Developmental processes of divergent genital morphologies in closely-related Ohomopterus ground beetles

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博士論文

Developmental processes of divergent genital morphologies in closely-related *Ohomopterus* ground beetles

（オオオサムシ亜属の多様な交尾器形態の発生過程の解明）

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Chapter 1

General introduction

General introduction

Insects constitute at least a half of global species diversity and their morphology is extremely diverse (Gullan & Cranston 2014). Thus, insects will be the important target for understanding the origins of biodiversity, and in particular the diversity of morphology. Sexual traits, such as male ornaments subjected to female choice, and male weapons used in male–male combat, are a typical example of divergent morphologies (Darwin 1871; Andersson 1994, Shuster & Wade 2003, Emlen 2008). Diversification may lead to exaggeration, enhancing the differences not only in the shape but also in the size among species. The mechanisms of diversification of these traits are relatively well studied in the context of sexual selection and speciation. However, less is known about the developmental mechanisms of sexual traits (Arnqvist 1994, Danielsson et al. 1999, Brooks 2002).

The genitalia are the most prominent sexual traits, and the diversity of genital morphology among species is a notable feature in animals that practice internal fertilization (Eberhard 1985). Additionally, in some lineages, genitalia show a marked

trend towards exaggeration (Eberhard 1985). These elaboration and diversification in male and female genital morphologies have been revealed to be associated with sexual selection and speciation. Postcopulatory sexual selection via sperm competition, cryptic female choice, and sexual conflict predominantly underlie the evolution and diversification of genitalia in most taxa (Eberhard, 1985, 2010, Hosken & Stockley 2004, Simmons 2014, Brennan & Prum 2015, Langerhans et al. 2016, Sloan & Simmons 2019). Natural selection against hybridization (Kawano 2002, 2004, Kameda et al. 2009, Kawakami & Tatsuta 2010, Hollander et al. 2013, Kosuda et al. 2016, Nishimura et al. 2022) and in response to ecological variation (Langerhans et al. 2016) may also result in divergent genital morphologies between closely related species or populations. Differences in male and female genital morphologies can hinder hybridization between closely related species as a mechanical and sensory reproductive isolation barrier (Sota & Kubota 1998, Tanabe & Sota 2008, Sota & Tanabe 2010, Bath et al. 2012, Kamimura & Mitsumoto 2012, Masly 2012, Wojcieszek & Simmons 2013, Richmond 2014, Barnard et al. 2017, Tanaka et al. 2018). Thus, selective factors leading to diversification of genital

morphology and the ecological and evolutionary consequences by divergence of genital morphology have been relatively well understood.

In contrast to the evolutionary processes shaping genital diversification, only limited examples are known about the morphogenesis of male and female genitalia. In fruit flies, the initial primordia of *Drosophila melanogaster* are a good experimental model for studies of morphogenesis and well-studied (Estrada et al. 2001). In particular, the focus has been on the differentiation between the male and female organs. The detailed process of genital disc development was originally investigated to explore how sex determination genes and homeotic genes direct the development of a population of cells into male or female terminalia. Recently, genetic studies focusing on the diversification of genital morphologies among species have identified that various genes or genomic regions responsible for species differences have been revealed (Masly et al. 2011, Tanaka et al. 2015, Hagen et al. 2019). Aside from fluit flies, genes contributing to the development of the genitalia have been investigated including the milkweed bug *Oncopeltus fasciatus*, the red flour beetle *Tribolium castaneum* (Aspiras et al. 2011), and

the dung beetles *Onthophagus taurus* and *O. binodis* (Macagno & Moczek 2015). Currently, however, few developmental studies have focused on genital morphologies concerning species diversification (e.g., Matsumura et al. 2013).

The exaggerated genital traits have been reported in some organisms (Gack & Peschke, 2005, Matsumura 2021, McCracken et al. 2001), and its evolutionary background has been revealed in several studies (Brennan et al. 2007, 2015, Kamimura 2005). For examples, in the earwig *Euborellia plebeja* (Dermaptera: Anisolabididae), the elongated male intromittent organ is as long as its body. The elongated male genital organ is used to remove rival sperm from the female sperm-storage organ (spermatheca), the length of which is twice that of the female body (Kamimura 2005). Such exaggeration in genital size may be promoted by sexual selection via cryptic female choice (Kamimura 2005). However, little is known about the processes that enable the development of such oversized genitalia.

In this thesis, I attempted to reveal the mechanisms of diversification in the shape and size of the genitalia especially from the developmental point of view. To this end, I

conducted a series of comparative and descriptive studies using the ground beetle species belonging to the subgenus *Ohomopterus* (Coleoptera, Carabidae, genus *Carabus*): *Carabus maiyasanus*, *C. iwawakianus* and *C. uenoi*, which have species-specific genitalia with evidence for coevolutionary divergence between the sexes (Ishikawa 1987, 1991, Sasabe et al. 2010, Fujisawa et al. 2019). In the chapter 2, I compared the developmental processes of the male and female genitalia between the *C. maiyasanus* and *C. iwawakianus* that had contrasting genital morphologies, and revealed the developmental processes leading to interspecific differences. In the chapter 3, I addressed the constraints on the evolution of exaggerated male genital morphology based on a comparative analysis of phenotypic covariation between the genitalia and other body parts using *C. maiyasanus*, *C. iwawakianu*s and *C. uenoi*. In the chapter 4, I investigated the developmental processes of *C. uenoi* that had extremely enlarged male genitalia to reveal the underlying mechanisms of the exaggeration of genital morphology. In the final chapter, I briefly summarized my main findings and discussed the evolution and diversification of sexual traits in the context of developmental process. This study is expected to provide a novel

insight into the mechanism of sexual trait diversification that leading to biological diversification.

Chapter 2

Heterochrony and growth rate variation mediate the development of divergent genital morphologies in closely related *Ohomopterus* ground beetles

2.1 Introduction

Genital morphology diverges more rapidly than other phenotypic traits among groups of animals with internal fertilization (Eberhard, 1985). Postcopulatory sexual selection via sperm competition, cryptic female choice, and sexual conflict predominantly underlie the evolution and diversification of genitalia in most taxa (Eberhard, 1985, 2010, Hosken & Stockley 2004, Simmons 2014, Brennan & Prum 2015, Langerhans et al. 2016, Sloan & Simmons 2019). Natural selection against hybridization (Kawano 2002, 2004, Kameda et al. 2009, Kawakami & Tatsuta 2010, Hollander et al. 2013, Kosuda et al 2016) and in response to ecological variation (Langerhans et al. 2016) may also result in divergent genital morphologies between closely related species or populations. Additionally, differences in male and female genital morphologies can hinder hybridization between closely related species as a mechanical and sensory reproductive isolation barrier (Sota & Kubota 1998, Tanabe & Sota 2008, Sota & Tanabe 2010, Bath et al. 2012, Kamimura & Mitsumoto 2012, Masly 2012, Wojcieszek & Simmons 2013, Richmond 2014, Barnard

et al. 2017, Tanaka et al. 2018). Thus, elaborate male and female genitalia are associated with sexual selection and speciation.

In contrast to the evolutionary processes shaping genital diversification, little is known about the morphogenesis of male and female genitalia in arthropods, except in fruit flies (Estrada et al. 2001). The initial primordia of *Drosophila melanogaster* are a good experimental model for studies of morphogenesis. Each one of the three genital primordia develop into external genitalia in each sex, depending on the genetic sex of the fly. Studies of the fruit fly have mainly focused on the morphogenesis of elaborate male genitalia or on differentiation between the male and female organs. The detailed process of genital disc development was originally investigated to explore how sex-determination genes and homeotic genes direct the development of a population of cells into male or female terminalia. Genetic studies focusing on the diversification of genital morphologies have identified various genes or genomic regions responsible for species differences (Masly et al. 2011, Tanaka et al. 2015, Hagen et al. 2019). Genes contributing to the development of genitalia have also been investigated in non-model species, including the

milkweed bug *Oncopeltus fasciatus*, the red flour beetle *Tribolium castaneum* (Aspiras et al. 2011), and the dung beetles *Onthophagus taurus* and *O. binodis* (Macagno & Moczek 2015). Few studies have described differentiation in the developmental processes generating genitalia between closely related species. Criocerinae leaf beetles are the first reported case of divergence in the development of the internal sac and flagellum in male intromittent organs (Matsumura et al. 2013).

Ground beetles belonging to the subgenus *Ohomopterus* (Coleoptera, Carabidae, genus *Carabus*) have species-specific genitalia with evidence for coevolutionary divergence between the sexes (Ishikawa 1987, 1991, Sasabe et al. 2010, Fujisawa et al. 2019). Genital morphologies in this group are related to sexual selection (Takami & Sota 2007, Takami et al. 2018) and speciation (Sota & Kubota 1998, Nagata et al. 2007, Takami et al. 2007, Kubota et al. 2013, Fujisawa et al. 2019). These species have genetic sex determination with male heterogamety (Serrano & Yadav 1984). The male has a sclerotized hook-like structure on the intromittent organ called the copulatory piece (CP, Fig. 2-1). The CP is a sclerotized projection in the wall of the endophallus (internal sac);

it is everted from the aedeagus (AD) in copula (Fig. 2-1). The female has a membranous pocket attached to the ventral wall of the bursa copulatrix (BC) called the vaginal appendix (VA, Fig. 2-1). In copula, the CP is inserted into the VA, and the male and female genitalia are rigidly coupled (Ishikawa 1987, Takami 2002). The CP plays roles in spermatophore deposition and sperm transfer (Takami 2003) as well as in the displacement of rival spermatophore (Takami 2007, Okuzaki and Sota 2014). A relatively long CP is more likely to manipulate fertilization and oviposition activity in females and to inflict fitness costs to the female, while a longer VA can mitigate these costs, consistent with sexually antagonistic coevolution of the CP and VA (Takami et al. 2018). Since the sizes and shapes of the CP and VA are species-specific, a mismatch between heterospecific genitals can prevent interspecific hybridization (Sota and Kubota 1998, Kubota et al. 2013). Thus, the diversification of genital morphologies via sexual selection and/or sexual conflict is relevant to mechanical isolating barrier between *Ohomopterus* species (i.e., lock-and-key, Shapiro and Porter 1989, Masly 2012, Fujisawa et al. 2019).

The CP and VA in *Ohomopterus* ground beetles have been studied in behavioral

and evolutionary contexts, including the identification of candidate genes or genomic regions associated with genital diversification (Sasabe et al. 2007, 2010, Fujimaki et al. 2014, Sota et al. 2018, Fujisawa et al. 2019, Nomura et al. 2019). An understanding of the morphogenesis of genitalia, including spatiotemporal differences between species, can provide a precise framework for analyses of the differential expression and functions of candidate genes. However, little is known about the developmental basis of *Ohomopterus* genitalia.

In this study, I evaluated the developmental processes underlying divergent male and female genital morphologies in *Ohomopterus* species. I observed the internal structures of the pupae of sister species with divergent genital morphologies, *Carabus maiyasanus* and *C. iwawakianus*, by X-ray micro-computed tomography (μCT). I describe the morphogenesis of species-specific genitalia and discuss the developmental events responsible for differentiation between species. Especially, two hypothetical developmental processes responsible for the morphological differentiation, heterochrony and growth rate variation, are focused on. Heterochrony is expected if the the timing of

the initial formation of a structure differ between species. Growth rate variation is expected if developmental speed of a structure differ or change between species.

2.2 Materials and methods

2.2.1 Sampling

C. maiyasanus has a long and hook-like CP, whereas *C. iwawakianus* has a short and broad CP. Females have VAs corresponding to the CP of conspecific males. The two species had similar body sizes (mean ± s.d.; *C. maiyasanus*: male, 23.01 ± 0.77 mm [N = 51], female, 23.42 ± 0.94 mm [N = 42]; *C. iwawakianus*: male, 23.31 ± 0.74 mm [N = 82], female, 23.97 ± 0.77 mm [N = 78]).

Adults were collected in Kobe, Hyogo, Japan (*C. maiyasanus*) and Suzuka, Mie, Japan (*C. iwawakianus*) during the reproductive season (early May to late June, 2017–2019) using pitfall traps. Collected beetles were immediately transferred to the laboratory. A male and a female were kept in a plastic jar (diameter, 10 cm; height, 10 cm) with moistened sphagnum and soil at the bottom (2 cm) and placed in an incubator (16L:8D,

20°C) to maintain sexual maturity. Beetles were fed minced beef every other day and allowed to mate and oviposit freely. Eggs were collected from the soil and kept in a plastic cup (diameter, 7 cm; height, 4 cm) with moistened soil and sphagnum. Hatched larvae were reared individually in the plastic cup under the same conditions. Larvae were fed megascolecid earthworms *ad libitum*. Satiated third instar larvae were moved to a plastic jar (6.5 cm diameter and 9 cm height) filled with soil (depth, 7 cm) for pupation. Larvae usually dug down into the soil to make a pupal room. If the pupal room was visible from the outside due to contact with the wall of the plastic jar, the time of pupation was observed directly. If the pupal room was hidden in the soil and not visible from the outside, the larva or prepupa was dug out and moved to an artificial pupal room to observe the time of pupation. Artificial pupal rooms were made of moistened tissue paper within a plastic cup placed in an incubator with no lights. Our preliminary examination indicated that pupae of the two species commonly spent 12 days until eclosion under the present conditions (20°C, N = 3 for *C. maiyasanus*, N = 3 for *C. iwawakianus*). Thus, pupae were sampled at 2, 4, 6, 8, and 10 days after pupation. Three male and three female pupae were

collected from each developmental stage, except at 2, 6, 8 and 10 days after pupation in female *C. iwawakianus* (N = 2). Pupae were sexed based on the morphology of the abdominal terminalia.

2.2.2 Infiltration, scanning, and analysis

The integument of each pupa was perforated with a fine pin and then fixed by FAE solution (formalin: acetic acid: ethanol = 6:1:16) for 1 day. Fixed pupae were dehydrated in a graded ethanol series and preserved in 70% ethanol. Fixed pupae were soaked in 1% ethanol iodine for 3–4 days. Infiltrated pupae were washed with 70% ethanol, mounted individually in a plastic tube (diameter, 1 cm; height, 5 cm), and submersed in 70% ethanol just before scanning.

X-ray micro-CT scans were performed using an XRadia Micro XCT-400 system (Carl Zeiss, Oberkochen, Germany). The acquisition parameters for scans were as follows: accelerating voltage = 150 kV, source current = 65 μA, exposure time = 10 sec; samples were rotated from -90° to 90°, with a rotation step of 0.01°. A total of 1800

images were obtained per sample, resulting in a voxel size of 6.43 μm. Scans were converted to stacks of digital image slices, which were reconstructed into three-dimensional (3D) images and analyzed using Amira 6.4 (FEI). Volume-rendered images were viewed from the longitudinally sliced plane of the object.

