Arthropod Structure & Development 80 (2024) 101360

Contents lists available at ScienceDirect

Arthropod Structure & Development

journal homepage: www.elsevier.com/locate/asd

Morphological and functional analyses for investigation of sexually selected legs in the frog legged beetle *Sagra femorata* (Coleoptera: Chrysomelidae)

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ARTICLE INFO

Article history:
Received 3 December 2023
Received in revised form
19 April 2024
Accepted 22 April 2024

Handling Editor: Dr G. Scholtz

Keywords:
Allometry
Exaggerated trait
Male–male combat
Microstructure
Weapon
Adhesion force

ABSTRACT

Mate choice and male–male combat over successful mating often cause disproportionate exaggeration of male trait relative to body size. However, the exaggeration is often not the only trait involved with male–male combat and mate choice: suites of co-expressed traits may function together as a coordinated unit. When this occurs, dimorphism may be expected for these additional, non-exaggerated, structures. *S. femorata* males have disproportionately large hind-legs used in male–male combat over females. During the fights, fore- and mid-legs are used to keep males in positions where advantageous for leverage. Because use of the exaggerated hind-legs is coordinated with the other legs, they will coevolve as a functional unit. Here, we show that 1) *S. femorata* has sexual size differences in all three legs; 2) males show positive allometry in the relative sizes of all three legs; and 3) microstructures of tarsi on the fore- and mid-legs are also sexually dimorphic. Despite these differences in the tarsal microstructure, 4) adhesion forces of the tarsi had no sexual difference in flat surface. The microstructure would be specialized on attaching elytra surface. These results suggest that the three pairs of legs function together during fighting behavior, with hind-legs employed primarily for fighting, and the fore- and mid-legs functioning to grip females, keeping males positioned on the back of the female during combat.

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1. Introduction

Many exaggerated traits are the result of sexual selection, which selects for individuals with greater reproductive success (Andersson, 1994; Emlen, 2008). Male ornaments attracting female (e.g., elaborate feathers in birds of paradise) (e.g., Frith et al., 1998; Ligon et al., 2018) and weapons of male–male combat (e.g., antlers of ungulates or horns and mandibles of beetles) (Zeh, 1987; Zeh et al., 1992; Kruuk et al., 2002; Lappin and Husak, 2005; Okada et al., 2006; Hongo, 2007) are typically expressed in one sex (often males), where the size and/or quality of the exaggerated trait determines individual fitness. It is important to recognize that expression of the trait is exaggerated in one sex due to the strong relationship between trait size and fitness, and restricted in the other sex because costs associated with large trait sizes prevents their expression (Kotiaho et al., 1998; Godin and McDonough, 2003; Allent and Levinton, 2007; Fuchikawa and Okada, 2013).

Exaggerated ornaments and weapons typically exhibit steep, positive allometric relationships between trait size and individual overall body size (Petrie, 1992; Simmons and Tomkins, 1996; Kodric-Brown et al., 2006; Emlen et al., 2012). Multiple sexually

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https://doi.org/10.1016/j.asd.2024.101360
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selected traits, including eye stalks of diopsid flies (Burkhardt et al., 1994), horns of beetles (McCullough et al., 2015), claws of crabs (Juanes et al., 2008), tails of widowbirds (Alatalo et al., 1988), and antlers of ungulates (Vanpé et al., 2007) show this steep, positive scaling relationship although some of these traits do not show positive allometry because of developmental and functional constraints (Bonduriansky, 2007; Voje, 2016). In fact, positive scaling pattern is so common that steep positive scaling relationships have been repeatedly suggested as an identifying characteristic of sexually selected ornaments and weapons (Kodric-Brown et al., 2006; Tomkins et al., 2010; O'Brien et al., 2018).

The study of ornaments and weapons has left us with a strong understanding of sexual dimorphism and scaling within sexually selected systems. However, the overwhelming majority of this work has focused exclusively on the ornaments and weapons *per se*, ignoring associated traits that may be under similar patterns of selection. Both male—male combat and female-choice courtship behavior may involve suites of co-expressed structures that function together with the exaggerated trait, forming a cohesive unit (Lande and Arnold, 1983). For example, in the broad horned flour beetle *Gnatocerus cornutus*, males use their mandibles as a weapon in combat (Okada et al., 2006), but also use their forelegs to anchor themselves and lift their opponent during these battles. In scenarios such as this, selection acts to increase the size of the focal trait but also enhances the performance of correlated, non-target traits, *i.e.* supportive traits. As a result, both focal and supportive traits should be under sex-specific patterns of selection. Indeed, evidence suggests the fore-legs of the broad horned flour beetle are strengthened and elongated in males, a result of their role as a supportive trait in mandible-focused combat (Okada et al., 2012).

Therefore, to understand the effects of these exaggerated traits on fitness and the role these patterns of selection play in weapon and ornament evolution, we must consider how selection acts on morphological characters correlated with the focal reproductive behavior. Here, using the frog legged beetle *Sagra femorata* (Drury, 1773) (Coleoptera: Chrysomelidae), we investigate the allometry of a sexually selected weapon (hind-legs) and the allometry and microstructure of its associated support traits (fore- and mid-legs).

S. femorata is naturally found in Southeast Asia and has invaded Japan since 2009 (Akita et al., 2011). The host in Japan is kudzu *Pueraria lobata* (Willd.) Ohwi subsp. *Lobata* (Fabales: Fabaceae). Frog legged beetles have enlarged, sexually dimorphic hind-legs, which males use in competition over direct access to females (Katsuki et al., 2014). Battles begin when a single male (intruder) encounters a mating pair. The intruder mounts the copulating pair, grasps the resident male by the hind-legs, and attempts to tear the mating pair apart (Katsuki et al., 2014). During combat, both males fix their bodies using their fore and mid-legs to grasp the substrate (plant stem) or elytra of the female or the rival male. As a result, the combative behavior of each contestant can be divided into two components in the manner of following Katsuki et al. (2014). Intruding males must 1) use their hind-leg weapons to tear the opponent from the mating female (primary function) and 2) use their fore and mid-legs to attach to the conspecific cuticle surface or substrate, ensuring sufficient force to dislodge the opponent (supportive function). Similarly, resident males must prevent separation from the female by using their hind-leg weapons to directly fend off intruders (primary function) and their fore and mid-legs to maintain position during competition (supportive function). Previous studies have reported that males with larger hind-legs tend to win the combat (O'Brien et al., 2017; O'Brien and Boisseau, 2018). In addition to the size of weapon, the ability of male frog legged

