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수의학박사 학위논문

**Three-dimensional reconstruction of  
male reproductive organs of  
East Asian bats**

동아시아 박쥐류 수컷 생식기관의 삼차원적 재구성

2021 년 8 월

서울대학교 대학원

수의학과 수의해부학 전공

손 준 혁

**A Dissertation for the Degree of Doctor of Philosophy**

**Three-dimensional reconstruction of  
male reproductive organs of  
East Asian bats**

August 2021

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# Three-dimensional reconstruction of male reproductive organs of East Asian bats

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이 논문을 수의학박사 학위논문으로 제출함  
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손준혁의 수의학박사 학위논문을 인준함  
2021 년 7 월

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# **Three-dimensional reconstruction of male reproductive organs of East Asian bats**

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## **ABSTRACT**

The reproductive organs of mammals include paired testes, epididymides, deferent ducts, and accessory genital glands, and these structures have interspecies differences. There are approximately 1,400 species of bats distributed along the tropical to temperate latitudes. Consequently, bats have one of the most diverse patterns of gametogenesis, mating behavior, fertilization, and birth among mammals. Comparative studies on the reproductive organs of bats may provide

helpful information because they reflect the growth, behavior, and evolutionary patterns of bats. In this study, male reproductive organs of 25 species and five families of bats in East Asia were observed, and the relationship between the morphology of the reproductive organs and the phylogenetic classification was examined.

Conventionally, the anatomy of bat genital organs has been studied using gross and microscopic observations. Here, we employed both histological and diffusible iodine-based contrast-enhanced computed tomography (diceCT) observations to study the detailed three-dimensional morphological structure of the male genital organs in bats. This is the first study to describe the entire reproductive organs of bats in detail.

In Chapter 1, our highly resolved three-dimensional reconstruction revealed that the male organs of *R. ferrumequinum* consist of paired testes, epididymides, deferent ducts, and five accessory genital glands. *R. ferrumequinum* is a hibernating species distributed in the temperate regions from Europe to Japan. The locations of the testis (external) and elongated epididymal tail are hallmarks of hibernating bats. We confirmed that the accessory genital glands of this species are composed of ampullary, vesicular, prostate, urethral, and bulbourethral glands. The boundary between the ampullary and vesicular glands has been difficult to identify in previous observations, but our diceCT imaging clearly differentiated the two. The ampullary gland is located at the terminal part of the deferent ducts, and the

vesicular gland lies distal to the ampullary glands. The prostate gland is circular and single lobed. The bulbourethral gland is paired, oval shaped, and lies on the bulbospongiosus muscle. This species possesses a single carrot-shaped urethral gland, which is not found in most chiropteran families. The presence of the urethral gland in this species and its secretions suggests that after copulation, this species is capable of forming a vaginal plug, which can seal the female's vaginal orifice to block the entrance of spermatozoa from other males. Given the presence of the urethral gland, elongated epididymal tail, and the fact that some individuals can terminate their hibernation and reactivate, it is possible that forced copulation on hibernating females may occur in *R. ferrumequinum*.

In Chapter 2, the male reproductive organs of East Asian bats were observed, and the morphological characteristics were compared. Twenty-five species and five families of bats were classified based on phylogenetic analysis and their relationship with male reproductive organs was observed using diceCT. The morphology of the male reproductive organs of these bats is different between species. The reproductive organs comprise paired testes, epididymides, deferent ducts, and accessory genital glands. The shape and location of testes, attachment site of the head of the epididymis, and elongation of the tail of the epididymis are different among species. The accessory genital glands usually consist of paired ampullary, vesicular, bulbourethral, and single prostate glands. Furthermore, the urethral gland is found in the Rhinolophidae family. The location of the testes varies

between species. They may be external to the abdominal muscles or inside the scrotum. The epididymis is attached to the testis. However, there are four different types of attachment sites of the epididymal head and elongation of the epididymal tail. Since the testis and epididymis are essential for breeding, these differences may be influenced by distribution and hibernation. For example, the species with an elongated epididymal tail may be able to maintain reproductive organ activity even during hibernation. Family Rhinolophidae, Vespertilionidae, and Emballonuridae have well-distinguished ampullary-vesicular glands. In contrast, Megadermatidae and Hipposideridae do not. In Megadermatidae and Hipposideridae, the end of the deferent duct becomes thick and enters the prostate gland directly as ampullary glands. The prostate gland is a single lobe that surrounds the urethra. The bulbourethral glands are paired and located lateral to the rectum or dorsal to the bulbospongiosus muscle. The prostate and bulbourethral glands are present in all species. The urethral gland is present only in Rhinolophidae and it contributes to the formation of a vaginal plug in the female reproductive tract. The presence of the urethral gland may be related to inter-male competition, as males are known to temporarily awaken during hibernation and force females to mate with them. In this chapter, we analyzed the relationship between the morphology of the reproductive organs and the classification based on phylogenetic studies. Morphological characteristics were related to phylogenetic classification using microCT imaging.

Reproductive organs are a very important specimen because ecological and phylogenetic features are reflected in them. However, there have not been many studies. Understanding reproductive organs, which are important for reproduction, is helpful in determining the phylogenetic relationships and ecological features of various bat species. The composition or structure of reproductive organs is very important in the study of bats. This information is essential to understand growth and reproductive patterns. In conclusion, the morphology of reproductive organs in bats differs depending on the reproductive pattern by habitat, and these differences appear in the phylogenetic classification of bats. The urethral gland is present in all species of Rhinolophidae, but not in other families. The epididymal tail in all Hipposideridae is short, however, *Asselicus* is long. In Vespertilionidae, only *Scotophilus* has a short tail. It is conceivable that species with long tails may be able to maintain reproductive organ activity during the hibernation period. The results of this research contribute to our understanding of bats.

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**Keywords: Chiroptera, Evolution, Reproductive organ, Convergence, Homology, Phylogenetic**

**Student Number: 2013-31125**

# TABLE OF CONTENTS

<b>ABSTRACT</b> .....	i
<b>CONTENTS</b> .....	vi
<b>LIST OF FIGURES</b> .....	viii
<b>LIST OF TABLES</b> .....	x
<b>GENERAL INTRODUCTION</b>	
General Characteristics of Bats (Order Chiroptera) .....	1
Breeding Patterns of Bats .....	1
Characteristics of Male Reproductive Organs of Bats .....	5
Evolution in anatomical techniques .....	15
Purpose of This Thesis .....	16
<b>CHAPTER 1.</b>	
<b>Three-dimensional and histological observations on male genital organs of greater horseshoe bat, <i>Rhinolophus ferrumequinum</i></b>	
Introduction .....	18
Materials .....	20
Methods .....	21
Results .....	23
Discussion .....	36

**CHAPTER 2.**

**Three-dimensional reconstruction of male reproductive organs of East Asian bats**

Introduction .....	46
Materials .....	55
Methods .....	60
Results .....	61
Discussion .....	71
<b>GENERAL CONCLUSION</b> .....	84
<b>LITERATURES CITED</b> .....	87
<b>ABSTRACT IN KOREAN</b> .....	116
<b>SUPPLEMENT</b> .....	120
<b>ACKNOWLEDGEMENTS</b> .....	145

## LIST OF FIGURES

Figure 1. Macroscopic observations of the genital organs in male greater horseshoe bat after removing the skin, muscles and other organs.

Figure 2. Schematic depiction of the gross morphology of the testis and epididymis.

Figure 3. The genital organs of male greater horseshoe bat.

Figure 4. Three-dimensional reconstruction of the genital organs for histological observations.

Figure 5. Histological image of the urethral gland stained by hematoxylin and eosin.

Figure 6. Phylogenetic relationship of bats in this study.

Figure 7. The testicular and epididymal morphology of the bats.

Figure 8. Three-dimensional reconstruction of genital organs of male bats.

8-1. *Rhinolophus ferrumequinum*

8-2. *Rhinolophus chaseni*

8-3. *Rhinolophus cornutus*

8-4. *Rhinolophus luctus*

8-5. *Rhinolophus macrotis*

8-6. *Rhinolophus malayamus*

8-7. *Rhinolophus paradoxolophus*

8-8. *Rhinolophus pearsonii*

8-9. *Rhinolophus pusillus*

- 8-10. *Rhinolophus steno*
- 8-11. *Rhinolophus thomasi*
- 8-12. *Megaderma lyra*
- 8-13. *Megaderma spasma*
- 8-14. *Aselliscus dongbacana*
- 8-15. *Coelops frithii*
- 8-16. *Hipposideros armiger*
- 8-17. *Hipposideros pomona*
- 8-18. *Murina hilgendorfi*
- 8-19. *Myotis frater*
- 8-20. *Myotis macrodactylus*
- 8-21. *Myotis petax*
- 8-22. *Pletocus sacrimontis*
- 8-23. *Scotophilus kuhlii*
- 8-24. *Vespertilio sinensis*
- 8-25. *Taphozous longinamus*

## **LIST OF TABLES**

Table 1. Morphology of bat male genitals.

Table 2. List of specimens analyzed in this study.

Table 3. Morphology of bat reproductive organs.

# **General Introduction**

## **General Characteristics of Bats (Order Chiroptera)**

Bats (Order Chiroptera) are the second-largest group of mammals in the world, with approximately 1,400 species, and are distributed from tropical to temperate latitudes (Simmons and Cirranello 2018; Wilson and Mittermeier 2019). Chiroptera is traditionally classified into two suborders, Megachiroptera (megabats) and Microchiroptera (microbats), based on their morphological and behavioral characteristics (Dobson 1875; Smith 1976; Jones et al. 2002). However, molecular and morphological studies and fossil evidence revealed a relationship of Megachiroptera with the Rhinolophoidea superfamily, which includes families belonging to Microchiroptera (Jones and Teeling 2006). As a result, bats have been newly classified as Yinpterochiroptera and Yangochiroptera, and a new taxonomy was established merely a decade ago (Springer 2013). Although the classification of bats has been established from molecular genetic studies, differences in reproductive patterns and reproductive organs have not been clarified. Considering reproductive strategies, bats have high interspecific variation in their genital morphology, which reflects the ecological diversity. However, the information of reproductive organs in the bat is still unclear.

## **Breeding Patterns of Bats**

Bats show an annual breeding pattern and are affected by the season and weather of the area they inhabit. According to Happold and Happold (1990), the breeding seasons and patterns vary interspecifically depending on the weather of a particular season; thus, geographic factors are important for breeding. In female bats, there are three types of reproductive patterns, which can be divided into two hibernating patterns and a non-hibernating pattern. The hibernating bats that live in temperate regions are seasonally mono-estrous and have parturition once each year (Jerret 1979; Oxberry 1979; Mori et al. 1982; Racey 1982; Kimura and Uchida 1983; Son et al. 1987), and they demonstrate two types of reproductive patterns (Oxberry 1979). In the Type I pattern, the bats mate in early autumn and enter hibernation soon after. During hibernation, the sperm is stored in the female reproductive tract (oviduct, utero-tubal junction, and uterus; Racey 1979), and ovulation and fertilization occur after emergence from hibernation in the spring. Gestation occurs in early summer, and this pattern is called the “sperm storage” or “delayed fertilization” pattern. In contrast, the Type II pattern is called the “delayed implantation” pattern. Although the bats also mate in early autumn in the Type II pattern, ovulation, fertilization, and initial embryogenesis occur before hibernation. The embryo stays in the female reproductive tract and begins implantation at the end of hibernation (Racey 1982; Kimura and Uchida 1983).

Bat species that live in the tropical zone demonstrate three patterns including seasonally monoestrous, seasonally polyoestrous, and non-seasonally

polyoestrous (Jerret 1979). According to Happold and Happold (1990), these patterns are further subdivided into 10 types. Seasonally monoestrous species give birth to one litter per year, with some giving birth only in certain seasons, whereas others doing so at any time of the year. In seasonally polyestrous species, cases include having two litters per year, having a pregnancy immediately after giving birth, and not having a litter again for a certain period after giving birth. Bats that give birth in succession include those that repeat pregnancy and birth within a specific period and those that give birth and repeat pregnancy all year round. The females who are pregnant all year round have a gestation period of approximately 5 to 6 months. There are three patterns in the species that deliver more than three litters per year, including patterns in which pregnancy and parturition are repeated within a specific period or throughout the year and one that allows a certain period before the next pregnancy. In the case of the non-seasonal polyestrous pattern, pregnancy and parturition occur at any time of the year. This has been described in Molossidæ, which are distributed in Africa. Research on bats at the 0° to 36° latitudes, moving into the temperate zone as the distance from the equator increases, showed that the reproductive pattern changed accordingly.

Male spermatogenesis in bats, like in other mammals, is season- and latitude-dependent (Happold and Happold 1989; Vivier and van der Merwe 1996; Bernard and Cumming 1997) and is associated with female reproductive patterns (Kruttsch 1979; Bernard and Cumming 1997). At temperate latitudes,

spermatogenesis begins in summer or early autumn, and spermatogenesis terminates from the mating period to hibernation (Clarke 1981). There are three patterns, and the patterns are divided into three types, “*Pipistrellus* pattern,” “*Myotis* pattern,” and “*Miniopterus* pattern.” (Gustafson 1979; Krutzsch 1979). In the *Pipistrellus* pattern, Leydig cells show activation during hibernation, whereas they demonstrate low-level activity in the *Myotis* pattern. In the *Miniopterus* pattern, the activity of the Leydig cells disappears after the mating period and Leydig cells do not show activation during hibernation. The accessory reproductive glands also demonstrate differences among the three types. In the *Pipistrellus* and *Pipistrellus* patterns, the activity of the accessory reproductive gland starts from the end of spermatogenesis, and the activity is maintained during hibernation. However, in the *Miniopterus* pattern, the activity finishes before hibernation, and the accessory reproductive gland is not active during hibernation (Gustafson 1979; Martin and Bernard 2000).

In the tropical zone, bats adopt daily torpor during the coldest or driest months; during this period, spermatogenesis is interrupted and suppressed (Jolly and Blackshaw 1987). This is closely related to male and female reproductive cycles (Krutzsch 1979; Bernard and Cumming 1997). When females are monoestrous, males also undergo spermatogenesis once a year (e.g., *Pipistrellus nanus*, O’Shea 1980). In polyestrous species with two litters per year, males also show two peaks in spermatogenesis (e.g., *Tadarida condylura*, Vivier and van der

Merwe 1996). In females that have three or more litters, or non-seasonal reproduction, males display an extended period of spermatogenesis (e.g., *Myotis albescens*, Myers 1977) or experience spermatogenesis throughout the year (e.g., *Myotis nigricans*, Myers 1977; *Haplonycteris fischeri*, Heideman 1989).

### **Characteristics of Male Reproductive Organs of Bats**

The male reproductive organs of bats are as diverse as those of other mammals, and they are associated with bat reproductive patterns that have seasonal variations (Kruttsch 2000). In addition, reproductive patterns are determined by latitude, weather, temperature, and food availability (Happold and Happold 1990). Testicular regression has been shown to differ between hibernating and non-hibernating species (Gustafson 1979; Kruttsch 1979, 2000; Racey 1979; Crichton 2000). Spermatogenesis in the seminiferous epithelium in the testes and storage of sperm in the tail of the epididymis have been reported, and the periods and duration are different depending on the species (e.g., *Nycticeius schlieffenii*, Merwe and Rautenbach 1987; *Pteropus vampyrus*, Morigaki et al. 2001; *Rhinolophus cornutus*, Morigaki et al. 2001; Kurohmaru et al. 2002; *Miniopterus schreibersi fuliginosus*, Kang and Lee 2004; *Myotis macrodactylus*, Lee and Mōri 2004; *Pipistrellus kuhlii*, Sharifi et al. 2004; *Platyrrhinus lineatus*, Beguelini et al. 2011; *Molossus molossus*, Morais et al. 2012; *Artibeus planirostris*, Beguelini et al. 2013; *Myotis nigricans*, Beguelini et al. 2014; *Sturnira lilium*, Morais et al. 2013, 2014; *Rhinolophus*

*ferrumequinum korai*, Lee 2018). In addition, the volume of the accessory reproductive glands changes depending on the vicissitudes of the season. This is because the amount of secretion correlates with the reproductive cycle (Racey and Tam 1974; Bernard 1985; Krutzsch et al. 1992).

Krutzsch (2000) observed the gross anatomy and histology of male reproductive organs in bats and reported that the reproductive organs in bats show an interspecies diversity (Table 1); however, they follow the normal mammalian pattern including paired testes, epididymides, deferent ducts, and accessory reproductive glands (König and Liebich 2013, Singh 2017, Feldhamer et al. 2020). The location of the testis varies from being intra-abdominal (elephants, anteaters, whales, tree sloths, and sales) or extra-abdominal with scrotal sacs (primates, artiodactyls, perissodactyls, and carnivores) or without (bats) to migrating (bats, rodents, ferrets, and rabbits). The bats also show inter-familial and inter-species differences in location or shape of reproductive organs and they do not necessarily reflect the phylogenetic relationship.

The location of the testis is divided into four categories; permanently abdominal, permanently inguinal or scrotal, migratory and external. *Mystacina tuberculata* (Lloyd 2001) which distribute to New Zealand, the testis and epididymis are not visible from external in all around the year. Other than, in Rhinopomatidae (*Rhinopoma kinneari*, Anand Kumar 1965; *Rhinopoma hardwickei*, Karim and Banerjee 1989) and Molossidae (*Tadarida aegyptiaca*,

Bernard and Tsita 1995), the testes are found in the abdomen. In *Taphozous longimanus* (Krishna 1983), Nycteridae (*Nycteris hispida* and *Nycteris luteola*, Matthews 1942) and Vespertilionidae (*Eptesicus furinalis*, *Histiotus velatus*, *Lasiurus blossevillii*, *Myotis albescens* and *Myotis nigricans*, Beguelini et al. 2012) show permanently scrotal testis, and also the epididymis is enclosed by the scrotum. Some bat species show testes migration from the inside to the outside of the abdomen (migratory), depending on the season (Krutzsch 1955; Kitchener 1980; Crichton and Krutzsch 1987). In *Taphozous hilli* (Kitchener 1980), the testes migrate from the abdomen to the external inguinal ring, and in *Mormopterus planiceps* (Crichton and Krutzsch 1987) and *Tadarida brasiliensis* (Krutzsch 1955) the testes migrate to the inguinal canal. The testicular position of *Taphozous georgianus* (Jolly and Blackshaw 1988) change from abdominal to the scrotal pouch. In addition, when both testes are located in the abdomen, their position is similar. However, if one testis descended to the scrotal pouch, the other one is in the inguinal canal or the abdominal entrance of the inguinal canal. Within this migratory type, spermatogenesis may vary depending on the location of the testes. In *Tadarida hindei*, spermatogenesis only occurs when the testes are outside the abdomen (Marshall and Corbet 1959), whereas in *Tadarida condylurus*, spermatogenesis can occur both inside and outside the abdomen (Mutere 1973). External testes are the most common in bats. This type can be further divided into three patterns, according to the testes location relative to the penis: lateral to the

penis (e.g., *Natalus stramineus*, Broadbooks 1961; Mitchell 1965, and *Rhinolophus megaphyllus*, Krutzsch et al. 1992), outside the external inguinal ring (e.g., *Thyroptera tricolor* and *Myzopoda aurita*, Krutzsch 2000), and inside the testicular pouch (e.g., *Macrotus waterhousii*, Krutzsch et al. 1976). The testis which locates external in the abdomen is shown between the skin and muscle. Under the skin, the testis is covered by tunica albuginea and this tunica allows the movement of testis under the skin. *Artibeus planirostris* (Beguelini et al. 2012) presents no scrotum, however, tunica vaginalis which covered testis and epididymis function in testicular movement between abdominal muscle and skin. The epididymis is divided into three parts: the head, body, and tail. After sperm is produced in the testis, maturation, concentration, protection, and storage of sperm is conducted in the epididymis (Beu et al. 2009; Robaire and Hinton 2015; Berton 2019; Sullivan et al. 2019). Spermatogenesis can take place during a specific season (*Mormopterus planiceps*, Crichton and Krutzsch 1987) or throughout the year (e.g., *Rhinolophus capensis*, Bernard 1985, 1986 and *Taphozous georgianus*, Jolly and Blackshaw 1987). In some hibernating species (e.g., *Pipistrellus pipistrellus* and *Nyctalus noctula*), androgenesis and functionality of the accessory genital glands are maintained to preserve the spermatozoa in their epididymal tail during hibernation (Racey and Tam 1974; Racey 1974; Racey 1979).

The accessory reproductive glands of bat comprise the ampullary, vesicular, prostate, bulbourethral glands, urethral or litter glands and para-anal

glands are present in specific species. The ampullary glands and vesicular glands produce substances (fructose, citric acid, sialic acid, and zinc) that are essential for ejaculation (Rajalaksmi and Prasad 1970; Krutzsch et al. 1976; Mokkapati and Dominic 1976). The ampullary glands are present in most bat species without *Carollia perspicillata*, *Glossophaga soricina* and *Phyllostomus discolor* (Martins et al. 2016). The vesicular glands are absent in *Myzopoda aurita* (Krutzsch 2000), *Thyroptera tricolor* (Krutzsch 2000), *Hipposideros fulvus* (Patil, 1968), *Scotophilus heathi* (Krishna and Singh 1997) and *Rhinolophus capensis* (Bernard 1985). In mammals, the ampullary glands are present in all domestic species except the boar, and the vesicular glands are common in humans, stallions, bulls, and rats but are absent in carnivores. The prostate gland shows a single pattern and it present in all bat species, and it is the same as humans and dogs; however, rodents have a multilobed pattern. This gland is divided into three parts including the dorsal, dorsolateral, and ventral lobes (e.g., Mongolian gerbil, Rochel et al. 2007). The secretion of the vesicular glands (e.g., *Pteropus poliocephalus*, Martin et al. 1995) and urethral gland (e.g., *Rhinolophus ferrumequinum*, Matthews 1937; *Rhinolophus hipposideros*, Gaisler and Titlbach 1964; Gaisler 1966; *Rhinolophus ferrumequinum nippon*, Oh et al. 1983; Uchida 1987; and *Rhinolophus ferrumequinum korai*, Lee 2019) forms a vaginal plug. In some mammals, the coagulating glands form copulatory plugs (Matthews 1942; Eadie 1948; Hartung and Dewsbury 1978; Voss 1979; Baumgardner et al. 1982; Oh et al. 1983; Breed

1990; Stockley 1997; Dixson 1998). The bulbourethral (Cowper's) glands are present on almost all bats without *Pteronotus parnellii* (Krutzsch 2000), and are generally paired, however, double-paired in the genus *Corynorhinus* and *Plecotus* (Krutzsch 2000). The para-anal glands have been reported in *Mormopterus planiceps* (Krutzsch and Crichton 1987), and locate on the lateral of the anal orifice, however, their function is unknown.

