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The development of extremely large male genitalia under spatial limitation

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Abstract

Extensive research in evolutionary biology has focused on the exaggeration of sexual traits; however, the developmental basis of exaggerated sexual traits has only been determined in a few cases. The evolution of exaggerated sexual traits may involve the relaxation of constraints or developmental processes mitigating constraints. Ground beetles in the subgenus *Ohomopterus* (genus *Carabus*) have species-specific genitalia that show coevolutionary divergence between the sexes. Here, we examined the morphogenesis of the remarkably enlarged male and female genitalia of *Carabus uenoi* by X-ray micro-computed tomography. The morphogenetic processes generating the male and female genitalia at the pupal stage were qualitatively similar to those in closely related species with standard genital sizes. Higher growth rates contributed to the exaggeration of both the male and female genital parts of *C. uenoi*, possibly related to a gene network commonly upregulated in both sexes. Additionally, the length of the copulatory piece (CP), the enlarged male genital part stored in the aedeagus (AD), reached close to that of the AD at the later developmental stages and thereafter decelerated to grew in parallel with the AD, suggesting a structural constraint on the CP by the outer AD. Then, unlike related species, the lengths of the CP and AD increased at eclosion, suggesting a mechanism leading to further elongation of the male genitalia. These observations suggest that a developmental process allows continuous growth of the male genitalia even under the spatial limitation. These results revealed the spatio-temporal dynamics of the development of exaggerated genital structures under structural constraints.

Keywords: aedeagus, *Carabus*, constraint, exaggerated trait, *Ohomopterus*

1 | INTRODUCTION

The exaggeration of sexual traits, such as weapons used in male-male combat and male ornaments subjected to female choice, has been an important topic of research in evolutionary biology (Andersson, 1994; Darwin, 1871; Emlen, 2008). The evolution of exaggerated sexual traits is determined by reproductive benefits as well as various costs or constraints (Emlen 2001; Jennions et al. 2001; Terada et al., 2023). Functional constraints affect the utility of exaggerated traits *per se* or decrease individual survival by holding the exaggerated traits (Godin & McDonough, 2003; Jennions et al., 2001; Langerhans et al., 2005; Weber et al. 2023). Genetic constraints, such as genetic correlations or pleiotropy, may hinder the independent evolution and exaggeration of a trait (Hansen, 2003; Johnsson et al., 2014). Structural constraints, such as competition for space and resources among traits, may necessitate coordination between the exaggerated trait and surrounding structures (Emlen, 2001; Matsumura & Yoshizawa 2010). Thus, the evolution of exaggerated sexual traits is expected to involve the relaxation of constraints or processes mitigating constraints (Emlen, 2001; Terada et al., 2023). Such processes may be detected in morphogenesis. However, the development of exaggerated sexual traits has only been elucidated in a few cases (Emlen et al., 2007; Moczek et al., 2007; Morita et al., 2019).

In animals with internal fertilization, genital morphology diversifies more rapidly than other traits and is one of the best examples of exaggerated sexual traits (Eberhard, 1985; Hosken & Stockley, 2004; Langerhans et al., 2016; Rowe & Arnqvist, 2012; Sloan & Simmons, 2019). Postcopulatory sexual selection via sperm competition, cryptic female choice, and sexual conflict predominantly underlie the evolution and diversification of genitalia in most taxa (Brennan & Prum, 2015; Eberhard, 1985, 2010;

Hosken & Stockley, 2004; Langerhans et al., 2016; Simmons, 2014; Sloan & Simmons, 2019). Natural selection against hybridization (Hollander et al., 2013; Kameda et al., 2009; Kawakami & Tatsuta, 2010; Kawano, 2002, 2004; 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. Additionally, differences in male and female genital morphologies can hinder hybridization between closely related species as a mechanical and sensory reproductive isolation barrier (Barnard et al., 2017; Bath et al., 2012; Kamimura & Mitsumoto, 2012; Masly, 2012; Richmond, 2014; Sota & Kubota, 1998; Sota & Tanabe, 2010; Tanabe & Sota, 2008; Tanaka et al., 2018; Wojcieszek & Simmons, 2013; Xia et al., 2023). Thus, rapid diversification of male and female genitalia is associated with sexual selection and speciation.