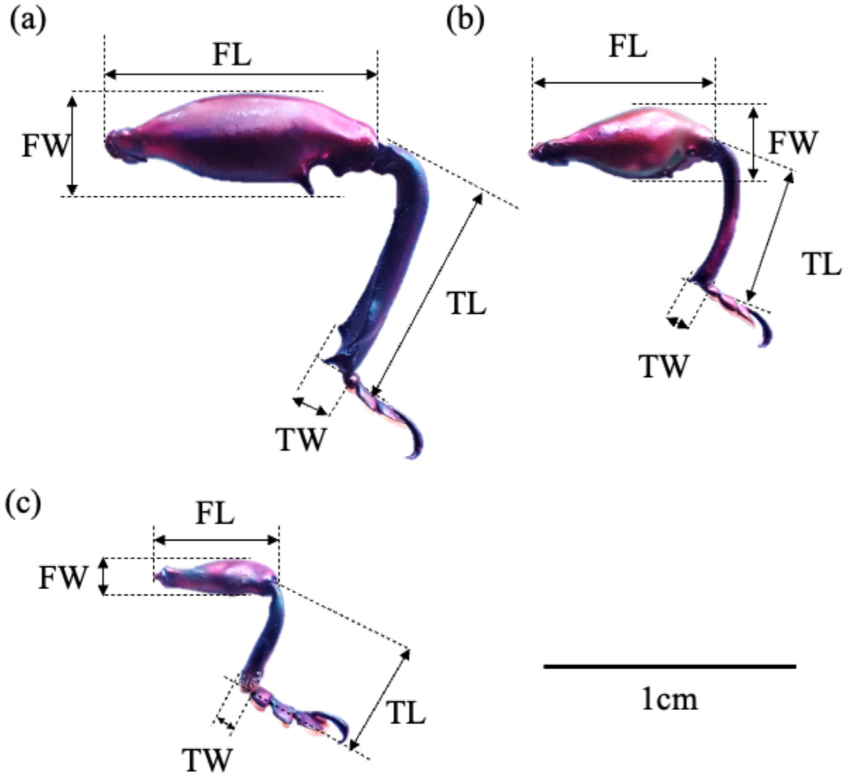

Fig. 1. Measured traits of (a) male hind-leg, (b) female hind-leg and (c) fore and mid-leg in both sexes. FL, femur length; FW, femur width; TL, tibia length; TW, tibia width.

beetles to affix their body to the substrate or conspecific is also an important factor determining the outcome of male–male competition. We therefore predict sex specific microstructures on the tarsi of male *S. femorata* to enhance attachment during combat, and associated sexual differences on leg adhesion force. This prediction is supported by observations in several Coleopteran species (especially in Chrysomelidae), where the setae of male tarsi are specialized to attach to females during mating (Voigt et al., 2008; Gloyna et al., 2014). In addition, we investigated whether supportive traits associated with combative behavior (*i.e.* mid- and fore-legs) follow a similar trend of hind-leg weapons which exhibit positive allometry in male beetles.

2. Materials and methods

2.1. Insects

S. femorata females lay eggs in the stems of kudzu *P. lobata*, where larvae grow and induce formation of a gall where they pupate. Larva overwinter within kudzu stems and adults emerge in the early summer.

Individuals used for morphological measurements of leg characters were collected from kudzu stems containing larvae from a population of *S. femorata* along the Sakanai River (Mie Prefecture, Japan) in January 2014. The kudzu stems were maintained at room temperature in plastic containers. As adults emerged, they were removed from the kudzu stem and the individuals were placed into a freezer. After individuals died, they were kept with silica gel for morphological measurement.

Individuals used in measurement of adhesion force of legs were collected along the Kushida River (Mie Prefecture, Japan) in January 2021. The galls including larvae were maintained at 10 °C in a refrigerator for keeping larval diapause until the time of measurement. To obtain adult individuals, the larvae were removed from kudzu stems and maintained individually in plastic bottles at 25 ± 2 °C until eclosion. After adults emerged, each individual was maintained at 25 ± 2 °C in a plastic cup with water and insect jelly (Pro Jelly, KB Farm, Saitama, Japan) as food and filter paper to keep their tarsi clean.

2.2. Measurement of leg and body size

To investigate sexual differences of each leg character, the left fore-, mid- and hind-legs were removed from each beetle. Each leg was then dissected to separate the femur and tibia. Femur length (FL) and width (FW) and tibia length (TL) and width (TW) were measured by digital vemier calipers (Degimatic Caliper, Mitutoyo Corporation, Japan). Measurements are shown in Fig. 1. Elytra length was measured and was used as an estimate of overall body size (Katsuki et al., 2014). Sixty individuals of each sex were used for morphological measurement.

2.3. Measurement of allometries of leg characters

To investigate whether leg characters have positive allometry, we adopted a power function based on the equation following Gould (1966):

$$y = mx^{\alpha}$$

where y and x are trait and body size, respectively, and α indicates the allometric slope. When $\alpha > 1$, relative trait size increases with body size (positive allometry).