Table 1. Morphology of bat male genitals

	Ampullary and vesicular glands	Number of Prostate lobes	Urethral gland	Morphology of bulbourethral gland	Testes location	Reference
<i>Artibeus lituratus</i>	absent	2	present as paraurethral glands	rounded to teardrop-shaped		Santos et al. 2018
<i>Artibeus planirostris</i>	absent	2	present as paraurethral glands	teardrop-shaped		Puga et al. 2012
<i>Cardioderma cor</i>			present			Matthews 1942
<i>Carollia perspicillata</i>	absent	3	present as paraurethral glands	teardrop-shaped		Martins et al. 2015
<i>Desmodus rotundus</i>	absent	2	present as paraurethral glands	teardrop		Martins et al. 2015
<i>Glossophaga soricina</i>	absent	3	present as paraurethral glands	teardrop		Martins et al. 2015
<i>Hipposideros caffer</i>		1	present			Martins et al. 2016
<i>Hipposideros fulvus</i>	only possess ampullary glands	1				Martins et al. 2015
<i>Hipposideros speoris</i>	only possess ampullary glands	1	present	cubic	scrotal	Menzies 1973
<i>Macrotus waterhousii</i>	clearly differentiated	1	absent	punching-bag-shaped	external	Patil 1968
<i>Miniopterus schreibersii</i>	clearly differentiated	1	absent	spherical or pear-shaped	external	Pal 1983
						Krutzsch et al. 1976
						Krutzsch and Crichton 1990

Table 1. continued

<i>Molossus molossus</i>	absent	2	present as paraurethral glands	teardrop-shaped		Christante et al. 2015
<i>Mormopterus planiceps</i>	clearly differentiated	1	absent	rounded	migratory	Crichton and Krutzsch 1987
<i>Myotis nigricans</i>	absent	3	absent			Negrin et al. 2014
<i>Myzopoda aurita</i>					external	Krutzsch 2000
<i>Natalus stramineus</i>					external	Broadbooks 1961; Mitchell 1965
<i>Noctilio albiventris</i>	absent	2	absent	teardrop-shaped		Beguelini et al. 2016
<i>Nycteris hispida</i>					scrotal	Matthews 1942
<i>Nycteris luteola</i>					scrotal	Matthews 1942
<i>Phyllostomus discolor</i>	absent	3	present as paraurethral glands	teardrop-shaped		Martins et al. 2015 Martins et al. 2016
<i>Pipistrellus dorreri</i>	only possess vesicular gland					Gadegone and Sapkal 1983
<i>Platyrrhinus lineatus</i>	absent	2	present as paraurethral glands	teardrop-shaped		Martins et al. 2015
<i>Rhinolophus capensis</i>	only possess ampullary glands	1	present		external	Bernard 1985
<i>Rhinolophus ferrumequinum</i>	clearly differentiated	1	present	oval	scrotal	

Table 1. continued

<i>Rhinolophus hipposideros</i>	clearly differentiated	1	present	teardrop-shaped		Gaisler 1966
<i>Rhinolophus megaphyllus</i>	clearly differentiated	1	present	oval	external	Krutzsch et al. 1992
<i>Rhinopoma hardwickei</i>					abdominal	Karim and Banerjee 1989
<i>Rhinopoma kinneari</i>	only possess ampullary glands	1	absent		abdominal	Anand Kumar 1965
<i>Rhynchonycteris naso</i>	absent	2	absent	teardrop-shaped		Beguelini et al. 2016
<i>Scotophilus heathii</i>	only possess ampullary gland	1	absent			Krishna and Singh 1997
<i>Sturnira erythromos</i>	absent	2	absent	teardrop-shaped		Miotti et al. 2018
<i>Sturnira lilium</i>	absent	2	absent	teardrop-shaped		Miotti et al. 2018
<i>Sturnira oporaphilum</i>	absent	2	absent	teardrop-shaped		Miotti et al. 2018
<i>Tadarida aegyptiaca</i>	clearly differentiated	1	present		abdominal	Bernard and Tsita 1995
<i>Tadarida Brasiliensis</i>	indistinguishable	1	absent		migratory	Krutzsch et al. 2002
<i>Tadarida mexicana</i>						
<i>Tadarida condylurs</i>					migratory	Mutere 1973
<i>Tadarida hindei</i>					migratory	Marshall and Corbet 1959
<i>Taphozous georgianus</i>	indistinguishable	1	absent			Jolly and Blackshaw 1987

Table 1. continued

<i>Taphozous hilli</i>						migratory	Kitchener 1980
<i>Taphozous longimans</i>	clearly differentiated	1	absent	teardrop-shaped		scrotal	Krishna 1983
<i>Thyroptera tricolor</i>	only possess ampullary glands					external	Krutzsch 2000

## **Evolution in anatomical techniques**

The use of microcomputed tomography (microCT) imaging in recent anatomical studies has demonstrated features that have not been clarified previously. Conventional studies of the anatomy of bat reproductive organs have been performed through gross and microscopic observation. However, although many details are obtained with these techniques, the detailed three-dimensional structure is poorly understood. In particular, it is difficult to clearly distinguish the boundaries of the glands using conventional methods. Therefore, microCT images are used to improve upon the previous techniques and reveal detailed structures. Furthermore, Gignac and Kley (2014) and Gignac et al. (2016) proposed that the bony structure and soft tissues could be studied in detail using the method of enhanced contrast through iodine staining (DiceCT). The advantage of DiceCT is that it can be used to identify soft tissue, such as muscle fiber direction, that could not be clearly observed with CT imaging. Furthermore, since both hard and soft tissue can be observed at the same time, the relationship between the tissues becomes confirmable by measuring the length and volume. In addition, once stained with iodine, the tissue can be restored to its original state with the use of sodium thiosulfate (Schmidbaur et al. 2015). This allows further observation macroscopically or microscopically. In bats, the method has been applied to the penis and baculum (Herdina et al. 2010, 2015a, 2015b), jaw muscle (Santana 2018), vascularization (Mrzilkova 2020), noseleaf (Gao 2019), skulls (Camacho 2019;

Giacomini 2019; Hedrick 2019), nasophonation (Curtis et al. 2019), prenatal cranial bone development (Nojiri et al. 2018, 2021), nasal turbinals (Ito et al. 2021), cochlea (Nojiri et al. 2021), and male reproductive organs (Sohn et al. 2021).

### **Purpose of This Thesis**

We present the reproductive organs of male bats in detail and compared these among the five Chiroptera families which are belonged to the suborder Yangochiroptera and Yinpterochiroptera in this study. At first, we clarify the basic anatomical information of genital organs by using conventional methods (macroscopic and microscopic observation). After this, we employ diceCT using iodine and microCT to demonstrate the detailed three-dimensional structure of the male soft-tissue genital organs of the bat for the first time. In chapter 1, we use *R. ferrumequinum* only, but in chapter 2, we use 25 species of bats which are classified by phylogenetic analysis. These characteristics will provide information on the relationship between reproductive organ patterns and reproductive strategies. It is expected that our study will show new results owing to the adoption of analytical approaches different from those relying on external measurement, cranial measurement, and phylogenetic analysis.

## **CHAPTER 1.**

**Three-dimensional and histological observations  
on male genital organs of greater horseshoe bat,**

***Rhinolophus ferrumequinum***

## INTRODUCTION

The greater horseshoe bat, *Rhinolophus ferrumequinum* belongs to the genus *Rhinolopus*, family Rhinolophidae, suborder Yinpterochiroptera, and is distributed across the Palearctic region from Europe to Japan (Csorba et al. 2003). They have a polygenous mating system, and parturition occurs in summer (Kuramoto 1977; Mori et al. 1982; Rossiter et al. 2000; Sano 2001). Mating occurs in the following autumn (Courrier 1927; Oxberry 1979; Oh et al. 1985a). After mating, the spermatozoa are stored in the female reproductive organs - oviduct, utero-tubal junction, or uterus - through the winter without fertilization (Gustafson 1979; Racey 1979; Mori et al. 1982). Fertilization occurs after they terminate their hibernation (delayed fertilization) (Matthews 1937). Although *Rhinolophus* is a large specious group in bats, the anatomy of its male genital organs, which is highly diverse, is still poorly described. As noted earlier, *Rhinolophus* possesses a urethral gland, which is not found in other bats (Krutzsich 2000). *Rhinolophus hipposideros* (Gaisler 1966) and *R. megaphyllus* (Krutzsich et al. 1992) have ampullary, vesicular, prostate, urethral and bulbourethral glands, while *R. capensis* is equipped only with ampullary glands (Bernard 1985). In the case of *R. landeri*, testes are reported to reach maximum size in the winter and shrink in the summer (Menzies 1973). Regarding *R. ferrumequinum*, only brief descriptions by Krutzsich (2000) are available, who reported on the structures of the testis and epididymis, although the

anatomy of the accessory genital glands was not described in detail. Given these, we present the detailed three-dimensional structure of the male soft-tissue genital organs of bats for the first time, with special reference to the accessory genital glands, and compared those with other bat species to provide insights into the phylogenetic and ecological patterns of the genital organs.

## **MATERIALS**

We used the genital organs of a fully mature, male great horseshoe bat, obtained on 29 June 2018 in Kiyotsu-kyo, Tokamachi city, Niigata Prefecture, Japan, under the capture permit and ethical approval from the Tokamachi City Government (Permission number 10KAN-63-1-3).

## **METHODS**

### **Gross Anatomy**

The bat was euthanatized by isoflurane overdose. The organs were fixed with 10% formaldehyde for 48 h, transferred to 70% ethanol, and then stained with 1%iodine in ethanol for 14 days before scanning (diceCT). The specimen was first observed macroscopically, and then scanned using a microCT system (inspeXio SMX-90CT Plus, Shimadzu Corp., Kyoto, Japan) with a 90kv source voltage and 100 mA source current; and the voxel size of the images was 34  $\mu\text{m}$ . We reconstructed the serial images of male genital organs using the Amira 5.2 software (Visage Imaging, San Diego, USA). The reconstructed 3D surface STL model generated and analyzed during the current study is available in the MorphoMuseum repository (<https://doi.org/10.18563/journal.m3.113>). All other datasets analyzed during the current study are available from the corresponding author on request.

### **Macroscopic Anatomy**

After scanning the genital organs, the sample was used for histological observation. The tissue sample of the genitals was dehydrated with a graded series of ethanol (70–100%), cleared in xylene, and embedded in paraffin. The paraffinembedded tissue were serially divided into 3- $\mu\text{m}$ -thick sections using a microtome (Reichert-Jung 2040, Leica Corp., Watzlar, Germany), and placed on

glass slides (Microscope Slides #1000612, Marienfeld Corp., Lauda-Königshofen, Germany). Deparaffinized sections were stained with hematoxylin and eosin (HE). After staining, the sections were dehydrated in graded series of ethanol, cleared in xylene, and covered with cover glass. Images of all genital organs were captured using a light microscope (BX51, Olympus Corp., Tokyo, Japan) equipped with a digital camera (DP71, Olympus Corp., Tokyo, Japan) connected to a computer.

## RESULTS

### Gross Anatomy

Using macroscopic observation, we confirmed that the male genital organs of *R. ferrumequinum* comprise five accessory genital glands, paired testes, epididymides, and deferent ducts (Fig. 1). The testis, epididymis, and part of the deferent duct were covered by the scrotum. The testes were located outside the abdomen. The epididymis was separated into three parts - head, body, and tail - which were attached to different regions of the testis. The head part starts on the middle region and medial surface of the testis, and continues to the body part as the epididymal duct, which runs to the tail along the medial surface of the testis. The tail of the epididymis showed a caudal elongation and a recurrently ascending turn into the testis (Fig. 2). Finally, the epididymal duct from the tail part continues as the deferent duct. The accessory genital glands included the ampullary, vesicular, prostate, urethral, and bulbourethral glands. The ampullary glands showed a thickening of the terminal part of the deferent duct, forming the ampulla of the deferent duct. They exhibited cone-shaped bodies, which were located dorsal to the urinary bladder. The vesicular glands lie distal to the ampullary glands, and were presented in a pair, rounded, and bilaterally symmetric in shape. The prostate gland was located dorsodistally to the complex of the ampullary-vesicular glands, surrounding the urethra and continuing to the urethral gland. The urethral gland

was carrot-shaped and encircling the urethra. The bulbourethral glands were located dorsal to the urethral gland, bulbospongiosus muscle, and both sides of the rectum.

The 3D model reconstructed from diceCT images (Fig. 3) clarified, with precision, the shape, location, and the internal structure of the genital organs. The urethra started from the urinary bladder, and the deferent ducts were joined at the prostate gland. After joining, the duct passed through the inside the urethral gland and reached the tip of the penis. In the penis, the corpus cavernosum formed the greater part of its inside, and the terminal of the urethra was capped by a small, bony ossicle - the os penis. The end of the corpus cavernosum and the origin of os penis were connected.

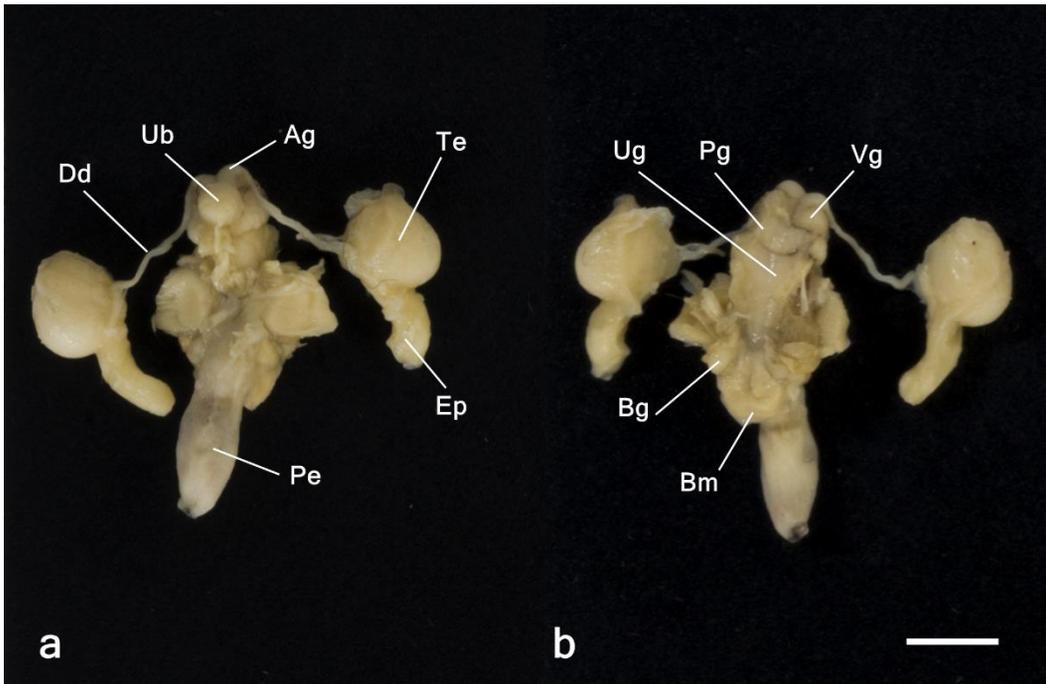


Fig. 1 Macroscopic observations of the genital organs in male greater horseshoe bat after removing the skin, muscles and other organs. **a** dorsal view. **b** ventral view. Ag, ampullary glands; Bg, bulbourethral glands; Bm, bulbospongiosus muscle; Dd, deferent duct; Ep, epididymis; Pe, penis; Pg, prostate gland; Te, testis; Ub, urinary bladder; Ug, urethral gland; Vg, vesicular glands. Scale bar: 1 cm.

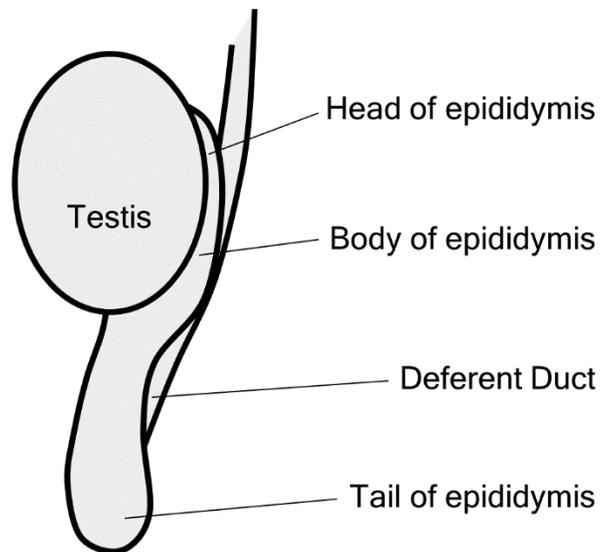
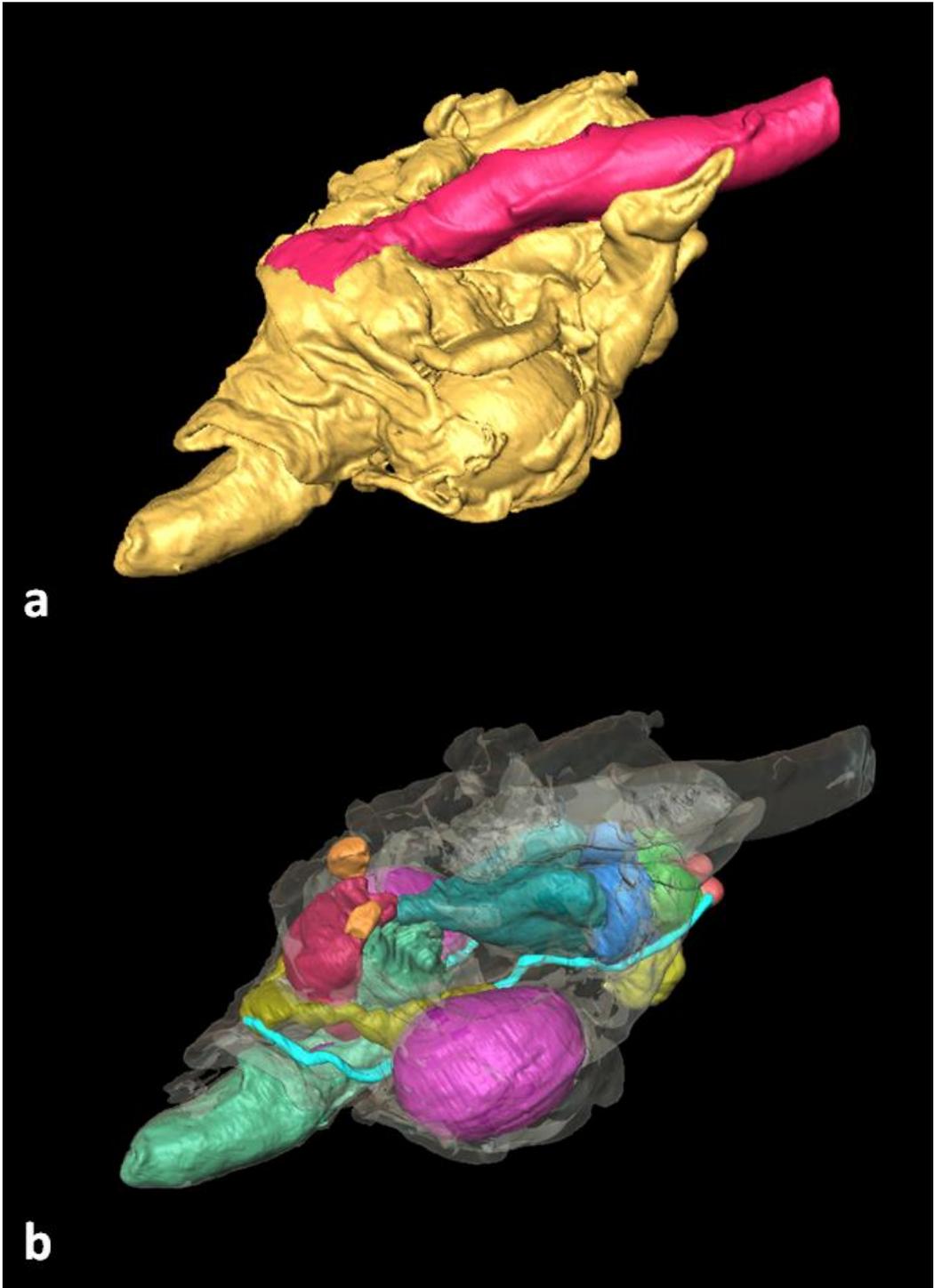


Fig. 2 Schematic depiction of the gross morphology of the testis and epididymis. The tail of the epididymis showed a caudal elongation and a recurrently ascending turn into the testis. The epididymal duct from the tail part continues as the deferent duct.



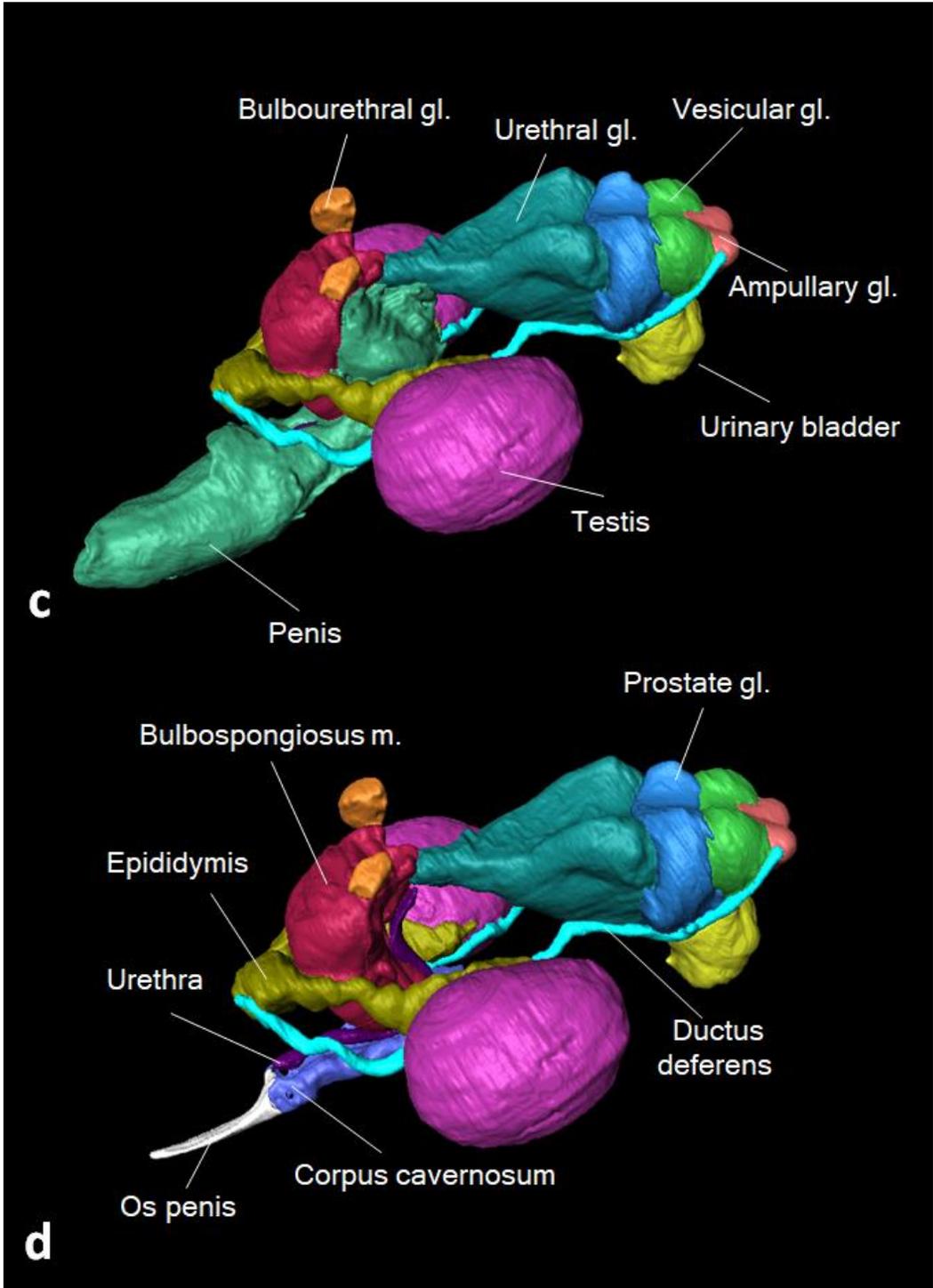


Fig. 3 The genital organs of male greater horseshoe bat. **a** Three-dimensional reconstruction, positions of organs in the pelvic region before removing of the skin, muscle, and other organs. **b** Position of genital organs, all organs are marked in different colors (skin, muscle and other organs removed). **c** Dorsolateral view and **d** dorsal view with penis removed. The *os penis*, urethra, and corpus cavernosum are located inside the penis.

## **Histological observations**

Histological observations of the male reproductive organs were conducted on six locations (the lines of a-g), as shown in Fig. 4. The terminal part of the deferent duct continued to the ampullary gland. The deferent duct was composed of a thick muscular wall. The acini of the ampullary glands were lined with pseudostratified epithelial cells with tall or round nuclei (Fig. 4b). The vesicular glands had an outer layer of connective tissue and consisted of multiple acini lined with the cuboidal epithelium with round nuclei (Fig. 4c). The prostate gland was lined with pseudostratified epithelium, which can be categorized as transitional epithelium. The prostatic urethra running inside the prostate gland was also lined with pseudostratified epithelium. The prostate gland and prostatic urethra were surrounded by fibromuscular tissue, acting as a support frame (Fig. 4d). The outer layer of the urethral gland was surrounded by a muscular capsule and the acini were lined with simple columnar epithelium without the fibromuscular tissue in the interstitial space (Fig. 4e). The outer layer of the bulbourethral gland consisted of striated muscular tissue (Fig. 4f). The acini were lined with tall columnar epithelial cells with rounded or pyramidal-shaped nuclei, located on the basement membrane. Luminal secretion was observed in all glands except the urethral gland. The penis was divided into two sections: the body and the penis glans. The corpus cavernosum was found in the body part and os penis was situated in the penis glans. The terminal part of the corpus cavernosum was connected to the os penis (Fig. 4g)

and the corpus cavernosum was composed of smooth muscles and elastic fibers, both of which were surrounded by the tunica albuginea. The paraurethral glands (glands of Littre) were not found in this species (Fig. 5).

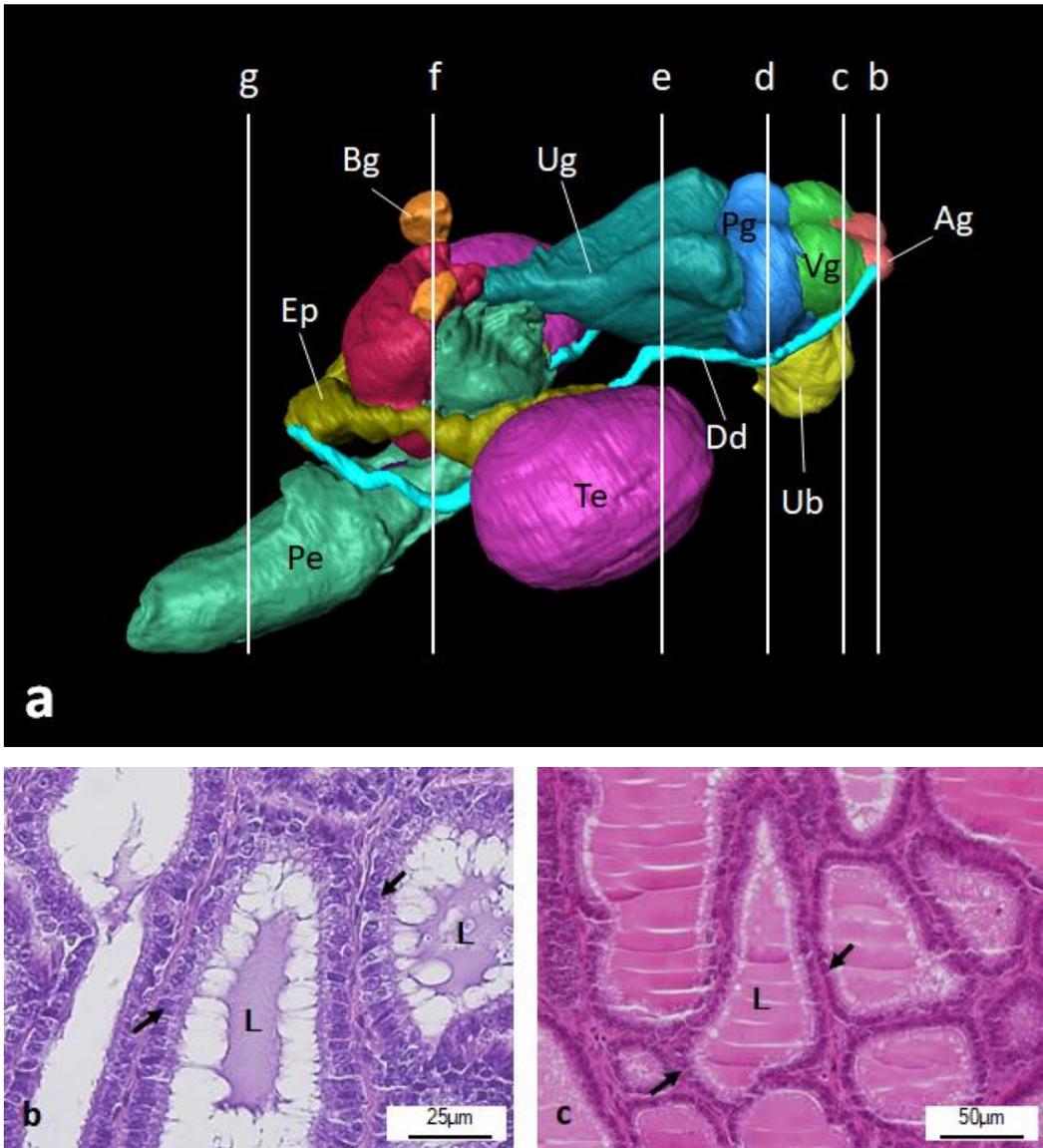


Fig. 4 Three-dimensional reconstruction of the genital organs for histological observations. **a** Histological observations were conducted at six locations. **b** Ampullary gland. The acinus was lined with pseudostratified epithelium (arrows). **c** Vesicular glands. The acinus was lined with simple columnar epithelium (arrows).