In contrast to the rapid diversification of genital shapes, the size of the genitalia shows less divergence among species (Rowe & Arnqvist, 2012), which may be due to stabilizing selection, as suggested by a negative static allometry (Eberhard et al., 1998, Eberhard, 2009). An exaggerated male genital size may be selected against in various contexts; for example, it may result in mismatches with the copulatory organs of conspecific females (the lock-and-key hypothesis, Masly, 2012; Shapiro & Porter, 1989) and/or may improperly stimulate the female sensory system and be disadvantageous in cryptic female choice (the one-size-fits-all hypothesis, Eberhard et al., 1998; Eberhard, 2009). Aside from this general trend, enlarged male primary genitalia is found in birds (McCracken et al., 2001) and insects (Gack & Peschke, 2005; Jałoszynski et al., 2014; Matsumura & Yoshizawa, 2010; Matsumura et al. 2021). In waterfowls, vaginal structures function as a barrier to phallus penetration, whereby directional selection may lead to the exaggeration of the male genital size (Brennan et al., 2007, 2015). Highly elongated male intromittent organs in insects function to deliver sperm into the deeply hidden female spermatheca and/or to remove rival sperm, thereby improving fertilization success under sperm competition (Simmons, 2001). Although the evolutionary processes shaping genital diversification and exaggeration have been well documented, the development of genital morphology in insects has only been investigated in a few taxa (Aspiras et al., 2011; Macagno & Moczek, 2015), with limited studies of taxa with extremely enlarged genitalia (Matsumura et al., 2013), except for fruit flies (Estrada & Sanchez-Herrero, 2001; Estrada et al., 2003; Hagen et al., 2019, 2020; Masly et al., 2011; Tanaka et al., 2015).

Ground beetles belonging to the subgenus *Ohomopterus* (Coleoptera, Carabidae, genus *Carabus*) show various degrees of exaggeration in the size and shape of genitalia (Figure 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* (Figure 2a). The female has a membranous pocket attached to the ventral wall of the bursa copulatrix (BC) called the vaginal appendix (VA) (Figure 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 (Fujisawa et al., 2019; Nishimura et al., 2022; Okuzaki & Sota, 2014; Takami & Sota, 2007; Takami et al., 2018; Xia et al., 2023).

In *Ohomopterus*, *Carabus uenoi* males have an extremely enlarged CP, as long as one-third of the male body, and females have a corresponding VA (Figures 1 and 2), providing an opportunity to examine the developmental processes contributing to extremely enlarged genitalia. The closely related species *C. iwawakianus* and *C. maiyasanus* have much smaller CPs and VAs with contrasting sizes and shapes (Figure 1) (Fujisawa et al., 2019). Interspecific differences in the sizes of the CP and VA between *C. iwawakianus* and *C. maiyasanus* are mediated by different underlying developmental mechanisms (Terada et al., 2021). The difference in CP size resulted from an advanced initial timing of development (i.e., heterochrony); thereby, the relatively longer CP in *C. maiyasanus* than in *C. iwawakianus* could be explained by a longer period of growth. The relatively longer VA in *C. maiyasanus* was attained by a higher growth rate than that in *C. iwawakianus*. These two hypothetical developmental processes (i.e., heterochrony and an increased growth rate) may also be responsible for the extremely enlarged genitalia in *C. uenoi*. Additionally, the structural or functional constraint on adult CP size is evident as the tip of the CP appears in the membranous opening of the AD in *C. uenoi* even when stored within the AD, different from other species (Figure 1). If the CP is longer than the present, it cannot be stored within the AD, and would hinder the insertion of the AD into the female genitalia, because the pointed tip and streamlined shape of the AD may be important in coercive mating with females showing rejection posture (Takami 2002). A recent morphometric analysis of adult genital sizes revealed that the sizes 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* was structurally constrained within the AD (Terada et al. 2023). It is possible that developmental processes can mitigate structural constraints on the extremely enlarged genital parts. Matsumura et al. (2013) described the morphogenesis of the exaggerated male genital organs of leaf beetles, in which the highly elaborate IS initially formed outside of the AD and subsequently retracted into the AD, possibly avoiding the spatial limitation.

In this study, we examined the developmental processes underlying the exaggeration of male and female genital sizes in *C. uenoi* by X-ray micro-computed tomography (μCT). We found that very high growth rates contribute to the exaggeration of both the male and female genital morphology. An earlier start of male genital development or longer pupal duration did not contribute to increased growth (i.e., there was no evidence for heterochrony). Additionally, we found that the elongation of the CP may be temporally constrained by the size of the AD, as indicated by decelerated growth of the CP and parallel growth of the CP and AD after the length of the CP reached close to that of the AD. We discuss the relevance of these developmental processes leading to the exaggeration of genital morphology.