2.4. Observation of microstructure of setae on each tarsus

To compare microstructure of the tarsus in fore-, mid- and hind-legs between sexes and legs, specimens of *S. femorata* were dried using silica gel at room temperature. Male and female tarsi were removed from the body and the microstructure of the tarsomeres was observed using Scanning Electron Microscopy (SEM) (VE-8800, KEYENCE). SEM images of whole area in tarsi were obtained at 1.0 kV and those of setae at from 1.7 to 3.0 kV, respectively. The obtained SEM images were processed the contrast and brightness by Adobe Photoshop (Adobe Inc, San Jose, CA)

2.5. Measurement of leg adhesion force

To investigate whether differences in tarsus structure influence attachment ability, adhesion force of legs was measured. To measure adhesion force of *S. femorata*, intact virgin females ($n = 9$) and males ($n = 6$) were tested. Fig. 2 shows a setup for measuring the adhesion force of the insects. The insect put on the mirror surface

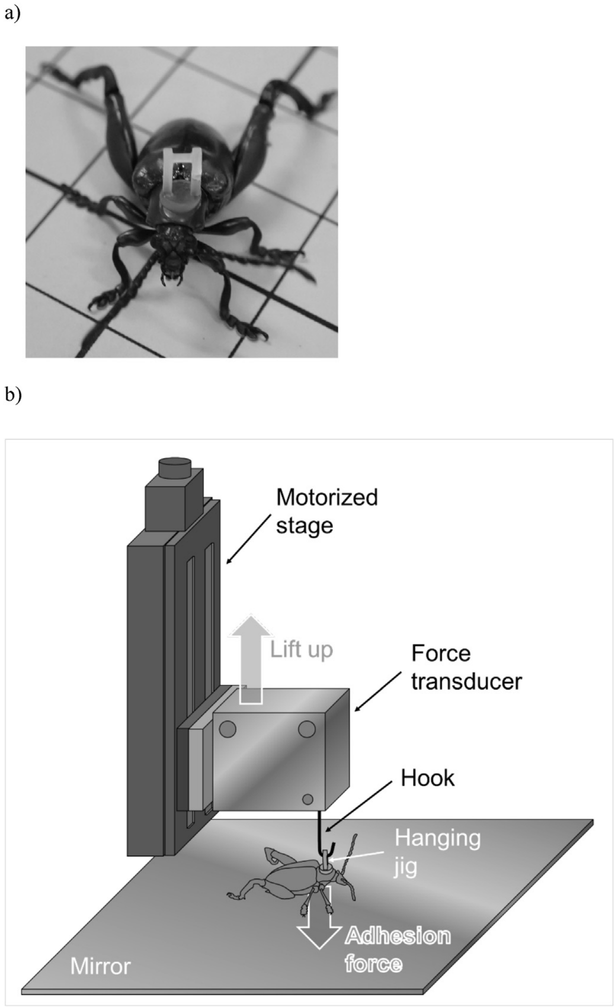

Fig. 2. Schematic illustration of the adhesion force measurement system. Each square under the insect is one centimeter square. *S. femorata* fixed hanging jig for adhesion force measurement (a). The insect connected to the force transducer was lifted up by the motorized stage from the mirror surface and the adhesion force between the legs and the mirror surface was measured (b).

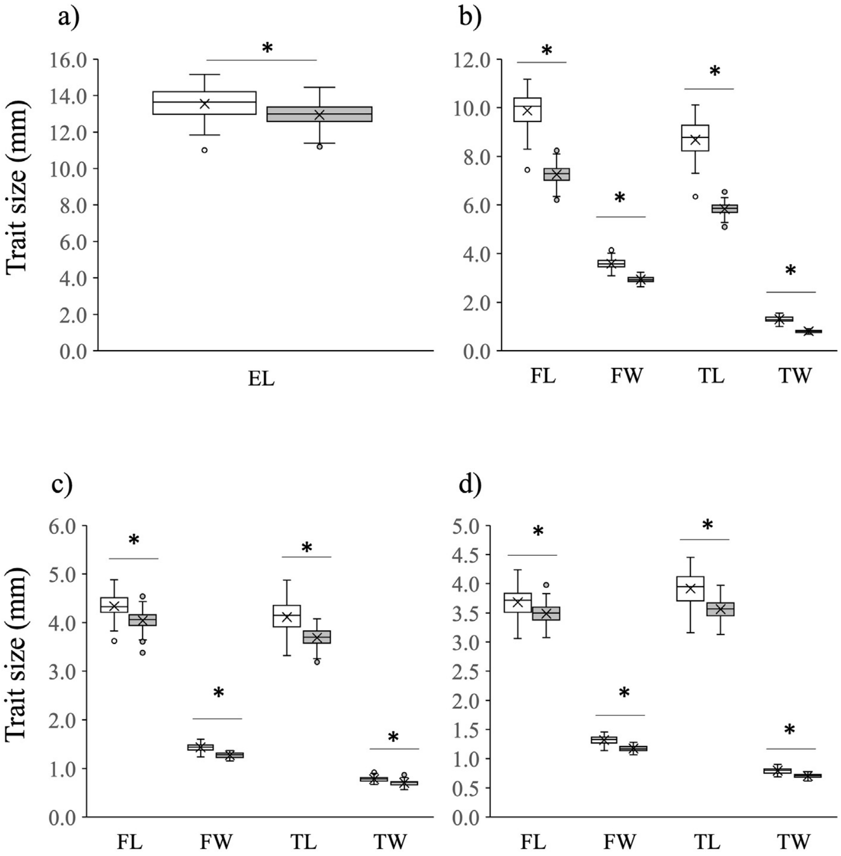

Fig. 3. Sexual size differences of a) elytra length (EL) as body size, femur length (FL), femur width (FW), tibia length (TL), and tibia width (TW) in b) hind-, c) mid- and d) fore-legs. Open (left) and grey (right) box plots show male and female, respectively. Cross mark shows mean value. Scales of y-axis are mm. Asterisk shows significant difference at $P < 0.0001$.

was lifted up and the detachment force between the insect and the mirror was defined as adhesion force. Before the experiment, a hanging jig was fixed on the prothorax by mixture of beeswax and pine resin (1:1) (Fig. 2a). Fig. S1a shows a schematic illustration of the hanging jig. The hanging jig was made of resin (Standard photopolymer resin, Elegoo Inc., Shenzhen, China) by 3D printer (MARS, Elegoo Inc.) and had a hole oriented along the body axis direction of the insect. By hooking the hole with the metal hook, the direction of the insect was fixed and the observation of the experiment became easier (Fig. S1).