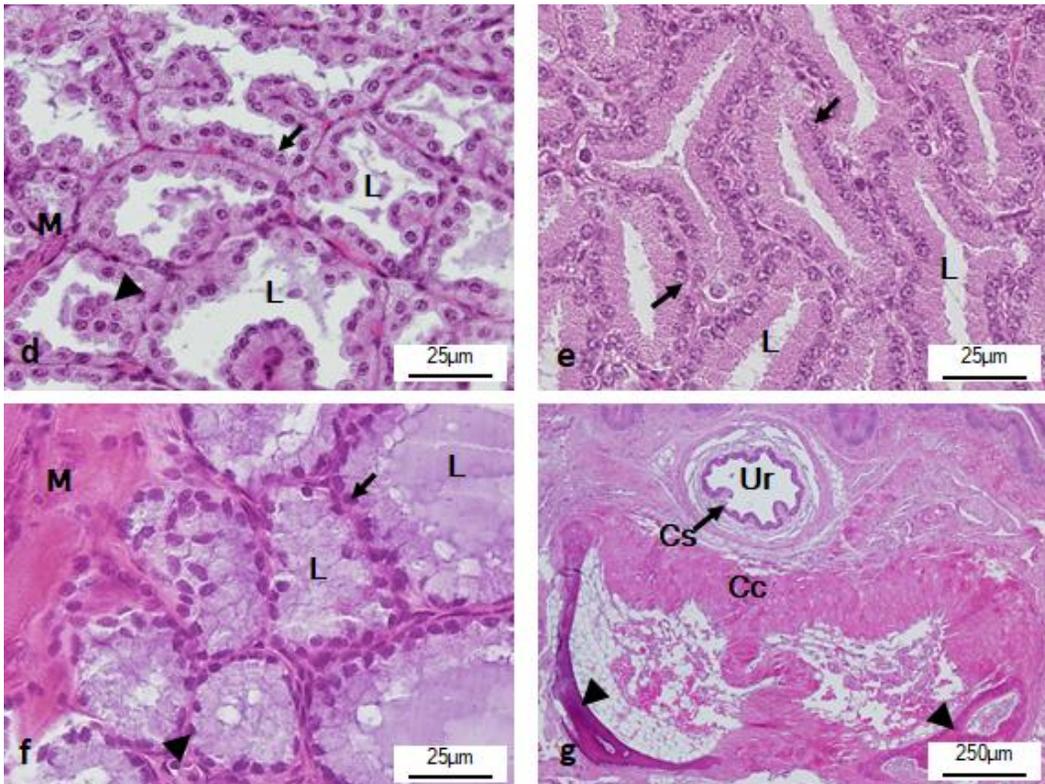


Fig. 4 continued. **d** Prostate gland. The acinus was lined by stratified (arrow) or pseudostratified (arrowhead) columnar epithelium. The fibromuscular tissue (M) was present as supporting connective tissue. **e** Urethral gland. The acinus was lined with simple columnar epithelium (arrows). **f** Bulbourethral glands. The striated muscle (M) surrounded the gland. The epithelium of acinus was lined with tall columnar cells with round (arrow) or pyramid-shaped (arrowhead) nuclei on the base. The luminal secretions (L) were present in all glands, except the urethral gland. **g** Penis. The terminal part of the corpus cavernosum (Cc) was connected to the *os penis* (arrowheads). The urethra (Ur) lied above the corpus cavernosum and lined with transitional epithelium. Ag, ampullary glands; Bg, bulbourethral glands;

Cc, corpus cavernosum; Dd, deferent duct; Ep, epididymis; Pe, penis; Pg, prostate gland; Te, testis; Ub, urinary bladder; Ug, urethral gland; Ur, urethra; Vg, vesicular glands.

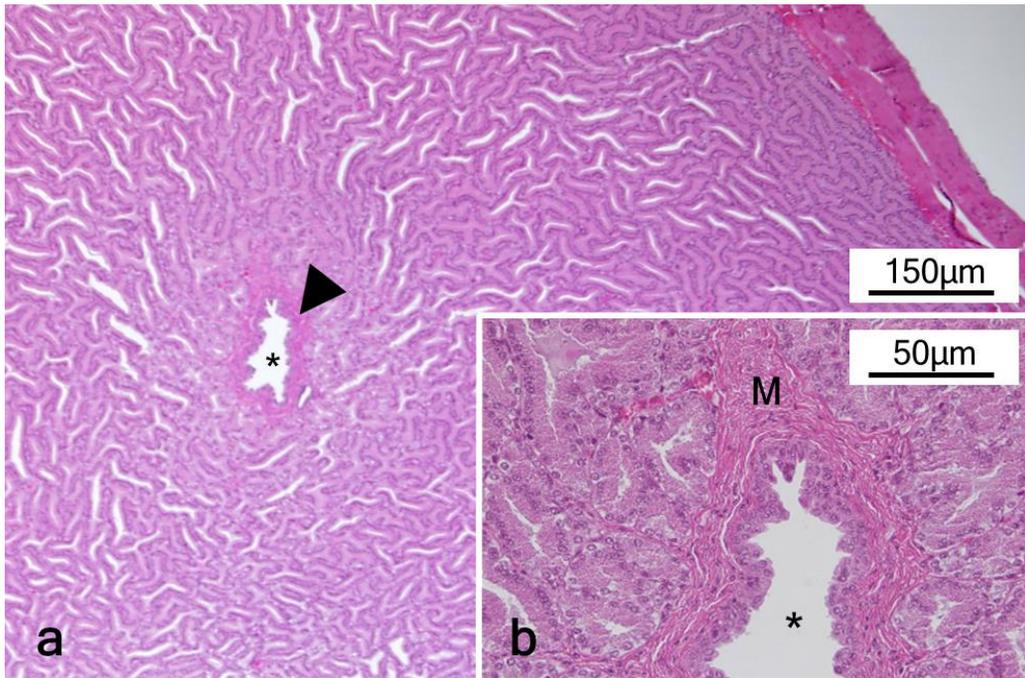


Fig. 5 Histological image of the urethral gland stained by hematoxylin and eosin.

**a** The uretra (arrow) passes through the inside the urethral gland. **b** A layer of muscular tissue (M) surrounds the lumen of urethra (\*), but the glands of Littré appears to be lacking in *R. ferrumequinum*.

## DISCUSSION

We found that the male genital organs of *R. ferrumequinum* are composed of the paired testes, epididymides, deferent ducts and accessory genital glands, consistent with the cases reported for *R. hipposideros* (Gaisler 1966) and *R. megaphyllus* (Krutzschnig et al. 1992). From the root to the shaft of the penis, the corpus cavernosum lies dorsal to the urethra. In the corpus cavernosum, there are blood vessels, smooth muscles, and elastic fibers responsible for erection. On the other hand, we found that the corpus spongiosum, which is one of the erection-related organs, is lacking in *R. ferrumequinum*. The *os penis* is found inside the penis glans. Its epithelium of the urethra is lined by transitional epithelium, suggesting its capability of detention and contraction (Eurell and Frappier 2006).

### Testes and epididymis

Among the four types (permanently abdominal, permanently scrotal, migratory and external), we confirmed that the testis's location of *R. ferrumequinum* can be classified as the external type, the most common pattern found in bats. Its testes are located lateral to the penis, and the testes are found under the skin, covering the inguinal part. The location of the testes influences spermatogenesis, as it is affected by body temperature and external temperature (Jolly and Blackshaw 1988). In hibernating species with testes permanently located

within the abdominal cavity (e.g. *R. hardwickei* Karim and Banerjee 1989; *Rhinopoma kinneari* Anand Kumar 1965), spermatogenesis is more active in the winter, when hibernation takes place. On the contrary, in hibernating species with testes located outside the abdominal cavity (external type), spermatogenesis does not occur during hibernation periods (Lee 2018). Among *Rhinolophus*, the activation of spermatogenesis is reported to occur during non-hibernating period in *R. cornutus* (August and October: Kurohmaru et al. 2002) and *R. capensis* (October to November: Bernard 1985). The external location of the testes in *R. ferrumequinum* implies that spermatogenesis does not occur during hibernation periods. Consistent with this prediction, it was reported that spermatogenesis takes place only during active seasons and does not occur during hibernation in Korean (Lee 2018) and Japanese populations (Oh et al. 1985b).

The tail part of the epididymis of *R. ferrumequinum* is elongated as in *R. capensis* (Bernard 1985). It is known that the tail of the epididymis is elongated in hibernating bats, such as *Pipistrellus kuhlii* (Sharifi et al. 2004), *Myotis daubentonii* (Encarnação et al. 2004), and *Neoromicia nanus* (van der Merwe and Stirnemann 2007). In addition, the tail of the epididymis is elongated in non-hibernating species, as seen in Emballonuridae (*Rhynchonycteris naso*) and Vespertilionidae (*Eptesicus furinalis*, *Histiotus velatus*, *Lasiurus blossevilli*, *Myotis albescens*, and *Myotis nigricans*) (Beguelini et al. 2012). According to Beguelini et

al. (2012), bat species with smaller testis tend to have an elongated epididymis tail, while those with larger testis are likely to show relatively shorter epididymis tails. Elongation of the tail part of the epididymis is, arguably, to be related to increased sperm storage (Beguelini et al. 2012).

### **The accessory reproductive glands of greater horseshoe bat**

The accessory genital glands of this species consist of the ampullary, vesicular, prostate, urethral and bulbourethral glands. One of the accessory glands, the ampullary glands, is the terminal part of the deferent duct, continuing to the vesicular glands. Previous studies on other bat species, such as *Taphozous georgianus* (Jolly and Blackshaw 1987), *Tadarida brasiliensis mexicana* (Kruttsch et al. 2002), *Carollia perspicillata*, *Glossophaga soricina*, and *Phyllostomus discolor* (Martins et al. 2016), point out that the ampullary and vesicular glands are indistinguishable by conventional macroscopic observations, and detailed descriptions have been lacking. However, our microCT imaging allowed us to clearly identify and differentiate the two. Additionally, the epithelium of these two glands appears to be clearly differentiated. The epithelium of the ampullary gland is lined with pseudostratified epithelium, which consists of tall columnar cells with ovoid nuclei and cuboidal cells with round nuclei. The apical surface of epithelial cells shows protrusions, suggesting that a secretion-related role (Eurell and Frappier 2006). The acini of vesicular glands are lined with simple cuboidal cells

and the lumen is filled with secretions. The vesicular gland secretion generally functions as an energy source for stored spermatozoa (Eurell and Frappier 2006).

The prostate gland is located dorsodistally to the complex of the ampullary-vesicular glands, continuing to the urethral gland. In other species, the prostate is separated into either two (*Artibeus planirostris* Puga et al. 2012; *Molossus molossus* Christante et al. 2015; *Desmodus rotundus* and *Platyrrhinus lineatus* Martins et al. 2015; *Noctilio albiventris* and *Rhynchonycteris naso* Beguelini et al. 2016; *Sturnira erythromos*, *Sturnira lilium* and *Sturnira oporaphilum* Miotti et al. 2018; *Artibeus lituratus* Santos et al. 2018) or three portions (*Myotis nigricans* Negrin et al. 2014; *Carollia perspicillata*, *Glossophaga soricina* and *Phyllostomus discolor* Martins et al. 2016). In contrast, we found that this gland constitutes a single structure in *R. ferrumequinum* (Fig. 3). The prostate was circular, and surrounding the urethra. Generally, in mammals, the prostate gland is single lobed, and its secretion is serous or seromucous (Eurell and Frappier 2006). The role of this secretion is, primarily, to activate ejaculated spermatozoa and neutralize the seminal plasma (Eurell and Frappier 2006), although the functional significance of lobe numbers is unclear and should be addressed in future research.

We confirmed that, in *R. ferrumequinum*, the bulbourethral glands are

located dorsal to the urethral gland and bulbospongiosus muscle, and bilaterally to the rectum. The bulbourethral glands consist of smooth and striated muscle fibers and the epithelium, which is lined with tall columnar cells. The ability of luminal secretion was confirmed in the bulbourethral glands, although the type of secretions was undetectable in this study. While the shape of bulbourethral glands can vary from punching bag (e.g. *Macrotus waterhousii* Krutzsch et al. 1976), to oval (e.g. *R. megaphyllus* Krutzsch et al. 1992), or teardrop-shaped (e.g. *Molossus molossus* Christante et al. 2015; *Artibeus lituratus* Santos et al. 2018), in *R. ferrumequinum*, it was oval. Most bats including, *R. ferrumequinum*, exhibit one pair of bulbourethral glands, except for *Corynorhinus rafinesquii*, which possesses two pairs of this gland (Pearson et al. 1952). According to Krutzsch (2000), the bulbourethral glands are generally located bilaterally to the rectum, anus, or near the base of the penis. In *R. ferrumequinum*, the duct of the bulbourethral glands is found on the bulbospongiosus muscle and is connected to the urethra, which is beneath this muscle. We confirmed that such topology is virtually similar to that of *R. megaphyllus* (Krutzsch et al. 1992).

The urethral gland in this species is a well-developed, carrot-shaped single structure, surrounded by muscular capsules. The epithelium of the gland is simple columnar epithelium; however, no glandular luminal secretion is found. Among bats, the urethral gland is reportedly found only in Rhinolophidae

(*Hippoideros caffer* Matthews 1942; *R. hipposideros* Gaisler 1966; *R. capensis* Bernard 1985; *R. megaphyllus* Krutzsch et al. 1992) and Megadermatidae (*Cardioderma cor* Matthews 1942), all of which belong to Yinpterochiroptera. It has been reported that, generally, the prostate gland is divided into multiple lobes, and the urethra that penetrates the prostate gland is surrounded by the paraurethral glands (e.g., *Desmodus rotundus*, *Platyrrhinus lineatus*, *Carollia perspicillata*, *Glossophaga soricina* and *Phyllostomus discolor* Martins et al. 2015; *Artibeus planirostris* Puga et al. 2012; *Molossus molossus* Christante et al. 2015). Although the paraurethral glands are also (occasionally) referred to as glands of Littre, or “urethral gland” (Beguelini et al. 2016), this should not be confused with the urethral gland described here. While the paraurethral glands lie in the mucosal and muscular wall stroma of the urethra, and are located between the prostate and prostatic urethra, the urethral gland is located outside the prostate gland (Krutzsch 2000), as seen in *R. ferrumequinum*. We confirmed that *R. ferrumequinum* does not possess the paraurethral glands (Fig. 5c). Phylogenetic distribution of the urethral and paraurethral glands is still largely unknown and requires further research.

The size of accessory genital glands, including the urethral gland, varies depending on the season, because the amount of secretions in the lumen corresponds to the reproductive cycle (Racey and Tam 1974; Bernard 1985; Krutzsch et al. 1992). The urethral gland of the *R. hipposideros* is non-functional

for about five months, around spring and summer (Gaisler 1966). In this species, a volume increase is observed from late summer onwards, peaking in autumn. During the winter, the gland enters a resting period and its secretion is arrested, making the lumen practically empty. It must be pointed out that the secretion from this gland forms a vaginal plug that seals the vaginal orifice, preventing sperm loss and blocking the entrance of sperm from other males (Matthews 1937; Gaisler and Titlbach 1964; Gaisler 1966; Oh et al. 1983; Uchida 1987; Lee in press). Similar plugs are known to exist in other mammals, such as rodents, eulipotyphlans, and marsupials (Ausitín and Short 1972; Rochel et al. 2007; Martin et al. 2011). It has been reported that *R. ferrumequinum* can interrupt hibernation (Sano 2001; Kim et al. 2019). In the Japanese *R. cornutus* species, evidence of forced mating during hibernation periods has been recently reported, and the functional significance of vaginal plug for deterring other males during hibernation period has been pointed out (Sato 2019). Thus, the urethral gland's capability of forming a firm vaginal plug is highly important for successful reproduction (Rossiter et al. 2000a). Whether *R. ferrumequinum* also engages in forced mating during hibernation is yet to be studied; however, the elongated epididymal tail in *R. ferrumequinum* suggests that an extended storage of spermatozoa during hibernation period takes place, and supports the possibility of such behavior in this species. Evaluating the occurrence of secretion in the accessory genital glands during the hibernation period is required, in order to confirm the possibility of extended storage of active spermatozoa, amid

hibernation and forced mating.

In this study, we performed the first microCT study of the soft-tissue male reproductive organs in bats, providing the first detailed three-dimensional description of the whole structure of the male genital in bats, using *R. ferrumequinum*. Until recently, observing and describing the intact accurate structure and composition of accessory genital glands was difficult, due to technical limitations. By using microCT imaging, we successfully described the detailed anatomy of the accessory genital glands in *R. ferrumequinum*. We concluded that the male reproductive organs of *R. ferrumequinum* comprise paired testes, epididymides, deferent ducts, and five accessory genital glands. The testes were located external to the abdomen and lateral to the penis, which is the hallmark of hibernating species. The epididymis is attached to the testis, and its tail part showed a caudal elongation with a characteristic turnback to the testis. The ampullary gland is located at the terminal part of the deferent ducts, and the vesicular gland lies distal to the ampullary glands. These two glands have been indistinguishable by conventional macroscopic observations, but our microCT imaging allowed us to identify their boundaries and confirm their anatomical differentiation. The presence of the urethral gland and its secretions strongly supports that this species is capable of forming a vaginal plug. Elongation of the tail part of the epididymis suggests increased sperm storage. Given that some individuals are known to halt their

hibernation and awaken, it is possible that forced copulation on hibernating females may occur. Studies on secretion status of the vesicular and prostate glands during hibernation period are required to test this hypothesis.

## **CHAPTER 2.**

### **Three-dimensional reconstruction of male reproductive organs of East Asian bats**

## INTRODUCTION

Bats (order Chiroptera) are the second-largest group of mammals, with approximately 1,400 species distributed over tropical to temperate latitudes (Simmons and Cirranello 2018; Wilson and Mittermeier 2019). Classification of this order has been based on morphology and behavior (Dobson 1875; Smith 1976; Jones et al. 2002), however, classification has changed significantly in the last decade (Jones and Teeling 2006; Springer 2013). Bats have been newly classified as Yinpterochiroptera and Yangochiroptera by molecular and morphological studies, as well as fossil evidence. Although bat classification has been established from molecular genetic studies, interspecific differences in reproductive patterns and reproductive organs have not been clarified. Considering reproductive strategies, bats have high interspecific variation in genital morphology, reflecting their ecological diversity. However, our understanding of reproductive organs in the bat is still incomplete.

Mammals have many types of reproductive patterns, with wide variation in reproductive organs morphology (Weir and Rowlands 1973, Kunz et al. 1996). The interspecific differences in reproductive patterns among mammals are manifested in fertilization, gestation, and parturition. After coitus, fertilization occurs and the fertilized eggs are implanted. However, each mammal has a unique reproductive strategy (Orr and Zuk 2014). In bats, the family Rhinolophidae and

Vespertilionidae, found in temperate zones, exhibit sperm storage, also called delayed fertilization or delayed ovulation (Wimsatt 1945; Oxberry 1979; Racey 1982; Bernard and Cumming 1997, Orr and Zuk 2013). Mating occurs in the fall before hibernation. After mating, the sperm is stored in the female reproductive tract, oviduct (Krutzsch et al., 1982; Mori et al., 1982, Racey et al., 1987), uterotubal junction (Uchida and Mori 1974; Mori and Uchina 1980), or uterus (Uchida et al. 1984) during hibernation; however, fertilization is blocked since ovulation has not occurred. In spring, after complete hibernation, females ovulated, leading to fertilization and implantation with offspring are born in early summer when the food availability is better (Racey, 1972, 1978; Speakman and Racey, 1986). Delayed fertilization is common in hibernating bats, living in temperate zones. Tropical bats do not hibernate, but also store sperm. This leads to birth at the time of the year when food is most available (Jones, 1972; McWilliam, 1987c, 1988a, b; Racey 1982). In delayed implantation, after mating, ovulation, and fertilization occur, the zygote remains in the female reproductive tract and only begins implantation at the end of hibernation (Aitken 1977 Although delayed fertilization/ovulation is only found in bat species, delayed implantation is common in bears, otters, armadillos, seals, and roe deer (Sandell 1990). According to Sandell (1990), delayed implantation occurs when the mother is lactating and the external environment seems to be less favorable for pregnancy and parenting. Interspecific differences have been observed in gestation periods in mice (20 days), bears (215

days), humans (270 days), and elephants (655 days) (Sacher and Staffeldt, 1974). Differences have also been observed in the interval of parturition and numbers or size of offspring. Parturition is related to the estrous cycle; monoestrous mammals can give birth once per year, which can be further classified as restricted seasonal, extended seasonal, or aseasonal. Polyestrous species also follow these three patterns but can give birth several times per year.

Breeding patterns in bats can be divided into hibernating and non-hibernating types. Hibernating species are distributed throughout temperate zones, with reproductive patterns appearing in the spermatogenesis cycle, decreasing in the fall and restarting in the spring (Racey and Tam, 1974; Racey, 1974b; Gustafson, 1979; Krutzsch and Crichton, 1987). Male and female reproductive patterns correspond, with reproductive cycles influenced by climate and food abundance (Krutzsch 1979; McWilliam, 1987; Bernard and Cumming, 1997). In tropical species, spermatozoa are produced during periods of rainfall and high food availability, with spermatogenesis peaks reflecting those of rainfall (Jones, 1972; McWilliam, 1987c, 1988a, b; Mutere, 1973a; McWilliam, 1987c). This suggests that food availability is an important factor in the male reproductive cycle. In addition, tropical species enter daily torpor during the coldest or driest periods, with spermatogenesis suppressed or interrupted (Jolly and Blackshaw, 1987).

The bat male reproductive organs have been described by Krutzsch (2000). They are not much different from those of common mammals (Thomson and

Marker 2006, König and Liebich 2013, Singh 2017, Feldhamer et al. 2020). They include paired testes, epididymides, deferent ducts, and accessory genital glands. Because of the large number of bat species, species differences abound. Differences in testes are location based. As reported by Krutzsch (2000), there are four major types: permanently abdominal, permanently inguinal or scrotal, migratory, and external. These differences are based on habitat, season, and breeding patterns. In the case of permanently abdominal species, the testes are located in the abdominal cavity and attached to the kidneys by suspensory ligaments (for example, *Rhinopoma kinneari*, Anand Kumar 1965; *R. hardwickei*, Karim and Banerjee 1989; *Tadarida aegyptiaca*, Bernard and Tsita 1995; and *Mystacina tuberculata*, Lloyd 2001). In permanently inguinal or scrotal species, the testes are permanently located in the scrotum, or in the inguinal region in species without a scrotum. The families Emballonuridae (*Taphozous longimanus*, Krishna 1983), Nycteridae (*Nycteris hispida* and *Nycteris luteola*, Matthews 1942), and Vespertilionidae (*Eptesicus furinalis*, *Histiotus velatus*, *Lasiurus blossevillii*, *Myotis albescens*, and *Myotis nigricans*, Beguelini et al. 2012) have permanently scrotal testes. Migratory testes move between the abdominal cavity and scrotum through the inguinal canal (*Tadarida brasiliensis mexicana*, Krutzsch 1955a and *Mormopterus planiceps*, Krutzsch and Crichton 1987). They are also found in the non-pigmented fascial sac formed by the extension of the external oblique muscle fascia external to the external inguinal ring (*Taphozous hilli*, Kitchener 1980, 1983 and *T. georgianus*,

Jolly and Blackshaw 1988). In species with external testes, they are located beside the penis (*Natalus stramineus*, Broadbooks 1961; Mitchell 1965, and *Rhinolophus megaphyllus*, Krutzsch et al. 1992), outside the inguinal ring (*Thyroptera tricolor* and *Myzopoda aurita*, Krutzsch 2000), and in the non-pigmented sac of the external spermatic fascia (e.g., *Macrotus waterhousii*, Krutzsch et al. 1976).