The CP and VA were measured throughout development. The size of the CP was defined as its maximum length (CPL, Fig. 2-1), as determined using the distance measure function in Amira (N = 3 for all developmental stages). The size of the VA was defined as the total length of the wall (VAL, Fig.2-4), as viewed on the longitudinally sliced pictures and measured using the freehand function in ImageJ 1.49m (Rasband 1992-2012) (N = 3 for all developmental stages except for 2, 6, 8 and 10 days after pupation in *C. iwawakianus*, N = 2). To estimate relative genital sizes by controlling variation in body size, the width of the 7th abdominal tergite was measured as a proxy of pupal body size using Amira. Genital parts of adult males and females were removed and measured based on digital images obtained using a digital camera mounted on a Leica S9i microscope (Leica Microsystems, Wetzlar, Germany) (*C. maiyasanus*: male, N = 47, female, N = 20;

C. iwawakianus: male , N = 70, female, N = 20).

To examine the developmental processes leading to genital shape differences between the species, the sizes of genital parts were compared between species by general linear models with species, developmental stage, and their interaction as explanatory variables. Statistical analyses were performed using JMP ver. 8 (SAS Institute, 2009).

2.3 Results

The morphogenesis of the male and female genitalia was qualitatively similar in *C. maiyasanus* and *C. iwawakianus*. However, I detected interspecific differences in the timing and rate of growth of genital parts, as described below.

2.3.1 Male genitalia

I did not detect apparent differences in the morphogenesis of the AD between the two species (Fig. 2-2). At 2 days after pupation, the apical portion of the AD was already formed and exposed at the abdominal terminalia. The basal portion of the AD was not yet

formed at this stage. Until 10 days after pupation, the basal portion of the AD elongated toward the internal abdomen. At 10 days after pupation, the shape of AD was similar to that of adults. The AD was not measured because there was no apparent morphological difference between species and the mature AD could not be fully viewed in the aperture.

The morphogenesis of the internal sac (excluding the CP) was also similar in the two species (Fig. 2-2). At 2 days after pupation, the ejaculatory duct ran longitudinally in the stem of the immature AD. The prospective internal sac was formed within the immature AD as a distal inflation of the ejaculatory duct. The internal sac was clearly distinguished from the ejaculatory duct at the part where the duct expanded abruptly. Until 10 days after pupation, the cavity surrounded by the internal sac increased along with the elongation of the AD, and the wall of the internal sac became thicker and folded. The length of the ejaculatory duct remained virtually constant at the stem of the AD. This process was similar in the two species.

The timing of CP formation differed between the two species (Fig. 2-3). The CP originated from a small protrusion of a part of the internal sac, which was directed

internally to the cavity surrounded by the internal sac within the AD. In *C. maiyasanus*, with a long-hooked CP, a small protrusion was observed 4 days after pupation. This protrusion exhibited gradual elongation until day 10, and the shape became similar to that of the adult. In *C. iwawakianus*, with a short-broad CP, a small protrusion was observed 6 days after pupation. This protrusion exhibited elongation and broadening until day 8, at which time the shape was similar to that of the adult.

2.3.2 Female genitalia

There were no apparent differences in the morphogenesis of the BC between species. The timing of BC formation was also similar, however, the growth rate differed between the two species after day 6 (Fig. 2-4). A small BC was observed 2 days after pupation, with substantial development until at least 10 days after pupation. The wall of the BC simply emerged by day 6 and became folded thereafter.

The VA originated from a small protrusion of the ventral wall of the BC. It was rudimentary 2 days after pupation in both species. In *C. maiyasanus*, the VA elongated

continuously until day 10 or later (Fig. 2-4). VA elongation was slightly slower in *C. iwawakianus* than in *C. maiyasanus* until day 10.

2.3.3 Interspecific differences in developmental processes

Increase in CPL differed between species as indicated by the significant interaction between species and stages (Table 2-1). Development of the CP of *C. maiyasanus* began between days 2 and 4, followed by continuous growth until day 10, at which point the length was similar to that of the adult CP (Fig. 2-5a). CP development in *C. iwawakianus* began between days 4 and 6, followed by continuous growth until day 8, at which point the length was similar to that of the adult CP. The lengths of the *C. maiyasanus* CP on days 4 and 6 were similar to those of *C. iwawakianus* CP on days 6 and 8, respectively. These results indicate that the interspecific difference in CP length arose at a relatively early pupal stage (i.e., day 4), after which the rates of growth were similar, with a 2-day delay in *C. iwawakianus*. Relative CPL showed similar results (Fig. 2-6).

Increase in VAL also differed between species as indicated by the significant

interaction between species and stages (Table 1). In both species, the VA started to develop before day 2, followed by growth until day 10, at which point the sizes did not differ from those of the adult VA (Fig. 2-5b). VA sizes were similar between species from days 2 to 6. On day 8, the VA increased substantially in size from day 6 in *C. maiyasanus* but not in *C. iwawakianus*. These results indicate that the interspecific difference in the VA arose at a relatively late pupal stage (i.e., day 8 and later) and could be attributed to constant and decreased growth rates in *C. maiyasanus* and *C. iwawakianus*, respectively. Relative VAL showed similar results (Fig. 2-6).

2.4 Discussion

I characterized the morphogenesis of species-specific genital parts in closely-related ground beetle species *C. maiyasanus* and *C. iwawakianus*. Aside from *Drosophila*, this is the first report of the developmental events leading to divergent genital morphologies related to reproductive isolation and speciation. The genital parts that are shared (i.e., not differentiated) between species, the AD in the male and BC in the female, were formed

at the early pupal stages, indicating that the development of these parts began prior to pupation. This is consistent with the development of genitalia in *Drosophila*, which starts during embryogenesis and continues throughout larval and pupal stages (Estrada et al. 2003). By contrast, the CP started to develop and differentiate between species specifically in the pupal stages, and VA differentiation was largely observed in the latter half of the pupal stages. These results suggest that the general morphology is shared between species at early developmental stages, but is followed by the modification of parts at later stages, leading to interspecific differences. This finding indicates that the early developmental stages are more strongly constrained (or canalized) than the later developmental stages. Stabilizing selection may have operated more strongly in the early developmental stages than in the later stages.

Our comparison of genital morphogenesis between species provides insight into the developmental processes underlying evolutionary differentiation in genitalia. In the male, the timing of the initial formation of the CP differed between the species. CP formation began at 4 and 6 days after pupation in *C. maiyasanus* and *C. iwawakianus*,

respectively, after which rates of growth were similar in the two species (Fig. 2-5a). Accordingly, heterochrony is involved in the differentiation of male genital parts. In the female, the timing of VA formation and initial growth rates were similar in the two species; however, at late stages, growth was more rapid in *C. maiyasanus* than in *C. iwawakianus* (Fig. 2-5b). The CP and VA were longer in *C. maiyasanus* than in *C. iwawakianus*, suggesting that *C. maiyasanus* needs to develop larger genital parts than *C. iwawakianus*. The developmental solution of this requirement differed between the sexes: a shift in the developmental schedule in males and a change in growth rate in females. Sexual difference in developmental constraint may play a role (Wylde & Bonduriansky 2020). These findings suggest that heterochrony and changes in growth rate are crucial developmental processes underlying the evolutionary diversification of genital morphologies. These conclusions are based on limited sample sizes, and I refrained from detailed statistical analyses for species differences. However, the observed interspecific differences in morphogenetic processes of male and female genital parts were unambiguous (Figs. 2-5 and 2-6).

The observed difference in developmental processes between sexes provides insights into the genetic factors underlying genital morphologies. Quantitative genetic and genomic analyses have suggested that genes responsible for species-specific genital morphologies in the two sexes are not in tight linkage (Sasabe et al. 2010, Fujisawa et al. 2019). Thus, genetic pathways resulting in the development of species-specific genital parts may differ between the sexes. This also indicates that species-specific morphological matching between the CP and VA may be a result of coevolution between the sexes driven by selective processes, such as natural selection against morphological mismatches in heterospecific mating (Sota and Kubota 1998) and intersexual selection and/or sexual conflict (Takami 2003, Takami et al. 2018).

In Coleoptera, Matsumura et al. (2013) described the detailed development of the male genitalia in leaf beetles (Chrysomelidae, Criocerinae). Highly elaborate internal sacs in Criocerinae leaf beetles initially form outside of the AD and subsequently retract into the AD, unlike in the ground beetles evaluated in this study. In ground beetles, the internal sac formed and developed within the AD (Fig. 2-2). The difference in the

morphogenesis of the male internal sac between suborders of Coleoptera (i.e., leaf beetles in Polyphaga and ground beetles in Adephaga) may allow to revise the recognition of homology in these genital parts between the suborders. Additionally, selection for an elaborate internal sac may be related to drastically altered morphogenetic processes. The findings in this study clarify a novel aspect of diversity in the most speciose insect order with marked diversification in external and internal morphologies.

The results of this study revealed the spatio-temporal dynamics of the development of divergent genital parts. These findings are expected to facilitate future gene expression and knockdown analyses using *Ohomopterus* ground beetles, which are currently based on broad tissue types and rough developmental stages, introducing uncertainties in the identification of candidate genes associated with divergence in genital morphologies (Fujimaki et al. 2014, Sota et al. 2018, Fujisawa et al. 2019, Nomura et al. 2019). These results improve our understanding of the precise developmental stages and tissues contributing to species-specific genital differentiation and will contribute to the construction a novel platform for evo-devo studies of the diversification of genital

morphologies.

Table 2-1. General linear models explaining variation in male and female genital sizes during pupal developmental periods between *Carabus maiyasanus* and *C. iwawakianus*.

	F	d.f.	*P*
Model for CPL (R^2 = 0.98)	500.9	11,135	<0.0001
Species	397.4	1,135	<0.0001
Stage	575.8	5,135	<0.0001
Species x stage	46.0	5,135	<0.0001
Model for VAL (R^2 = 0.94)	70.8	11,54	<0.0001
Species	42.4	1,54	<0.0001
Stage	99.3	5,54	<0.0001
Species x stage	11.0	5,54	<0.0001

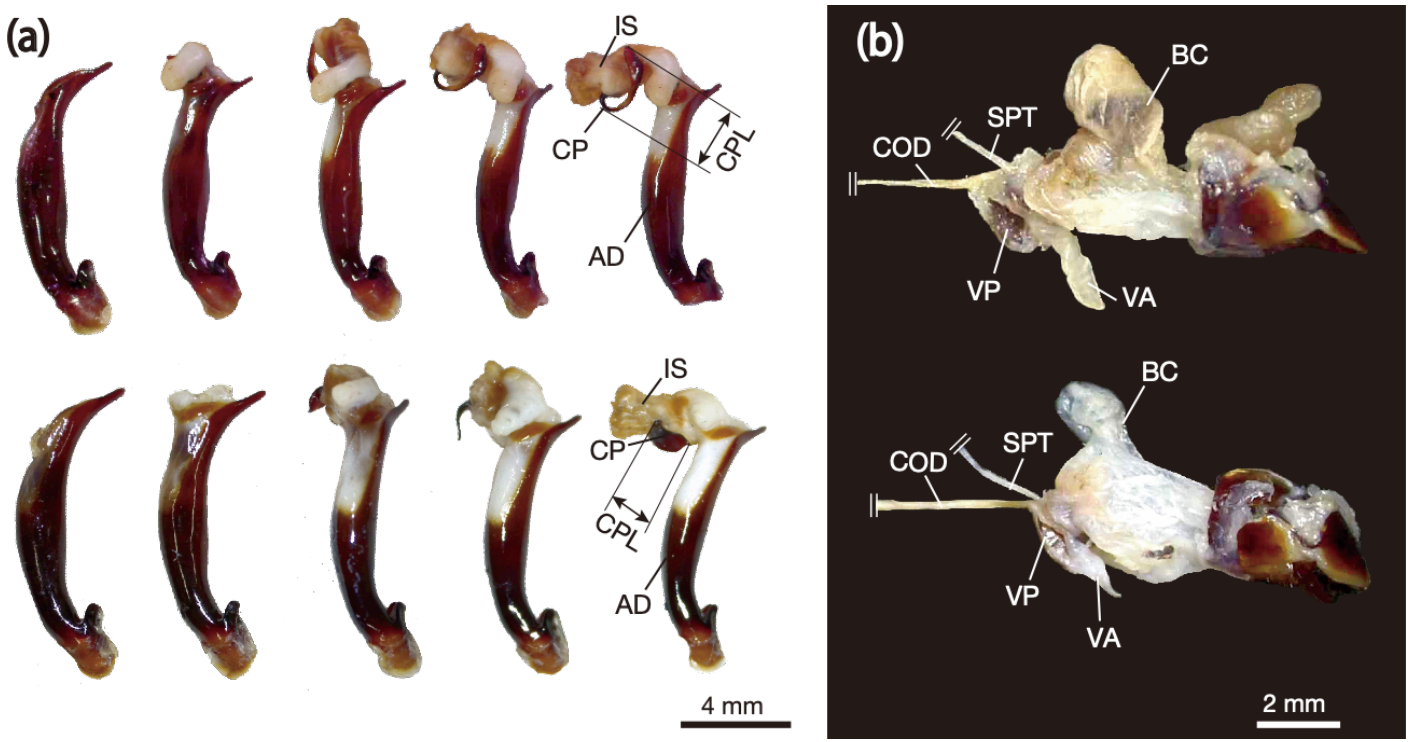

Fig. 2-1. (a) Male genitalia of *Carabus maiyasanus* (upper) and *C. iwawakianus* (lower), showing internal sacs everting from the aedeagus (left to right); AD: aedeagus; CP: copulatory piece; CPL: copulatory piece length; IS: internal sac. (b) Female internal reproductive organ of *C. maiyasanus* (upper) and *C. iwawakianus* (lower); BC: bursa copulatrix; COD: common oviduct; SPT: spermatheca; VA: vaginal appendix; VP: vaginal apophysis.