The hanging jig of female or male was hooked by a metal hook connected to a capacitance type force transducer system (Force sensor, B20-003, THK Precision Co., Ltd., Tokyo, Japan) (Force sensor amplifier, FSA201C, THK Precision Co., Ltd.) (range, 0–100 mN; resolution, 20 μN). To measure adhesion force, each individual was placed on a flat mirror (KH03-3716, KOHNAN SHOJI Co., Ltd., Osaka, Japan; Fig. 2b). The mirror was cleaned with ethanol before each measurement. The force transducer was lifted at a speed of 1 mm/s with a motorized stage (motorized stage, KS161-35, SURUGA SEIKI Co., Ltd., Shizuoka, Japan) (motor controller, NI USB-6211, National Instruments Co., Texas, USA) (motor driver, GDB-5F30V1, Melec Inc., Tokyo). Simultaneously, the insect connected to the force transducer with the hanging jig and the metal hook was lifted, and downward force (the sum of the adhesion force of the insect's legs and the gravitational force due to the insect's weight) was measured during the lifting. A data acquisition system (data acquisition device, NI USB-6211, National Instruments Co.) (data acquisition software, LabVIEW, 2020 ver. 20.0, National Instruments Co.) was used for storing force signals. The adhesion force was determined by subtracting the gravitational force resulting from body weight from the downward force. Measurements of adhesion force were conducted from three to five trials per individual. The experiments were carried out at room temperature (23.5–27.0 °C) and humidity (60.0–66.5 %). Behaviors and position of legs were recorded by digital camera (Olympus Tough TG-6, OM Digital Solutions Corporation, Tokyo, Japan). The sampling rate during date acquisition was 1 kHz. The obtained force signals were run through a low pass filter (end of pass band, 50 Hz; start of reject band, 100 Hz) by commercial analysis software (Igor Pro ver. 6.22J, Wave Metrics, Lake Oswego, OR, USA). The maximum peak force measured at the moment when the fore legs or the middle legs detached from the mirror surface was defined as the leg adhesion force. By checking position of all legs against measured adhesion force during recording, failed trials were removed. Average of maximum adhesion force was used for statistical analysis.

2.6. Statistics

To examine whether the slopes of trait size versus body size differed between the sexes, we used general linear models (ANOVA). Each leg character (length and width of femur and tibia in fore-, mid- and hind-leg) was used as a response variable, and sex, body size and the interaction between the two were used as

Table 1
Results of general linear models in each leg character. Bold effect is interaction between sex and body size on a trait with significant difference.

trait	effects	*df*	SS	*F*	*P*	Coefficient ±s.e.
Hind-leg						
Femur length	Sex	1	124.723	3123.887	<0.0001	−1.104 ± 0.020
	Body size	1	29.562	740.439	<0.0001	0.677 ± 0.025
	Sex * Body size	1	1.819	45.558	<0.0001	−0.168 ± 0.025
	error	116	4.631			
Femur width	Sex	1	6.952	739.368	<0.0001	−0.261 ± 0.010
	Body size	1	2.847	302.801	<0.0001	0.210 ± 0.012
	Sex * Body size	1	0.038	4.050	0.0465	−0.024 ± 0.012
	error	116	16.902			
Tibia length	Sex	1	156.039	3858.912	<0.0001	−1.235 ± 0.020
	Body size	1	23.612	583.948	<0.0001	0.605 ± 0.025
	Sex * Body size	1	2.451	60.602	<0.0001	−0.195 ± 0.025
	error	116	4.691			
Tibia width	Sex	1	5.103	1513.286	<0.0001	−0.223 ± 0.006
	Body size	1	0.320	94.945	<0.0001	0.070 ± 0.007
	Sex * Body size	1	0.038	11.405	0.001	−0.024 ± 0.007
	error	116	0.391			
Mid-leg						
Femur length	Sex	1	0.371	39.033	<0.0001	−0.060 ± 0.010
	Body size	1	5.285	555.868	<0.0001	0.286 ± 0.012
	Sex * Body size	1	0.026	2.742	0.101	0.020 ± 0.012
	error	116	1.103			
Femur width	Sex	1	0.355	252.363	<0.0001	−0.059 ± 0.004
	Body size	1	0.345	245.160	<0.0001	0.073 ± 0.005
	Sex * Body size	1	0.003	2.168	0.144	−0.007 ± 0.005
	error	116	0.163			
Tibia length	Sex	1	1.512	106.156	<0.0001	−0.122 ± 0.012
	Body size	1	5.599	393.048	<0.0001	0.295 ± 0.015
	Sex * Body size	1	0.056	3.910	0.0504	−0.029 ± 0.015
	error	116	1.652			
Tibia width	Sex	1	0.051	42.242	<0.0001	−0.022 ± 0.003
	Body size	1	0.169	141.112	<0.0001	0.051 ± 0.004
	Sex * Body size	1	0.000	0.001	0.9714	−0.000 ± 0.004
	error	116	0.139			
Fore-leg						
Femur length	Sex	1	0.061	5.496	0.021	−0.024 ± 0.010
	Body size	1	3.675	330.863	<0.0001	0.239 ± 0.013
	Sex * Body size	1	0.001	0.050	0.824	−0.003 ± 0.013
	error	116	1.288			
Femur width	Sex	1	0.293	196.876	<0.0001	−0.054 ± 0.004
	Body size	1	0.260	174.425	<0.0001	0.063 ± 0.005
	Sex * Body size	1	0.005	3.222	0.075	−0.009 ± 0.005
	error	116	0.173			
Tibia length	Sex	1	0.995	64.053	<0.0001	−0.099 ± 0.012
	Body size	1	4.140	266.649	<0.0001	0.253 ± 0.016
	Sex * Body size	1	0.169	10.895	0.001	−0.051 ± 0.016
	error	116	1.801			
Tibia width	Sex	1	0.113	185.302	<0.0001	−0.033 ± 0.002
	Body size	1	0.127	208.540	<0.0001	0.044 ± 0.003
	Sex * Body size	1	0.001	0.951	0.332	−0.003 ± 0.003
	error	116	0.071			

explanatory variables. These analyses were conducted by JMP version 11 (SAS Institute, 2013).

For analyses of static allometry, all traits were log-transformed. We assumed that variable errors of x- and y-axies were equal, and conducted major axis (MA) regression by using the R package “lmodel2” (Legendre, 2022). In MA regression, 95 % confidence intervals are also provided. When the interval between 95 % confidence intervals didn't include 0, we determined that the allometric slope was significantly different from zero.

To investigate whether sexes differed in tarsal adhesion force, mean adhesion force was compared between sexes by Welch test. These analyses were conducted by R Ver 4.1.3 (R Core R Development Core Team, 2022).