The epididymis is divided into three parts: head, body, and tail. After sperm is produced in the testis, its maturation, concentration, protection, and storage occur in the epididymis (Beu et al. 2009; Robaire and Hinton 2015; Berton 2019; Sullivan et al. 2019). Spermatogenesis can occur during a specific season (*Mormopterus planiceps*, Crichton and Krutzsch 1987) or throughout the year (*Rhinolophus capensis*, Bernard 1985 1986 and *Taphozous georgianus*, Jolly and Blackshaw 1987). In some hibernating species (e.g., *Pipistrellus pipistrellus* and *Nyctalus noctula*), androgenesis and function of the accessory genital glands are maintained to preserve the spermatozoa in the epididymal tail during hibernation (Racey and Tam 1974; Racey 1979).

The accessory reproductive glands of bats include the ampullary, vesicular, prostate, bulbourethral, and urethral or Littre glands; para-anal glands are present in some species. The ampullary and vesicular glands produce substances (fructose, citric acid, sialic acid, and zinc) essential for ejaculation (Rajalaksmi and Prasad 1970; Krutzsch et al. 1976; Mokkapati and Dominic 1976). In mammals, the ampullary glands are present in all domestic species except the boar; the vesicular

glands are common in humans, stallions, bulls, and rats, but are absent in carnivores (Setchell and Breed 2006; Prins and Lindgren 2015; Singh 2017). In bats, ampullary glands are present in most species, except for *Carollia perspicillata*, *Glossophaga soricina*, and *Phyllostomus discolor* (Martins et al. 2016). The vesicular glands are absent in *Myzopoda aurita* (Krutzschn 2000), *Thyroptera tricolor* (Krutzschn 2000), *Hipposideros fulvus* (Patil 1968), *Scotophilus heathi* (Krishna and Singh 1997), and *Rhinolophus capensis* (Bernard 1985). The prostate gland is consistently present in all bat species examined to date, as well as in humans and dogs; in rodents, however, the prostate is multilobed pattern (Prins and Lindgren 2015). This gland is divided into three lobes: dorsal, dorsolateral, and ventral (as in the Mongolian gerbil, Rochel et al. 2007). The bulbourethral (also known as Cowper's) glands are present on almost all bats except *Pteronotus parnellii* (Krutzschn 2000), and are generally paired; however, they are double-paired in the genera *Corynorhinus* and *Plecotus* (Krutzschn 2000). Para-anal glands have been reported in *Mormopterus planiceps* (Krutzschn and Crichton 1987) and are located on the lateral side of the anal orifice; however, their function is unknown. Secretions of the vesicular (e.g., *Pteropus poliocephalus*, Martin et al. 1995) and urethral (e.g., *Rhinolophus ferrumequinum*, Matthews 1937; *Rhinolophus hipposideros*, Gaisler and Titlbach 1964; Gaisler 1966) glands in bats form a vaginal plug that stores the sperm. In addition, a vaginal plug can be formed by semen (*Miniopterus schreibersii fuliginosus*, Mōri and Uchida 1981) or

keratinization of the vaginal epithelium (*Chalinolobus gouldii*, Kitchener 1975; *Scotophilus heathi*, Krishna and Dominic 1978). Many other mammals have vaginal plugs that work in the same way, blocking the vagina and preventing sperm leakage and mating with other males (Matthews 1942; Eadie 1948; Hartung and Dewsbury 1978; Voss 1979; Baumgardner et al. 1982; Breed 1990; Stockley 1997; Dixon 1998).

Previous phylogenetic classifications primarily focused on flight morphology (Thomas and Suthers 1972; Bullen and McKenzie 2001), the possession of a complex laryngeal echolocation system (Simmons 1998), and reproductive organ morphology (Krutzsch 1979; Barclay 1994). These studies were sufficient to study the physiology and ecology of bats but failed to yield unambiguous phylogenies. Therefore, phylogenetic relationships between 916 extant species and nine recently extinct bat species have been estimated (Jones et al. 2002). This study has since become a useful tool for phylogenetic comparisons and macroevolution studies. Currently, DNA sequencing is the main approach, and as a result, the superfamily Rhinolophoidea, which was previously classified as Microchiroptera, has been shown to belong to the suborder Megachiroptera. This led to a major change in the classification of bats (Jones and Teeling 2006). This has led to a major change in classification (Jones and Teeling, 2006), with two suborders: Yangochiroptera and Yinpterochiroptera (Springer 2013).

In this chapter, we describe our use of diffusible iodine-based contrast-

enhanced computed tomography (diceCT) to study the detailed three-dimensional morphological structure of the bat male genitalia, using 25 species belonging to five families of East Asian bats. The species used in this study have already been extensively investigated through DNA analysis and classified. The family Megadermatidae has two classifications: one is based on mitochondrial DNA (Kaňuch et al. 2015) and the other is based on hyoid morphology (Griffiths 1992). The family Hipposideridae has recently been classified using molecular studies as well as individual measurements, with an increasing number of member species (Thong et al. 2012; Tu et al. 2015). In addition, the morphologies of frontal sacs and noseleaves are also being compared (Foley et al 2017). As Rhinolophidae has a large number of species, there have been studies employing through molecular biology and skull measurements to identify regional species previously thought to be monospecific (Volleth et al. 2015; Ith et al. 2016; Soisook et al. 2016; Liu et al. 2019; Demos et al. 2019). Other studies have classified species based on DNA collected from museum samples (Bailey et al. 2016). Vespertilionidae has been reconstructed using DNA sequences and morphological analysis of skull shape (Eger et al. 2018; Tu et al. 2018) and rhinaria (Hutterer et al. 2019), which resulted in the identification of new species. Emballonuridae has been studied using a combination of morphology and genetics (Lim et al. 2010). Interspecific comparisons of reproduction analyze reproductive patterns and morphologies of the os penis; however, no other reproductive organs have been examined. This is

the first study to describe and compare the entire reproductive system of multiple bat species. Our analysis of genital morphologies provides insights into the phylogenetic and ecological patterns of the genital organs.

## MATERIALS

In this study, 25 species of bats were fully matured and healthy specimens without disease and they were performed by using DiceCT (Table 2). The classification by phylogenetic relationship is demonstrated in Figure 6. Two Megadermatidae (*Megaderma spasma*, *M. lyra*; hereafter megadermatid), four Hipposideridae (*Aselliscus dongbacana*, *Coelops frithii*, *Hipposideros armiger*, *H. pomona*; hereafter hipposiderid), eleven Rhinolophidae (*Rhinolophus ferrumequinum*, *R. luctus*, *R. thomasi*, *R. pearsonii*, *R. malayanus*, *R. megaphyllus*, *R. stheno*, *R. macrotis*, *R. paradoxolophus*, *R. cornutus*, *R. pusillus*; hereafter rhinolophid), seven Vespertilionidae (*Murina hilgendorfi*, *Myotis frater*, *M. macrodactylus*, *M. petax*, *Plecotus sacrimontis*, *Vespertilio sinensis*, *Scotophilus kuhlii*; hereafter vespertilionid) and Emballonuridae (*Taphozous longimanus*; hereafter emballonurid) (Figure 6). The bat samples used in this study belong to the curatorial collections of the Institute of Ecology and Biological Resources of Vietnamese Academy of Science and Technology (IEBR), The University of Tokyo Hokkaido Forest (UTHF), and the National Museum of Nature and Science, University Museum of University of Tokyo (UMUT) and Kanagawa Prefectural Museum of Natural History (KPM).

Table 2. List of specimens analyzed in this study

Family	Species	Location	Storage	ID
Megadermatidae	<i>Megaderma lyra</i>	Nui Chua, Ninh Thuan, Viet Nam	IEBR	VN-17-535
	<i>Megaderma spasma</i>	Nui Chua, Ninh Thuan, Viet Nam	IEBR	VN17-371
Hipposideridae	<i>Aselliscus dongbacana</i>	Trung Khanh, Cao Bang, Viet Nam	IEBR	VTTu15-013
	<i>Hipposideros armiger</i>	Pu Luong, Thanh Hoa, Viet Nam	IEBR	VN11-0490
	<i>Hipposideros pomona</i>	Nui Chua, Ninh Thuan, Viet Nam	IEBR	VN17-364
	<i>Coelops frithii</i>	Son Tra, Da Nang, Viet Nam	IEBR	VN19-196
Rhinolophidae	<i>Rhinolophus chaseni</i>	Tuy Phong, Binh Thuan, Viet Nam	IEBR	VN15-055
	<i>Rhinolophus cornutus</i>	Unknown, Japan	NMNS	Kahaku23082
	<i>Rhinolophus ferrumequinum</i>	Niigata, Japan	UMUT	JP18-006
	<i>Rhinolophus luctus</i>	Mae Taw, Pyin Oo Lwin, Myanmar	UTHF	B-19
	<i>Rhinolophus macrotis</i>	Copia, Son La, Viet Nam	IEBR	VN11-0089

Table 2. continued

	<i>Rhinolophus malayanus</i>	Ngoc Lac, Thanh Hoa, Viet Nam	IEBR	VN11-0413
	<i>Rhinolophus paradoxolophus</i>	Ngoc Lac, Thanh Hoa, Viet Nam	IEBR	VN11-0442
	<i>Rhinolophus pearsonii</i>	Trung Khanh, Cao Bang, Viet Nam	IEBR	VTTu15-014
	<i>Rhinolophus pusillus</i>	Trung Khanh, Cao Bang, Viet Nam	IEBR	VTTu15-005
	<i>Rhinolophus stheno</i>	Ba Vi, Ha Noi, Viet Nam	IEBR	VN11-0178
	<i>Rhinolophus thomasi</i>	Copia, Son La, Viet Nam	IEBR	VN11-0058
Vespertilionidae	<i>Murina hilgendorfi</i>	Wakayama, Japan	UTHF	FK13-07
	<i>Myotis frater</i>	Hokkaido, Japan	UTHF	FK17-24
	<i>Myotis macrodactylus</i>	Kagoshima, Japan	UTHF	FK09-01
	<i>Myotis petax</i>	Hokkaido, Japan	UTHF	FK15-84
	<i>Pletocus sacrimontis</i>	Hokkaido, Japan	UTHF	FK15-61

Table 2. continued

	<i>Scotophilus kuhlii</i>	Pyinmana, Nay Pyi Taw, Myanmar	UTHF	B-03
	<i>Vespertilio sinensis</i>	Kanagawa, Japan	KPM	KPM3993
Emballonuridae	<i>Taphozous longimanus</i>	Ywar Taw, Nay Pyi Taw, Myanmar	UTHF	B-10

Note: The abbreviations are as follows: IEBR, The Institute of Ecology and Biological Resources of Vietnamese Academy of Science and Technology; KPM, Kanagawa Prefectural Museum of Natural History; NMNS; National Museum of Nature and Science; UMUT, The University Museum of University of Tokyo; UTHF, The University of Tokyo Hokkaido Forest.

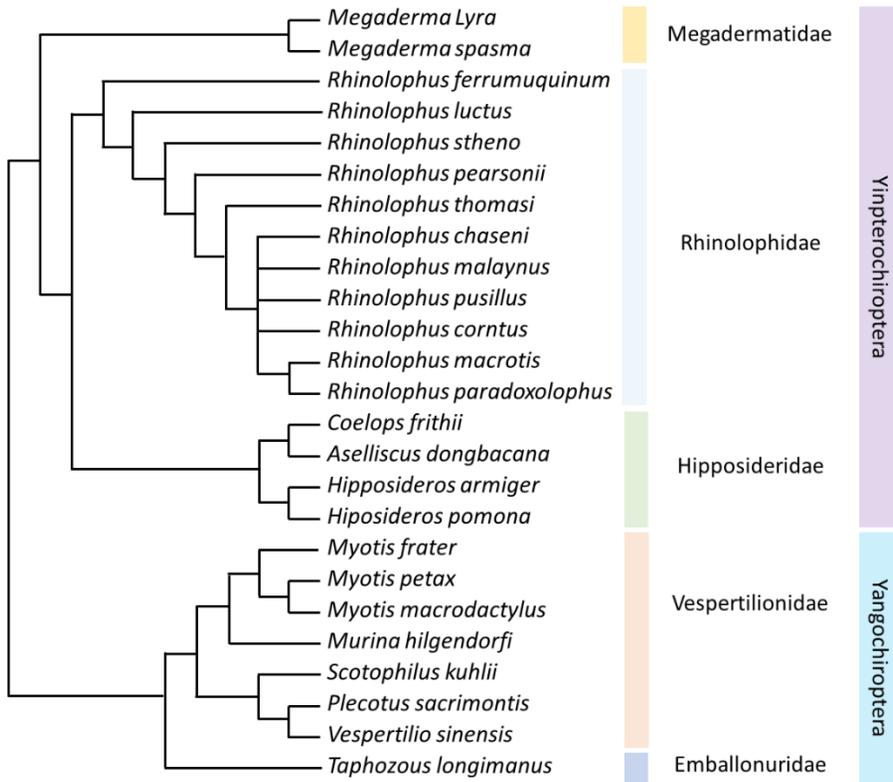


Fig. 6. Phylogenetic relationship of bats species used in this study. The phylogenetic framework is based on Upham et al. (2019).

## METHODS

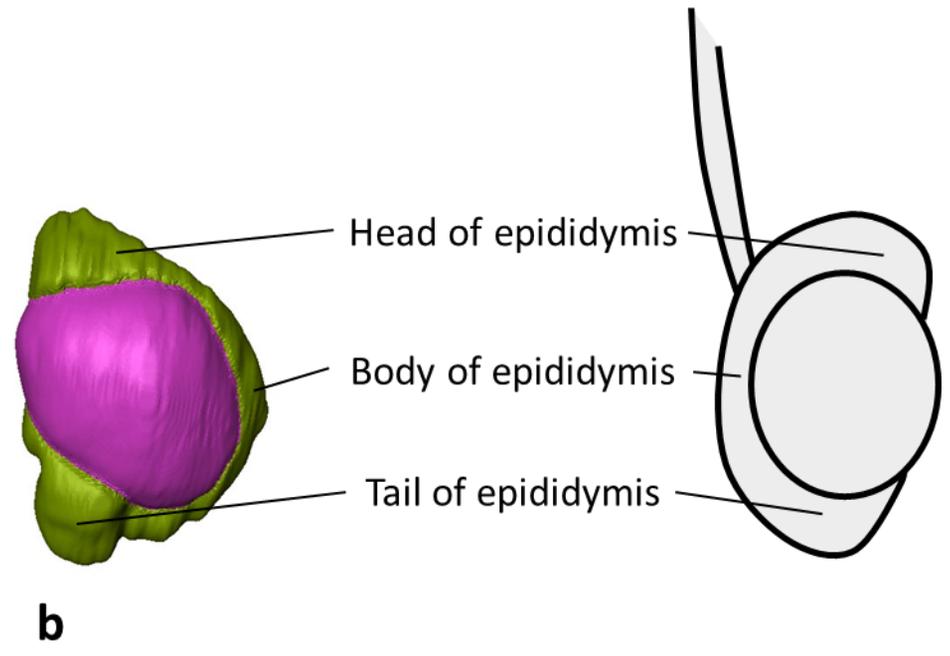
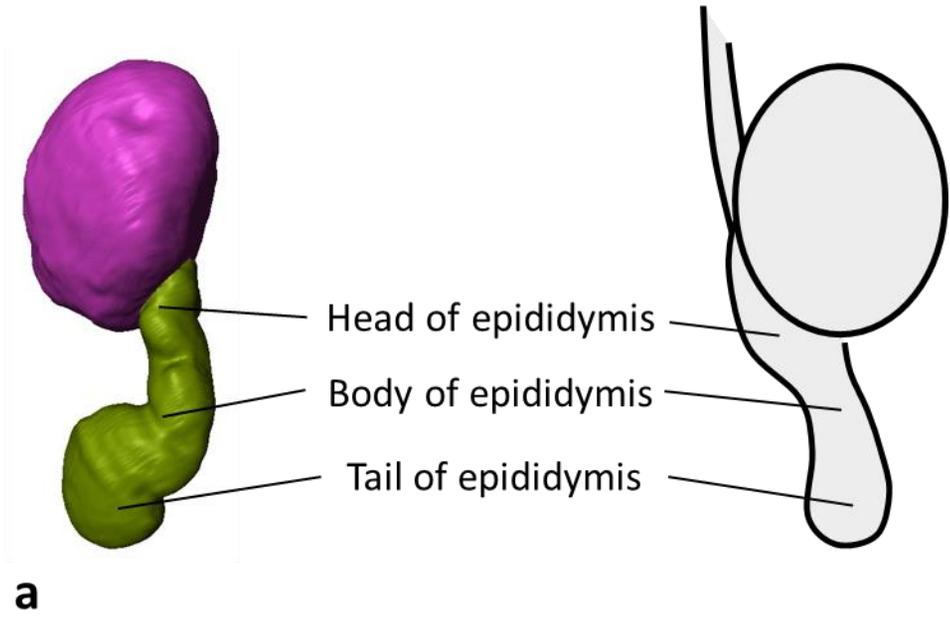
The specimens for diceCT (diffusible iodine-based contrast-enhanced computed tomography) were fixed with 10 % formaldehyde, transformed to 70% ethanol, and then stained with 1% iodine in ethanol for 14 days before scanning (Gignac et al. 2016)). The whole body of all specimens was scanned using a microCT system (system inspeXio SMX-90CT plus, Shimadzu Corporation, Tokyo) with a 90kv source voltage and 100mA source current. After that, we reconstructed the male reproductive organs including testes, epididymis, deferent duct, accessory genital glands by manual segmentation. Three-dimensional reconstructions and all measurements were conducted in Amira 5.2 software (Visage Imaging, San Diego, USA). This iodine staining technique is reversible and allows specimens once stained with iodine to be restored to their original state using sodium thiosulfate (Schmidbaur et al. 2015). With this reusability, gross anatomical observation through dissection, microscopic observation after staining the tissues and three-dimensional observation using microCT images become possible (Sohn et al. 2021). After staining with iodine, CT imaging shows the soft tissue clearly, and this allows to be clearly distinguished and observe the soft and hard (skeletal) tissue that composed a single specimen, and to investigate the relationship. This gives a surprising amount of information from one specimen.

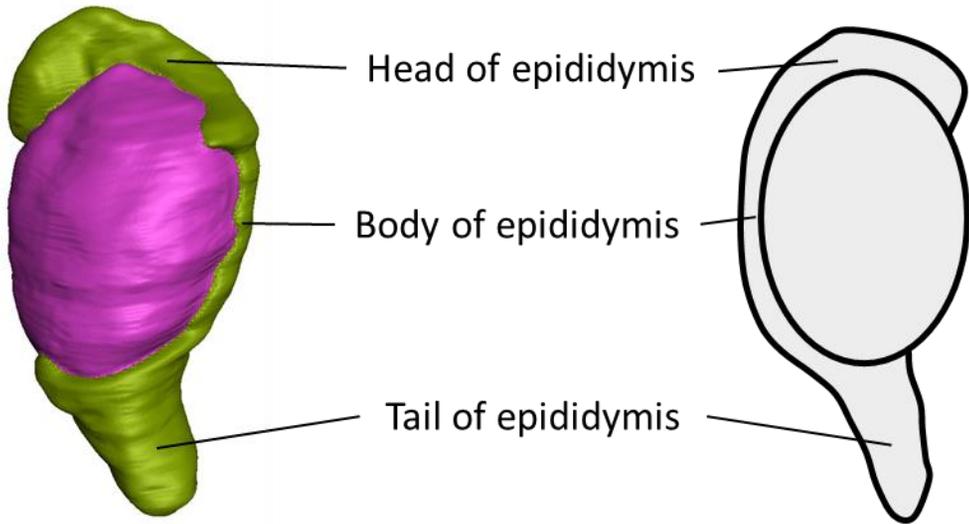
## RESULTS

In the present study, 25 bats displayed clear variation in the male genital morphology (Table 3). Using three-dimensional reconstructions, we confirmed that bat male reproductive organs of bats include paired testes, epididymides and deferent ducts, and accessory genital glands (Fig. 8). The testes were located outside the abdominal wall; however, their specific locations varied. The testes of all megadermatids, hipposiderids, rhinolophids, emballonurid, and two vespertilionids (*Myotis frater* and *Myotis petax*) were located outside the abdominal muscles and were covered by the skin. Testes shapes were ellipsoidal (rhinolophids and vespertilionids) and flattened circular (megadernatids, hipposiderids, and emballonurid). The testes of *Myotis macrodactylus* and *Plecotus sacrimontis* were located lateral to the base of the penis. In *Murina hilgendorfi*, *Vespertilio sinensis*, and *Scotophilus kuhlii*, the testes are located caudal to the back of the base of the penis and enclosed in a scrotum, giving the appearance of a pouch.

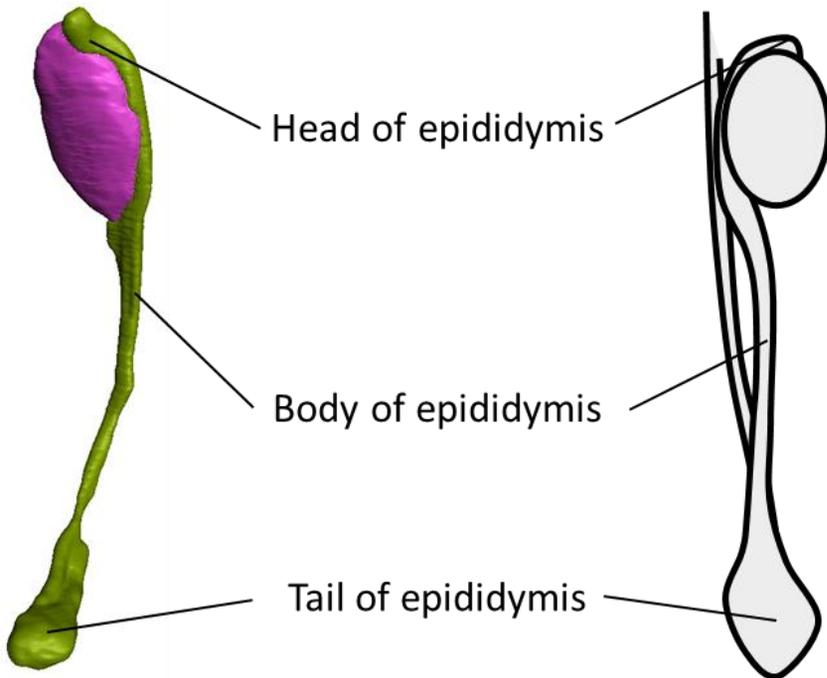
The epididymis was divided into three parts: head, body, and tail (caput, corpus, and cauda). The head of the epididymis is attached to the testis and continues along the body and tail. Four testes-epididymis configurations were observed (Fig. 7). In the first, the head of the epididymis was attached to the middle of the testis (Fig. 7a); the tail was not attached to the testis and had an elongated caudal region. This pattern was observed only in the family Rhinolophidae. In the

second configuration, the epididymis covered the testis, and the tail section did not have an elongated caudal region (Fig. 7b). This configuration was observed in one emaballonurid (*T. longimanus*), one verpertilionid (*S. kuhlii*), three hipposiderids (*Hipposideros armiger*, *Hipposideros pomona*, and *Coelops frithii*), and two megadermatids (*Megaderma lyra* and *Megaderma spasma*). The tail of the epididymis was also elongated in the third type of configuration; however, the epididymal head was attached to the top of the testis (Fig. 7c). This pattern was observed in six vespertilionids (*M. hilgendorfi*, *M. frater*, *M. petax*, *M. macrodactylus*, *P. sacrimontis*, and *V. sinensis*). The final type has a long, elongated tail (Fig. 7d). The length of the tail section was longer than that of the epididymal tail of the rhinolophid. This type was observed only in *Aselliscus dongbacana*.





**c**



**d**

Fig. 7. Three-dimensional reconstruction and schematic depiction of the gross morphology of the testis and epididymis. **a** The epididymal head was attached to the middle of the testis, and the tail part was elongated to the caudal. **b** The epididymal head was attached to the superior pole of the testis, and the tail of the epididymis did not show an elongation. **c** The epididymal head started from the superior pole and the body and tail showed a short elongation to the caudal. **d** The epididymal head was attached to the superior head, however, the size of the testis was smaller than other species. The epididymal body was elongated and the tail located far from the testis.

The accessory genital glands also showed multiple patterns of location, shape, and size. Generally, bats possess ampullary, vesicular, prostate, and bulbourethral glands; however, in some bats, these organs are not well differentiated. A urethral gland was found in *Rhinolophus*, but no in other species. The deferent duct, which continues from the tail of the epididymis, became thicker at the end, formed an ampulla at the terminal part, and entered the ampullary glands. The paired ampullary glands were located dorsal to the urinary bladder and attached to the vesicular glands as a single integrated organ. The shape of the glands varied among species, including spheres, ellipses, horns, cones, columns, or teardrop shapes.

Vesicular glands were not clearly identified in any species, because the differentiation between them and the ampullary glands was unclear. In megadermatids and hipposiderids, glands that continued from the end of the deferent duct were observed; however, no differences in their internal structures were found. In vespertilionids, *P. sacrimontis* also lacked these glands. In the species in which these glands occur independently, their positions relative to the other glands were highly variable. The vesicular glands were located dorsolaterally to the ampullary glands in *T. longimanus*; however, in *M. hilgendorfi* and *S. kuhlii*, they were ventral to the ampullary gland. The vesicular glands were also observed medial to and between the ampullary glands. The vesicular glands were located dorsolaterally to the ampullary glands in *M. petax* and *M.*

*macrodactylus*, ventral in *V. sinensis*, and caudodorsal in *M. frater*. In megadermatids and hipposiderids, in which vesicular glands were not observed, the deferent ducts formed ampulla at their termini; however, the differentiation of these glands was impossible. The shapes of these glands varied from circular to bilaterally symmetric and rounded.