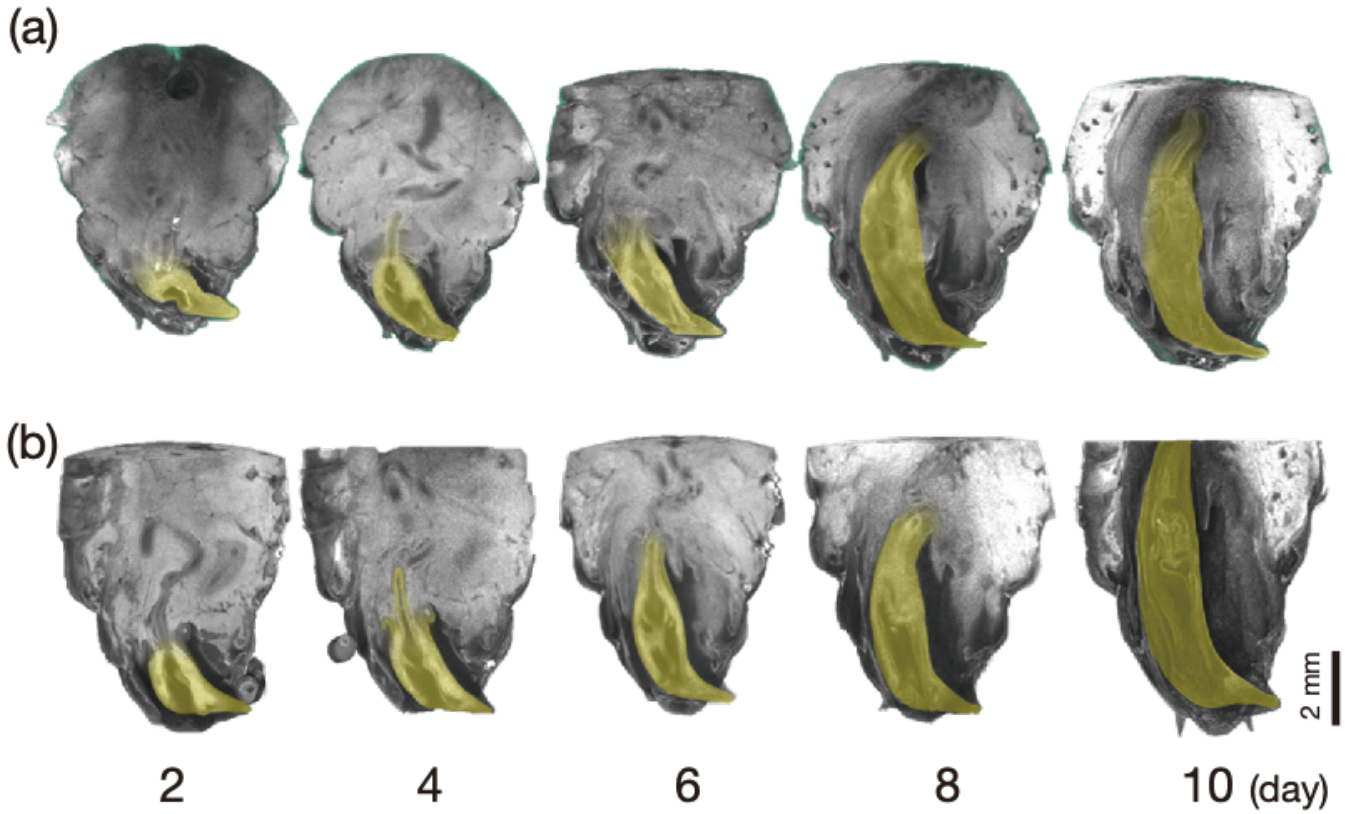

Fig. 2-2. Morphogenetic processes of AD and IS therein (yellow). (a) *Carabus maiyasanus*; (b) *C. iwawakianus*.

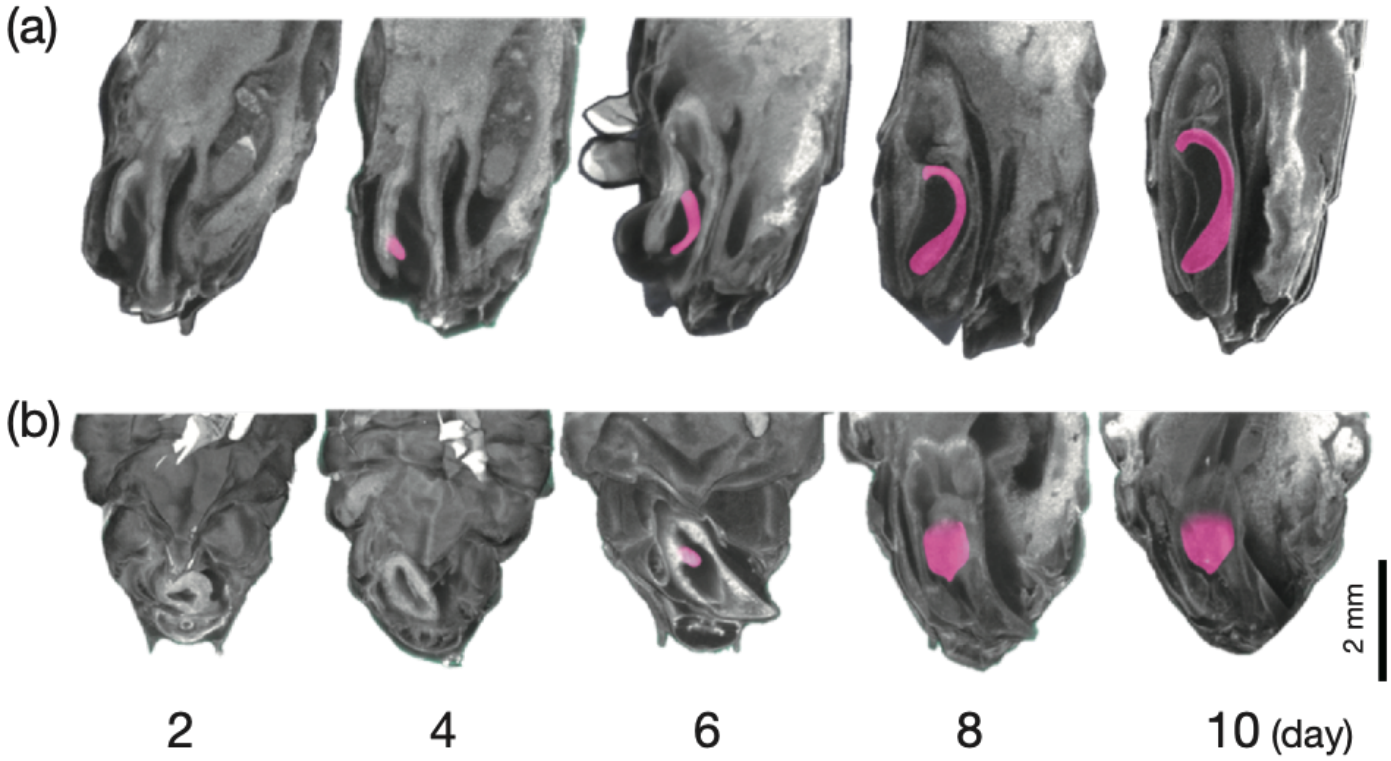

Fig. 2-3. Morphogenesis of CP (magenta). (a) *Carabus maiyasanus*; (b) *C. iwawakianus.*

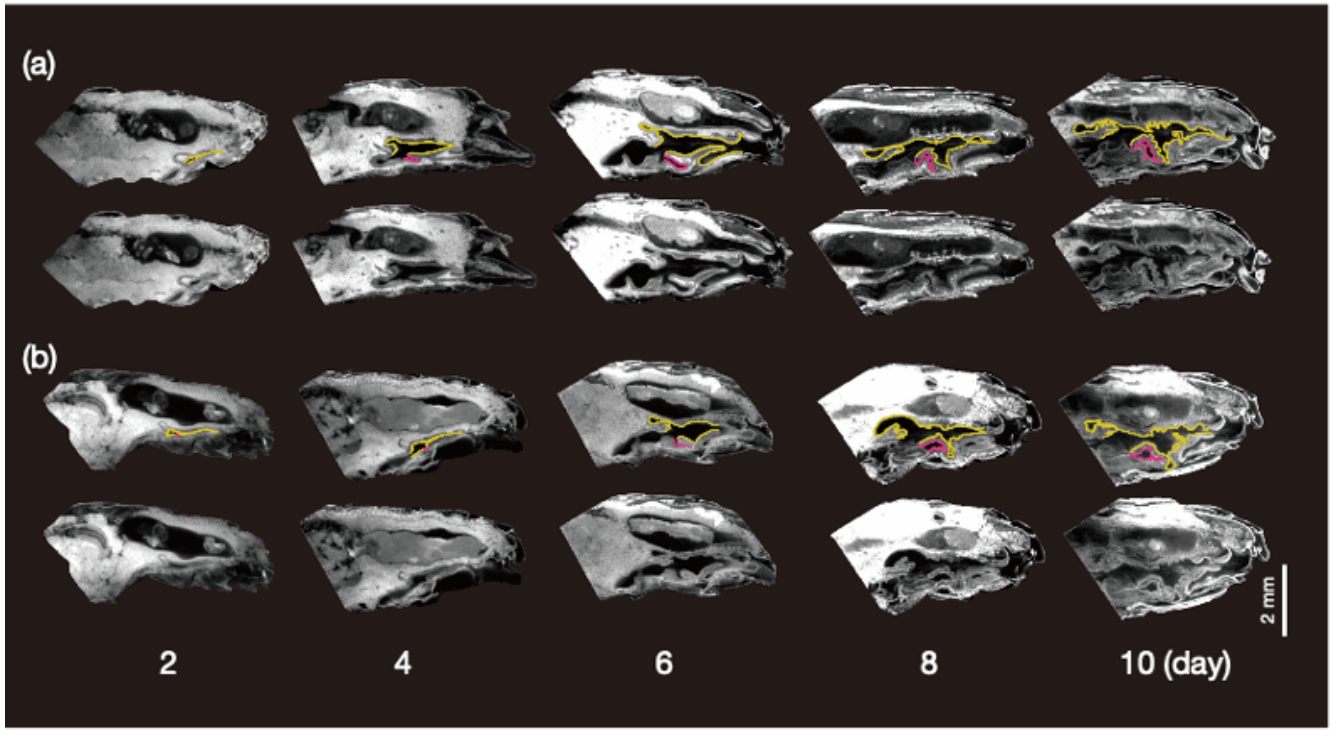

Fig. 2-4. Morphogenesis of the female genitalia, (a) *C. maiyasanus*; (b) *C. iwawakianus*. BC (yellow) and VA (magenta) are indicated in upper pictures, which correspond to the lower raw pictures. VA length (VAL) was defined as the length of magenta trace.

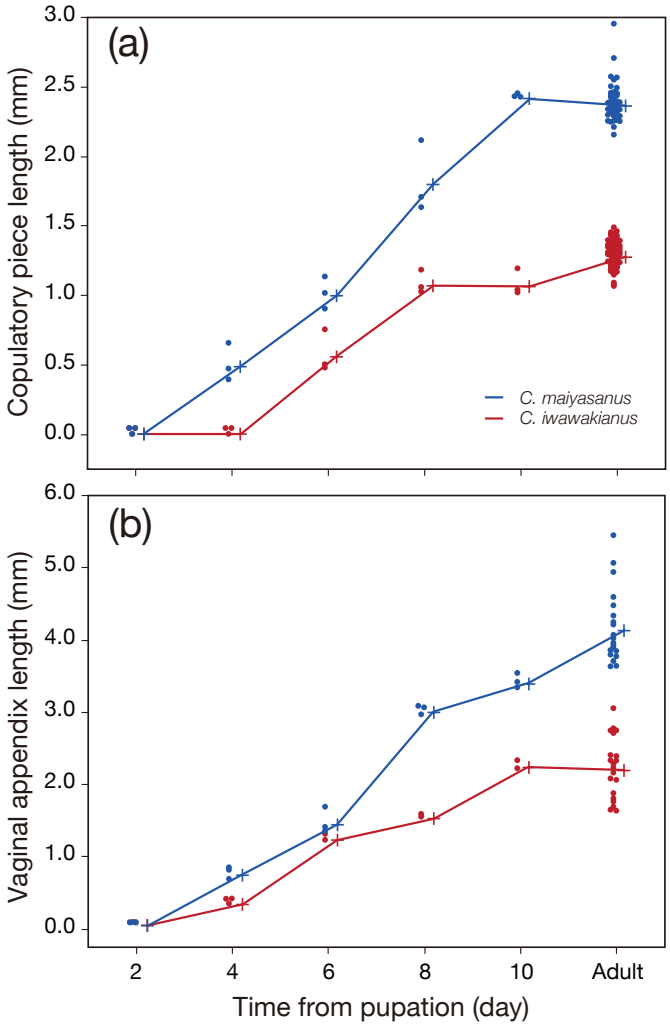

Fig.2-5. Variation in genital sizes during development in *Carabus maiyasanus* (blue) and *C. iwawakianus* (red). (a) CPL; (b) VAL.

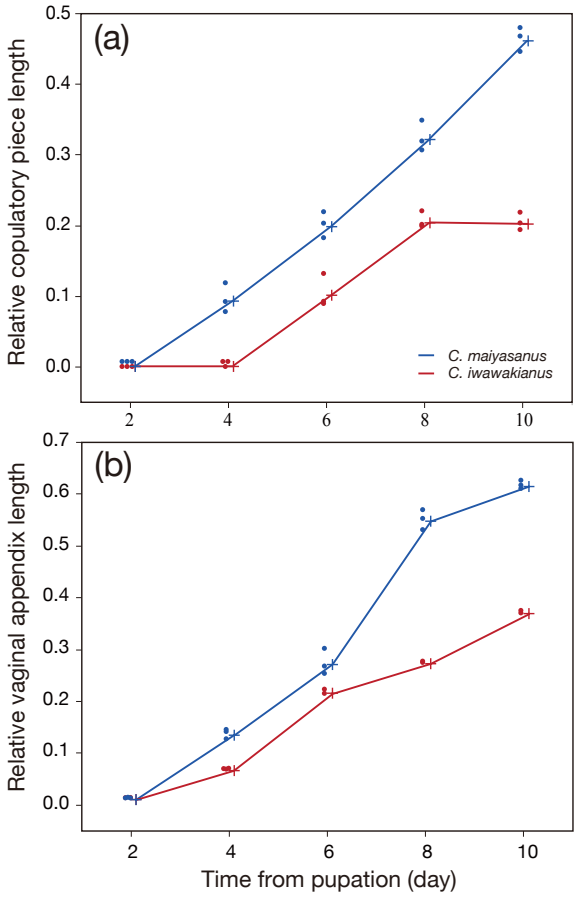

Fig. 2-6. Variation in relative genital sizes (i.e., genital size divided by body size) during development in *Carabus maiyasanus* (blue) and *C. iwawakianus* (red). (a) relative CPL; (b) relative VAL.

Chapter 3

Functional, genetic, and structural constraints on the exaggeration and diversification of male genital morphology in *Ohomopterus* ground beetles

3.1 Introduction

Male ornaments subjected to female choice and male weapons used in male–male combat are typical examples of exaggerated sexual traits (Darwin 1871, Andersson 1994, Shuster & Wade 2003, Emlen 2008). The evolution of exaggerated sexual traits may involve the relaxation of various constraints. Functional constraints affect the utility of exaggerated traits *per se* or decrease individual survival by holding the exaggerated traits. For example, exaggerated sexual traits may attract the attention of potential predators and hinder antipredator behavior (Jennions et al. 2001, Godin et al. 2003, Langerhans et al. 2005). Genetic constraints, such as genetic correlations or pleiotropy, may hinder the independent evolution and exaggeration of a trait (Hansen 2003). For example, a combination of pleiotropy and linkage mediates genetic regulatory variation in the development of male ornaments (comb and bone) in the chicken, implying that there are constraints on comb mass (Jhonsson et al. 2014). Structural constraints, such as competition for space and resources among traits, may necessitate coordination between the exaggerated trait and surrounding structures. For example, the production of horns

reduces the size of neighboring morphological structures in beetles (Emlen 2001), suggesting that horn development is constrained by competition among structures in the head. These constraints can be detected by phenotypic covariation between traits (Armbruster et al. 2013). However, few studies have comprehensively investigated the effects of these constraints on the evolution of exaggerated sexual traits. Comparative analyses of related species with and without exaggerated sexual traits are expected to be useful to evaluate the tradeoff between constraints and exaggeration (e.g., Satomi et al. 2021, Fasanelli et al. 2022).