3. Results

Mean sizes of each leg character are shown in Fig. 3. Trait size increased with body size in both sexes, and males were significantly larger than females across all measured traits. In addition, all hind-leg traits and the tibia length of fore-legs had a significant interaction with sex and body size (Table 1, Fig. 4). The tibia length of mid-legs also had a marginally significant interaction with sex and body size ($P = 0.0504$; Table 1).

Five leg traits, FL, TL and TW in hind-leg, and TL of mid- and fore-legs had positive allometries with body size in males, while females only had positive allometry on tibia length of the mid-leg (Table 2).

Tarsal morphology with setae for both sexes is shown in Fig. 5. The tarsus of *S. femorata* is constructed of five tarsomeres and a claw (Figs. 1 and 5A). The lower surface of first, second, and third basal tarsomeres in both sexes are covered in setae (Fig. 5B and C). Setae of these tarsomeres display sex-specific differences in fore- and mid-legs but not in hind-legs. Almost all setae of tarsomeres in female legs, and of the male hind-legs, are bifid setae with spatulate

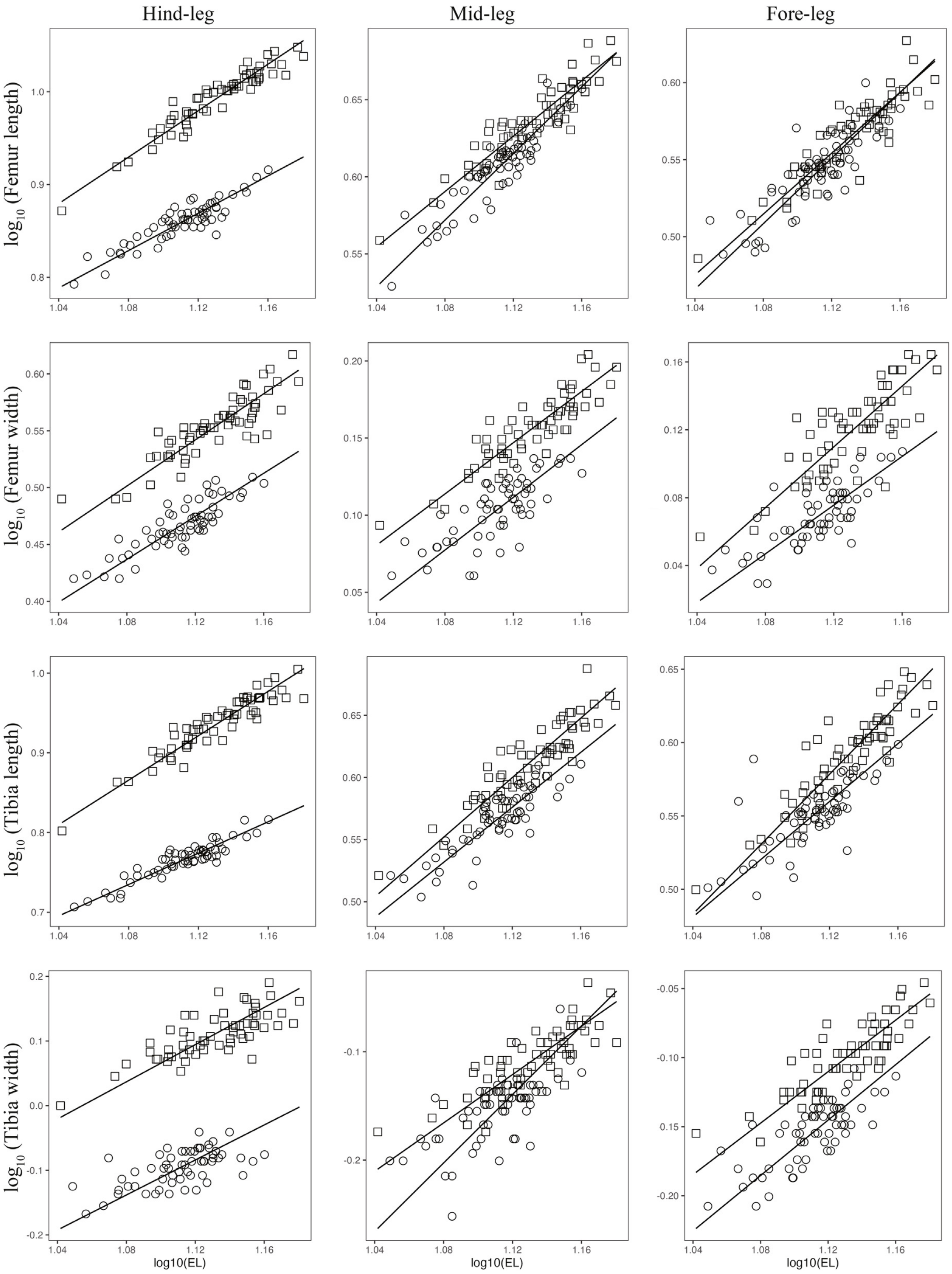

Fig. 4. Relationships between body size and each leg character of fore-, mid- and hind-leg. X-axis is elytra length (EL) as body size and y-axis is trait size of each leg character. Left, central and right figures show hind-, mid- and fore-leg characters, respectively. Each point shows individual size. Circle and Square symbols show female and male, respectively.

setules (Fig. 5B and D, 6A and B). On the other hand, setae of tarsomeres of male fore- and mid-legs have discoidal terminal pads (Fig. 6C and D) on the centre of each tarsomere, and bifid spatulas, similar to those of females, surrounding the area of discoidal setae (Fig. 5C and E).

Mean adhesion forces were 5.67 mN (s.e. = 0.60) in female and 6.59 mN (s.e. = 1.02) in male, respectively. We found no significant sexual difference of adhesion force by tarsus on the flat surface

Table 2
Allometric slope (α), its confidence intervals and the correlation coefficient (r). Bold traits have positive allometry.

