Barbara, Metscher Brian D. 2011. Talk: What to do with a penis bone: histological and x-ray microtomographical approaches to baculum variation, function, and ontogeny. In: Hutson AM, Lina PHC, editors. XIIth European Bat Research Symposium – Programme, Abstracts, List of Participants. Gamtos tyrimų cantras, Vilnius. Litauen, 22nd - 26th August 2011: p 28

Herdina Anna Nele, Pokorny Petra, Metscher Brian D., Herzig-Straschil Barbara, Hilgers Helge, Plenk Jr. Hanns. 2010. Talk: Histomorphologie und Funktion von Fledermaus-Penisknochen. 9. Tagung: Zellbiologie, Histologie und Embryologie, University of Veterinary Medicine Vienna, 10th - 11th December 2010

Herdina Anna Nele, Pokorny Petra, Metscher Brian D., Herzig-Straschil Barbara, Hilgers Helge, Plenk Jr. Hanns. 2010. Talk, invited speaker: Funktionelle Histomorphologie des Penisknochens von Fledermäusen. Herbsttagung 2010 of Arbeitskreis Fledermäuse Sachsen-Anhalt e.V. and Landesreferenzstelle für Fledermausschutz Sachsen-Anhalt, Schloß Mansfeld, 19th - 21st November 2010

Herdina Anna Nele. 2010. Talk, invited speaker: Determination von *Plecotus* Arten anhand des Penises. Herbsttagung 2010 of Arbeitskreis Fledermäuse Sachsen-Anhalt e.V. and Landesreferenzstelle für Fledermausschutz Sachsen-Anhalt, Schloß Mansfeld, 19th - 21st November 2010

Herdina Anna Nele, Plenk Jr. Hanns, Pokorny Petra, Hilgers Helge, Herzig-Straschil Barbara, Metscher Brian D. 2010. Poster: Looking at baculum function from a histomorphological perspective. In: Horáček I, Benda P, editors. 15th IBRC – the Conference

Manual: Programme, abstracts, list of participants. Volume of abstracts of the 15th International Bat Research Conference, held in Prague, 23rd - 27th August 2010, Symposium: Morphology. p 169
Student Award for the Best Poster Presentation

Herdina Anna Nele, Metscher Brian D., Plenk Jr Hanns, Herzig-Straschil Barbara, Pokorny Petra, Hilgers Helge. 2009. Non-destructive 3D imaging of microstructures in whole small vertebrates using microCT. In: Cremer S, Schrempf A, Heinze J, editors. Deutsche Zoologische Gesellschaft, 102. Annual Meeting, Regensburg, 25th - 28th September 2009. p 134