Male genital morphology diverges more rapidly than other phenotypic traits among groups of animals with internal fertilization (Eberhard 1985). Postcopulatory sexual selection via sperm competition, cryptic female choice, and sexual conflict predominantly underlie the evolution and diversification of genitalia in most taxa (Eberhard 1985, 2010, Hosken & Stockley 2004, Simmons 2014, Brennan & Prum 2015, Langerhans et al. 2016, Sloan & Simmons 2019). Natural selection against hybridization (Kawano 2004, 2006, Kameda et al. 2009, Kawakami & Tatsuta 2010, Hollander et al.

2013, Kosuda et al. 2016, Nishimura et al. 2022) and in response to ecological variation (Langerhans et al. 2016) may also result in divergent genital morphologies between closely related species or populations. The remarkable diversity of male genital morphology, determined by multiple selective contexts as well as structural (Gack & Peschke 2005, Matsumura et al. 2013) and genetic (Aspiras et al. 2011, Genevcius et al. 2020, Hagen et al. 2020) factors, provides an ideal opportunity for examining constraints on sexual trait exaggeration.

Extreme variation in male genital morphology is likely to be stabilized due to functional constraints. Since male genital organs are usually stored within the male body, natural selection via predation may operate infrequently (cf. Langerhans et al. 2005). However, exaggerated male genitalia may have various fitness consequences; for example, it may result in mismatches with female copulatory organs (the one-size-fits-all hypothesis, Eberhard et al. 1998). Extreme-sized male genitalia may lead to a physical mismatch with the female genitalia, or may be subjected to cryptic female choice, possibly resulting in reduced fertilization success. Under this type of functional constraint,

reduced variation in genital size and resultant weak scaling relationships between genital and body sizes (i.e., negative allometry) are expected (Eberhard et al. 1998). On the other hand, some organisms have acquired extremely exaggerated male copulatory organs as a result of sexually antagonistic coevolution. In a species of waterfowl, the male phallus is almost half a meter long (McCracken et al. 2001). In waterfowls, vaginal structures function as a barrier to phallus penetration, suggesting that directional selection for male phenotypes able to penetrate the female barrier is likely responsible for the exaggeration of the male genitalia (Brennan et al. 2007, 2015). Such directional selection is expected to result in increase in genital size variation by increaseing exaggerated phenotypes, leading to steep scaling relationships, as in male ornamental and weapon traits (Kodric-Brown et al. 2006). Thus, a change in the selective regime from stabilizing selection (or selection against exaggeration) to directional selection (or selection for exaggeration) may be seen as a relaxation of functional constraint against exaggeration.

Phenotypic variation in male genital morphology can be constrained, or modulated, by genetic and developmental factors. The male copulatory organs in insects

are hypothesized to be homologous to appendages in other body segments, such as antennae, jaws, and legs, as evidenced by the regulatory roles of appendage-patterning genes in genitalia development (Aspiras et al. 2011, Macagno & Moczek 2015, Nomura et al. 2021). In addition, genetic correlations have been detected between the male genitalia and other body parts (Arnqvist & Thornhill 1998) and between the male and female genitalia (Simmons & Garcia-Gonzalez 2011). These genetic constraints are also expected to lead to significant phenotypic correlations between genitalia and other morphological traits. Exaggeration of external body parts may be limited by natural selection (e.g., by behavioral changes or predation), thereby indirectly constraining the exaggeration of internal organs, such as genitalia, via genetic correlation. Alternatively, appendages in insect body segments are morphologically so diverse among antennae, jaws, legs, and genitalia, and the genetic constraints on these appendages may have already been relaxed. If this is the case, no signs of constraints are expected to be detected. For example, the condition-dependent expression of shared genes may facilitate the exaggeration of genitalia, resulting in a decrease in phenotypic correlations and a

relaxation of genetic constraint.

Structural constraints may limit the exaggeration of genital morphology. Males have to accommodate unwieldy structures, copulatory organs, in a limited space (i.e., the abdomen) and manipulate them acutely during copulation (Jałoszynski et al. 2014). Anatomical specializations and/or mechanisms to accommodate and efficiently manipulate the genitalia may be necessary prior to, or in concert with the evolution of exaggerated genitalia (Briceño et al. 2011, Eberhard 2005, Gack & Peschke 2005, Matsumura & Yoshizawa 2010, 2012). The flagellum of Criocerinae leaf beetles is an extremely elongated intromittent organ. These beetles have acquired a specialized structure to store the flagellum in the abdominal cavity, and this promotes effective insertion and retraction. The acquisition of the novel trait surrounding the intromittent organ may facilitate the evolution of highly elongated genitalia (Matsumura et al. 2014). The coevolution of genital parts and surrounding structures may result in covariation, indicating that the exaggeration of the genitalia requires the evolution of surrounding structures and is not free from structural constraints.

Ground beetles belonging to the subgenus *Ohomopterus* (Coleoptera, Carabidae, genus *Carabus*) have species-specific genitalia with evidence for coevolutionary divergence between the sexes (Ishikawa 1987, 1991, Sasabe et al. 2010, Fujisawa et al. 2019). Genital morphologies in this group are related to sexual selection (Takami & Sota 2007, Okuzaki & Sota 2014, Takami et al. 2018) and speciation (Sota & Kubota 1998, Fujisawa et al. 2019, Nishimura et al. 2022). The male has a sclerotized hook-like structure on the intromittent organ called the copulatory piece (CP, Fig. 3-1). The CP is a sclerotized projection in the wall of the endophallus (internal sac); it is everted from the aedeagus (AD) in copula (Fig. 3-1). The female has a membranous pocket attached to the ventral wall of the bursa copulatrix (BC) called the vaginal appendix (VA). In copula, the CP is inserted into the VA, and the male and female genitalia are rigidly coupled (Ishikawa 1987, Takami 2002). The CP plays roles in spermatophore deposition and sperm transfer (Takami 2003) as well as in the displacement of rival spermatophores (Takami 2007; Okuzaki & Sota 2014). A relatively long CP is more likely to manipulate fertilization and oviposition activity in females and to inflict fitness costs to the female, while a longer VA

can mitigate these costs, consistent with sexually antagonistic coevolution of the CP and VA (Takami et al. 2018). Interspecific differences in the size of CP and VA are mediated by differences in developmental timing in males and in growth rate in females, respectively (Chapter 2, Terada et al. 2021).

In *Ohomopterus*, *Carabus uenoi* has an extraordinarily enlarged CP, reaching a third of the male body length (Fig. 1). By contrast, the closely related species *C. maiyasanus* and *C. iwawakianus* have much smaller hook-like and broad CPs than those of *C. uenoi* (Fig. 3-1). This exaggeration and interspecific variation in genital morphology may be due to sexual selection via sperm competition and/or sexual conflict (Takami & Sota 2007, Takami et al. 2018) and natural selection for species isolation (Nishimura et al. 2022). Therefore, the genital exaggeration in *C. uenoi* is hypothesized to be a result of change in selective regimes, i.e., relaxation of functional constraint. The analysis of gene expression in the development of the CP and VA indicated that genes associated with appendage development were enriched in a comparison between *C. uenoi* and other two species, suggesting contribution of appendage development genes in genital development

and diversification (Nomura et al. 2021). Phylogenomic analysis revealed the relationships among the three species could be described as (*C. iwawakianus*, (*C. maiyasanus*, *C. uenoi*)), suggesting that the exaggeration of the male genitalia occurred after the divergence of *C. maiyasanus* and *C. uenoi* (Fig. 3-1, Fujisawa et al. 2019). These species are suitable materials for analyses of constraints and the exaggeration of the male genitalia.

In this study, I evaluated the constraints on the evolution of exaggerated male genital morphology based on a comparative analysis of phenotypic covariation between traits using *Ohomopterus* ground beetles. I compared correlation coefficients for sizes and genital parts and other body parts among species. Functional constraints due to mismatches with female genitalia were examined by comparing allometric scaling relationships. Genetic constraints were examined based on correlation coefficients between sizes of genital parts and other appendage-derived organs. Structural constraints were examined based on correlation coefficients between measurements of abdominal and genital parts. I discuss the roles of functional, genetic, and structural constraints on

the exaggeration of male genitalia.

3.2 Materials and Methods

3.2.1 Sampling

Adult males were collected in Mt. Kongo-san, Osaka, Japan (*C. uenoi*), Kobe, Hyogo, Japan (*C. maiyasanus*), and Suzuka, Mie, Japan (*C. iwawakianus*) during the reproductive season (early May to early July, 2012) using pitfall traps. Collected beetles were immediately transferred to the laboratory and fixed with ethyl acetate. Males were dissected to remove the aedeagus, CP, and other body parts (see below) for morphological measurements. The carcasses of beetles were pinned and dried.

3.2.2 Measurement and correlation structure

To quantify phenotypic variation and covariation, five body, two appendage, and four genital dimensions were measured. Body dimensions were the prothorax length (TL), prothorax width (TW), elytra length (EL), abdomen width (ABW), and abdomen depth

(ABD, defined as the maximal distance from the top of elytra to the bottom of abdominal sternum) and were measured by digital calipers (0.01 mm increments). The appendage parameters were the 5th antennal segment length (ATL) and right hind tibia length (HTL). Genital dimensions were the aedeagus length (ADL), aedeagus width (ADW), CP length (CPL), and CP width (CPW). These parts were separated from the body and stuck on drafting paper, and the maximal length or width was measured on images obtained by a digital camera attached to a microscope (Leica EZ4HD) using ImageJ to the nearest 0.01 mm. To minimize measurement error, each part was measured five times (10 times for relatively small parts, ATL and CPW), and the average value was used for analyses. These measurements were highly repeatable ($R^2 > 0.98$), except for measurements of ATL and HTL in *C. maiyasanus* ($R^2 = 0.60$ and 0.74, respectively) and CPW in *C. uenoi* ($R^2 = 0.89$). A total of 30 males of *C. uenoi*, 30 males of *C. maiyasanus*, and 30 males of *C. iwawakianus* were measured.

To capture and compare overall correlation structures across the traits among species, pairwise Pearson's correlation coefficients (r) and P-values were calculated

across five body size, two appendage size, and four genital size parameters for *C. iwawakianus*, *C. maiyasanus*, and *C. uenoi*. The correlation matrix for each species included 55 off-diagonal elements, consisting of 21 correlation coefficients between external (body and appendage) parts, 6 between genital parts, and 28 between external and genital parts. Differences in correlation coefficients among species were evaluated using the package *psych* (Revelle 2022) in R ver. 4.1.2. False discovery rates for multiple comparisons (three comparisons among species) were corrected using the Benjamini and Hochberg (1995) method.

3.2.3 Functional constraint

The one-size-fits-all hypothesis, which proposes that exaggeration in the male genitalia is constrained by the morphological fit with the typical female genitalia, predicts negative allometry of male genital sizes (Eberhard et al. 1998). However, relatively steep allometric slopes are also predicted in species with exaggerated male genitalia as a result of the relaxation of functional constraint. To examine this, allometric slopes of four

genital dimensions (ADL, ADW, CPL, and CPW) were evaluated in the three species by ordinary linear regression and major axis regression based on $\log_{10}$-transformed values. Differences in allometric slopes among species were tested by general linear models based on the pooled dataset across the three species, in which genital size was used as the dependent variable, and the proxy of body size, species, and their interaction term were included as independent variables. A significant interaction indicated a difference in allometric slopes among species.

C. maiyasanus and *C. iwawakianus* have similar body lengths, while *C. uenoi* is slightly larger (distance from the anterior margin of the labrum to the apices of the elytra in male, mean ± s.d.; *C. maiyasanus*: 23.01 ± 0.18 mm [N = 51], *C. iwawakianus*: 23.31 ± 0.74 mm [N = 82], *C. uenoi*: 26.34 ± 0.47 mm [N = 30]) (Takami & Sota 2007). Unlike other ground beetle species, *C. uenoi* males are longer than females (Sota et al. 2000). This interspecific variation in the sex difference in body lengths could be due to the elongation of the abdomen in *C. uenoi* as a direct consequence of the exaggeration of the male genitalia. Thus, rather than body length (including abdomen length), TL was used

as a proxy of body size in the analysis of allometry.

3.2.4 Genetic constraint

The male genitalia of beetles (the median lobe and parameres) are serially homologous to other appendages, which are commonly regulated by appendage-patterning genes in development (Macagno & Moczek 2015, Nomura et al. 2021). The median lobe corresponds to the aedeagus in *Ohomopterus* species. If trait exaggeration was enabled by the relaxation of genetic and developmental constraints based on a shared morphogenetic basis across appendages, the sizes of male genitals and other appendages are expected to show weaker covariances in species with more exaggerated male genitalia.

Pairwise correlation coefficients between genital (ADL, ADW, CPL and CPW) and appendage (ATL and HTL) sizes were compared among species using the *psych* package in R. Additionally, the canonical correlation coefficient (CCC) between the set of genital sizes and the set of appendage sizes in each species was calculated using the *CCA* package in R. CCC represent maximal correlation between two sets of variables, so

that we can evaluate covariation between two body parts. The greater CCC indicates the stronger covariation. For comparisons among species, the 95% confidence interval of the CCC was calculated by bootstrapping using the *boot* package in R with 1000 replicates.

3.2.5 Structural constraint

If a body part is stored within an outer part, the exaggeration of the inner part is expected to be constrained by the size of the outer part. The male genitalia of *Ohomopterus* ground beetles exhibit this type of nested structure: the CP is stored within the AD, and the AD including the CP is stored within the abdomen (Fig. 3-1). This type of structural constraint predicts that the exaggeration of inner parts requires modification (i.e., enlargement) of the outer parts, resulting in size covariation between inner and outer parts.

Correlations between sizes of inner and outer parts were evaluated. ABW and ABD were used as indicators of abdominal size. EL was also used as a measure of the length of the abdomen because the elytra entirely cover the dorsal surface of the

functional abdomen in beetles (including meso- and metathoraces). AD size was measured by ADL and ADW, and the size of the innermost part, CP, was determined by the CPL and CPW. Four types of nested relationships were evaluated: abdomen vs. AD and CP, abdomen vs. AD, abdomen vs. CP, and AD vs. CP. Pairwise correlation coefficients were compared. Additionally, the CCC between the sizes of the outer and inner structures and 95% confidence intervals were calculated as explained above and compared between the three species.

3.3 Results

3.3.1 Correlation structure

The overall correlation structure among traits was similar in *C. iwawakianus* and *C. maiyasanus*; however, it differed between these two species and *C. uenoi*, especially for correlations involving genital traits (Table 3-1). Relatively strong and significant correlations were detected between body dimensions in *C. iwawakianus* and *C. maiyasanus*; however, these correlations tended to be weak for *C. uenoi*. Among genital

dimensions, ADL was moderately correlated with body dimensions in the three species; however, it was not significantly correlated with other genital dimensions in *C. iwawakianus* and *C. maiyasanus*. ADW, CPL, and CPW were only weakly correlated with each other in *C. iwawakianus* and *C. maiyasanus*. By contrast, relatively strong correlations were detected between CPL and other body and genital dimensions in *C. uenoi*. The correlation coefficients between CPL and TL were significantly greater in *C. uenoi* than in *C. iwawakianus* ($P = 0.015$) and in *C. maiyasanus* ($P < 0.001$), even after correction of false discovery rate ($P < 0.05$, Table 3-1). The correlation coefficients between ABD and EL and between HTL and EL were significantly lower in *C. uenoi* than in *C. maiyasanus* ($P = 0.030$ in both cases); however, significance was not retained after controlling for the false discovery rate ($P > 0.05$, Table 3-1). Parameter values are summarized in Table 3-3.