Trait	α	Confidence intervals	r
Male			
Hind-leg			
Femur length	**1.253**	**1.155–1.362**	**0.954**
Femur width	1.014	0.872–1.178	0.869
Tibia length	**1.393**	**1.264–1.540**	**0.936**
Tibia width	**1.438**	**1.156–1.822**	**0.760**
Mid-leg			
Femur length	0.899	0.798–1.011	0.912
Femur width	0.830	0.721–0.953	0.884
Tibia length	**1.194**	**1.058–1.351**	**0.907**
Tibia width	1.115	0.921–1.357	0.807
Fore-leg			
Femur length	0.989	0.870–1.124	0.900
Femur width	0.896	0.735–1.088	0.804
Tibia length	**1.192**	**1.053–1.354**	**0.903**
Tibia width	0.932	0.786–1.102	0.843
Female			
Hind-leg			
Femur length	1.008	0.887–1.144	0.901
Femur width	0.945	0.805–1.108	0.856
Tibia length	0.984	0.883–1.096	0.925
Tibia width	1.355	0.981–1.935	0.622
Mid-leg			
Femur length	1.081	0.962–1.216	0.914
Femur width	0.854	0.675–1.073	0.754
Tibia length	1.102	0.943–1.291	0.860
Tibia width	**1.580**	**1.204–2.149**	**0.679**
Fore-leg			
Femur length	1.064	0.892–1.273	0.830
Femur width	0.718	0.571–0.889	0.768
Tibia length	0.985	0.773–1.255	0.739
Tibia width	1.002	0.799–1.258	0.760

($t = 0.35$, $P = 0.74$, Fig. 7).

4. Discussion

Numerous studies have reported a strong, positive relationship between male sexually selected traits and reproductive success, focusing on the important role trait size *per se* plays in male–male combat (Emlen, 1997; Okada et al., 2006, 2011; Judge and Bonanno, 2008). Recent studies, however, shed light on the integrated evolution of overall morphology resulting from strong sexual selection (Okada et al., 2012; Pizzo et al., 2012; Palaoro and Peixoto, 2022). In addition to exaggerated traits *per se*, morphologically and functionally relevant support traits are also modified during evolution. Male frog legged beetles, *S. femorata*, not only have larger hind-legs compared to females (Katsuki et al., 2014) but also larger mid- and fore-legs (*i.e.* supportive structures; Fig. 3). In male leg characters, five leg traits (femur length, tibia length and tibia width in hind-legs, and tibia length of mid- and fore-legs) showed positive allometric relationships (Table 2). In addition, these traits had significant interactions with body size and sex (Table 1). These sex differences in regression slope suggest that relative growth of these traits differs between sexes. It is consistent with the widespread pattern that traits with positive allometries function in the context of sexual selection (e.g., Okada et al., 2011; Heuring and Hughes, 2019).

Exaggeration of a trait through sexual selection leads to other changes in phenotype based on functional and developmental integrations of morphological traits (Tomkins et al., 2005; Cheverud, 1996; Zelditch and Goswami, 2021). Through the integrations, the exaggeration of weapon couples with parallel changes of other traits supporting the weapon (Okada and Miyatake, 2009), while the exaggeration of sexually selected trait constrains the supportive traits due to morphological trade-offs (Tomkins et al., 2005). In *S. femorata*, the male mid- and fore-legs are used to attach to females and rival males or the substrate during battle (Katsuki et al., 2014; O'Brien et al., 2017). Positive allometries detected in these supportive traits (male fore- and mid-tibia) are considered as the consequence of functional integration with the primary exaggerated trait (male hind-leg). This conclusion is further supported by sexual differences in the microstructure of the tarsi on the mid- and fore-legs. Females have bifid setae with spatulate setules in all legs (Fig. 6A and B). Males have similar bifid setae on the tarsi of hind-legs, but the tarsi of male fore- and mid-legs have setae with discoidal structure on the centre of each tarsomere (Fig. 6C and D), and bifid setae on the distal part (Fig. 5C–E). The setae of Chrysomeloidea fall into five categories: (i) Simple adhesive setae for plant climbing, (ii) setae with spatulate plate for plant climbing, (iii) spatulate setae bearing setules for plant climbing, (iv) bifid setae with plates for plant climbing and (v) disco-setae used in pairing. Among these five types of setae, only disco-setae are used in attachment to females (Stork, 1981). In the Colorado potato beetle *Leptinotarsa decemlineata*, for example, males have setae with discoidal terminal part, which are specialized for attachment to females during copulation (Voigt et al., 2008). Similarly, the bifid setae and discoidal setae observed on *S. femorata* tarsi were consistent with type (iv) and (v) setae, respectively. We therefore conclude that discoidal terminal setae specifically found on the tarsi of male fore- and mid-legs are specialized for attachment to females during copulation. Discoidal setae are often found in Coleopteran species, and are typically selected for through mating behavior (Stork, 1981; Voigt et al., 2008; Gloyna et al., 2014). Same trend was found from SEM observations of male discoidal setae and elytra surface in female but also in male (Fig. S2). In case of *S. femorata*, discoidal structure of male fore- and mid-leg tarsi are also a likely consequence of intra-sexual selection, as males use fore- and mid-legs to attach their body to the back of female during combat as well as mating. Alternatively, the structure of tarsal setae on all female legs and hind-legs and surrounding area of fore- and mid-leg tarsi in male are used for general plant climbing (Figs. 5 and 6).

Although the sexes differed in tarsus microstructure, we could not detect any differences in adhesion forces between males and females based on our specific experimental design. In this study, we evaluated the adhesion force of tarsus microstructure on a flat adhesion surface on which we removed the increase in pushing (holding) force normally caused by the legs and the claws (Fig. S3a). However, in the natural situation, males hold the round-shaped females' backs. In this posture, both of holding force and frictional force are also generated (Fig. S3b). Further study is needed how sex-specific microstructure plays a role in the adhesion in the natural situation including the situation which a male mounting on a female.

One leg character of female, which is tibia length of mid-leg, shows positive allometry. At the moment, we are not able to evaluate why tibia length of female mid-leg shows positive allometry and the advantage of disproportional scaling relative to body size. However, it is possible the female trait shows positive allometry when female mid-leg acts on the holding host plant during oviposition and the successful mating. Indeed, it has been reported that some of naturally selected traits show positive allometric slope (Voje, 2016). Both of allometric measurement and revealing the behavioral or morphological function of focal traits are needed to estimate selection pressure for the traits.