The prostate gland was located between the urinary bladder and the ampullary-vesicular gland complex, except for in *T. longimanus*, in which it was located caudally to the complex. The urethra, beginning from the urinary bladder, is surrounded by the prostate gland and is called the prostatic urethra.

The urethral gland was observed only in the family Rhinolophidae. This was a single, carrot-shaped gland surrounded by a muscular wall, located between the prostate gland and the root of the penis. This gland also surrounded the urethra, which continued from the prostate gland.

The bulbourethral gland was found attached laterally to the rectum; however, in *T. longimanus*, the gland was not observed beside the rectum. It was located at the terminal section of the rectum in *M. frater*, *P. sacrimontis*, and *V. sinensis*, and in the middle section of the rectum in *M. hilgendorfi*, *M. petax*, *M. macrodactylus*, and *S. kuhlii*. The bulbourethral gland was surrounded by a muscular wall, with its duct entering the urethra.

Table 3. Morphology of bat reproductive organs

Species	Ampullary and Vesicular glands	Urethral gland	Testis location	Elongation of Epididymal tail	Location of Epididymal head
<i>Megaderma lyra</i>	unclearly differentiated	absent	external (outside of the abdominal muscle)	absent	superior pole
<i>Megaderma spasma</i>	unclearly differentiated	absent	external (outside of the abdominal muscle)	absent	superior pole
<i>Aselliscus dongbacana</i>	unclearly differentiated	absent	external (outside of the abdominal muscle)	present	superior pole
<i>Hipposideros armiger</i>	unclearly differentiated	absent	external (outside of the abdominal muscle)	absent	superior pole
<i>Hipposideros pomona</i>	unclearly differentiated	absent	external (outside of the abdominal muscle)	absent	superior pole
<i>Coelops frithii</i>	unclearly differentiated	absent	external (outside of the abdominal muscle)	absent	superior pole
<i>Rhinolophus chaseni</i>	clearly differentiated	present	external (outside of the abdominal muscle)	present	middle region
<i>Rhinolophus cornutus</i>	clearly differentiated	present	external (outside of the abdominal muscle)	present	middle region
<i>Rhinolophus ferrumequinum</i>	clearly differentiated	present	external (outside of the abdominal muscle)	present	middle region
<i>Rhinolophus luctus</i>	clearly differentiated	present	external (outside of the abdominal muscle)	present	middle region

Tabel 3. continued

<i>Rhinolophus macrotis</i>	clearly differentiated	present	external (outside of the abdominal muscle)	present	middle region
<i>Rhinolophus malayanus</i>	clearly differentiated	present	external (outside of the abdominal muscle)	present	middle region
<i>Rhinolophus paradoxolophus</i>	clearly differentiated	present	external (outside of the abdominal muscle)	present	middle region
<i>Rhinolophus pearsonii</i>	clearly differentiated	present	external (outside of the abdominal muscle)	present	middle region
<i>Rhinolophus pusillus</i>	clearly differentiated	present	external (outside of the abdominal muscle)	present	middle region
<i>Rhinolophus stheno</i>	clearly differentiated	present	external (outside of the abdominal muscle)	present	middle region
<i>Rhinolophus thomasi</i>	clearly differentiated	present	external (outside of the abdominal muscle)	present	middle region
<i>Murina hilgendorfi</i>	clearly differentiated	absent	external (caudal to the penis and enclosed in a scrotum)	present	superior pole
<i>Myotis frater</i>	clearly differentiated	absent	external (outside of the abdominal muscle)	present	superior pole
<i>Myotis macrodactylus</i>	clearly differentiated	absent	external (lateral to the base of the penis)	present	superior pole
<i>Myotis petax</i>	clearly differentiated	absent	external (outside of the abdominal muscle)	present	superior pole

Tabel 3. continued

<i>Pletocus sacrimontis</i>	unclearly differentiated	absent	external (lateral to the base of the penis)	present	superior pole
<i>Scotophilus kuhlii</i>	clearly differentiated	absent	external (caudal to the penis and enclosed in a scrotum)	absent	superior pole
<i>Vespertilio sinensis</i>	clearly differentiated	absent	external (caudal to penis and enclosed in a scrotum)	present	superior pole
<i>Taphozous longimanus</i>	clearly differentiated	absent	external (outside of the abdominal muscle)	present	superior pole

## DISCUSSION

The reproductive patterns of bats have been classified as monoestrous or polyestrous, as well as seasonal or nonseasonal (Fleming et al. 1972; Happold and Happold 1990). Their reproductive strategies depend on the climate they inhabit (Wilson 1979). Hibernation is a reproductive strategy that bats employ to survive in some temperate climates in which hot and cold seasons coexist (Gustafson 1979; Oxberry 1979). Bats in tropical climates do not undergo prolonged hibernation; however, they do undergo short periods of torpor during the dry and cold seasons (Jolly and Blackshaw 1987). These reproductive features are reflected throughout the reproductive organs.

In the testis, a difference was observed post-spermatogenesis in the activation of the Leydig cell and accessory reproductive glands. In non-hibernating tropical species, the male reproductive cycle depends on the female reproductive cycle. When females have two estrous cycles, the spermatogenesis peak is twice as high. The breeding cycle of the male is not affected by the season and is also dependent on the reproductive cycle of females. In this study, we sampled six families belonging to the suborders Yangochiroptera and Yinpterochiroptera. We included 25 species distributed from Southeast to East Asia, in both tropical and temperate climates. Despite their different habitats, the reproductive organs of bats include paired testes, epididymides, different ducts, and accessory reproductive

glands, as in other mammals (Krutzschs 2000). The accessory genital glands were divided into ampullary, vesicular, prostate, bulbourethral, and urethral glands, and were diverse. Several differences between species we observed; the urethral gland was only present in Rhinolophidae, and the distinction between ampullary and vesicular glands was unclear in Megadermatidae and Hipposideridae. The corpus cavernosum was found dorsal to the urethra from the root of the shaft of the penis. The os penis was found inside the glans penis.

### **Testis and epididymis**

Among the four types of testes (permanently abdominal, permanently scrotal, migratory, and external), it was confirmed that the bats used in this study could be classified into external and permanently scrotal types. The most common pattern found in bats was the external type; however, specific positions varied. The testes were located on the outside of the abdominal muscles and under the external skin, extending from around the external inguinal ring to the base of the penis. The testes can move in this way because they, along with the epididymis, are suspended by the testicular suspensory ligament from the kidney. These ligaments allow the testes to move under the skin. The tunica vaginalis that surrounds the testes and epididymis further smoothens this movement. The external position was observed in megadermatids, hipposiderids, rhinolophids, emballonurids, and two vespertilionids (*M. frater* and *M. petax*). The testes of the other two vespertilionids

(*M. macrodactylus* and *P. sacrimontis*) were found lateral to the base of the penis. However, these two types may be the same as the testes may have moved to any area lateral to the external side of the abdomen. The testes were also located inside the scrotum, as observed in *M. hilgendorfi*, *V. sinensis*, and *S. kuhlii*. The scrotum has a thermoregulatory function that keeps the testis temperature below body temperature. Previous studies have suggested that a body temperature higher than that of the testes affects sperm status. Although high body temperature does not affect sperm maturation, it influences spermatogenesis (Bedford, 2008). Therefore, the presence of the scrotum can reduce spermatogenesis disruption.

Between the testis and epididymis, the attachment site of the epididymal head to the testes and elongation of the epididymal tail differed between species. Among mammals, long-term sperm storage in reproductive organs is observed in male and female bats found in temperate zones (Racey, 1975; Crichton, 2000). Sperm production in male bats begins in late summer and completes in early fall. When mating occurs, the sperm moves to the tail section of the epididymis and the testis begins to regress. After mating is complete, bats enter hibernation and the sperm is stored at the tail of the epididymis; this function is maintained even during hibernation (Miller 1939, Racey 1972, 1974, 1995, Gustafson, 1979). Mating in some species continues throughout winter (*Myotis lucifugus*, Racey et al. 1987) using sperm stored in the epididymal tail. The activity of accessory genital glands during hibernation is maintained by a low level of androgenesis, which enables

mating during hibernation (Racey and Tam 1974, Racey 1974).

Rhinolophids, hipposiderids (*A. dongbacana*), and vespertilionids except for *S. kuhlii* all showed elongation of the epididymal tail (Fig. 7a, 7c, and 7d). In the elongated epididymal tail, the sperm is stored in the testis. During hibernation, the testes regress, and the sperm that were made in the testis are stored in the elongated epididymal tail. The degree of elongation in *M. petax* and *V. sinensis* was similar and longer than in other bats (Fig. 7c). *A. dongbacana* displayed the longest epididymal body in this study (Fig. 7d). These differences also seem to be related to the temperature regulation of the testis and epididymis. According to Bedford (2008), the long epididymal body keeps the epididymal tail away from the testis, lowering the temperature of the tail and facilitating sperm storage. In one vespertilionid (*S. kuhlii*), emballonurid (*T. longimanus*), megadermatids, and hipposiderids except for *A. dongbacana*, no elongated epididymal tail was observed, but the epididymis covered a part of the testis (Fig. 7b). The whole body of the epididymis dorsolateral to and attached to the testis. The epididymal head of rhinolophids was attached to the mid-side of the testis, with only the head section attached. In the other rhinolophids, *R. megaphyllus* (Kruttsch et al. 1992) also displayed this pattern (Fig. 7a). However, the epididymal head as well as the body section in other species was attached to the superior pole of the testis (Fig. 7b, 7c, and 7d). The relationship between the attachment site and the length of the epididymal tail is unclear; however, the elongation is likely to be related to

hibernation, allowing for sperm storage and the activity of accessory reproductive glands during hibernation. Recently, in *R. ferrumequinum* and *R. cornutus* evidence of interrupted and forced mating during hibernation periods has been reported (Sano 2001, Kim et al. 2019, Sato 2019).

### **Accessory genital glands**

The accessory genital glands of bats consist of the ampullary, vesicular, prostate, bulbourethral, and urethral glands. One of the accessory glands, the ampullary glands, is the terminal section of the deferent duct that continues to the vesicular glands. Rhinolophids, emballonurids, and six vespertilionids displayed this pattern. Previous studies on other rhinolophids, such as *R. megaphyllus* (Krutzsch et al. 1992) and *R. hipposideros* (Gaisler 1966) have demonstrated that the ampullary and vesicular glands are distinguishable through conventional macroscopic observation; however, those in *R. capensis* are not. In vesper bats, both glands have been observed (*Corynorhinus Mexicanus*, León-Galván et al. 2005), only ampullary (*Scotophilus heathi*, Krishna and Singh 1997), only vesicular (*Pipistrellus dormer*, Gadegone and Sapkal 1983), and neither has been observed, as in *Myotis nigricans* (Negrin et al. 2014). The ampullary-vesicular complex of *Pipistrellus hesperus* (Krutzsch 1975) is also indistinguishable. In emballonurids, *Rhynchonycteris naso* (Beguelini et al. 2016) did not possess these glands; *Thyroptera tricolor* (Krutzsch 2000) only possessed ampullary glands; and

*Taphozous georgianus* (Jolly and Blackshaw 1987) possessed glands that were indistinguishable. In domestic mammals, the ampullary gland is present in ruminants, horses, and dogs, but not in cats or pigs (Singh 2017). The function of the secretions of this gland is to reduce chemical compounds (ergothioneine) and nourish the sperm (fructose). The vesicular glands are common in rodents, ruminants, humans (Lindholmer et al. 1974, Stegmayr and Ronquist 1982), and boars (Tso and Lee 1980); however, they are absent in carnivores (Setchell and Breed 2006; Prins and Lindgren 2015). The secretions of this gland (fructose and citric acid) are major components of semen that contribute to the formation of the copulatory plug in mice (Mangels et al 2016, Noda and Ikawa 2019). Morphologically, the ampulla and vesicular glands are difficult to distinguish through macroscopic observation. In this study, all rhinolophids, six vespertilionids, and one emballonurid showed a well-differentiated structure (Table 3). These species displayed a sulcus between glands or different shapes and internal constituents on microCT images, which allowed us to identify and differentiate the two glands. However, in two megadermatids, four hipposiderids, and one vespertilionid, there were no differences observed through either macroscopic or microCT imaging. MicroCT images revealed only one internal pattern of the glands.

The prostate gland is located dorsodistally to the urinary bladder and the ampullary-vesicular gland complex. It was present in all species in this study, surrounding the urethra. The secretions of this gland provide increased survival,

viability, and motility to spermatozoa (Rochel et al. 2007). The number of lobes of the prostate gland in mammals varies widely. Rodents (five in Mongolian gerbils; four in mice; three in rats), insectivores (two in musk shrews), and lagomorphs (two in rabbits) have a multilobed prostate gland. Nonhuman primates also have two lobes; however, their shape is a single mass (Lewis et al., 1981). In bats, the prostate gland is composed of two (*Artibeus planirostris*, Puga et al. 2013; *Desmodus rotundus* and *Platyrrhinus lineatus*, Martins et al. 2015; *Molossus molossus*, Christante et al. 2015; *Noctilio albiventris* and *Rhynchonycteris naso*, Beguelini et al. 2016; *Sturnira erythromos*, *Sturnira lilium*, and *Sturnira oporaphilum*, Miotti et al. 2018; *Artibeus lituratus*, Santos et al. 2018) or three distinct regions (*Myotis nigricans*, Negrin et al. 2014; *Carollia perspicillata*, *Glossophaga soricina*, and *Phyllostomus discolor*, Martins et al. 2016). In contrast, we found that this gland constituted a single structure in all species in the present study. The number of prostate lobes varies among mammals, although the functional significance of lobe numbers remains unclear.

We confirmed that the bulbourethral glands, consisting of smooth and striated muscle fibers, were located dorsally to the bulbospongiosus muscle and bilaterally to the rectum. Luminal secretion was confirmed in the bulbourethral glands, although the type of secretion was undetectable in this study. The ducts from these glands carry secretions to the urethra. Their shape can vary from that of a punching bag (e.g., *Macrotus waterhousii*, Krutzsch et al. 1976), to oval (e.g., *R.*

*megaphyllus*, Krutzsch et al. 1992; *R. ferrumequinum*, Sohn et al. 2021), or teardrop-shaped (e.g., *Molossus molossus*, Christante et al. 2015; *Artibeus lituratus*, Santos et al. 2018). Most bats exhibit one pair of bulbourethral glands, except for *Corynorhinus rafinesquii*, which possesses two pairs (Pearson et al. 1952). According to Krutzsch (2000), the bulbourethral glands are generally located bilaterally to the rectum, anus, or near the base of the penis. The duct of the bulbourethral glands is found on the bulbospongiosus muscle and is connected to the urethra, which is beneath this muscle. The morphologies observed in this study were an oval, spherical, teardrop, and punching bag, similar to those of other bat species.

The urethral gland is found only in rhinolophids and is a well-developed, carrot-shaped single structure surrounded by a muscular capsule. Among bats, the urethral gland has been reported in Rhinolophidae (*R. hipposideros*, Gaisler 1966; *R. capensis*, Bernard 1985; *R. megaphyllus*, Krutzsch et al. 1992), Hipposideridae (*Hipposideros caffer*, Matthews 1942), and Megadermatidae (*Cardioderma cor*, Matthews 1942), all of which belong to Yinpterochiroptera. However, the urethral gland was not found in Hipposideridae and Megadermatidae. During mating, sperm is transferred with additional secretions from the male into the female reproductive organs; these substances contribute to the formation of the mating plug. This plug is also called a vaginal plug or copulation plug, and works to prevent multiple mating (Martan and Shepherd 1976) and function in sperm storage during

hibernation (Orr and Zuk 2014). In Rhinolophidae, the vaginal plug has been reported in *R. hipposideros hipposideros* (Gaisler 1966), *R. ferrumequinum* (Ransome, 1973; Fenton 1984; Rossiter et al. 2000), *R. clivosus* (Bernard 1983), *R. ferrumequinum nippon* (Oh et al. 1983), *R. capensis* (Bernard 1985), *R. ferrumequinum korai* (Kim and Oh 1991) and *R. cornutus* (Sato 2019); the urethral gland is also found in these species. However, although the urethral gland is present in *R. megaphyllus* (Krutzsich et al. 1992), a vaginal plug is not observed. The secretion of the urethral gland is considered a coagulating material that contributes to the formation of a plug that seals the vagina. Its origin varies between species. In bat species, the mating plug of rhinolophids originates from a urethral gland, however, the mating plug in the other species is derived from the vaginal epithelium (*Pipistrellus pipistrellus*, Courrier 1924; *Myotis lucifugus lucifugus*, Reeder 1939; *Chalinolobus gouldii*, Kitchener 1975; *Scotophilus heathi*, Krishna and Dominic 1978; *Mormopterus planiceps*, Crichton and Krutzsich 1987), female genitalia (*Miniopterus schreibersii fuliginosus*, Mōri and Uchida 1981), connective tissue hyperplasia (*Nyctalus noctula*, Grosser 1903, Racey et al. 1975), and semen and vaginal epithelium (*Tritoma brasiliensis*, Davis et al. 1962, Keeley and Keely 2004; *Nyctophilus geoffroyi*, Hosken 1997; *Nyctophilus gouldi*, Phillips and Inwards 1985). In other animals, the mating plug is also found in insects (Orr and Rutowski 1991), natricine snake (*Thamnophis sirtalis*, *Thamnophis butleri*, and *Natrix taxipilota*, Devine 1975), primates (Dixson and Anderson 2002), rodents (Martan

and Shepherd 1976; Voss 1979; Michener 1984; Dewsbury 1988; Sahwagmeyer and Parker 1990; Koprowski 1992; Carballada and Esponda 1993), and carnivores (Jia et al. 2002). According to Mangels et al. (2016), in mice, when a copulatory plug was not formed by mating with a first male, the female allowed a second male. In *R. ferrumequinum* and *R. cornutus*, the vaginal plug is formed in female reproductive organs and contributes to sperm storage until fertilization occurs and prevents multiple mating events during the hibernation period. However, it has been reported that both species can interrupt hibernation (Sano 2001; Kim et al. 2019; Sato 2019); they may have been forced to mate during hibernation.

The phylogenetic relationships among the 25 species used in this study showed interspecific differences in the male reproductive organs. The male reproductive organs of the bat species showed morphological differences in the testis-epididymis and constitution of the accessory genital glands. The epididymis was elongated caudally, and this structure has been observed in all Rhinolophidae and Vespertilionidae (Racey 1982). The bats distributed in temperate zones store spermatozoa after completing spermatogenesis, and sperm move to the elongated epididymal tail. Rhinolophidae used in this study were not located only in temperate zones; however, all species showed the same morphology in the epididymis. The elongated epididymal tail was also present in six Vespertilionidae. Other hibernating vespertilionids, including *Pipistrellus* (Racey and Tam 1974), *P. subflavus* (Krutzsch and Crichton 1986), *P. kuhlii* (Sharifi et al. 2004), *Myotis*

*daubentonii* (Encarnacao et al. 2004), *Corynorhinus mexicanus* (Leon-Galvan et al. 2005), and *Neoromicia nanus* (Van der Merwe and Stirnemann 2007) also have an elongated tail. All of these species are distributed throughout temperate zones; however, the tropical *Scotophilus kuhlii* has a short tail. The other species, megadermatids, hipposiderids, and emballonurid, which are located in tropical zones, also possessed short tails. In hipposiderids, the elongated epididymal tail was present in *Aseliscus dongbacana*; however, the testes were smaller and the body of the epididymis was longer than that of rhinolophids and vespertilionids. The length was very similar to that of *Myotis albescens* (Beguelini et al. 2012) which is a non-hibernating bat. In *M. albescens*, an elongated tail was present, and regression of the seminiferous tubules and sperm storage was found. These data suggest that, whether or not *A. dongbacana* undergoes torpor, this species has sperm storage in the epididymal tail.

In accessory genital glands, the constitution varied among species. Some species have both ampulla and vesicular glands; however, this does not apply to all species. The main secretions of these glands are fructose (Racey 1974; Mokkaapati and Dominic 1976; Crichton et al. 1981; Krishna and Dominic 1982; Krutzsch and Crichton 1986; Krishna and Singh 1997) and citric acid (Mokkaapati and Dominic 1976), which contribute to the maintenance of pH (Mann and Lutwak-Mann 1981) and sperm motility (Videla et al 1981). Megadermatids, hipposiderids, and one vespertilionid showed no clear differentiation; however, the function was thought

to be no different. The prostate gland was present in all species and did not have multiple lobes. The morphology was rounded and the urethra was surrounded by this gland, which secretes the materials directly into the prostatic urethra. Bulbourethral glands were also present in all the species, while specific locations varied. These glands lay bilaterally to the rectum and anus, the bulb of the penis, or dorsally to the bulbospongiosus muscle.

In this study, the morphology of the male reproductive organs was demonstrated through diceCT, and its relationship with phylogenetic studies was analyzed. The morphology showed interspecific differences and relied on species distribution. The bats located in tropical zones, megadermatids, hipposiderids, and emballonurid, were associated with the absence of an elongated epididymal tail. Testis and epididymis morphologies seem to be related to the storage, production, and capacitation of spermatozoa (Bedford 2008), and their morphologies are very similar to those of non-hibernating bats (Beguelini et al 2012). Although they do not have an elongated epididymal tail, a relatively large testis was present. Rhinolophidae, which are distributed in tropical to temperate zones, possess an elongated epididymal tail and urethral gland. The vaginal gland is found in *Rhinolophus*, and the precise functions of the urethral gland are unclear; however, it seems to contribute to the formation of the vaginal plug along with secretions of the other accessory genital glands. In *R. ferrumequinum* and *R. cornutus*, evidence of awakening from hibernation has been reported and forced copulation with

hibernating females may occur.

## General Conclusion

To determine the characteristics of the reproductive organs of bats, three types of the anatomical study were conducted and the following results were obtained, after which the relationship with phylogenetic studies was analyzed.

In this study, the soft tissue of male reproductive organs in bats was analyzed through a microCT study, which provided the first detailed three-dimensional description of the whole structure of the male genital system in bats. Until recently, it was difficult to observe and describe the intact structure and composition of accessory genital glands due to technical limitations. By using microCT imaging and iodine, enhanced contrast imaging allowed us to successfully describe the detailed anatomy of the accessory genital glands (diceCT; Gignac et al. 2016). The study demonstrated that a pair of testes, epididymides, deferent ducts, and four to five accessory genital glands comprise the male reproductive organs of bats.

The testes were located externally to the abdomen or inside the scrotal sac. External testes were covered by the tunica vaginalis along with the epididymis, allowing for the movement of the testes from under the abdominal skin to the inside of the scrotum. Due to this movement, the location of the testes changes between the breeding and non-breeding seasons.

The epididymis was attached to the testes, and its tail showed caudal

elongation with a characteristic turnback to the testes as the deferent duct. Four morphological patterns were observed between the testes and epididymis. First, the epididymal head was attached to the middle of the testis, and the epididymal tail was elongated. This was observed in *Rhinolophus* and is the hallmark of hibernating species. The sperm, which is produced before mating, is stored in the tail of the epididymis. The second type consisted of the epididymal head attached to the superior pole of the testes, and no elongation of the epididymal tail. This configuration was observed in non-hibernating species, and is similar to mammals that produce sperm all year round. The epididymal head of the third type was attached to the superior pole of the testes; however, the epididymal body was elongated. The last type had an elongated epididymal tail with small testes. The epididymal tail descended far from the testes. The length of the epididymis allows the temperature of the caudal part of the epididymis to lower, which helps to store sperm.

The ampullary gland was located at the terminal part of the deferent ducts, and the vesicular gland was located distal to the ampullary glands. These two glands were indistinguishable through conventional macroscopic observations, but our microCT imaging allowed us to identify their boundaries and internal constituents and confirmed their anatomical differentiation.

The presence of the urethral gland and its secretions strongly suggested that this species is capable of forming a vaginal plug; it was found only in

*Rhinolophus*. Elongation of the tail of the epididymis suggests increased sperm storage. Given that it has already been reported that some individuals interrupt hibernation and awaken in *R. ferrumequinum* and *R. cornutus*, it is possible that forced mating of hibernating females may occur in these species.

This study presents detailed three-dimensional imaging of the male reproductive organs of bats. The morphology of the specimens was described more precisely by using the diceCT technique (Gignac et al. 2014) than through conventional dissections. Comparing these morphological characteristics with phylogenetic relationships can contribute to classification. Although reproductive organ morphologies have not been used to classify bats, the classification we produced was similar to that based on phylogeny. In this study, we confirmed that anatomical observation using microCT is a more accurate and convenient method than conventional methods; it indicated that the morphology of reproductive organs can provide information for phylogenetic classification.

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## 국문초록

포유류의 생식기관은 한 쌍의 고환, 부고환, 정관 그리고 덧생식샘을 포함하며, 이들의 구조는 종마다 다르다. 박쥐는 열대에서 온대에 걸쳐 약 1,400종이 서식하고 있다. 그렇기 때문에 박쥐의 배우자 형성, 교미 행동, 수정, 출산 패턴은 포유류 중에서도 가장 다양하다. 박쥐 생식기관의 비교 연구는 박쥐의 성장, 생동, 진화 패턴을 반영하고 있으며, 우리에게 도움이 되는 정보를 줄 수 있다. 본 연구에서는 동아시아에 서식하는 25종 5과의 박쥐 수컷 생식기관을 관찰하여 생식기의 형태와 계통 분류와의 관계를 조사하였다.

전통적으로 박쥐 생식기의 해부학적 구조는 육안과 현미경에 의한 관찰로 연구가 진행되었었다. 본 연구에서는 조직학적 관찰과 diffusible iodine-based contrast-enhanced computed tomography (diceCT) 관찰을 함께하여 박쥐 수컷 생식기의 상세한 3차원 형태 구조를 조사하였다. 본 연구는 박쥐 생식기 전체를 상세하게 기술한 첫 연구다.