2 | MATERIALS AND METHODS

2.1 | Sampling

Adults of *C. uenoi* were collected on Mt. Kongosan or Mt. Katsuragisan on the border between Osaka and Nara prefectures, Japan during the reproductive season (late 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 (depth, 2 cm) 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 plastic cups under the same conditions. Larvae were fed megascolecid earthworms *ad libitum*. Satiated third instar larvae were moved to a plastic jar (diameter, 6.5 cm; height, 9 cm) 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 moved to an artificial pupal room to observe the time of pupation. Artificial pupal rooms were made of moistened OASIS® floral foam within a plastic cup placed in an incubator with no lights. Our preliminary examination indicated that the time to eclosion was typically 13 days 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). Pupation was checked daily; the day on which pupation was discovered was defined as day 1. Pupae were sexed based on the morphology of the abdominal terminalia.

2.2 | Infiltration, scanning, and analysis

The integument of each pupa was perforated with a fine pin and then fixed using FAE solution (formalin: acetic acid: ethanol = 6:1:16) for at least 1 day (up to 1 week). 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 and mounted individually in a plastic tube (diameter, 1 cm; height, 5 cm) just before scanning.

X-ray µCT scans were performed using the 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 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 (Thermo-Fisher, Waltham, MA, USA). Volume-rendered images were viewed from the longitudinally or latitudinally sliced plane of the object. The segmentation function in Amira was applied to CPs at day 8 or later for visualization.

To quantify the growth of genital parts and to investigate the relevance of the developmental constraints on the CP by the outer AD, the sizes of the CP and the AD, estimated as the maximum length (CPL and ADL), were measured throughout pupal development using the distance measure function in Amira. To estimate relative genital sizes adjusted to variation in body size, the width of the seventh abdominal tergite was also measured as a proxy of pupal body size using Amira. Genital parts of adult males were removed and measured based on digital images obtained using a digital camera implemented in a Leica S9i microscope (Leica Microsystems, Wetzlar, Germany) (N = 30) (Terada et al., 2023). Male genital sizes of two related species, *C. iwawakianus* and *C. maiyasanus*, were evaluated for comparison based on a previous study (Terada et al., 2021). To examine growth rate difference in the male genitalia between *C. uenoi* and its closest relative *C. maiyasanus* (Figure 1), a general linear model (GLM) was constructed with CPL in developmental stages as the objective variable, and species, developmental stage and their interaction as explanatory variables. A significant interaction was expected if growth rates differed between species. Similarly, growth rate difference between the CP and AD in *C. uenoi* was also examined using a GLM with CPL or ADL in developmental stages as the objective variable, and genital part (CP or AD), developmental stage and their interaction as explanatory variables. Statistical analysis was performed using JMP ver. 14 (SAS Institute, 2018). In the female, VA development was described only by µCT images and the size of the VA was not measured because its structure was complex and difficult to quantify (see Results).

3 | RESULTS

3.1 | Male genitalia

At 2 days after pupation, the AD was already formed and the apical portion was exposed at the abdominal terminalia (Figure 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. These processes were similar to those in the related species *C. iwawakianus* and *C. maiyasanus* (Terada et al. 2021). The extremely curved and twisted base of the AD, which was specific to *C. uenoi* (Figure 2a), formed between 8 and 12 days after pupation.

The CP originated from a small protrusion of a part of the IS and directed internally to the cavity surrounded by the IS within the AD (Figure 3b). The small protrusion was observed 4 days after pupation, similar to *C. maiyasanus* but earlier than *C. iwawakianus* (Figures 4 and S1) (Terada et al., 2021). Then, the CP showed accelerated elongation until 8 days after pupation; the growth rate was greater than those of the CPs of *C. iwawakianus* and *C. maiyasanus* (Figures 4 and S1). The significantly greater growth rate in *C. uenoi* than in *C. maiyasanus* was confirmed by the GLM analysis (interaction between species and developmental stage: absolute CPL, $F_{1,28} = 67.07$, $P < 0.0001$; relative CPL, $F_{1,28} = 66.15$, $P < 0.0001$). The growth rate of the CP was also greater than that of the AD in *C. uenoi* (interaction between genital part and developmental stage: absolute length, $F_{1,30} = 13.99$, $P = 0.0008$; relative length, $F_{1,30} = 9.50$, $P = 0.0044$); thereby, CPL became close to ADL at 8 days after pupation and the growth rate of the CP started to decelerate (Figures 4 and S1). At this stage, a structure with complex folding formed in the distal portion of the CP in one of three replicate samples; this sample had the smallest ADL and CPL (Figures 3b, 4 and S1). This structure was not conspicuous in the remaining two samples at day 8 with a longer AD and CP; however, the CPs were generally shrunk (Figure S2). A cross-sectional view of the genitalia at day 8 showed that the CP was thick and 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 folded or shrunk structure in the CP almost disappeared (Figures 3, 4 and S1). A cross-sectional view at 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 and AD elongated gradually in parallel until adult eclosion (Figure 4). A cross-sectional view at 12 days after pupation showed that the CP became far thinner and occupied only a part of the cavity surrounded by the IS. The CPL (and ADL) of *C. uenoi* increased at eclosion, different from *C. iwawakianus* and *C. maiyasanus* (Figure 4).