Exaggeration of trait incurs cost such as increased metabolic rate for maintenance and predation risk (Somjee et al., 2018; O'Brien et al., 2019; Okada et al., 2021). Previous studies have shown that larger hind legs of male *S. femorata* include heavier leg muscle for

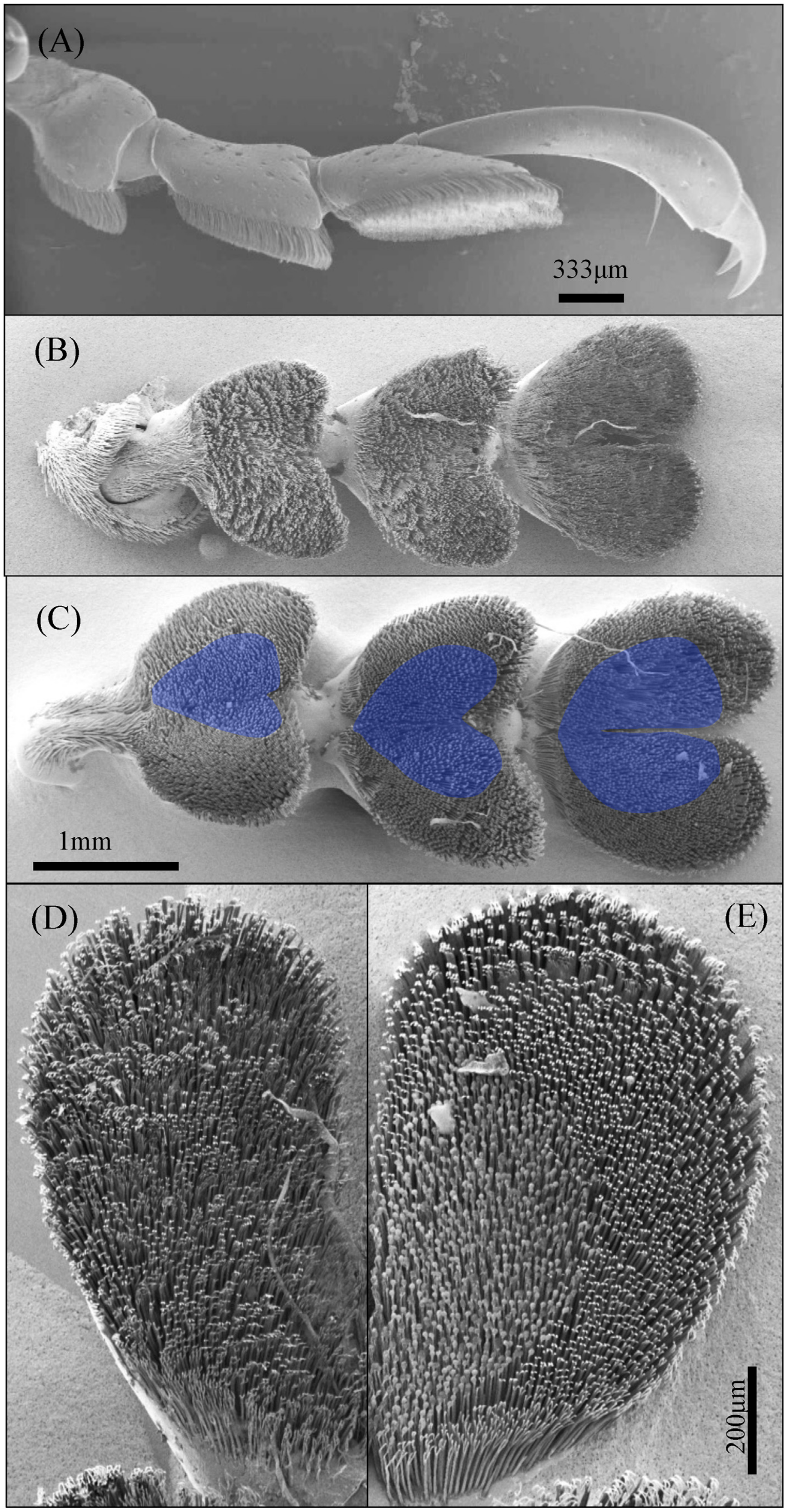

Fig. 5. Whole of tarsus (A). The underside of first, second, and third basal tarsomeres of fore legs of *Sagra femorata* female (B) and male (C). (D) Female tarsomere is covered in setae with bifid spatulate setules in all legs. (E) Male tarsomeres of fore- and mid-legs are cover in discoidal setae at centre of each tarsomere (blue area in C) and bifid spatulas, similar to those of females, surrounding the area of discoidal setae.