제 1 장에서는 관박쥐의 수컷 생식기가 한쌍의 고환, 부고환, 정관, 그리고 5가지의 덧생식샘으로부터 구성된다는 것을 3차원 재구축을 통해 나타냈다. 관박쥐는 유럽에서 일본에 걸친 온대 지역에 분포하는 동면을 하는 종이다. 외부에 위치한 고환의 위치와 부고환 꼬리의 신장은 동면을 하는 박쥐의 특징이다. 본 종의 덧생식샘은 팽대샘, 정낭샘, 전립샘, 요도샘, 망울요도샘으로 구성되며 것을 확인하였다. 지금까지의 관찰로는 팽대샘과 정낭샘의 경계를 특정하는 것이 어려웠으나, 이번 연구에서 사용된 diceCT로 인해 두 구조를 명확하게 구별 할 수 있었다. 팽대샘은 정관의 말단부에 위치하며, 정낭샘은

팽대샘의 원위부에 위치한다. 전립샘은 원형이며 하나의 엽으로 이루어져있다. 망울요도샘은 한쌍이며 구형이며 망울해면체근 위에 놓여있다. 이 종은 당근 모양을 한 요도샘을 가지고 있으며, 다른 대부분의 박쥐 종에서는 발견되지 않는다. 이 종에서 요도샘의 분비물은 교미 후에 암컷의 생식기에 질전을 형성하여, 암컷의 질 입구를 막음으로써 다른 수컷의 정자 침입을 막을 수 있다고 생각된다. 요도샘의 존재나 부고환꼬리의 신장, 그리고 동면 중에 깨어나 활동하는 개체가 있는 것으로 보아, 관박쥐는 동면 중에 암컷에게 강제 교미가 행해질 가능성이 있다고 생각된다.

제 2 장에서는 동아시아 박쥐의 수컷 생식기를 관찰하고 그 형태의 특징을 비교하였다. 계통학적 분석에 의한 분류된 25종 5과의 박쥐를 diceCT를 사용하여 수컷 생식기와의 관련성을 관찰하였다. 이들 박쥐의 수컷 생식기의 형태는 종에 따라 다르다. 생식기는 한 쌍의 고환, 부고환, 정관, 덧생식샘으로 구성된다. 고환의 모양과 위치, 부고환 머리의 부착 부위, 부고환 꼬리의 신장 정도는 종에 따라 다르다. 덧생식샘은 일반적으로 한 쌍의 팽대샘, 정낭샘, 망울요도샘, 그리고 전립샘으로 이루어진다. 추가로 요도샘은 관박쥐과에서 발견된다. 고환의 위치는 종에 따라 다르다. 복부 근육의 외측이 있거나, 음낭의 내부에 존재하였다. 부고환의 고환에 붙어 있다. 하지만 부고환 머리의 부착부위와 부고환 꼬리의 신장 정도는 4가지 다른 타입이 있다. 고환과 부고환은 번식에 필수이므로 이들의 차이는 분포하는 지역이나 동면의 영향을 받는다고 생각된다. 예를 들어 부고환 꼬리가 긴 종은 동면 중에도 생식기관의 활동을 유지할 수 있는 가능성이 있다. 관박쥐과와 애기박쥐과, 보자기날개박쥐과에서는 팽대샘과 정낭샘의 구분이 잘 되었다. 한편 위흡혈박쥐과와

앞코박쥐과에서는 정관의 말단 부위가 두꺼워지며, 이 부분이 팽대샘이 되어 전립샘으로 직접 들어가게 된다. 전립샘은 요도를 둘러싸고 있는 하나의 옆으로 구성되어있다. 요도망울샘은 한 쌍이며, 곧창자의 외측 혹은 망울해면체근 등쪽에 위치한다. 전립샘과 망울요도샘은 모든 종에 존재한다. 요도샘은 관박쥐과에만 존재하며, 암컷 생식기에 질전형성에 기여한다. 요도샘의 존재는 수컷이 동면 중에 일시적으로 깨어나, 암컷에게 강제로 교미를 하는 것이 알려져 있는 것으로부터, 수컷들 사이의 경쟁과 관계하고 있을 가능성이 있다. 이 장에서는 생식기의 형태와 계통학적 연구에 기반한 분류와의 관계를 분석하였다. 형태학적 특징은 microCT 화상을 이용하여 계통학적 분류와 관련지었다.

생식기는 생태나 계통의 특징이 반영되기 때문에 매우 중요한 표본이다. 하지만 그에 대한 연구는 많이 진행되고 있지 않다. 생식에 중요한 생식기를 이해하는 것은 다양한 박쥐종의 계통 관계나 생태적 특징을 밝히는데 도움이 된다. 생식기의 구성 및 구조는 박쥐 연구에 있어 매우 중요하다. 이 정보는 성장과 생식의 패턴을 이해하는데 있어 필수다. 결론으로 박쥐의 생식기의 형태는 서식지에 의한 번식 패턴에 따라 다르며, 이 들 차이는 박쥐의 계통학적 분류에 나타난다. 요도샘은 관박쥐과에 존재하지만, 다른 종에는 없다. 모든 앞코박쥐과의 부고환 꼬리는 짧았지만 테이트삼지창코박쥐속 (*Aselliscus*) 에서는 길다. 애기박쥐과에서는 노랑박쥐속 (*Scotophilus*) 만 짧은 부고환 꼬리를 가진다. 긴 부고환 꼬리를 가진 종은 동면 기간 중에도 생식 기관의 활동을 유지하는 가능성이 크다고 생각된다. 본 연구의 성과는 박쥐를 이해하는데 공헌한다.

주요어: 박쥐, 수컷생식기. 진화, 융합, 계통학, 상동성

학번: 2013-31125

## SUPPLEMENT

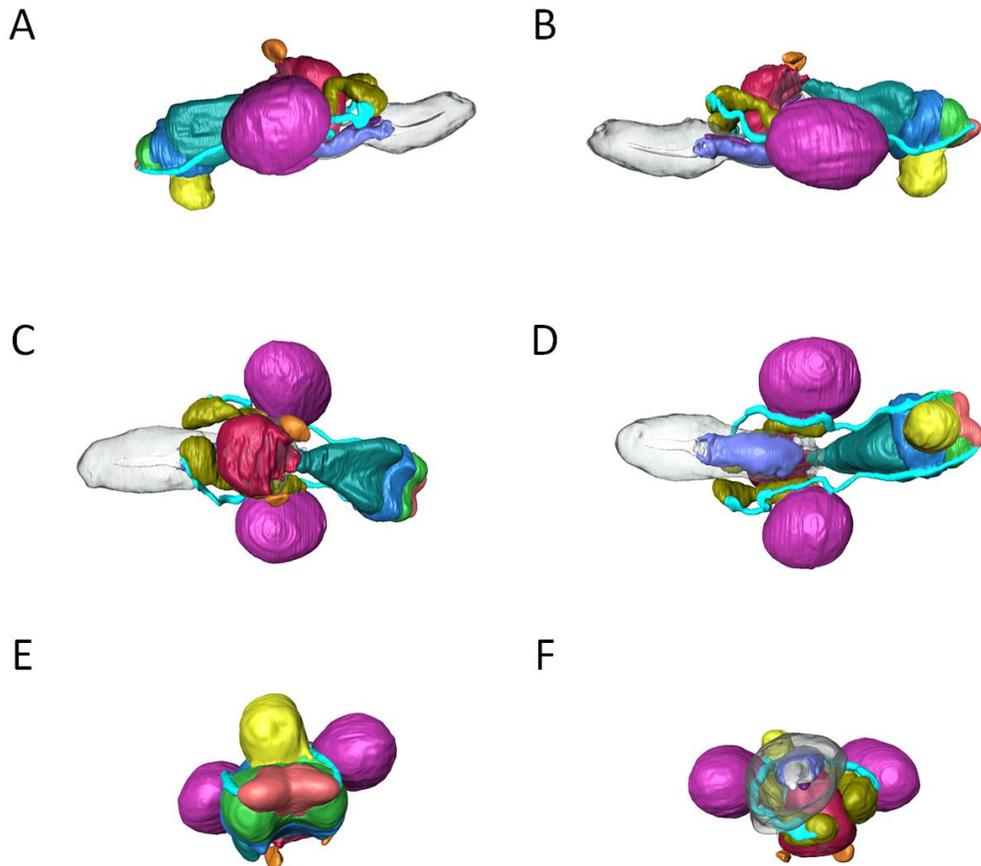


Fig 8-1. The male genital organs of *Rhinolophus ferrumequinum*. A, left lateral; B, right lateral; C, ventral; D, dorsal; E, anterior; and F, posterior view.

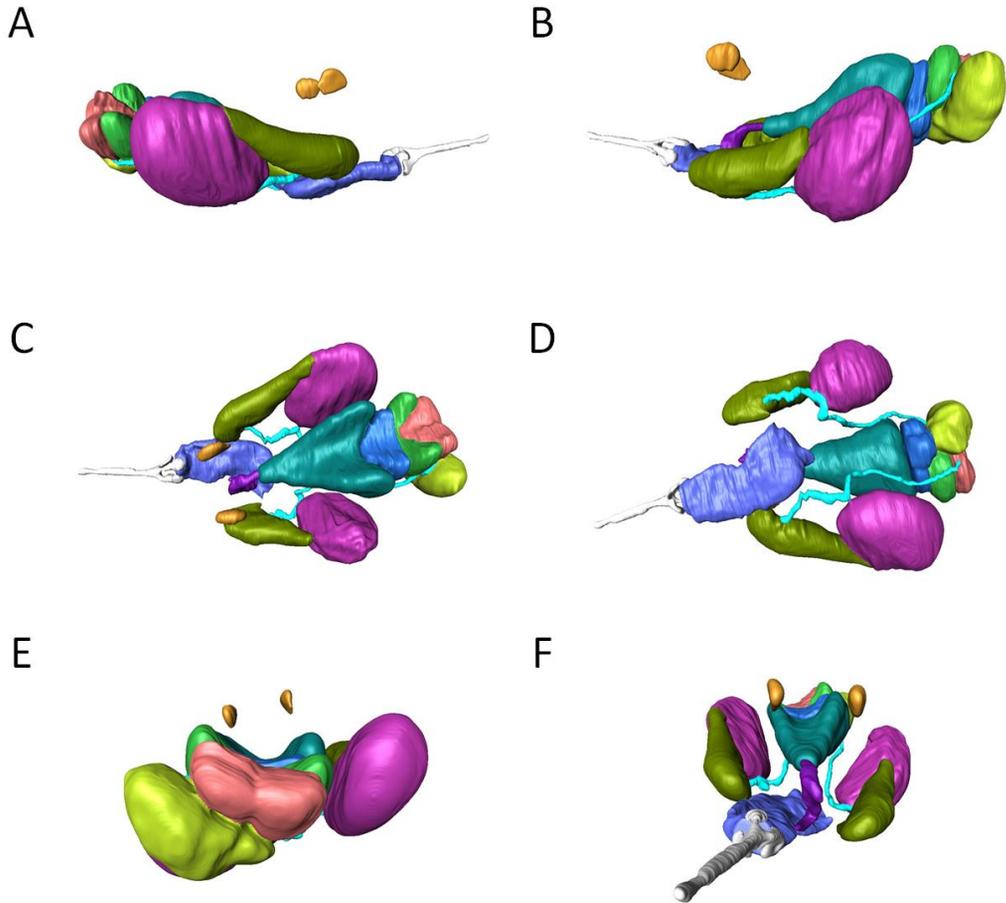


Fig 8-2. The male genital organs of *Rhinolophus chaseni*. A, left lateral; B, right lateral; C, ventral; D, dorsal; E, anterior; and F, posterior view.

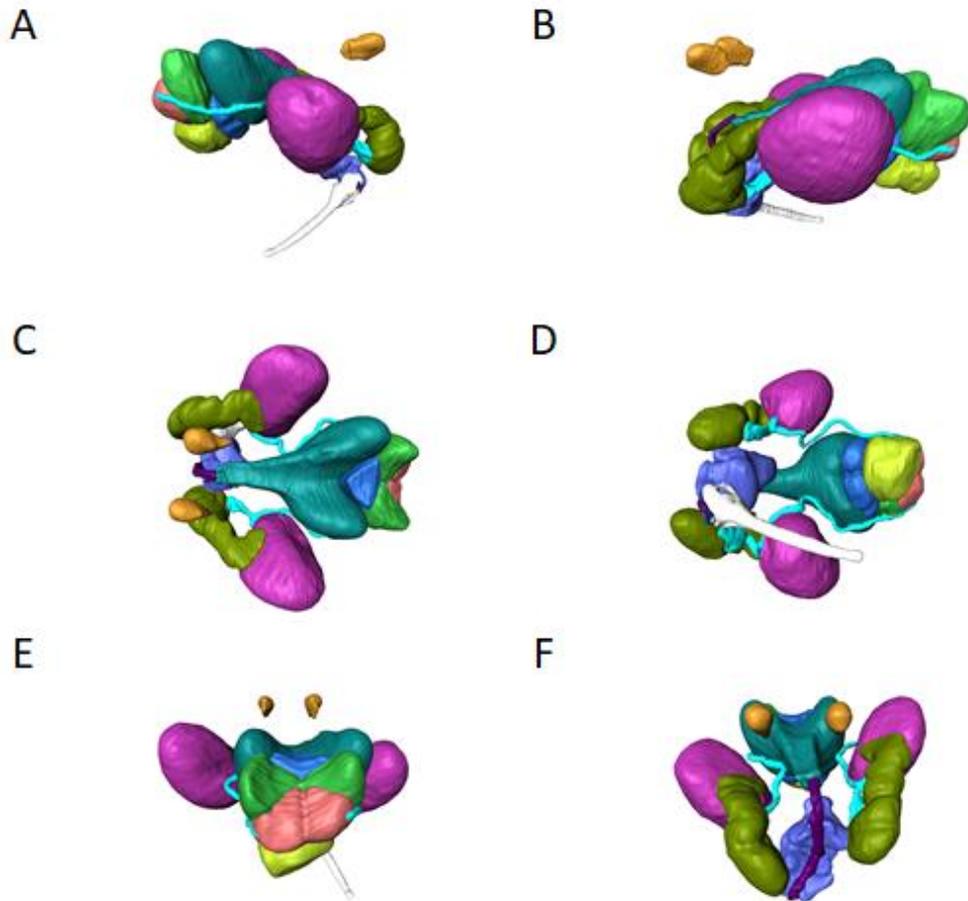


Fig 8-3. The male genital organs of *Rhinolophus cornutus*. A, left lateral; B, right lateral; C, ventral; D, dorsal; E, anterior; and F, posterior view.

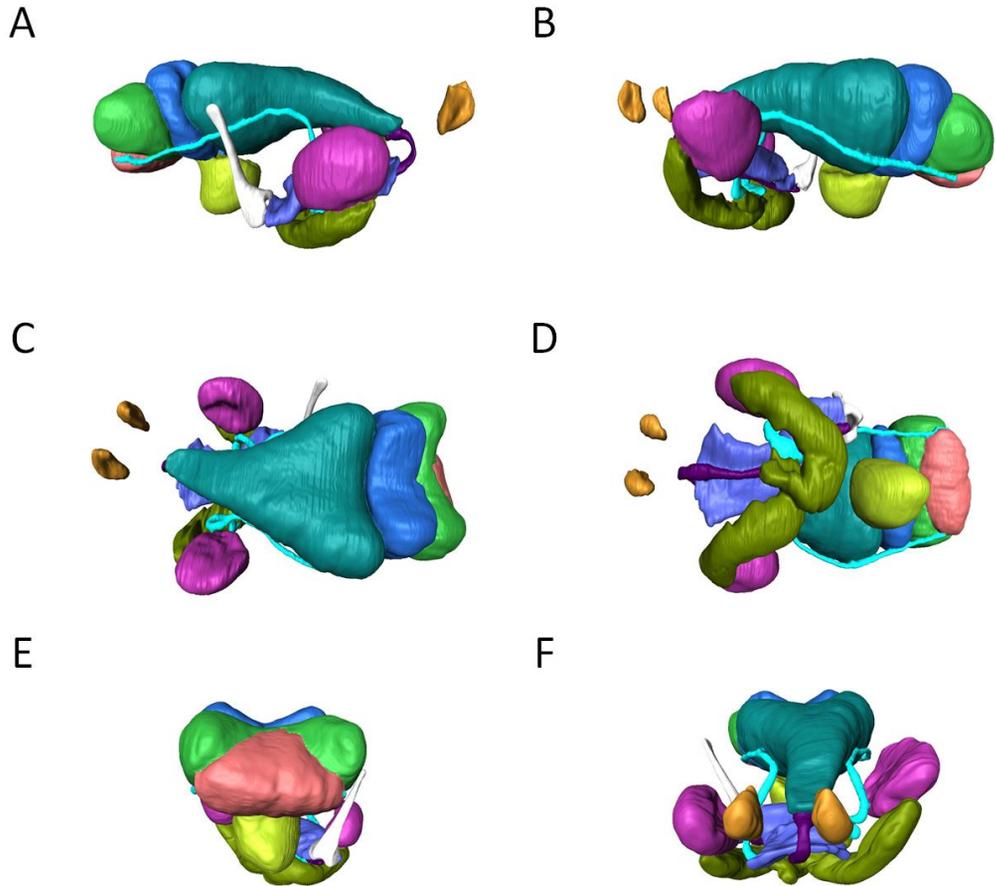


Fig 8-4. The male genital organs of *Rhinolophus luctus*. A, left lateral; B, right lateral; C, ventral; D, dorsal; E, anterior; and F, posterior view.

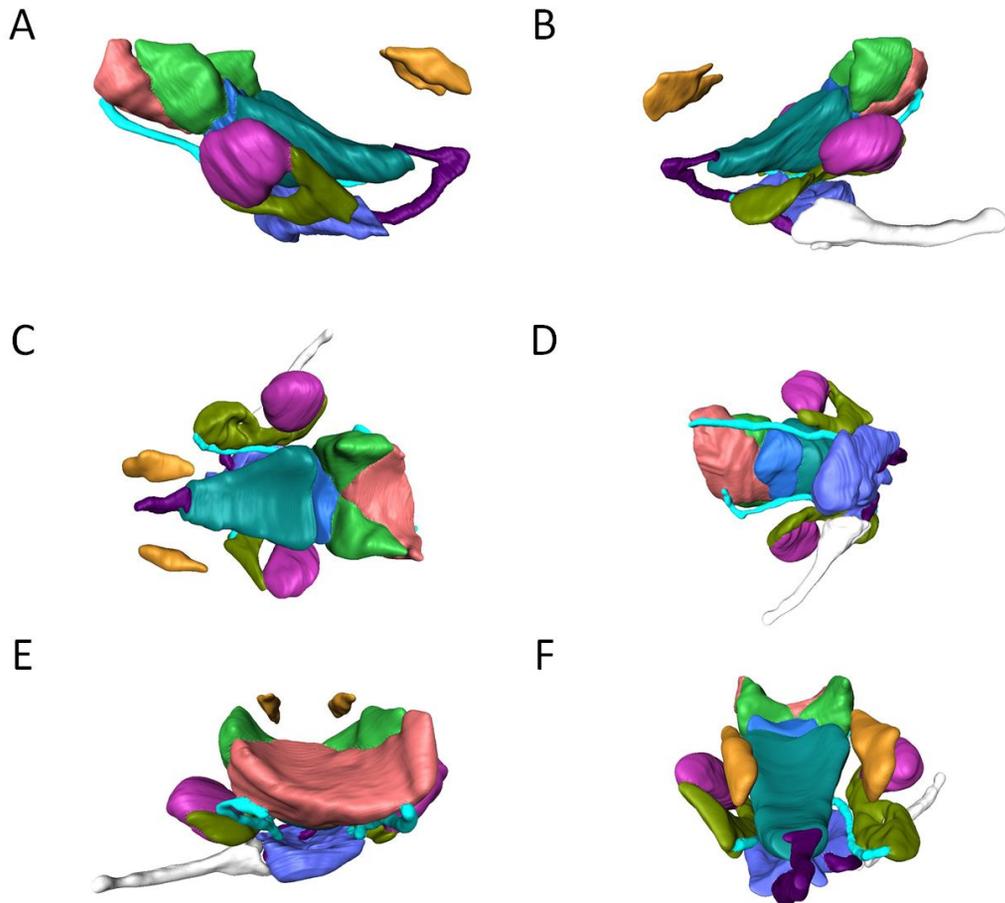


Fig 8-5. The male genital organs of *Rhinolophus macrotis*. A, left lateral; B, right lateral; C, ventral; D, dorsal; E, anterior; and F, posterior view.

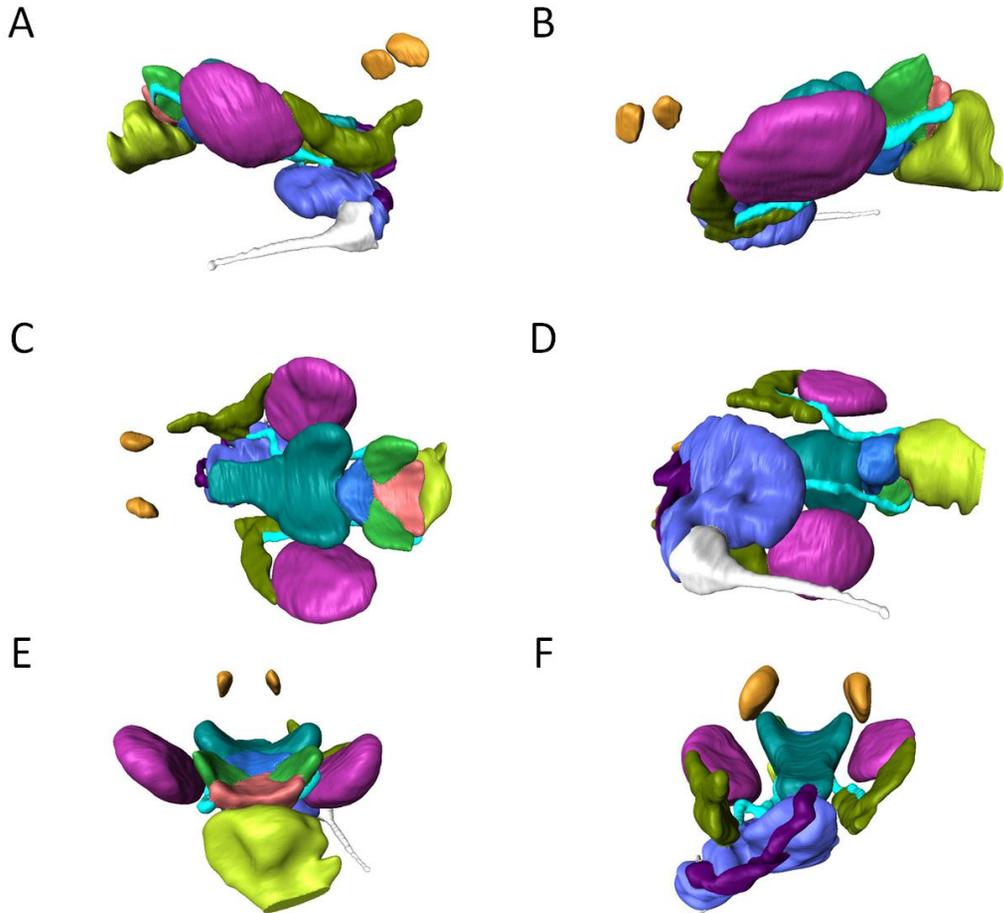


Fig 8-6. The male genital organs of *Rhinolophus malayamus*. A, left lateral; B, right lateral; C, ventral; D, dorsal; E, anterior; and F, posterior view.

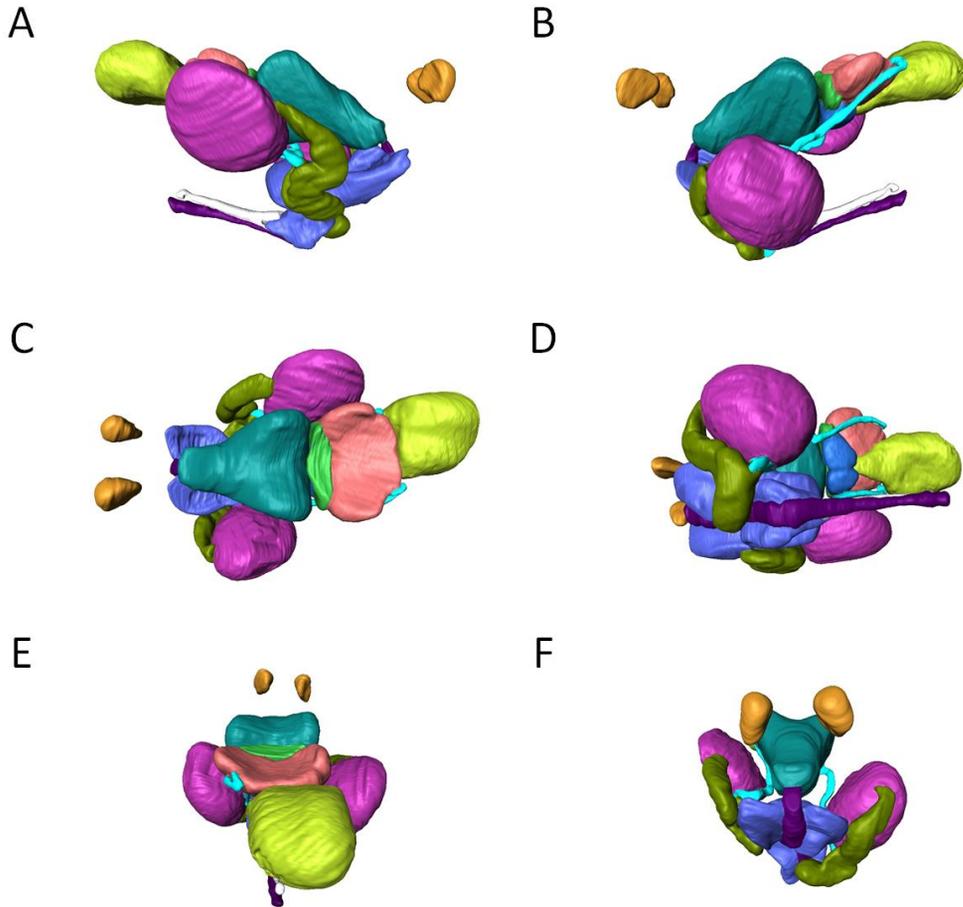


Fig 8-7. The male genital organs of *Rhinolophus paradoxolophus*. A, left lateral; B, right lateral; C, ventral; D, dorsal; E, anterior; and F, posterior view.