3.3.2 Functional constraint

The allometric slopes of ADL showed significant departures from zero in all

three species, as revealed by OLS regression analyses (Table 3-2). The allometric slope of CPL was significantly non-zero only in *C. uenoi*. The remaining two genital dimensions (ADW and CPW) revealed non-significant slopes. All four significant allometric slopes were less than 1.0 in MA regression analyses, indicating negative allometry. However, the 95% confidence intervals of these MA slopes included 1.0 (Table 3-2).

Allometric slopes of CPL differed significantly among species (GLM, interaction between body size and species, $P = 0.0354$). *C. uenoi* with the most exaggerated male genitalia showed the steepest OLS slope, as predicted by the hypothesis that the relaxation of functional constraint facilitates exaggeration. There were no significant differences in allometric slopes in other three genital dimensions ($P > 0.14$).

3.3.3 Genetic constraint

Pairwise correlation coefficients between genital (ADL, ADW, CPL, and CPW) and other appendage (ATL and HTL) sizes were not significantly different from zero at

the 5% level in the three species, except for those between ADL and ATL in the three species and that between ADL and HTL in *C. maiyasanus* (Table 3-1).

Inconsistent with the hypothesis that exaggeration in male genitalia was facilitated by release from the genetic constraint based on a common morphogenetic basis across appendages, correlations between genital and other appendage sizes tended to be weaker in species with the least exaggerated male genitalia. Although pairwise correlation coefficients between sizes of genitalia and other appendages did not differ between species (Table 3-1), the CCC between genital sizes and appendage sizes was significantly smaller in *C. iwawakianus*, in which the CCCs of *C. maiyasanus* and *C. uenoi* were not within the 95% confidence intervals of the CCC for *C. iwawakianus* (Fig. 3-2).

3.3.4 Structural constraint

In *C. iwawakianus*, significant pairwise correlation coefficients were detected between inner genital parts and outer parts for ADL vs. three abdominal dimensions and for ADW vs. EL and ABD but not for CP sizes vs. others (Table 3-1). In *C. maiyasanus*,

correlations were significant only for ADL vs. EL and were marginally nonsignificant for ADL vs. ABD and ABW (Table 3-1). In *C. uenoi*, correlations were significant in ADW vs. EL and in CPL vs. EL, ABW, ABD, and ADL and were marginally nonsignificant in ADL vs. EL and ABW (Table 3-1).

As opposed to the hypothesis that the exaggeration of the inner genital part is facilitated by a relaxation of structural constraint related to the size of the outer part, the inner genital parts were most strongly constrained by the outer parts in *C. uenoi* with the most exaggerated male genitalia (Fig. 3-3). Although there were no significant differences in pairwise correlation coefficients between abdominal and genital dimensions across species (Table 3-1), the CCCs between the AD and CP were significantly greater in *C. uenoi* than in other two species; the estimated CCC for *C. uenoi* fell outside of the 95% CI for the other species (Fig. 3-3). The CCCs between the abdomen and CP were also significantly greater in *C. uenoi* than in other two species for comparisons in both directions (Fig. 3-3). The CCCs between the abdomen and four dimensions of AD and CP were greater in *C. uenoi* than in *C. iwawakianus* in one direction (Fig. 3-3). The CCCs

between the abdomen and AD measurements did not differ among species (Fig. 3-3).

3.4 Discussion

I hypothesized that the exaggeration of sexual traits is possible by the relaxation of various constraints. To test this hypothesis, three types of constraints were examined: functional, genetic, and structural. Correlations between body and genital dimensions were analyzed and compared among closely related *Ohomopterus* ground beetle species (*C. iwawakianus*, *C. maiyasanus*, and *C. uenoi*) with variation in the degree of exaggeration in male genital morphology. The results indicated that the relaxation of functional constraints (but not the two other constraint types) may be responsible for the exaggeration of male genital morphology. Below, I discuss the effects of each type of constraint on the exaggeration of male genitalia in detail.

The one-size-fits-all hypothesis predicts that stabilizing selection favors intermediate male genital sizes and negative allometric scaling (Eberhard et al. 1998). Although the possibility of isometry was not statistically excluded, all four significant

allometric slopes (three for ADL and one for CPL in *C. uenoi*) for genital dimensions tended to be negative, providing support for the one-size-fits-all-hypothesis. Interestingly, most genital dimensions other than above four were not significantly related to body size (Tables 3-1 and 3-2), suggesting that genital sizes vary almost independently from body sizes, especially in species with less exaggerated genitalia. For CPL, allometric slopes varied significantly among species, and the steepest OLS slope was detected in *C. uenoi* with the most exaggerated male genitalia. This result indicates that selection against relatively larger genitalia is weaker in *C. uenoi* than in the other two species, suggesting that the function of the exaggerated male CP in *C. uenoi* is relatively weakly constrained. Weak correlations observed in species with relatively small genital parts (*C. iwawakianus* and *C. maiyasanus*) could be explained by measurement errors given the small genital sizes; however, high repeatability in genital sizes for these species suggests that the effect of measurement errors was limited. In *C. insulicola*, a species in *Ohomopterus* ground beetles including the three species in question, sexual conflict between male manipulation of female oviposition activity and female resistance against male manipulation generates

selection for a longer male CP and female VA (Takami et al. 2018). Secondary contact between closely related species, including *C. iwawakianus*, *C. maiyasanus* and *C. uenoi*, may also generate reinforcing selection favoring a longer CP (Nishimura et al. 2022). Note that the longer VA may also be favored in these two processes, as a counter adaptation in sexual conflict and for species isolation in reinforcement, thereby coevolution between the sexes may be promoted. These processes favoring a longer CP may facilitate release from functional constraints via stabilizing selection and drive the evolution of exaggerated male genitalia.

Since the appendages of the body segments of insects, including male genitalia, share a common genetic basis, I hypothesized that the exaggeration of male genitalia was constrained by genetic correlations among appendages across body segments. However, our results did not support this hypothesis; the pairwise correlation coefficients between genital and other appendage sizes were mostly non-significant and did not differ between species. Additionally, comparison of the CCCs between genital and other appendage sizes revealed that *C. iwawakianus* with the least exaggerated male genitalia was most weakly

constrained among the species. Therefore, exaggerated male genitalia in *C. uenoi* cannot be explained by relaxed genetic and developmental constraints. As discussed above, weak correlations in *C. iwawakianus* also cannot be explained by measurement errors.

Nested configurations of body and genital parts (i.e., outer and inner parts) are often found in male insect genitalia, such as the aedeagus and its associated internal sac (Tuxen 1970), where exaggeration in the inner part may be constrained by the size of outer part (i.e., structural constraint). The CCC between the sizes of the outer and inner genital parts was greater in *C. uenoi* than in the other two species, suggesting that structural constraints on genital parts were strongest in the species with the most exaggerated male genitalia. This result was not consistent with the hypothesis that a relaxation of structural constraint facilitates the exaggeration of the male genitalia. In other words, the male genitalia of *C. iwawakianus* and *C. maiyasanus* are not exaggerated even under weak structural constraints. Interestingly, although males are generally smaller than females in ground beetles, including *Ohomopterus* species, males of *C. uenoi* are slightly larger than females (Sota et al. 2000). This may be a necessary compensation

of the exaggeration of male genitalia in *C. uenoi*.

Collectively, the exaggeration of the male genitalia of *C. uenoi* may be facilitated by relaxed functional constraint, in which selective regimes may change from stabilizing selection to directional selection favoring a longer CP. However, this was not the case for AD. Genetic and developmental constraints based on a common genetic architecture across appendages may have little effects on the exaggeration and diversification of male genitalia in *Ohomopterus*. Constraints by the outer structures are also unlikely to limit exaggeration of the male genitalia. Alternatively, sexual selection and/or reinforcing natural selection may have led to the exaggeration of the CP of *C. uenoi*, until it reached the spatial capacity of the outer parts and became constrained. Our findings provide insight into sexual trait exaggeration and its underlying constraints.

Table 3-1. Pearson's correlation coefficients (upper triangle) and *P*-values (lower triangle, not corrected for false discovery rate) across five body, two appendage, and four genital dimensions of *Carabus iwawakianus*, *C. maiyasanus*, and *C. uenoi*. Black, dark grey, light grey, and white cells indicate $0.7 < r$, $0.5 < r < 0.7$, $0.3 < r < 0.5$, and $r < 0.3$, respectively. For comparisons among species, different letters indicate significant differences between species after correction for the false discovery rate ($P < 0.05$).

C. iwawakianus	TL	TW	EL	ABW	ABD	ATL	HTL	ADL	ADW	CPL	CPW
TL		**0.808**	**0.788**	**0.647**	**0.765**	**0.498**	0.236	**0.465**	0.118	0.165[a]	-0.002
TW	<.0001		**0.808**	**0.745**	**0.791**	**0.610**	**0.513**	**0.549**	0.249	0.168	-0.003
EL	<.0001	<.0001		**0.668**	**0.859**	**0.677**	**0.493**	**0.459**	**0.367**	0.019	0.176
ABW	<.0001	<.0001	<.0001		**0.702**	**0.492**	**0.310**	**0.615**	0.231	0.279	0.153
ABD	0.000	<.0001	<.0001	<.0001		**0.592**	**0.422**	**0.429**	**0.370**	0.224	0.077
ATL	0.005	0.000	<.0001	0.006	0.001		**0.584**	**0.405**	0.169	-0.019	0.162
HTL	0.209	0.004	0.006	0.095	0.020	0.001		0.286	0.174	0.114	-0.074
ADL	0.010	0.002	0.011	0.000	0.018	0.026	0.125		0.147	0.181	0.176
ADW	0.533	0.186	0.046	0.219	0.044	0.372	0.359	0.438		0.150	0.224
CPL	0.383	0.375	0.922	0.135	0.234	0.921	0.549	0.338	0.430		-0.055
CPW	0.991	0.987	0.353	0.421	0.687	0.393	0.699	0.354	0.235	0.772	

Table 3-1. Continued.

C. maiyasanus	TL	TW	EL	ABW	ABD	ATL	HTL	ADL	ADW	CPL	CPW
TL		**0.804**	**0.701**	**0.616**	**0.793**	**0.659**	**0.506**	**0.454**	**0.334**	0.024[a]	0.004
TW	<.0001		**0.731**	**0.647**	**0.687**	**0.590**	**0.409**	**0.430**	**0.373**	0.223	-0.063
EL	<.0001	<.0001		**0.770**	**0.682**	**0.876**	**0.647**	**0.574**	0.236	0.193	-0.035
ABW	0.000	0.000	<.0001		**0.704**	**0.702**	**0.445**	**0.324**	0.165	0.229	0.096
ABD	<.0001	<.0001	<.0001	<.0001		**0.682**	**0.437**	**0.350**	0.070	0.246	0.003
ATL	<.0001	0.001	<.0001	<.0001	<.0001		**0.661**	**0.539**	0.181	0.131	0.116
HTL	0.004	0.025	0.000	0.014	0.016	<.0001		**0.428**	0.130	0.206	-0.056
ADL	0.012	0.018	0.001	0.081	0.058	0.002	0.018		-0.008	0.284	0.081
ADW	0.072	0.043	0.209	0.385	0.712	0.339	0.493	0.967		0.236	-0.006
CPL	0.901	0.237	0.308	0.223	0.190	0.489	0.276	0.128	0.734		-0.409
CPW	0.985	0.741	0.853	0.615	0.987	0.540	0.768	0.670	0.977	0.025	

Table 3-1. Continued.

C. uenoi	TL	TW	EL	ABW	ABD	ATL	HTL	ADL	ADW	CPL	CPW
TL		**0.621**	**0.566**	**0.471**	**0.480**	**0.540**	0.292	**0.608**	0.208	**0.696**[b]	-0.061
TW	0.000		**0.660**	**0.582**	**0.751**	**0.521**	0.244	**0.348**	0.108	**0.632**	-0.101
EL	0.001	<.0001		**0.322**	**0.722**	**0.581**	**0.436**	**0.350**	0.022	**0.417**	-0.114
ABW	0.009	0.001	0.082		**0.451**	0.269	0.093	**0.361**	**0.497**	**0.594**	0.074
ABD	0.001	<.0001	<.0001	0.012		**0.507**	0.229	0.296	-0.047	**0.487**	-0.066
ATL	0.002	0.003	0.001	0.152	0.004		**0.595**	**0.464**	-0.079	**0.356**	-0.006
HTL	0.117	0.194	0.016	0.624	0.224	0.001		0.056	0.036	0.217	-0.269
ADL	0.000	0.060	0.058	0.050	0.113	0.010	0.771		0.177	**0.501**	0.218
ADW	0.571	0.908	0.005	0.804	0.679	0.851	0.360	<.0001		0.254	0.092
CPL	<.0001	0.000	0.022	0.001	0.006	0.054	0.249	0.005	0.704		0.114
CPW	0.748	0.594	0.551	0.698	0.728	0.976	0.150	0.247	0.629	0.550	

TL, prothorax length, TW, prothorax width, EL, elytra length, ABW, abdomen width, ABD, abdomen depth, ATL, 5th antennal segment length, HTL, right hind tibia length, ADL, aedeagus length, ADW, aedeagus width, CPL, copulatory piece length, CPW, copulatory piece width.

Table 3-2. Allometric slopes of four genital dimensions in the three species. Estimates based on ordinary least squares (OLS) and major axis (MA) regressions are shown. Confidence intervals for the MA slope are indicated only when the slope of the scaling relationship determind by OLS regression is significant.

Species	Trait	Allometric slope (OLS)	*P*	Allometric slope (MA)	95% Confidence interval
C. iwawakianus	ADL	0.342	0.0079	0.720	0.293/1.771
	ADW	0.155	0.58	1.470	
	CPL	0.224	0.41	1.436	
	CPW	-0.004	0.99	-1.402	
C. maiyasanus	ADL	0.178	0.012	0.392	0.144/1.068
	ADW	0.360	0.074	1.090	
	CPL	0.018	0.89	0.697	
	CPW	0.034	0.93	2.103	
C. uenoi	ADL	0.415	0.0003	0.673	0.392/1.158
	ADW	0.299	0.24	1.360	
	CPL	0.687	<0.0001	0.989	0.647/1.512
	CPW	-0.123	0.83	-2.937	

ADL, aedeagus length, ADW, aedeagus width, CPL, copulatory piece length, CPW, copulatory piece width

Table 3-3. Summary of all measurements.