3.2 | Female genitalia

The VA originated from a small protrusion of the ventral wall of the BC posterior to the vaginal apophysis (Figure 5). The BC was small and the VA was rudimentary 2 days after pupation. The BC continued to grow and the VA continued to elongate until 6 days after pupation, and then the BC and VA began to form complex folded structures from day 8. The moderately folding BC was found in *C. iwawakianus* and *C. maiyasanus* at later stages (day 10) (Terada et al. 2021). The highly developed folded structure in the VA was specific to *C. uenoi* and continued to grow until adult eclosion, although the apparent length of the VA did not change substantially. In addition to folded structures, elongated VAs were occasionally bent laterally, resulting in difficulty in aligning a longitudinal plane on which the actual size of the VA was measured, different from the case of *C. iwawakianus* and *C. maiyasanus* (Terada et al., 2021). Thus, quantification of VA size in *C. uenoi* and quantitative comparison with the related species were abandoned to avoid suffering from incorrect measurements and large errors.

4 | DISCUSSION

4.1 | Developmental processes leading to interspecific differences

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 a µCT analysis of pupal stages. It was hypothesized that heterochrony and/or an increased growth rate are responsible for the development of extremely enlarged genitalia (Terada et al. 2021). The CP of *C. uenoi* started to develop between days 2 and 4 in the pupal stages, as observed in *C. maiyasanus* with a shorter but long CP (the second longest among the three species compared) (Figures 1, 4 and S1). Thus, early growth did not contribute to the development of the large male genitalia, despite the role of this mechanism in differentiation between *C. maiyasanus* and *C. iwawakianus* (Terada et al., 2021). The start of initial CP development may be limited because coordinated or preceding development of the surrounding structures, such as the AD and IS, is necessary. The AD and IS were immature at 2 days after pupation (Figure 3a) and therefore CP formation may be difficult. While the timing of development of the CP was not advanced, the pupal stage of *C. uenoi* (13 days) was 1 day longer than those of *C. maiyasanus* and *C. iwawakianus* (12 days, Terada et al., 2021), possibly contributing to the elongation of the CP in *C. uenoi* until just before eclosion. However, there was little growth of the AD and CP from days 10 to 12 (Figures 4 and S1), suggesting that the posteriorly extended development did not contribute substantially to the enlargement of the male genitalia. By contrast, the growth rate of the CP was significantly higher in *C. uenoi* than in *C. maiyasanus,* especially until 10 days after pupation (Figures 4 and S1). Additionally, ADL and CPL of *C. uenoi* increased conspicuously at eclosion (from day 12 to the adult stage), different from results in the closely related *C. iwawakianus* and *C. maiyasanus* (day 10 to the adult stage) (Figure 4). This suggests that another mechanism enables the elongation of the male genitalia at eclosion, such as the unfolding of beetle horns at pupation (Emlen et al., 2007; Moczek et al., 2007; Morita et al., 2019). From the phylogenetic point of view (Figure 1), the development of the enlarged CP may have evolved in *C. uenoi* as mediated mostly by an increased growth rate and possibly by further elongation at eclosion via an unidentified mechanism.