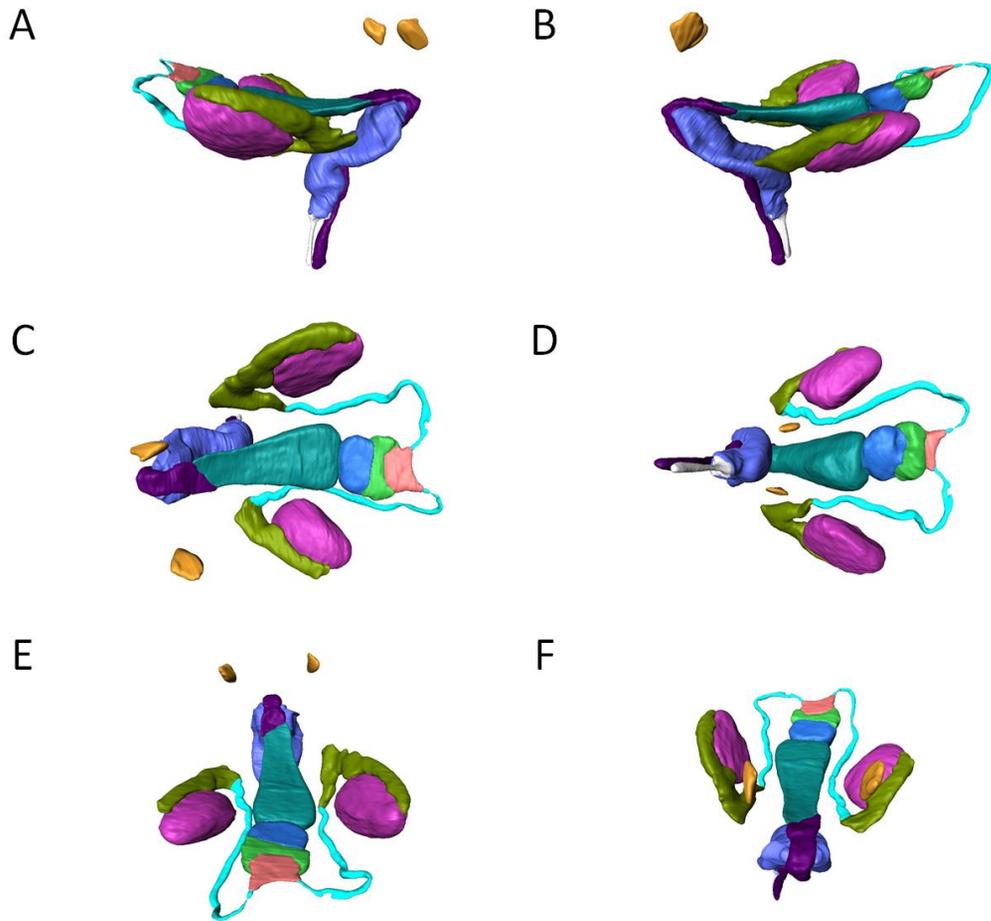
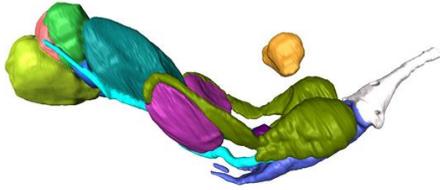
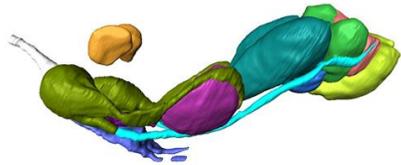


Fig 8-8. The male genital organs of *Rhinolophus pearsonii*. A, left lateral; B, right lateral; C, ventral; D, dorsal; E, anterior; and F, posterior view.

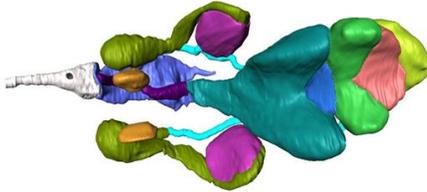
A



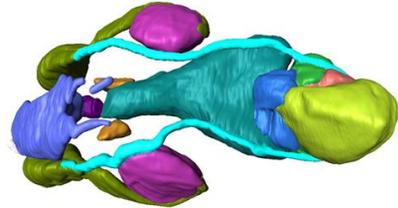
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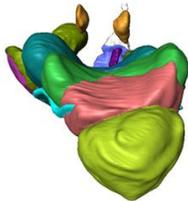
C



D



E



F

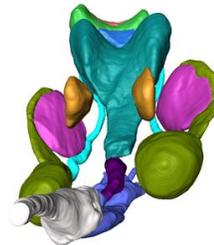


Fig 8-9. The male genital organs of *Rhinolophus pusillus*. A, left lateral; B, right lateral; C, ventral; D, dorsal; E, anterior; and F, posterior view.

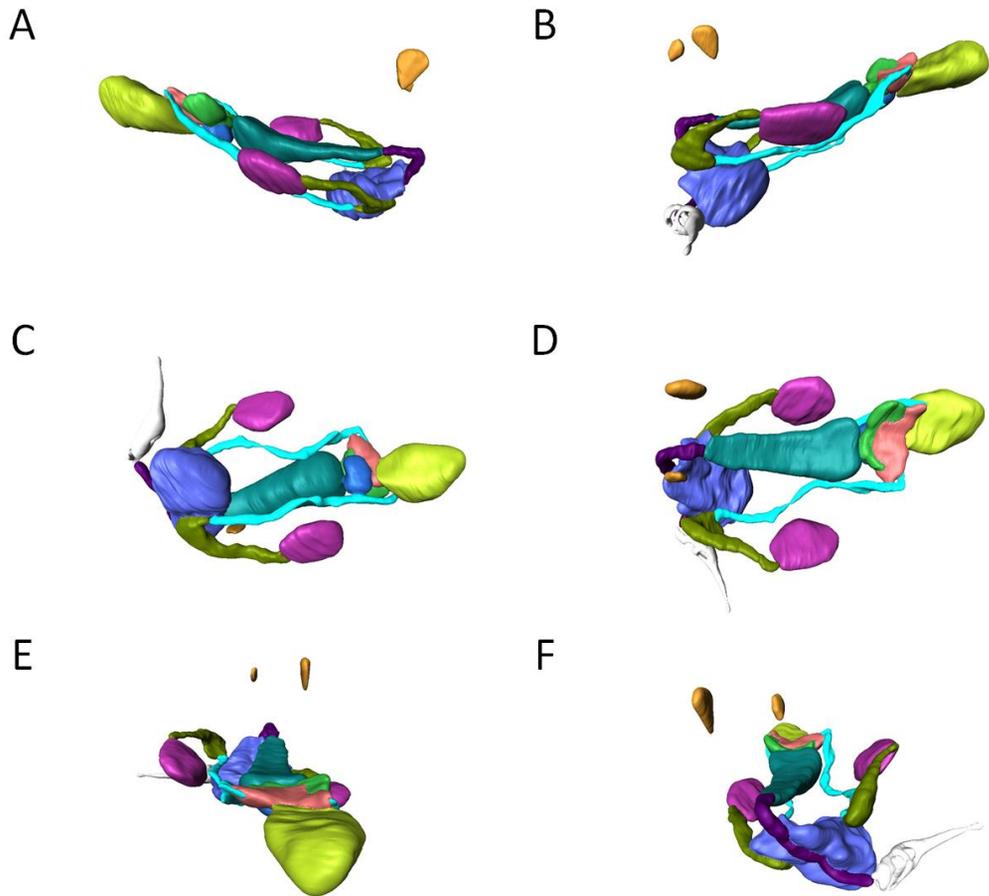


Fig 8-10. The male genital organs of *Rhinolophus steno*. A, left lateral; B, right lateral; C, ventral; D, dorsal; E, anterior; and F, posterior view.

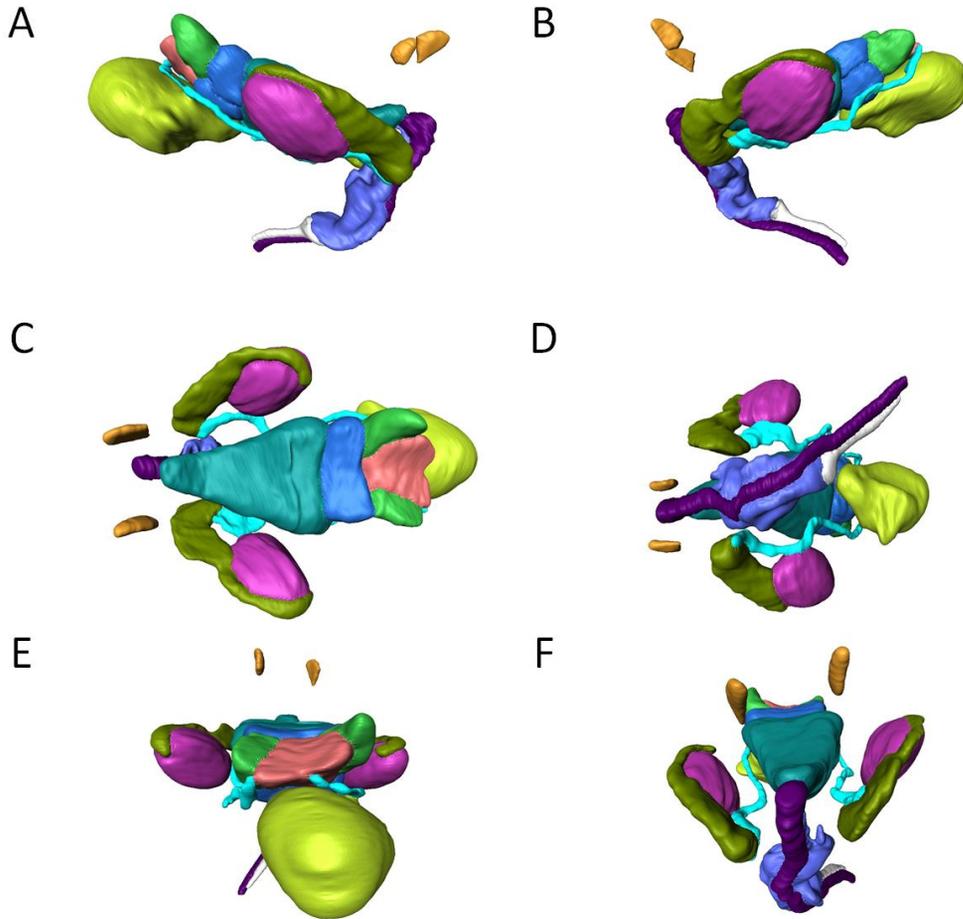


Fig 8-11. The male genital organs of *Rhinolophus thomasi*. A, left lateral; B, right lateral; C, ventral; D, dorsal; E, anterior; and F, posterior view.

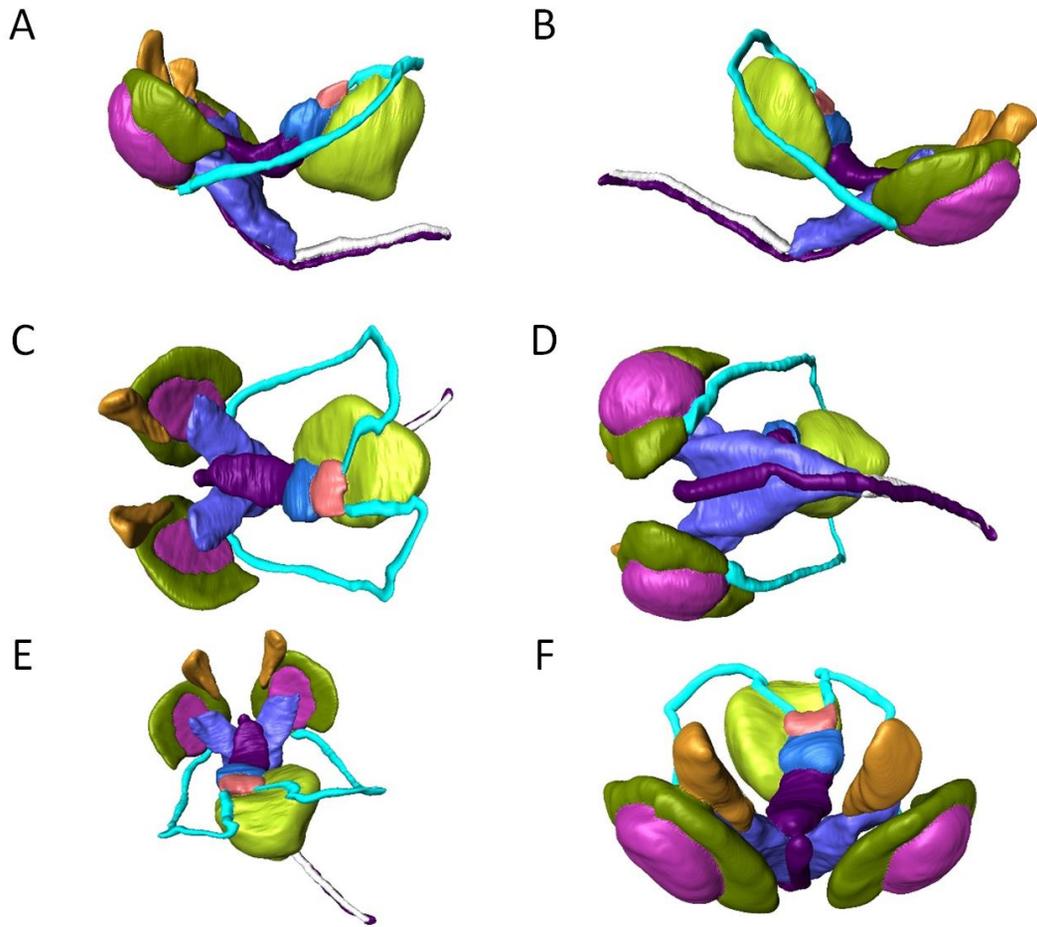


Fig 8-12. The male genital organs of *Megaderma lyra*. A, left lateral; B, right lateral; C, ventral; D, dorsal; E, anterior; and F, posterior view.

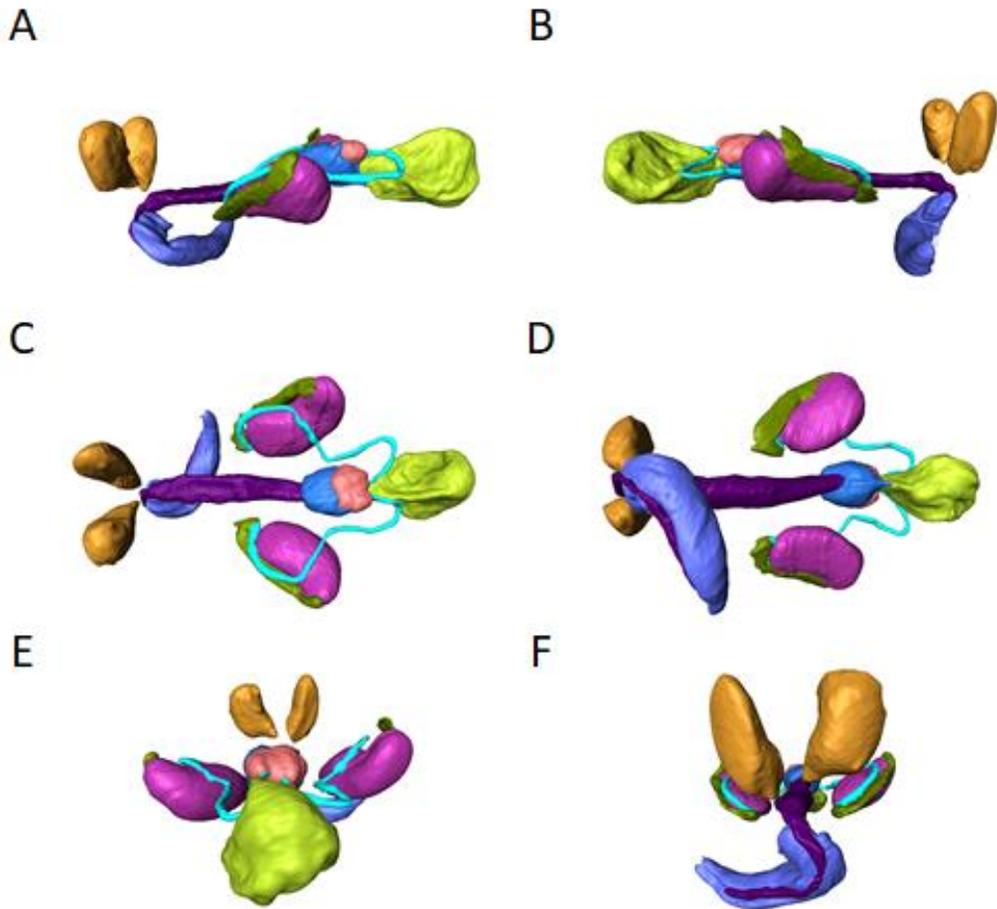


Fig 8-13. The male genital organs of *Megaderma spasma*. A, left lateral; B, right lateral; C, ventral; D, dorsal; E, anterior; and F, posterior view.

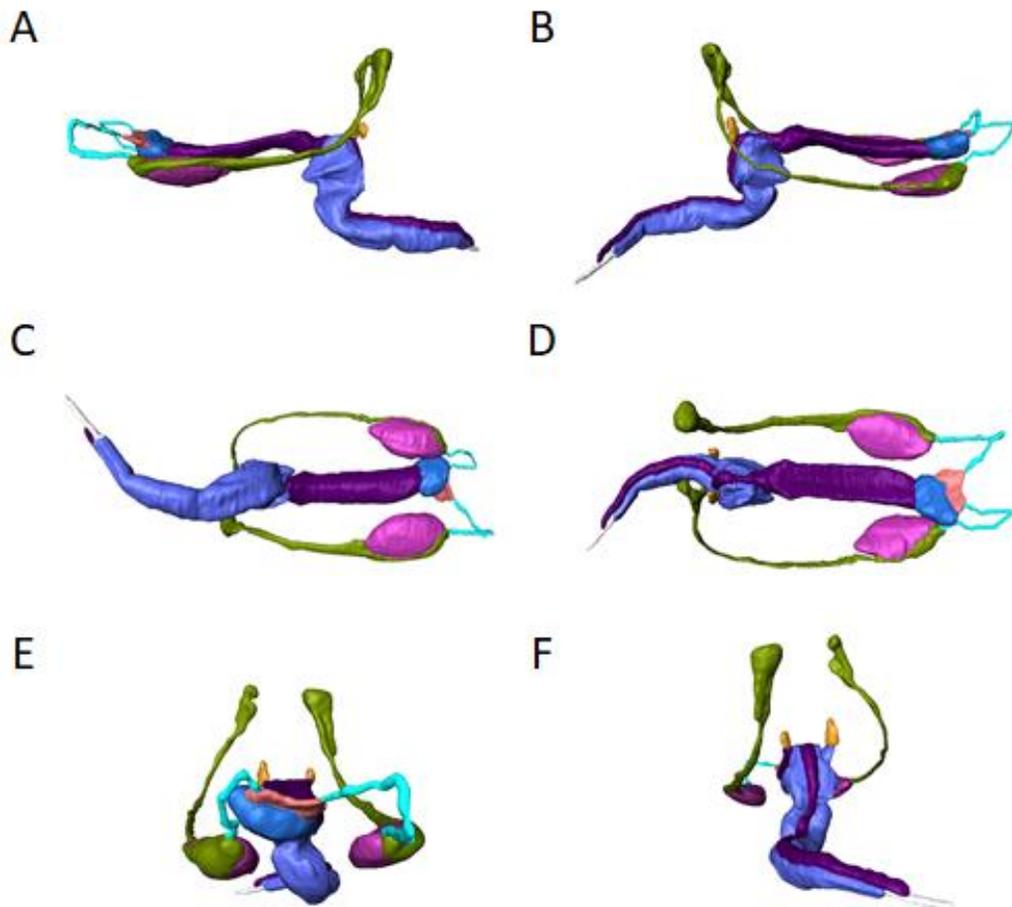
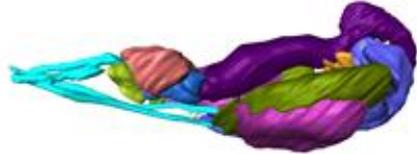


Fig 8-14. The male genital organs of *Aselliscus dongbacana*. A, left lateral; B, right lateral; C, ventral; D, dorsal; E, anterior; and F, posterior view.

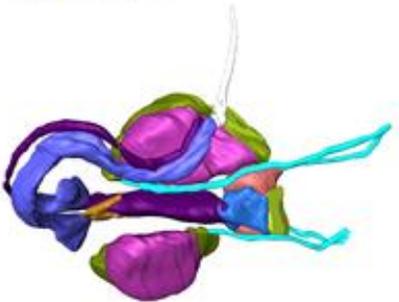
A



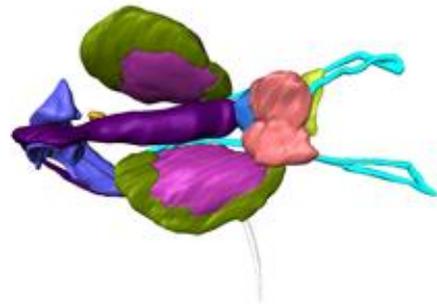
B



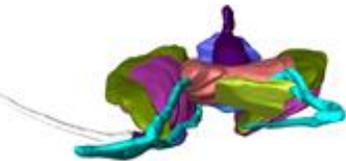
C



D



E



F



Fig 8-15. The male genital organs of *Coelops frithii*. A, left lateral; B, right lateral; C, ventral; D, dorsal; E, anterior; and F, posterior view.

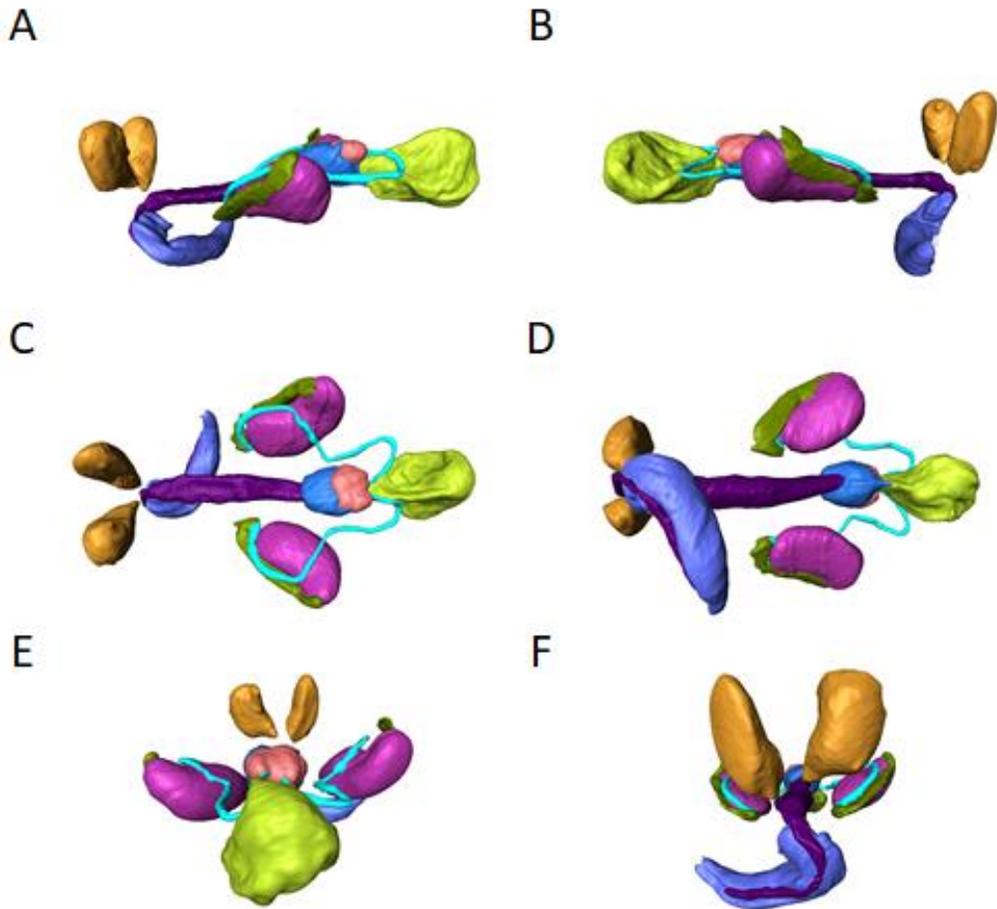


Fig 8-16. The male genital organs of *Hipposideros armiger*. A, left lateral; B, right lateral; C, ventral; D, dorsal; E, anterior; and F, posterior view.