C. iwawakianus	TL	TW	EL	ABW	ABD	ATL	HTL	ADL	ADW	CPL	CPW
Average	4.94	6.48	15.76	6.45	8.88	7.73	6.41	7.56	1.35	1.31	0.73
Standard deviation	0.18	0.30	0.45	0.20	0.36	0.29	0.26	0.20	0.07	0.07	0.04
C. maiyasanus	TL	TW	EL	ABW	ABD	ATL	HTL	ADL	ADW	CPL	CPW
Average	4.68	6.05	15.47	6.27	8.48	7.49	6.10	7.29	1.37	2.31	0.32
Standard deviation	0.23	0.32	0.54	0.27	0.38	0.31	0.44	0.14	0.07	0.08	0.03
C. uenoi	TL	TW	EL	ABW	ABD	ATL	HTL	ADL	ADW	CPL	CPW
Average	5.36	7.09	17.97	7.19	10.00	8.72	6.79	9.57	2.25	8.53	0.64
Standard deviation	0.18	0.23	0.40	0.22	0.30	0.29	0.20	0.21	0.10	0.27	0.06

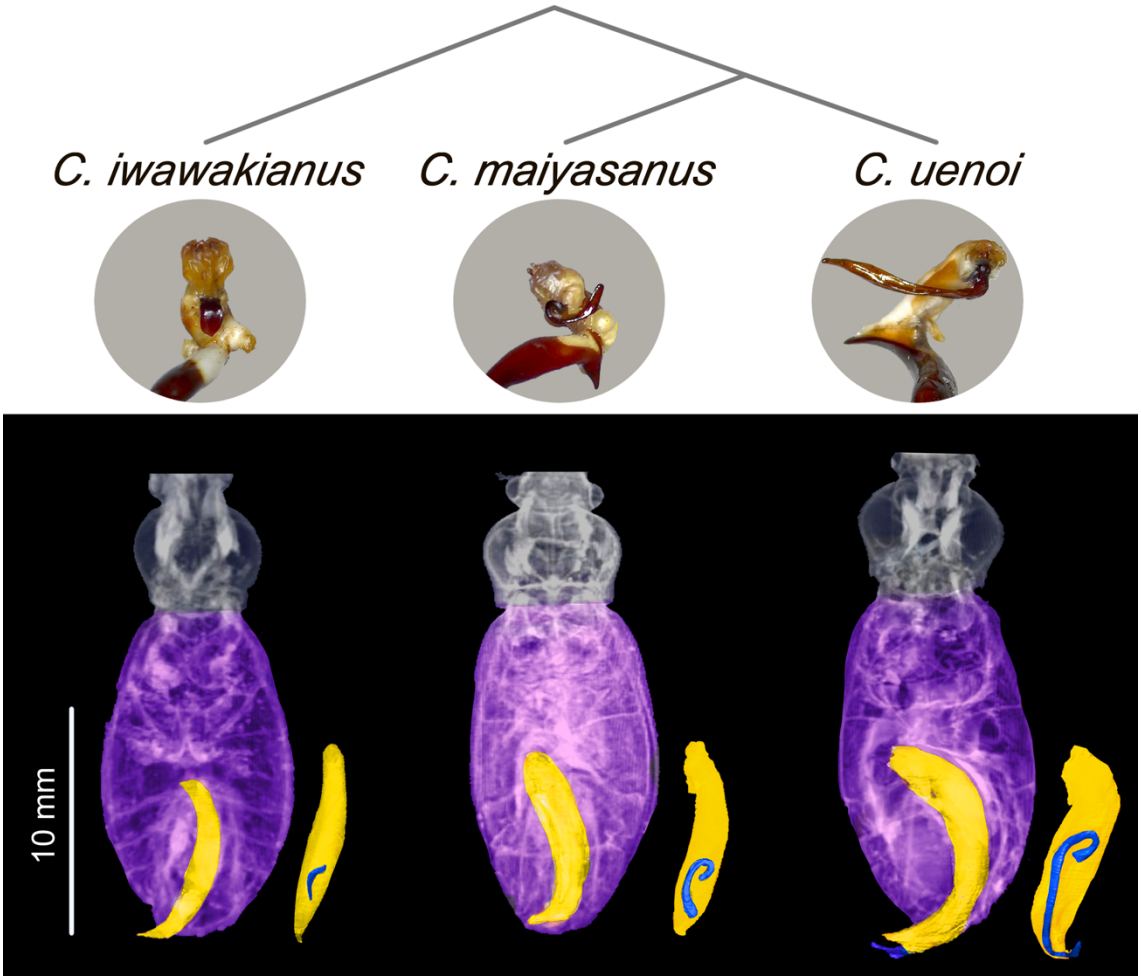

Fig. 3-1. Phylogenetic relationships among *Carabus iwawakianus*, *C. maiyasanus*, and *C. uenoi* and configurations of body and genital parts. Male genitalia are shown in circles; the endophallus and copulatory piece (CP) are everted from the aedeagus (AD). Nested configuration consisting of the functional abdomen including the meso- and metathoraces (purple), aedeagus (yellow), and copulatory piece (blue) are depicted in dorsal view based on μCT scan.

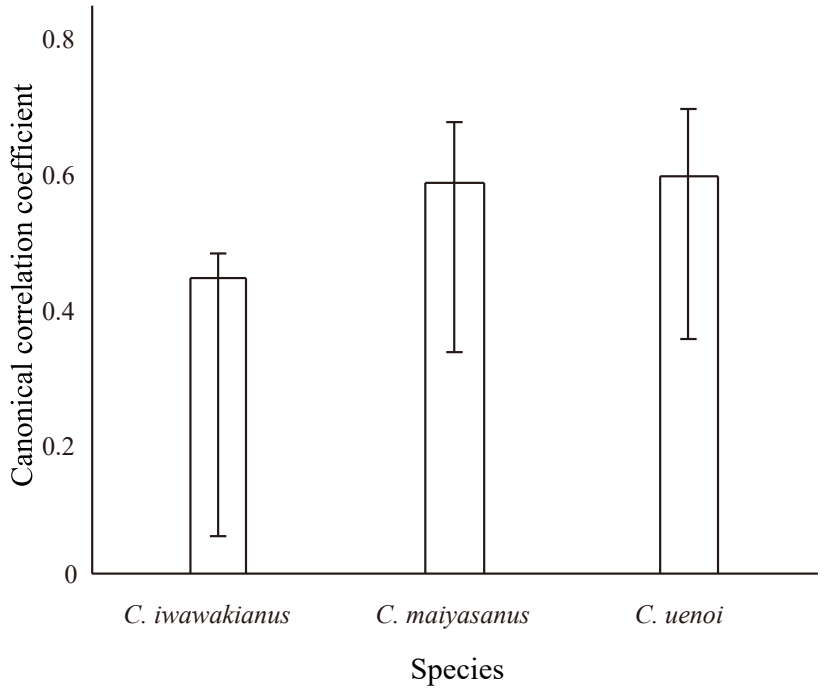

Fig. 3-2. Canonical correlation coefficients between genital and appendage sizes and 95% confidence intervals in *C. iwawakianus*, *C. maiyasanus*, and *C. uenoi*.

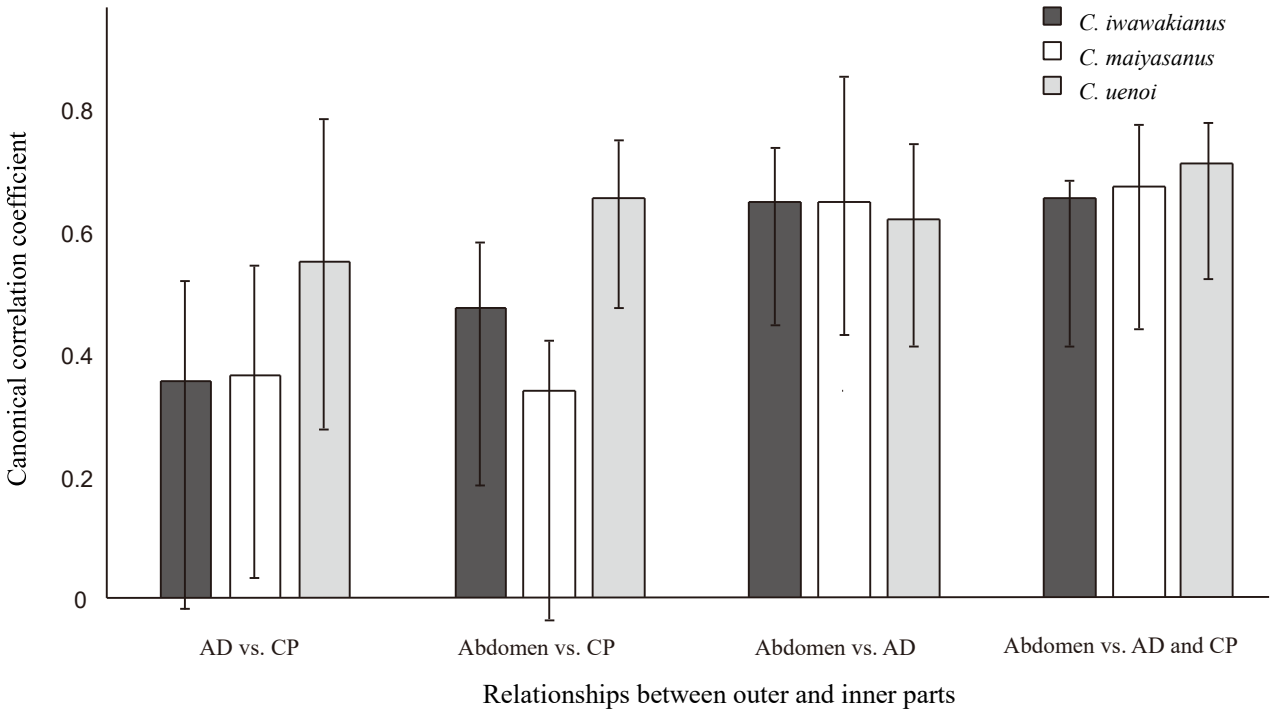

Fig. 3-3. Canonical correlation coefficients between the sizes of the inner genital parts and outer parts and 95% confidence intervals in *C. iwawakianus*, *C. maiyasanus*, and *C. uenoi*. AD, aedeagus; CP, copulatory piece.

Chapter 4

Temporal folding mediates the development of huge genitalia under spatial limitation

4.1 Introduction

Sexual trait exaggeration is a common phenomenon in nature (Anderson 1994). Examples include exaggeration of the weapon traits used in male–male combat and the male ornament traits subjected to female choice (Darwin 1871, Andersson 1994, Shuster & Wade 2003, Emlen 2008). While the evolutionary processes behind the exaggeration of sexual traits have been well investigated in the context of sexual selection, the developmental basis of the exaggerated sexual traits has been elucidated only in limited cases (Jennions et al. 2001, Godin et al. 2003, Langerhans et al. 2005). In some horned beetles, the exaggerated male horn was initially formed as the folded tubes of epidermis and then it was unfolded to elongate in pupal stages, which resulted primarily from genetic modifications to the patterning processes that control cell proliferation (Emlen et al. 2007, Morita et al. 2019). The shape of the genital morphology in animals with internal fertilisation is known to diversify more rapidly than other traits (Eberhard 1985), being one of the best examples of exaggerated sexual traits. However, in insects, developmental

process of the genital morphology is investigated only in limited taxa (Aspiras et al. 2011, Macagno & Moczek 2015, Matsumura et al. 2013) except for fluitflies (Estrada et al., 2001, Hagen et al. 2019, Masly et al. 2011, Tanaka et al. 2015).

In contrast to the rapid diversification of the shape of the genitalia, the size of the genitalia is known to be less diversified among species and more likely to have a negative allometry within a species (Eberhard 2010). The exaggerated male genital size may be selected against in various contexts; for example, it may result in mismatches with the copulatory organs of the conspecific females (the lock-and-key hypothesis, Masly 2012); and/or may inproperly stimulate the female sensory system and be disadvantageous in cryptic female choice (the one-size-fits-all hypothesis, Eberhard et al. 1998). However, exaggerated male genital size is known in some species, such as waterfowls (McCracken et al. 2001). In the waterfowls, vaginal structures function as a barrier to phallus penetration, suggesting that directional selection for the male phenotypes that can penetrate the female barrier is likely responsible for the exaggeration of the male genital size (Brennan et al. 2007, 2015). In insects, extremely elongated male

genitalia are found in some beetles and earwigs (Gack & Peschke, 2005, Matsumura et al. 2021). However, the developmental processes that enable such oversized genitalia has been less studied.

Ground beetles belonging to the subgenus *Ohomopterus* (Coleoptera, Carabidae, genus *Carabus*) show varied degree of exaggeration in the size and shape of the genitalia (Fig. 4-1), with evidence for coevolutionary divergence between the sexes (Ishikawa 1987, 1991, Sasabe et al. 2010, Fujisawa et al. 2019). The male has a hook-like structure on the intromittent organ called the copulatory piece (CP). The CP is a sclerotized projection in the wall of the endophallus (internal sac, IS); it is everted from the aedeagus (AD) in copula (Fig. 4-2a). The female has a membranous pocket attached to the ventral wall of the bursa copulatrix (BC) called the vaginal appendix (VA) (Fig. 4-2b). Females have VAs corresponding to the CP of conspecific males. The CP is inserted into the VA in copula, and the male and female genital parts are rigidly coupled (Ishikawa 1987, Takami 2002). These male and female genital parts play roles in sexual selection and speciation (Takami & Sota 2007, Okuzaki & Sota 2014, Takami et al. 2018, Fujisawa et

al. 2019, Nishimura et al. 2022). In *Ohomopterus*, *C. uenoi* has an extremely enlarged CP, reaching a third of the male body length, and the corresponding VA (Figs. 4-1 and 4-2). By contrast, closely related species *C. iwawakianus* and *C. maiyasanus* have much smaller CPs and VAs than those of *C. uenoi* (Fig. 4-1). The morphogenesis of the male and female genitalia in *C. iwawakianus* and *C. maiyasanus* have already been elucidated (Chapter 2, Terada et al. 2021). Thus, elucidating the morphogenesis of the extremely enlarged genitaia of *C. uenoi* allows us to compare among the species for understanding the developmental processes of the exaggeration of the genital morphology.

In Chapter 2, substantial interspecific differences in the size of the CP and VA between *C. iwawakianus* and *C. maiyasanus* were revealed to be mediated by different underlying developmental mechanisms. The difference in CP size resulted from the advanced initial timing of the development (i.e., heterochrony), thereby the relatively longer CP in *C. maiyasanus* grew for the longer time than in *C. iwawakianus*. By contrast, development of the relatively longer VA in *C. maiyasanus* was attained by the greater growth rate than in *C. iwawakianus*. These two hypothetical developmental processes

(i.e., heterochrony and increased growth rate) may also be responsible for the extremely enlarged genitalia in *C. uenoi*. Additionally, the recent morphometric analysis of adult genital sizes revealed that the lengths of the AD and CP covaried more strongly in *C. uenoi* than in *C. iwawakianus* and *C. maiyasanus*, suggesting that the elongation of the CP of *C. uenoi* reached the spatial capacity of the outer part (i.e., AD) and became constrained (Fig. 4-1) (Chapter 3). Such a structural constraint on the extremely enlarged genital parts may result in further modification of the developmental process. For example, Matsumura et al. (2013) reported the morphogenesis of the exaggerated male genital organs of the leaf beetles (Chrysomelidae, Criocerinae), in which the highly elaborate internal sac was initially formed outside of the aedeagus and subsequently retracted into the aedeagus, possibly avoiding spatial limitation by the aedeagus.