For the female genitalia, the interspecific difference in VA size between *C. iwawakianus* and *C. maiyasanus* could be explained by a difference in growth rate (Terada et al., 2021). In the present study, a quantitative comparison among species was not possible because the VA size in *C. uenoi* was difficult to quantify owing to its structural complexity. Detailed three-dimensional quantification of the VA size and comparison among species are warranted in future study. However, the initial development of the VA was similar among the three species: the rudimentary VA was already formed 2 days after pupation (Figure 5) (Terada et al., 2021). Then, different from other two species, the VA of *C. uenoi* elongated rapidly and formed a complex folded structure (Figure 5). These findings suggest that the growth rate of the VA is higher in *C. uenoi* than in *C. iwawakianus* and *C. maiyasanus*, supporting the hypothesis that the interspecific difference in VA size was due to growth rate variation (Terada et al., 2021). It is unclear whether the longer pupal period contributed to the development of the extremely large female genital part. Unlike the male CP, the folded structure of the female VA was not fully expanded during late pupal stages (note that the adult VA in Figure 2b was manually expanded for visual purposes); however, is expanded when the CP is inserted *in copula* (Ishikawa, 1987).

4.2 | Structural constraint and its possible mitigation

In some horned beetles, an exaggerated male horn initially forms as a folded tube of epidermis in larval stages and then it is unfolded to elongate in pupal stages, resulting primarily from genetic modifications to the patterning processes that control cell proliferation (Emlen et al., 2007; Moczek et al., 2007; Morita et al., 2019). This process enables the development of exaggerated beetle horns within a limited space in larval bodies. In the case of the extremely enlarged male genitalia in a ground beetle, the growth of the CP of *C. uenoi* is also structurally constrained by the size of the outer AD (Terada et al., 2023). This constraint was also suggested by the present result, as the growth rate of the CP started to decelerate after the day 8 when CPL reached close to ADL and resulted in parallel growth of the CP and AD (Figure 4). We hypothesized that the development of the CP of *C. uenoi* was modified in response to this structural constraint. We observed that the CP of *C. uenoi* formed a folded structure 8 days after pupation when the length of the CP was likely to be limited by the internal capacity of the AD (Figure 3). However, since only one of three samples at day 8 showed this structure (Figure S1), this observation could be an anomaly. Alternatively, the observed decelerated growth rate of the CP as well as parallel growth of the CP and AD could be a developmental process that attains predetermined adult genital sizes, not relate to structural constraint. However, further growth of the CP and AD at eclosion indicates that the male genitalia do not fully enlarge in pupal stages. This suggests that decelerated growth in later pupal stages reveal a contrary effect on attaining adult genital sizes. Thus, an unidentified process, or constraint due to spatial capacity of the pupal abdomen, may determine the maximal male genital size (principally the size of the AD), and it may have a downstream effect (i.e., constraint) on the growth of the CP within the AD. Further study with increased sample sizes, as well as examination of the internal capacity of the abdomen and the morphogenetic process at eclosion is warranted to examine whether the above-mentioned developmental process is related to the mitigation of the structural constraint on the extremely enlarged male genitalia.

4.3 | Genetic background

Interestingly, we observed that both the CP and VA commonly showed greater growth rates than the related species at the same developmental stages, 8 days after pupation and later (Figures 3, 4 and 5; Terada et al. 2021). Recent comparative transcriptome analysis of genital development in *C. uenoi*, *C. iwawakianus,* and *C. maiyasanus* revealed that genes involved in organ size control are commonly upregulated in males and females at the late pupal stages (Nomura et al., 2021). This implies that synchronous growth of the CP and VA are a result of modifications to the common gene network in males and females. These findings provide insights into the developmental processes of exaggerated genital morphologies showing coevolutionary diversification between the sexes. These results also improve our understanding of the precise developmental stages and tissues involve in species-specific genital differentiation and exaggeration, contributing to the construction of a novel platform for studies on the diversification and exaggeration of the genital morphologies from an evo-devo perspective.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests.

DATA AVAILABILITY STATEMENT

The data in this study are available in Dryad.