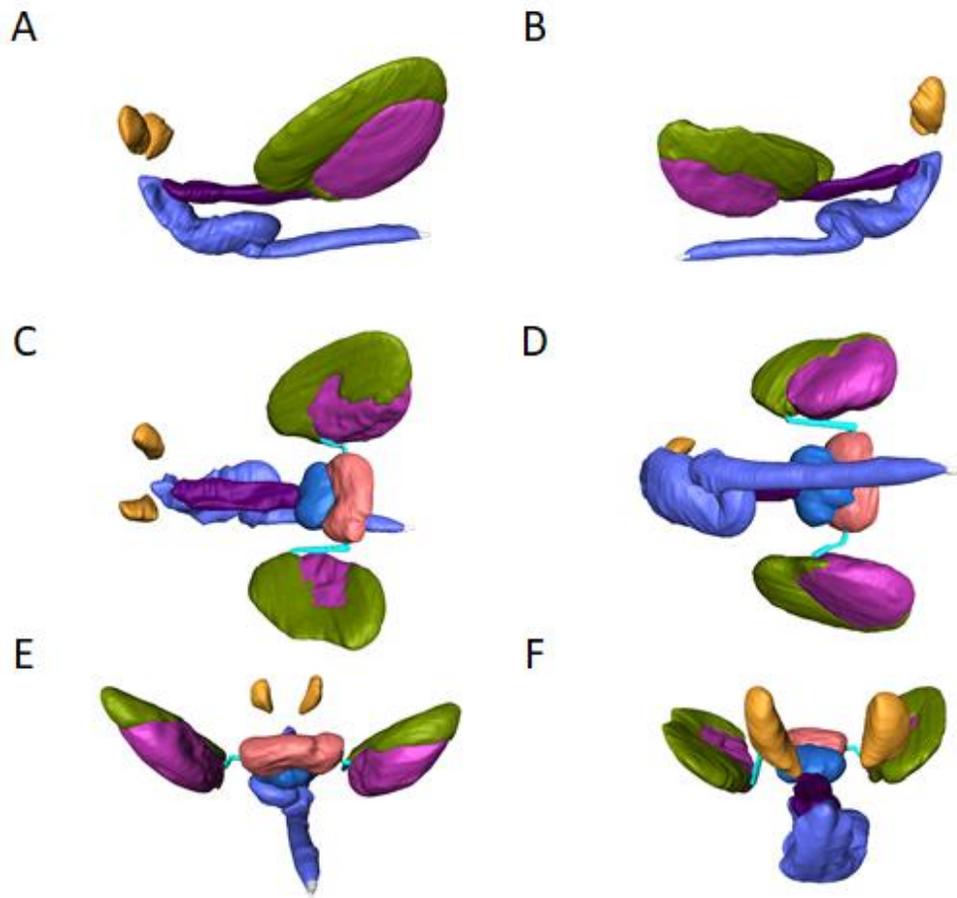


Fig 8-17. The male genital organs of *Hipposideros pomona*. A, left lateral; B, right lateral; C, ventral; D, dorsal; E, anterior; and F, posterior view.

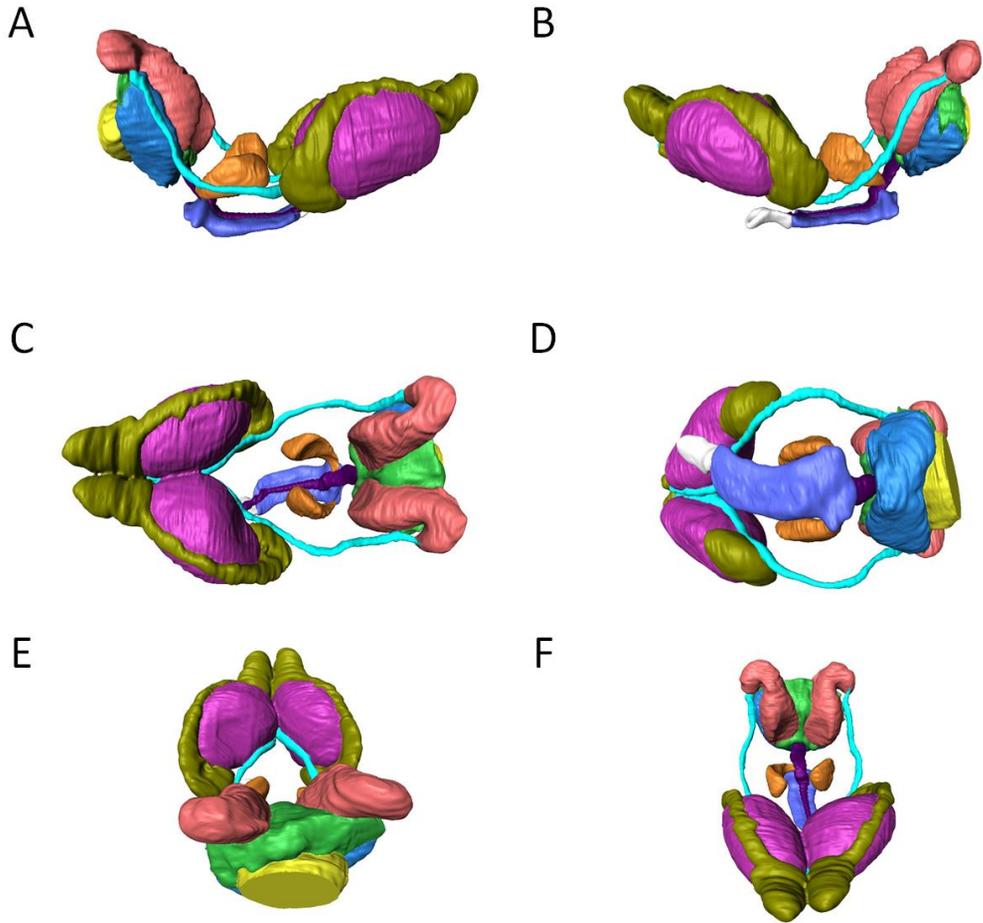


Fig 8-18. The male genital organs of *Murina hilgendorfi*. A, left lateral; B, right lateral; C, ventral; D, dorsal; E, anterior; and F, posterior view.

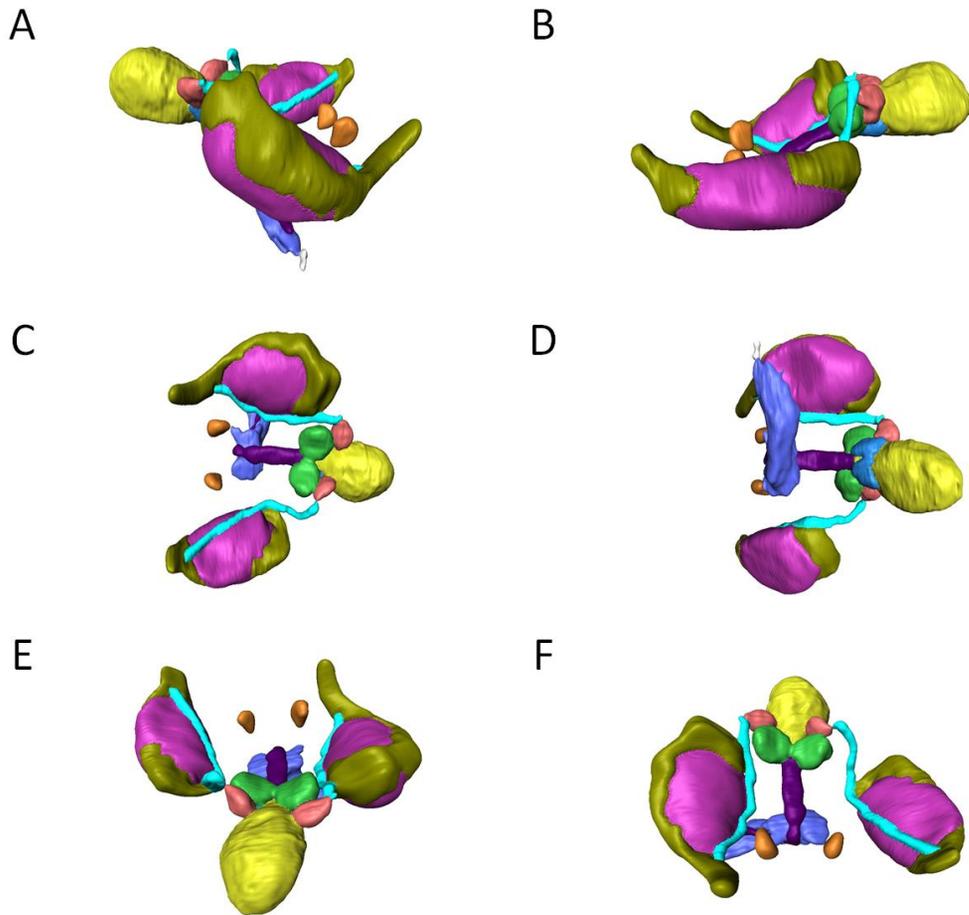


Fig 8-19. The male genital organs of *Myotis frater*. A, left lateral; B, right lateral; C, ventral; D, dorsal; E, anterior; and F, posterior view.

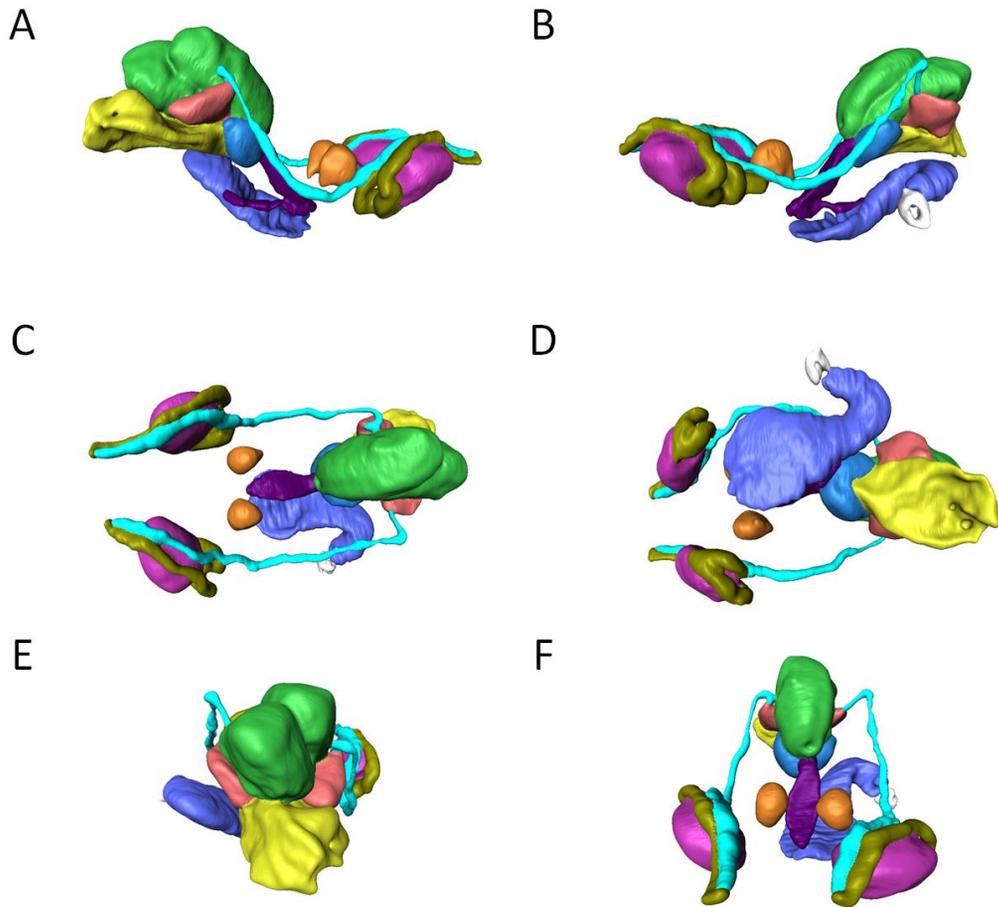


Fig 8-20. The male genital organs of *Myotis macrodactylus*. A, left lateral; B, right lateral; C, ventral; D, dorsal; E, anterior; and F, posterior view.

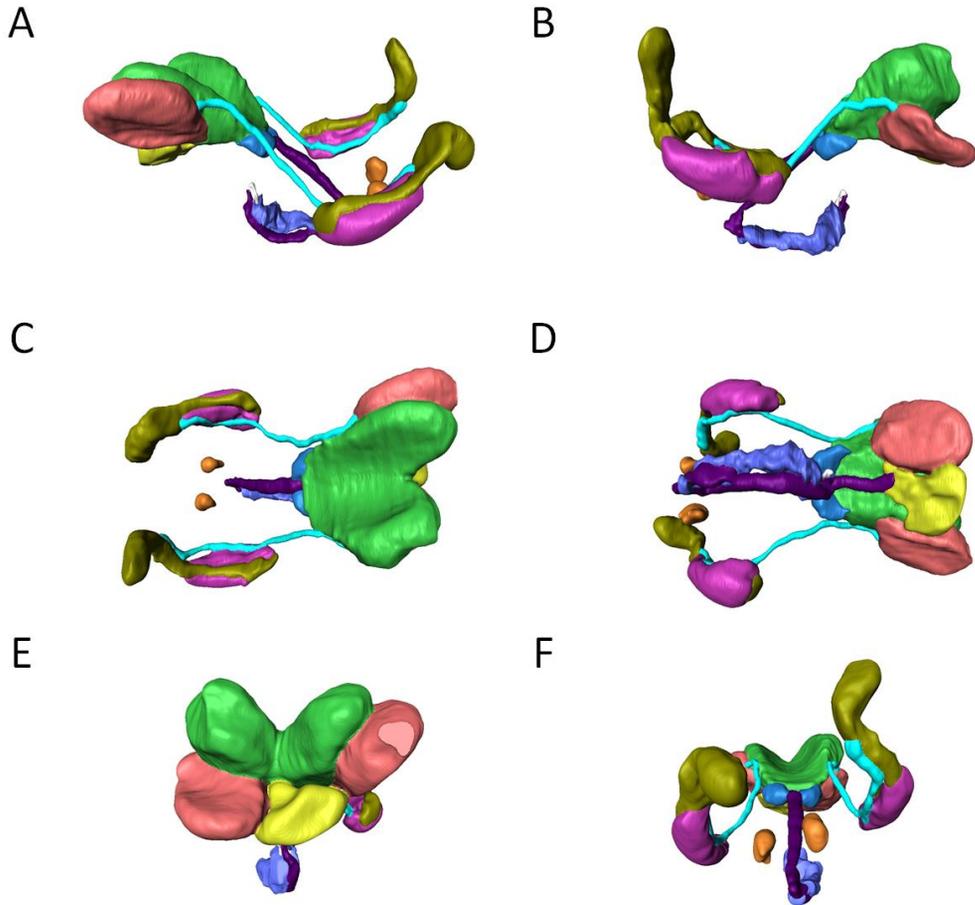
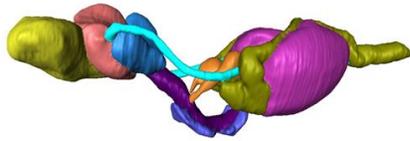
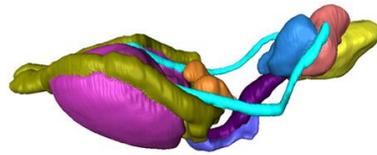


Fig 8-21. The male genital organs of *Myotis petax*. A, left lateral; B, right lateral; C, ventral; D, dorsal; E, anterior; and F, posterior view.

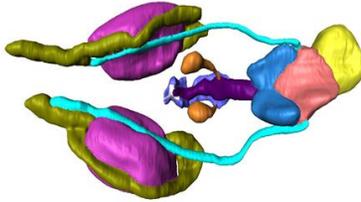
A



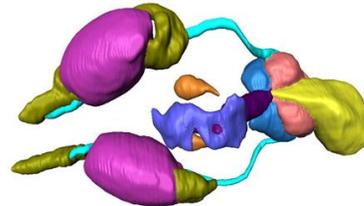
B



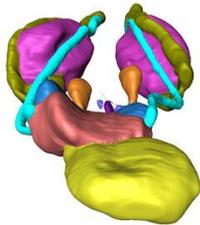
C



D



E



F

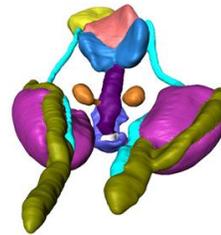


Fig 8-22. The male genital organs of *Pletocus sacrimontis*. A, left lateral; B, right lateral; C, ventral; D, dorsal; E, anterior; and F, posterior view.

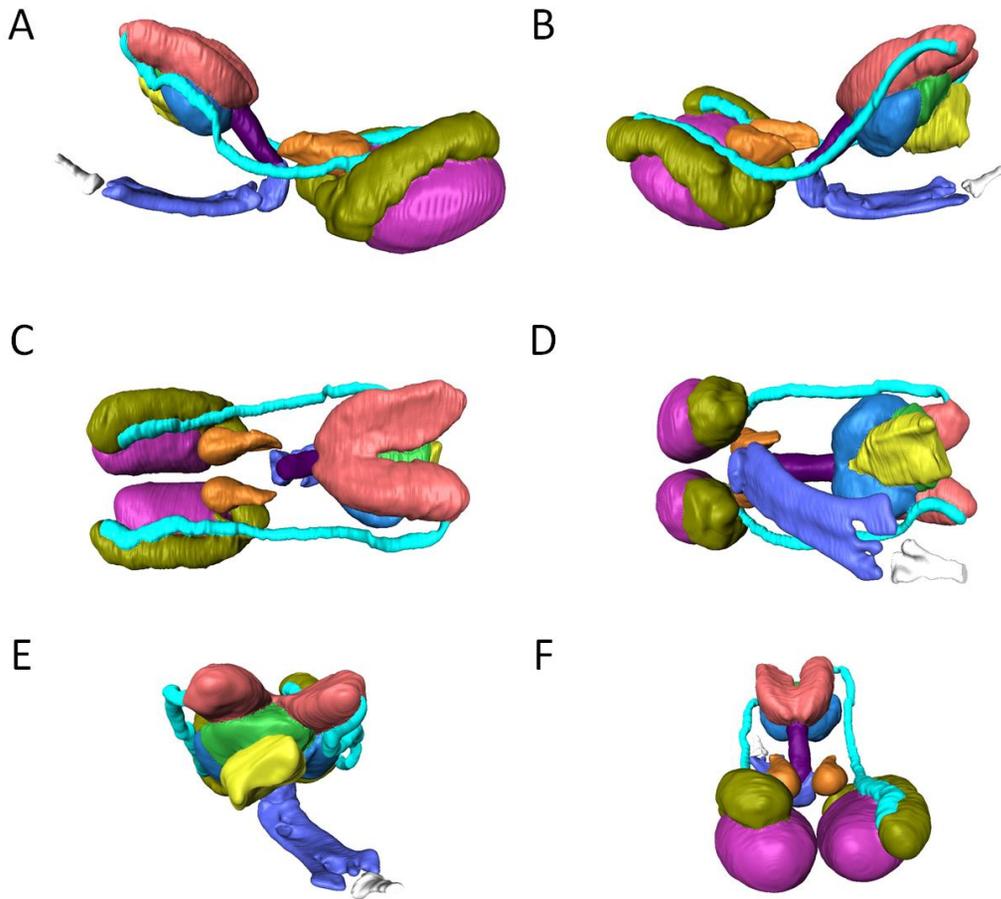


Fig 8-23. The male genital organs of *Scotophilus kuhlii*. A, left lateral; B, right lateral; C, ventral; D, dorsal; E, anterior; and F, posterior view.

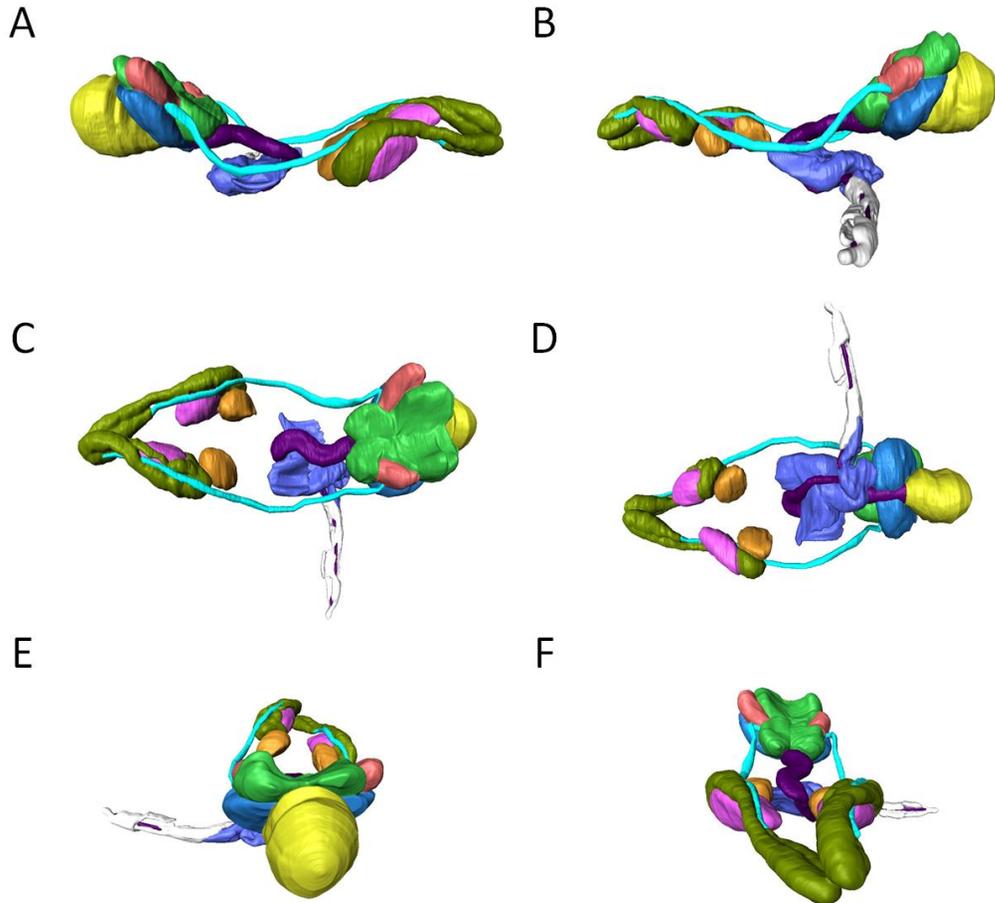


Fig 8-24. The male genital organs of *Vespertilio sinensis*. A, left lateral; B, right lateral; C, ventral; D, dorsal; E, anterior; and F, posterior view.

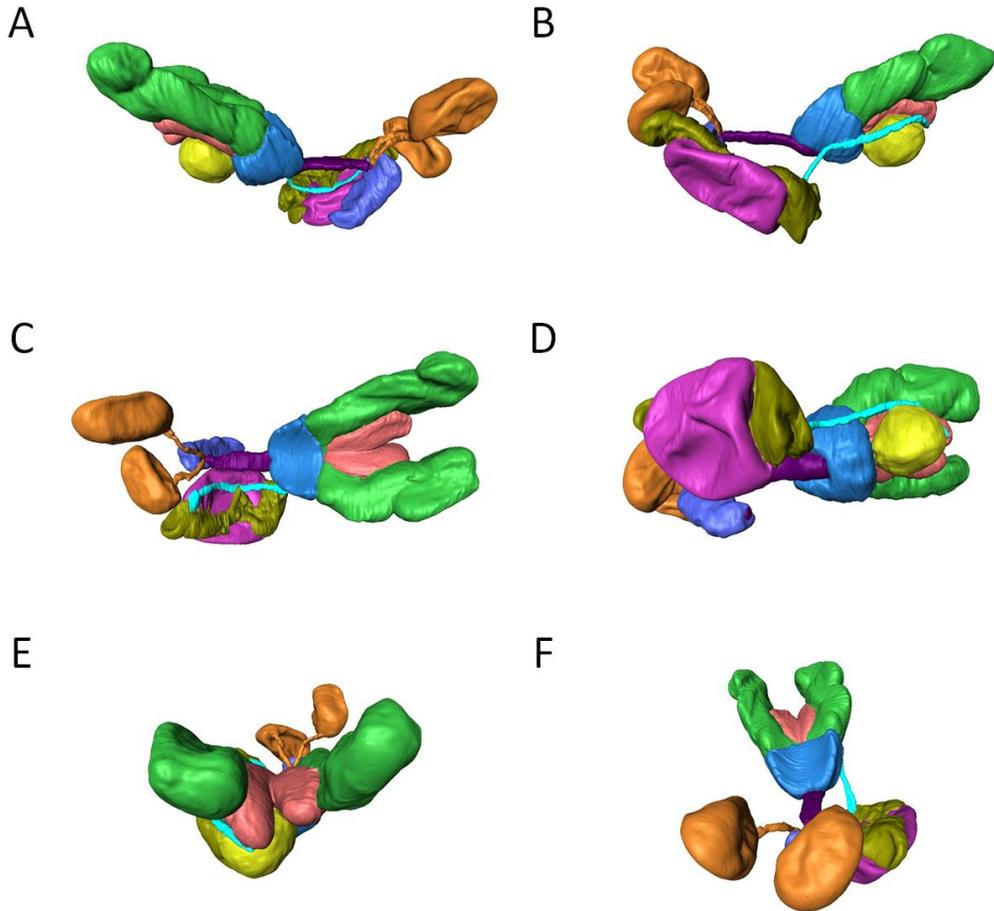


Fig 8-25. The male genital organs of *Taphozous longinamus*. A, left lateral; B, right lateral; C, ventral; D, dorsal; E, anterior; and F, posterior view.

## **ACKNOWLEDGEMENTS**