In this study, I examined the developmental processes underlying exaggeration of the male and female genital sizes in *C. uenoi* by X-ray micro-computed tomography (μCT). The morphogenesis of species-specific male and female genitalia was described. Very high growth rates were found to contribute to the exaggeration of both the male and

female genital morphology, and longer pupal duration also contributed to increase developmental time for further growth, possibly as another form of heterochrony. Additionally, I found that the elongation of CP was temporally constrained by the size of AD, but the CP continued to grow and resulted in temporal folding structure. I discuss the relevance of these developmental processes leading to the exaggeration of genital morphology.

4.2 Materials and Methods

4.2.1 Sampling

Adults of *C. uenoi* were collected on Mt. Kongo-san, Osaka, Japan during the reproductive season (early May to early July, 2017 to 2021) using pitfall traps. A male and a female were kept in a plastic jar (diameter, 10 cm; height, 10 cm) with moistened sphagnum and soil at the bottom (2 cm in depth) and placed in an incubator (long-daylight condition of 16L:8D at 20°C) to maintain sexual maturity. Beetles were fed minced beef every other day and allowed to mate and oviposit freely. Eggs were collected from the

soil and kept in a plastic cup (diameter, 7 cm; height, 4 cm) with moistened soil and sphagnum. Hatched larvae were reared individually in the plastic cup under the same conditions. Larvae were fed megascolecid earthworms *ad libitum*. Satiated third instar larvae were moved to a plastic jar (6.5 cm diameter and 9 cm height) filled with soil (depth, 7 cm) for pupation. Larvae usually dug down into the soil to make a pupal room. If the pupal room was visible from the outside due to contact with the wall of the plastic jar, the time of pupation was observed directly. If the pupal room was hidden in the soil and not visible from the outside, the larva or prepupa was dug out and moved to an artificial pupal room to observe the time of pupation. Artificial pupal rooms were made of moistened tissue paper within a plastic cup placed in an incubator with no lights. Our preliminary examination indicated that pupae of the species commonly spent 13 days until eclosion under the present conditions (20°C, N = 2). Thus, pupae were sampled at 2, 4, 6, 8, 10 and 12 days after pupation (male: N = 3 for all developmental stages except for 2 and 12 days after pupation, N = 2; female: N = 3 for 2 and 6 days after pupation and N = 2 for other developmental stages). I checked pupation daily, and determined the day

on which pupation was discoverd as day 1 because of a potential delay from pupation to discovery. Pupae were sexed based on the morphology of the abdominal terminalia.

4.2.2 Infiltration, scanning, and analysis

The integument of each pupa was perforated with a fine pin and then fixed by FAE solution (formalin: acetic acid: ethanol = 6:1:16) for 1 day. Fixed pupae were dehydrated in a graded ethanol series and preserved in 70% ethanol. Fixed pupae were soaked in 1% ethanol iodine for a week. Infiltrated pupae were washed with 70% ethanol, mounted individually in a plastic tube (diameter, 1 cm; height, 5 cm), and submersed in 70% ethanol just before scanning.

X-ray micro-CT scans were performed using an XRadia Micro XCT-400 system (Carl Zeiss, Oberkochen, Germany). The acquisition parameters for scans were as follows: accelerating voltage = 150 kV, source current = 65 μA, exposure time = 10 sec; samples were rotated from -90° to 90°, with a rotation step of 0.01°. A total of 1800 images were obtained per sample, resulting in a voxel size of 6.43 μm. Scans were

converted to stacks of digital image slices, which were reconstructed into three-dimensional (3D) images and analyzed using Amira 6.4 (FEI). Volume-rendered images were viewed from the longitudinally or latitudinally sliced plane of the object.

To investigate the relevance of the developmental constraints on the CP by its outer structure, the AD, the sizes of the CP and the AD were measured throughout pupal development. The sizes of the CP and AD were defined as its maximum length (CPL, ADL), as determined using the distance measure function in Amira. Genital parts of adult males were removed and measured based on digital images obtained using a digital camera mounted on a Leica S9i microscope (Leica Microsystems, Wetzlar, Germany) (N = 30, Chapter 3). Male genital sizes of two related species, *C. iwawakianus* and *C. maiyasanus*, were shown for comparison based on the previous study (Chapter 2, Terada et al. 2021). In the female, the development of the VA was described only by μCT images and the size of the VA was not measured because its structure was complex and difficult to measure (see Results).

4.3 Results

4.3.1 Male genitalia

At 2 days after pupation, the AD was already formed and the apical portion exposed at the abdominal terminalia (Fig. 4-3a). The basal portion was not yet formed at this stage. The basal portion of the AD gradually elongated toward the internal abdomen until adult eclosion. The extremely curved basal portion of the AD, which was specific to this species (Fig. 4-2a), was formed along the ejaculatory duct between 8 and 12 days after pupation.

The CP originated from a small protrusion of a part of the internal sac, which was directed internally to the cavity surrounded by the IS within the AD (Fig. 4-3b). A small protrusion was observed 4 days after pupation, which was same to *C. maiyasanus* but earlier than *C. iwawakianus* (Fig. 4-4). Then, the CP acceleratedly elongated until 8 days after pupation, the growth rate of which was greater than in the AD of *C. uenoi* as well as in the CPs of *C. iwawakianus* and *C. maiyasanus* (Fig. 4-4). At 8 days after pupation in *C. uenoi*, CPL became close to ADL, as if the growth of the CP was limited by the size of AD (Fig. 4-4). At this stage, complex folding structure was formed in the

distal portion of the CP (Figs. 4-3b). The cross-sectional view of the genitalia showed that the CP mostly occupied the cavity surrounded by the IS, and the CP and IS mostly filled the interior of the AD. At 10 days after pupation, the base of the CP moved internally along with the growth of the basal portion of the AD, whereby CPL and ADL increased in parallel and the folding structure in the CP almost disappeared (Figs. 4-3 and 4-5). The cross-sectional view on 10 days after pupation showed that the CP became thinner and the CP and IS occupied only a part of the interior of the AD. Then, the CP continued gradual elongation in parallel with that of the AD until adult eclosion (Fig. 4-4). The cross-sectional view on 12 days after pupation showed that the CP became far thinner and occupied only a part of the cavity surrounded by the IS.

4.3.2 Female genitalia

The VA originated from a small protrusion of the ventral wall of the BC posterior to the vaginal apophysis (Fig. 4-5). It was rudimentary 2 days after pupation. The VA continued to elongate until 6 days after pupation, and then began to form complex folded structures

from day 8. This structure hindered measuring the actual size of the VA. The folded structure continued to grow until adult eclosion, although the apparent length of the VA did not change largely.

4.4 Discussion

To elucidate the developmental processes leading to exaggerated genital sizes, the morphogenesis of the extremely large male and female genital parts in the ground beetle *C. uenoi* was examined based on micro-CT analysis, and compared with two closely related species with ordinary genital sizes, *C. iwawakianus* and *C. maiyasanus*. It was hypothesized that heterochrony and/or increased growth rate are responsible for the development of the extremely enlarged genitalia. The CP of *C. uenoi* started to develop between the 2nd and 4th days in the pupal stages, which was same to *C. maiyasanus* with the shorter but second longest CP (Figs. 4-3 and 4-5). Thus, early start of growth did not contribute to the development of the large male genitalia, different from the finding in the previous study (Chapter 2, Terada et al. 2021). The start of CP development may be

limited to advance, because coordinated or preceded development of the surrounding structures, such as the AD and IS, is necessary. The AD and IS were inmature at 2 days after pupation (Fig. 4-3a), in which the CP may be difficult to be formed. While the timing of the CP started to develop was not advanced, the pupal stage of *C. uenoi* (13 days) was one day longer than that of *C. maiyasanus* and *C. iwawakianus* (12 days, Chapter 2, Terada et al. 2021), contributing to the further elongation of the CP in *C. uenoi* until just before eclosion (Fig. 4-4). Such an extention of developmental time may be regarded as another type of heterochrony. Additionally, the growth rate of the CP was obviously higher in *C. uenoi* than in *C. maiyasanus* and *C. iwawakianus* especially until 10 days after pupation (Fig. 4-4). These results indicate that the exaggeration of CP size was enabled mostly by increase in growth rate during posteriorly extended developmental time.

The previous study suggested that the growth of the CP of *C. uenoi* is structurally constrained by the size of the outer part, AD (Chapter 3, Terada et al. 2023), allowing me to hypothesize that the developmental process of the CP of *C. uenoi* was modified in

response to this structural constraint. As expected, the CP of *C. uenoi* formed a complex folding structure 8 days after pupation when the length of the CP reached to the maximal capacity of the AD (Fig. 4-3). This developmental process was specific to *C. uenoi* and were not observed in closely related *C. maiyasanus* and *C. iwawakianus* (Chapter 2, Terada et al. 2021). At this stage, the growth of the CP may have continued via continusous cell proliferation even within the limited space of the AD, and the resultant excessive tissue may be folded as observed (Fig. 4-3). Thereafter, the base of the CP moved proximally along with the development of the AD, whereby the folding structure of the CP was gradually pulled and expanded. This interpretation is corroborated by the fact that the CP became gradually thinner from 8 to 12 days after pupation (transectional views in Fig. 4-3). This process may facilitate the development of extremely large genital parts, because it requires active cell proliferation even under spatial limitation.

For the female genitalia, interspecific difference in VA size between *C. iwawakianus* and *C. maiyasanus* was revealed as a result of difference in growth rate (Chapter 2, Terada et al. 2021). In the present study, quantitative comparison among

species was not possible because measurement of VA size in *C. uenoi* was difficult due to structural complexity. However, the initial development of the VA was similar in time among the three species: the rudimentary VA had already been formed 2 days after pupation (Fig. 4-5, Chapter 2, Terada et al. 2021). Then, different from other two species, the VA of *C. uenoi* elongated rapidly, and formed a complex folding structure (Fig. 4-5). These findings suggest that the growth rate of the VA may be higher in *C. uenoi* than in *C. iwawakianus* and *C. maiyasanus*, supporting the hypothesis that interspecific difference in VA size was due to growth rate variation (Chapter 2, Terada et al. 2021). Additionally, as in males, the longer pupal period may also contribute to the development of the extremely large female genital part. Unlike the male CP, the folding structure of the female VA is not fully expanded in adults (note that the VA in Fig. 4-2b was manually expanded for visual purpose), but is expanded when the CP is inserted in copula (Ishikawa 1987).

It is interesting that both the CP and VA commonly formed folding structures in a same developmental stage, 8 days after pupation (Figs. 4-3 and 4-5). Recent

transcriptome analysis in *C. uenoi* in compared with *C. iwawakianus* and *C. maiyasanus* revealed that genes involved in organ size control were commonly upregulated in male and female late pupal stages (Nomura et al. 2021). This implies that synchronous growth of the CP and VA, and construction of similar folding structures are a result of genetic modification to the gene network that is common to the male and female. These findings provide insights into the development processes of exaggerated genital morphologies showing coevolutionary diversification between the sexes. These results also improve our understanding of the precise developmental stages and tissues contributing to species-specific genital differentiation and exaggeration, contributing to the construction a novel platform for evo-devo studies of the diversification and exaggeration of the genital morphologies.

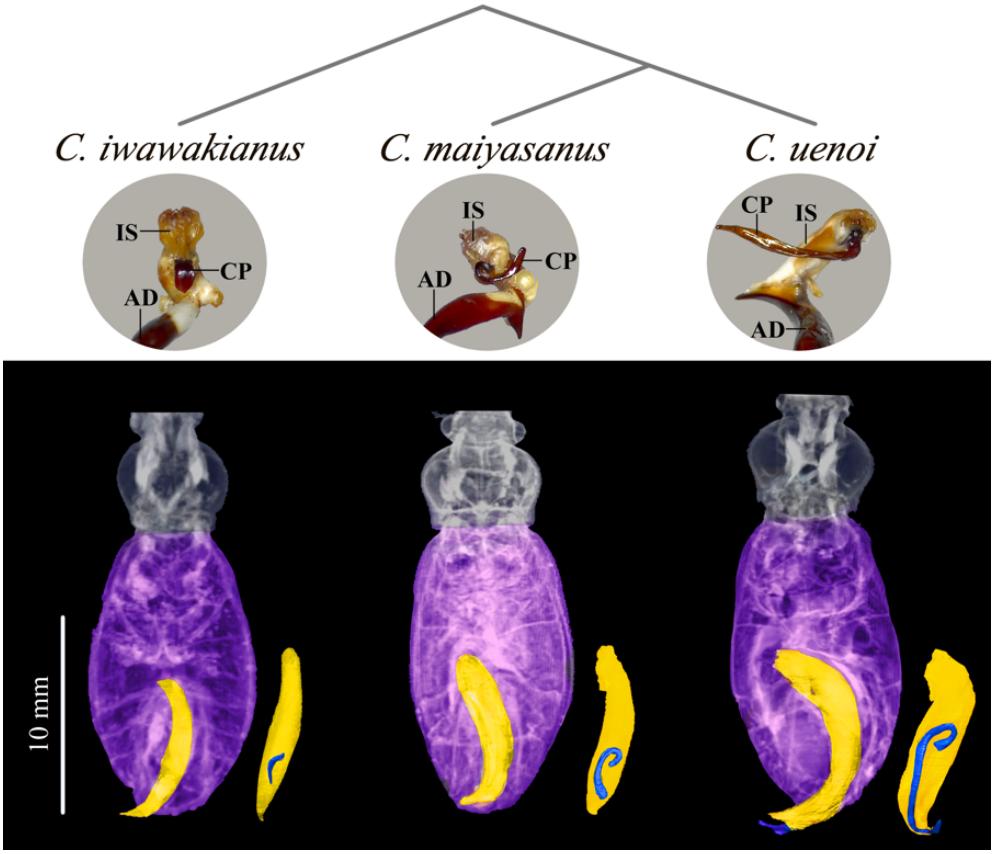

Fig. 4-1. Phylogenetic relationships among *Carabus iwawakianus*, *C. maiyasanus*, and *C. uenoi* and configurations of body and genital parts. Male genitalia are shown in circles; the internal sac (IS) and copulatory piece (CP) are everted from the aedeagus (AD). Nested configuration consisting of the functional abdomen including the meso-and metathoraces (purple), AD (yellow), and CP (blue) are depicted in dorsal view based on μCT scan (cited from Terada et al. 2023).

Fig. 4-2. (a) Male genitalia of *Carabus uenoi*, showing internal sacs everting from the aedeagus (left to right); AD: aedeagus; CP: copulatory piece; IS: internal sac. (b) Female internal reproductive organ of *C. uenoi*; BC: bursa copulatrix; COD: common oviduct; SPT: spermatheca; VA: vaginal appendix; VP: vaginal apophysis.