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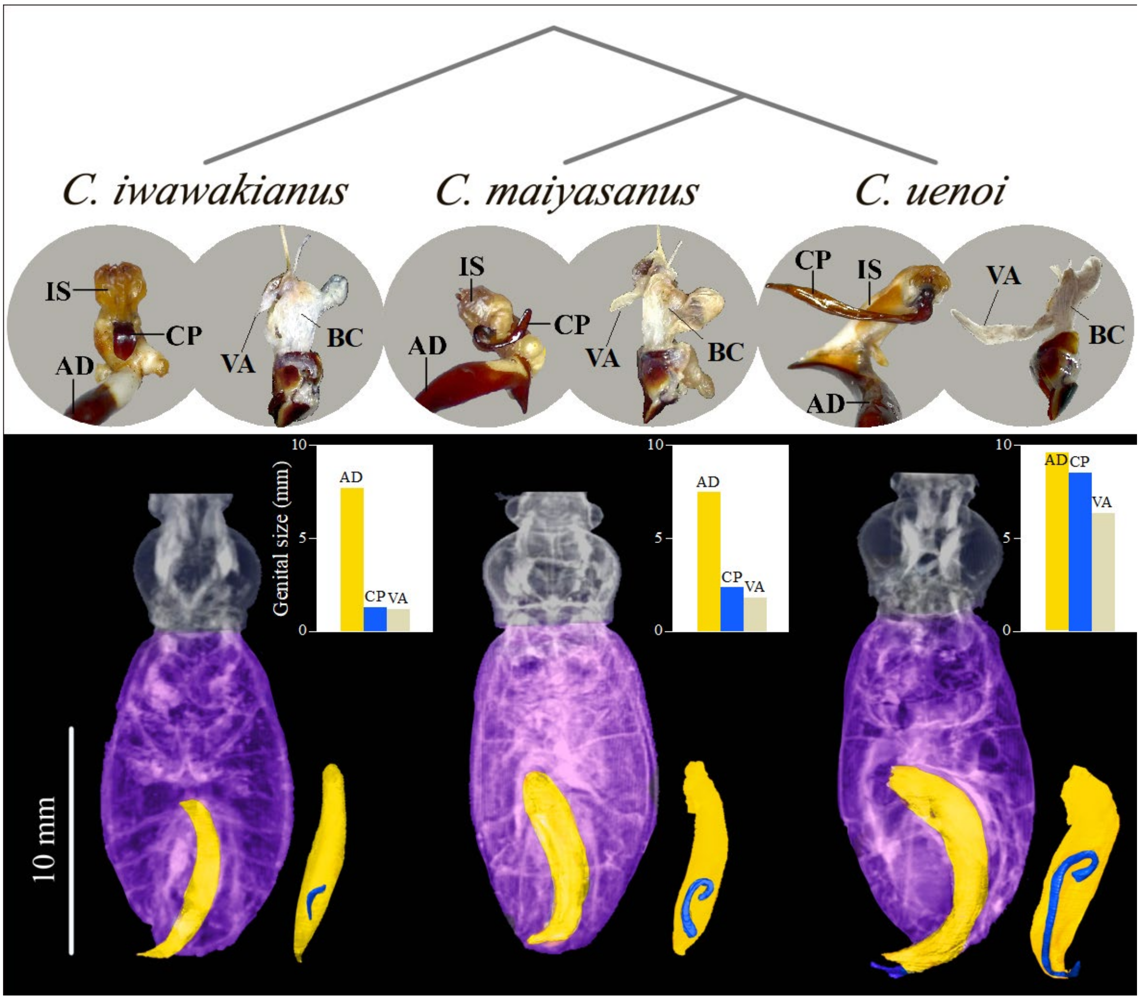

Fig. 1. Phylogenetic relationships between *Carabus iwawakianus*, *C. maiyasanus*, and *C. uenoi* and configurations of body and genital parts. Male and female genitalia are shown in circles; the internal sac (IS) and copulatory piece (CP) are everted from the aedeagus (AD); the vaginal appendix (VA) is attached to the ventral wall of the bursa copulatrix (BC). Nested configurations consisting of the functional abdomen including the meso-and metathoraces (purple), AD (yellow), and CP (blue) are depicted in dorsal view based on µCT scans (from Terada et al. 2023). The mean lengths (in millimeters) of the adult AD (ADL), CP (CPL), and VA (VAL) are shown (from Nishimura et al. 2022).

Fig. 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.