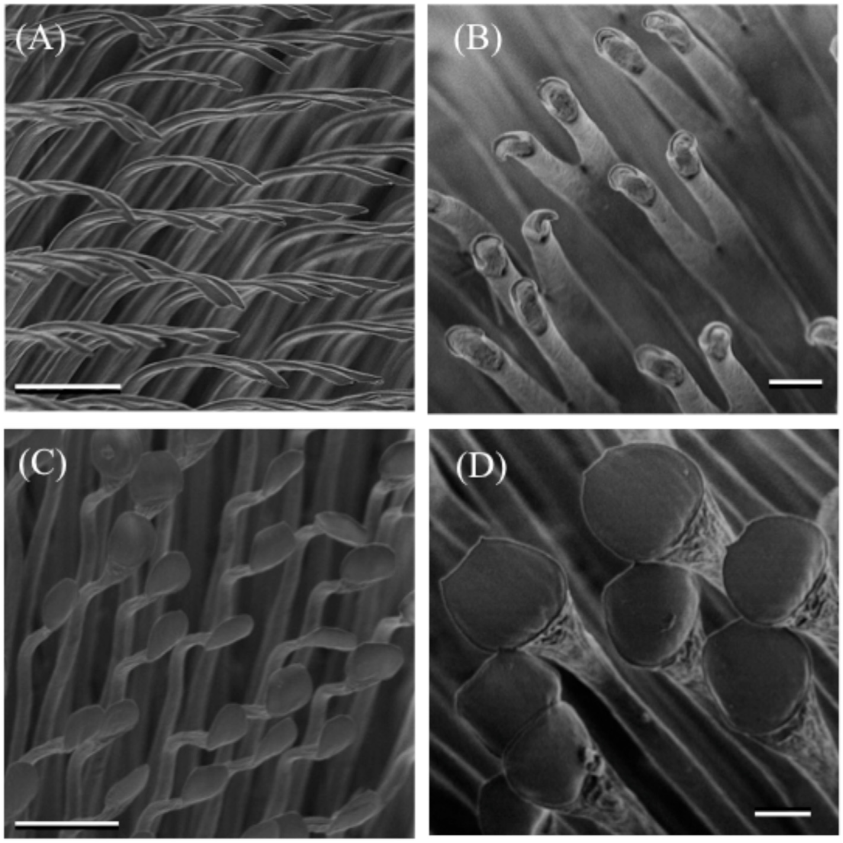

Fig. 6. Sex-specific microstructures of setae in *S. femorata* tarsus. (A, B) bifid setae with spatulate setules observed in all female tarsi and male hind-tarsus. (C, D) setae with discoidal pad observed only in males. Scales are 20 μm (A, C) and 5 μm (B, D), respectively.

the mechanical advantage (O'Brien and Boisseau, 2018), but increases of weapon size and the muscle mass also incur the increased resting metabolic rate (O'Brien et al., 2019). Therefore, increased metabolic rate would be the cost of exaggeration of legs in *S. femorata* and it suggests that the metabolic cost constrains the exaggeration of other leg characters exhibited non-positive allometry. Dinh (2022) has reported the finding that exaggerated traits with positive allometry are facilitated by increased investment in exoskeleton and reduced investment in muscle in snapping shrimp and fiddler crabs. Mid- and fore-leg characters showing positive allometries in *S. femorata* may be relatively inexpensive for increasing their size. However, all of these traits were relatively larger in males compared to the legs of females. It is suggested that sexual size differences of traits related to male–male combat can enhance the function of a weapon during battle (Kojima and Lin, 2017). We suggest that larger fore- and mid-legs in *S. femorata* males also function in male–male combat, by helping a male position himself so that he can squeeze his massive hindlegs to maximal advantage. Additionally, these larger leg characters in males could facilitate effective locomotion, given the disproportionally increased size of their hind legs. Further study is needed to investigate whether size of fore- and mid-legs and microstructures of tarsomeres affect mechanical advantage of male hind-legs and outcome of male–male combat.

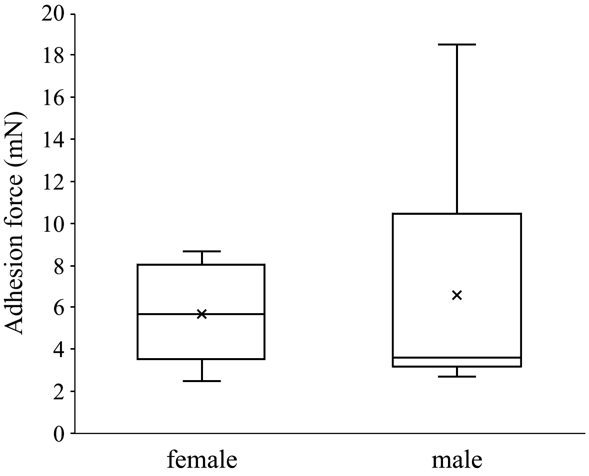

Fig. 7. Adhesion force in males and females. Bold line and cross mark in each box shows median and mean adhesion force of the sex, respectively. Bars are SD.

It should be noted that weapon traits involved in male–male combat function as honest signals not only for male ability in fighting assessment but also male quality in female mate choice (Hongo, 2003; Berglund et al., 1996; Emlen et al., 2012; Suzaki et al., 2013; McCullough and O'Brien, 2022). This is because that males with weapons may provide fitness benefits to females (Suzaki et al., 2013; Okada et al., 2014): they may protect the female during and after mating or allow access to superior resources (direct benefit). Additionally, mating with them provide genetic benefit when weapon traits are heritable (indirect benefit). If hind legs function the signals in this beetle, mate choice and male—male combat act in a reinforcing manner. In this case, the evolution of positive allometry may be facilitated in the hindleg because of the exaggeration of weapons being favoured more than we thought. The stronger impact is expected to promote positive allometry in the fore- and mid-legs because both traits are supportive traits to advertise the hind legs for the potential mates and rival males. It is needed to investigate whether exaggerated hind legs in *S. femorata* act as the signals for fighting assessment and mate choice in future studies.

In conclusion, males of the frog legged beetle *S. femorata*, display positive allometry in hind-leg size as a primary weapon, and in fore- and mid-legs as supportive traits. Microscopic analyses identified sex- and module-specific microstructures in the tarsomeres of fore- and mid-legs. We propose that these structures have been specialized to keep males in position on the backs of females during combat, although no differences in adhesion force were observed between the sexes in our experimental design. Our findings provide morphological evidence that selection acts not only on primary weapon traits used during male—male competition, but also on other behaviorally and functionally relevant support traits in different scales such as the trait size and the microstructure. This encourages further investigation into which traits are linked to exaggerated weapons and how the morphology and function of support traits respond to selection primarily acting on the focal trait.

CRediT authorship contribution statement

Masako Katsuki: Writing – original draft, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Kaoru Uesugi:** Writing – original draft, Methodology, Formal analysis, Data curation. **Tomoyuki Yokoi:** Writing – original draft, Investigation, Conceptualization. **Takane Ozawa:** Writing – original draft, Methodology, Data curation. **Devin M. O'Brien:** Writing – original draft. **Douglas J. Emlen:** Writing – original draft. **Kensuke Okada:** Writing – original draft, Formal analysis. **Yasukazu Okada:** Writing – original draft, Formal analysis.

Declaration of competing interest

All authors declare no conflicts of interest.

Acknowledgements

We thank to Technology Advancement Center microscopy section, Graduate School of Agricultural and Life Sciences, The University of Tokyo for assistance of measurement of microstructures using SEM. This work was supported by Japan Society for the Promotion of Science (Grants-in-Aid for JSPS Fellow 19J40100 and KAKENHI 22K06387) and Hokuto Foundation for Bioscience to MK and Yamada Science Foundation to YO.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.asd.2024.101360.

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