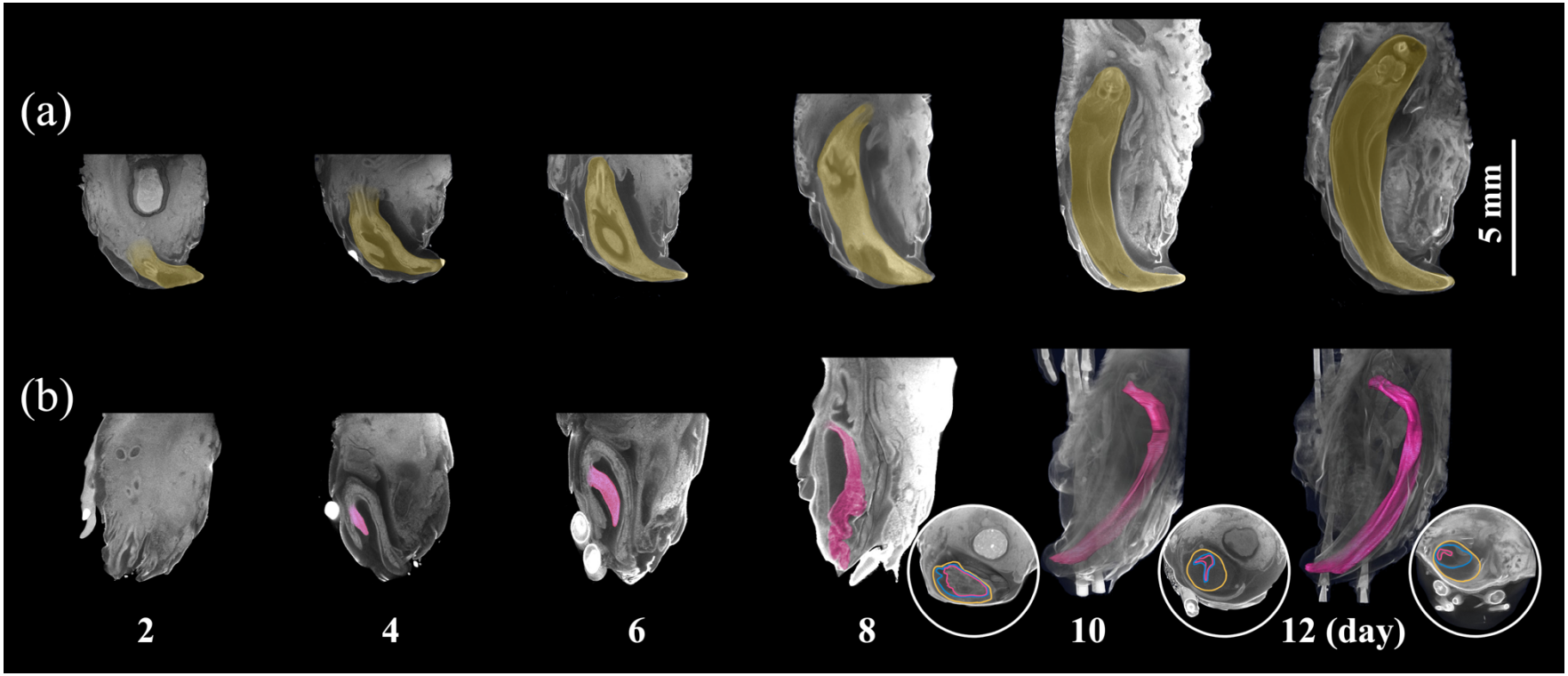

Fig. 4-3. Morphogenesis of the male genitalia of *Carabus uenoi*, (a) aedeagus (yellow), (b) copulatory piece (magenta). The interior of the white circle shows a cross-sectional view between the 8th and 9th abdominal segments; internal sac (blue).

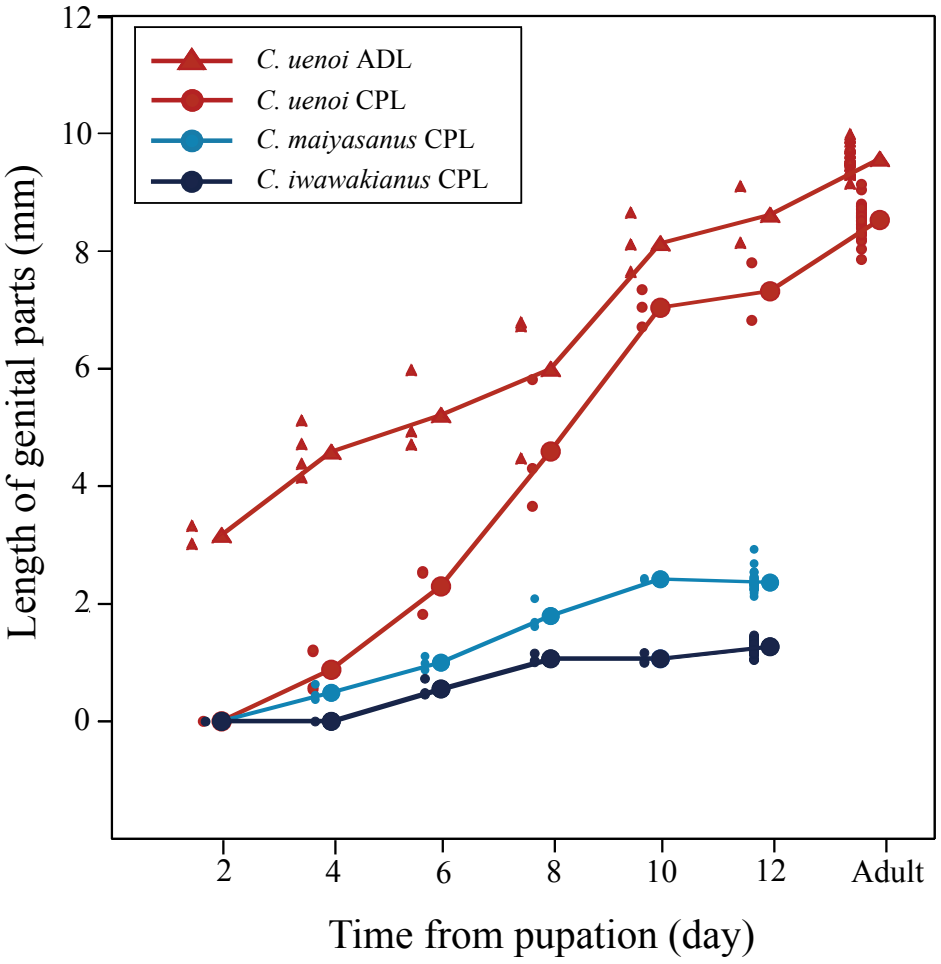

Fig. 4-4. Variation in genital sizes during development in *Carabus uenoi* (red), *C. maiyasanus* (light blue) and *C. iwawakianus* (navy blue). Circles and triangles refer to CP and AD, respectively. Small and large plots refer to individual and mean values, respectively. Data in 12 days for *C. maiyasanus* and *C. iwawakianus* are of adults.

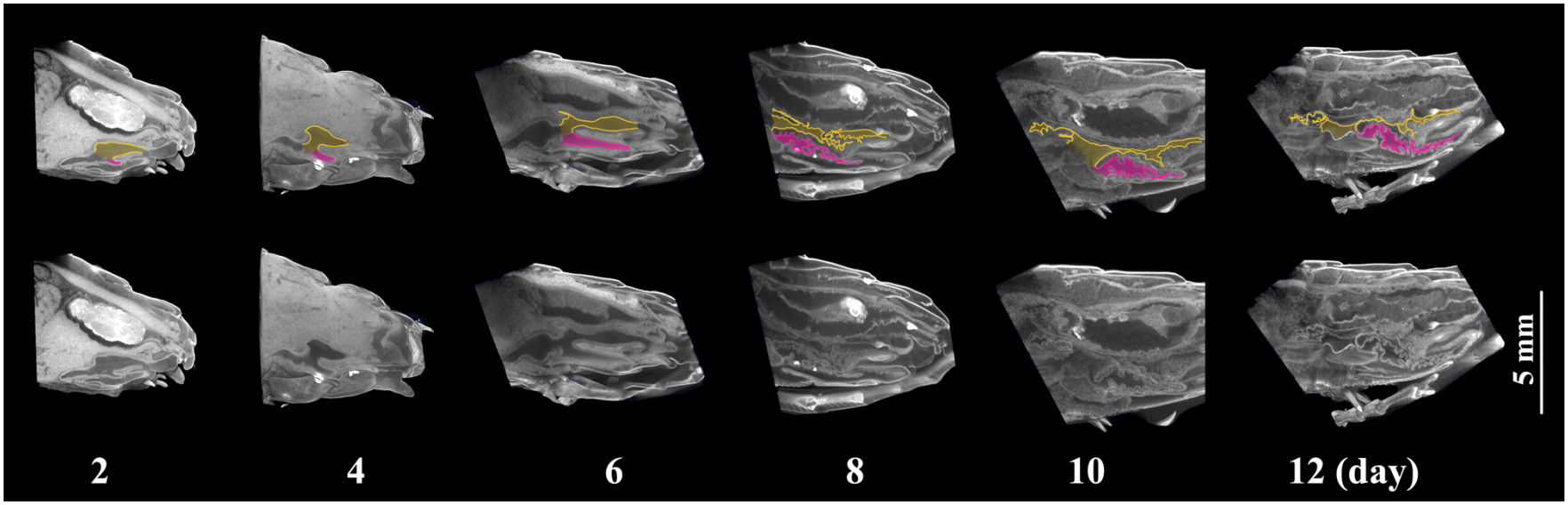

Fig. 4-5. Morphogenesis of the female genitalia of *Carabus uenoi*. The bursa copulatrix (yellow) and vaginal appendix (magenta) are indicated in upper pictures, which correspond to the lower raw pictures.

Chapter 5

General discussion

5.1 Summarization of the findings

In this thesis, I examined the developmental mechanisms of the evolutionary diversification of genital morphology based on analyses of morphogenesis and phenotypic (co)variation. The ground beetle subgenus *Ohomopterus* with species-specific male and female genitalia was the target of this study. Below, I briefly summarize the main findings of each chapter, then I discuss the evolutionary diversification of sexual traits from the developmental point of view.

In Chapter 2, I characterized the morphogenesis of species-specific male and female genital parts based on X-ray microcomputed tomography, using closely related species with divergent male and female genital morphologies, *C. maiyasanus* and *C. iwawakianus*. The results revealed the spatio-temporal dynamics of development of species-specific genital parts, providing a novel insight into the developmental process of the diversification of genital morphologies. Specifically, heterochrony and growth rate difference played relevant roles in generating interspecific differences in genital

morphology in the male and female, respectively.

In Chapter 3, I hypothesized that the exaggeration of sexual traits was possible by the relaxation of various constraints, and examined functional, genetic, and structural constraints on the evolution of exaggerated male genital morphology based on a comparative analysis of phenotypic covariation between the genitalia and other body parts using closely related species with divergent genital morphologies, *C. maiyasanus*, *C. iwawakianus* and *C. uenoi*. The exaggerated male genitalia in *C. uenoi* was related to relaxation of functional constraint, as revealed by a steeper allometric slope than in other species. By contrast, genetic constraint based on a shared genetic basis for the male genitalia and other appendages may have little effect on diversification in the male genitalia. Structural constraints were strongest in *C. uenoi* with the most exaggerated male genitalia, suggesting that the observed constraint was a result of exaggeration.

In Chapter 4, I examined the morphogenesis of the exaggerated male and female genitalia in *C. uenoi* by X-ray microcomputed tomography. The result of Chapter 2 allowed me to hypothesize that the shift of developmental timing (heterochrony) and

increased growth rate play roles in the development of the extremely enlarged genitalia. The result of Chapter 3 suggested that structural constraint on the extremely enlarged genital parts may result in further modification of the developmental process. The results revealed that the higher growth rate was a factor underlying the exaggeration of the genital sizes. Relatively longer pupal period enabled further growth of the genitalia until just before eclosion, suggesting a contribution of heterochrony, while it was different from advanced developmental timing as observed between *C. iwawakianus* and *C. maiyasanus* in Chapter 2. Additionally, relatively low growth rate in the AD resulted in temporal structural constraint on CP growth, on which the CP formed a complex folding structure within a limited spatial capacity of the AD. This structure was interpreted as a result of continuous cell proliferation, probably contributing to the development of exaggerated genital morphology.

5.2 Significance and future directions

In contrast to the evolutionary studies on sexual traits, very few studies have been

conducted for the developmental processes of sexual traits, especially those related to speciation. This study provided a pioneering case of the developmental study on divergent and exaggerated genital morphologies, which have been confirmed to be related to speciation. The findings in this thesis will contribute to studies on the genetic background of the sexual traits. For example, detailed description of the process of morphogenesis may provide a precise spatiotemporal platform for gene expression studies. Additionally, developmental processes resulting in interspecific differences and exaggeration of the genital morphology can help to design a novel research agenda that seeks genetic modification of gene networks corresponding to those developmental processes.

The results of the studies in this thesis propose several future challenges. Although my studies focused on interspecific difference in genital morphologies, intraspecific geographical variation in male and female genital morphologies are prominent in *Ohomopterus* ground beetles (Ishikawa 1991). Geographical variation within a species is important because it corresponds to the initial stages of evolutionary diversification and speciation. For example, divergence in genital traits is greater between

populations in contact with closely related species than between allopatric populations of *C. maiyasanus* and *C. iwawakianus*, as known to reproductive character displacement (RCD) (Nishimura et al. 2022). Additionally, geographical variation in genital morphologies within *C. maiyasanus* due to RCD led to reproductive isolation among allopatric populations within the species, revealing the ongoing process of genital evolution and speciation (Xia et al. 2023). The comparison of developmental processes of genital morphologies between geographical lineages within a species may tighten the link between the ongoing evolutionary process and its underlying developmental process of the sexual traits that contribute to speciation.

Carabus uenoi has the largest male and female genitalia across *Carabus* species on the earth (Ishikawa 1960). Based on the analysis of morphological variation, I revealed that relatively strong directional selection for the large male genitalia may resulted in the extremely enlarged male genitalia in this species (Chapter 3). Developmental processes responsible for the enlarged male and female genitalia were also revealed based on micro CT analysis (Chapter 4). In addition, genetic background of the development of enlarged

male and female genitalia were also suggested by transcriptome analyses (Nomura et al. 2021). However, the functional and behavioral background leading to the exaggeration of genital morphology in *C. uenoi* is still poorly known. Natural selection for enhanced reproductive isolation (Nishimura et al. 2022) and/or sexual selection or sexual conflict leading to run-away or chase-away coevolution between the sexes (Takami et al. 2018) may be responsible for this peculiar evolutionary event. Behavioral study of mating processes and functional study of the enlarged genitalia of *C. uenoi* will improve our understanding of the evolution of sexual trait exaggeration with more detailed discussion of its developmental background.

In conclusion, the results of this thesis revealed the developmental processes leading to diversification and exaggeration of sexual traits. Only a few studies have addressed this topic based on both the evolutionary and developmental perspectives. Here I tackled this topic using *Ohomopterus* ground beetles with divergent genital morphologies, and provided novel insights into the developmental and evolutionary mechanisms of diversification of sexual traits. These findings will contribute to the

understanding of the origin of morphological diversity.

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