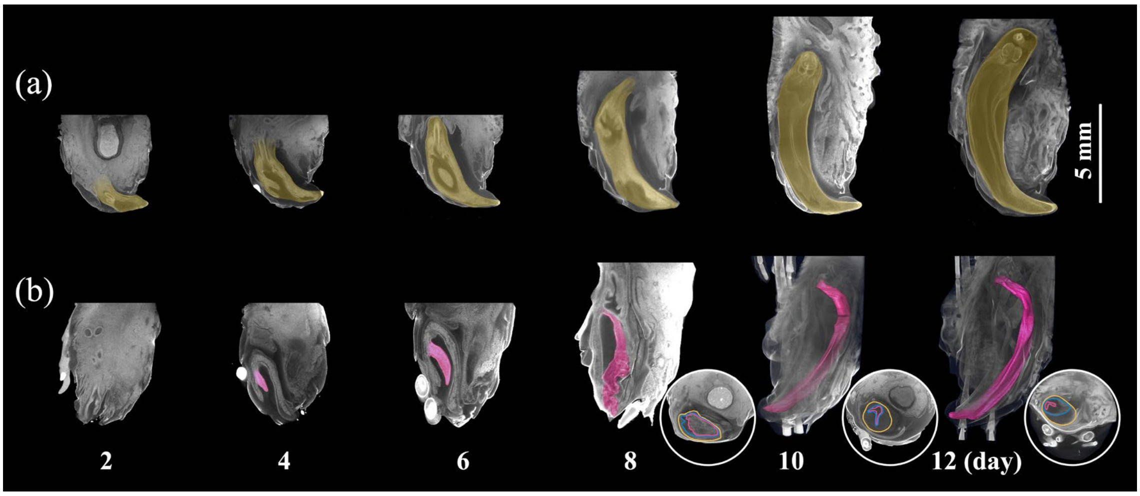

Fig. 3. Morphogenesis of the male genitalia of *Carabus uenoi*, (a) aedeagus (yellow), (b) copulatory piece (magenta). The white circle shows a cross-sectional view between the 8th and 9th abdominal segments; internal sac (blue).

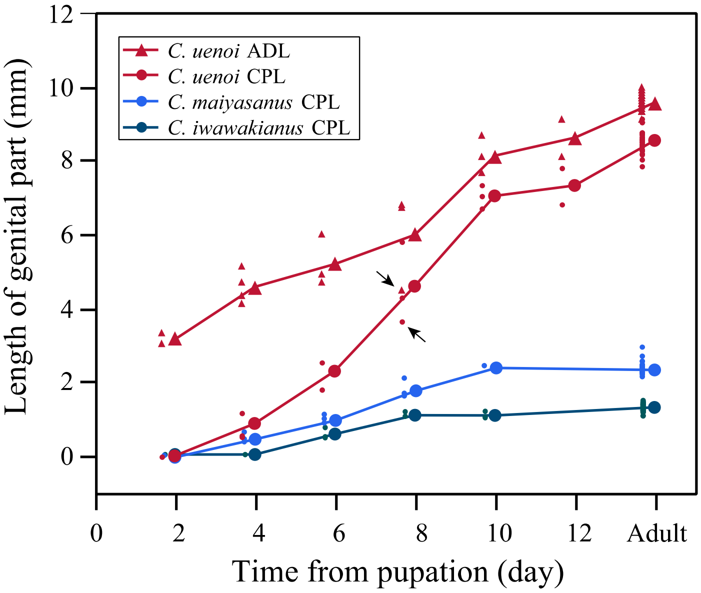

Fig. 4. Changes in male genital sizes during development in *Carabus uenoi* (red), *C. maiyasanus* (light blue), and *C. iwawakianus* (navy blue) (data for the latter two species were obtained from Terada et al., 2021). Circles and triangles indicate the CPL and ADL, respectively. Small and large points indicate individual and mean values, respectively. Arrows refer to an individual on day 8, shown in Figure 3b.

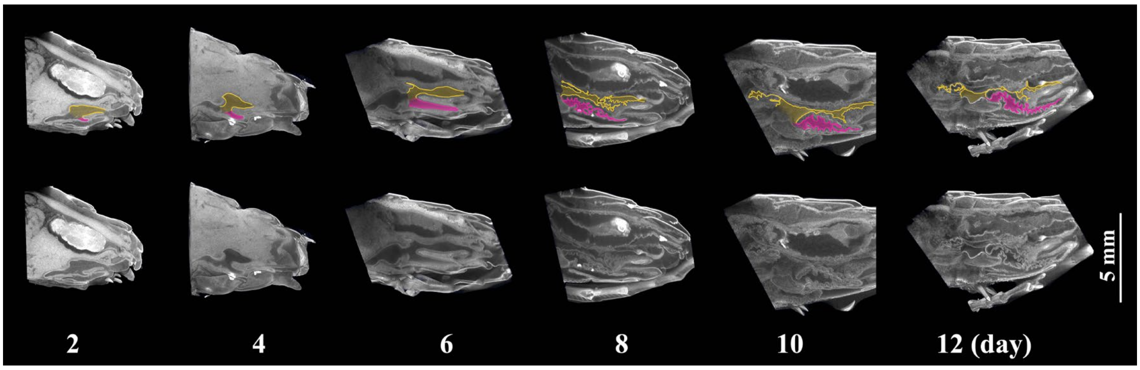

Fig. 5. Morphogenesis of the female genitalia of *Carabus uenoi*. Sagittal views of bursa copulatrix (yellow) and vaginal appendix (magenta) are shown.

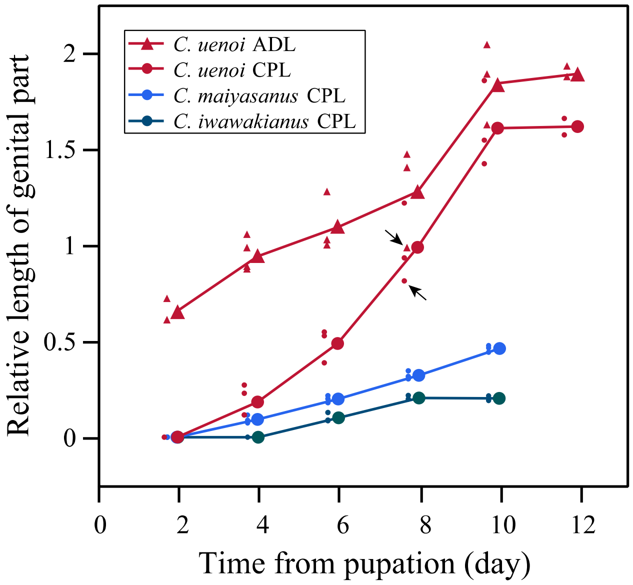

Fig. S1. Changes in relative male genital sizes during development in *Carabus uenoi* (red), *C. maiyasanus* (light blue) and *C. iwawakianus* (navy blue) (data for the latter two species were obtained from Terada et al., 2021). Genital sizes were divided by body size (the width of 7th abdominal tergite) to obtain relative values. Circles and triangles indicate the relative CPL and ADL, respectively. Small and large points indicate individual and mean values, respectively. Arrows refer to an individual on day 8, shown in Figure 3b.

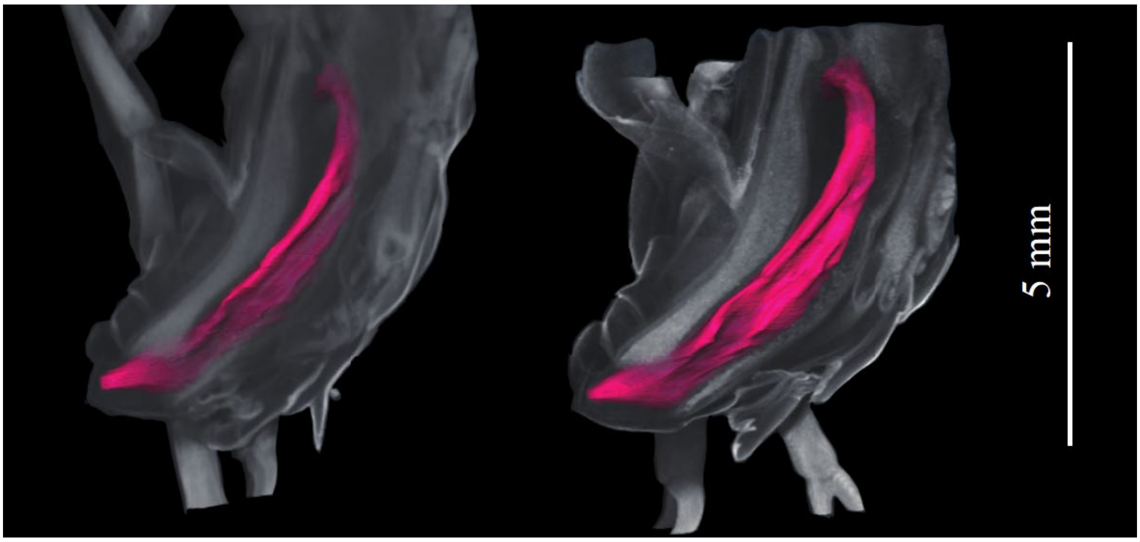

Fig. S2. The copulatory piece (CP) (magenta) at the day 8 pupal stage of *Carabus uenoi*. Two individuals (different from that in Figure 3) are shown. The CP showed shrinkage.