

A bilateral gynandromorph of *Discoelius dufourii* (Hymenoptera, Vespidae, Zethinae): morphology and mating behaviour

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Abstract

A gynandromorph is an organism combining both female- and male-type tissues. While the vast majority of studies have described the morphology of gynandromorphs, their sexual behaviours remain underexplored. We studied a predominantly bilateral gynandromorph specimen of the predatory wasp *Discoelius dufourii* (Hymenoptera: Vespidae: Zethinae) reared from a trap-nest. In addition to describing the morphology, we explored the gynandromorph's response to a conspecific female, comparing it with the behavioural interactions between normal males and females of *D. dufourii* and the phylogenetically related *D. zonalis*. For most specific behavioural elements, males of both *Discoelius* species exhibited a greater frequency and a higher probability of occurrences of the behaviour than did females. However, aggression and plopping behaviours were prevalent in females. The gynandromorphic specimen demonstrated behavioural activities similar to those of a male, including a relatively frequent mate body antennation and an attempt to mount a female. However, it approached the female less frequently in comparison to conspecific normal males, and it did not demonstrate substrate antennation and jumping, typical of *Discoelius* male mating behaviour. Based on the parameters of the nest cell, the probability of the development of the gynandromorphic specimen from an unfertilized (male) egg was higher than the probability of development from a fertilized (female) egg.

Keywords

Aggression, antennation, development, mounting, sex-specific behaviour

Introduction

Gynandromorphy is a developmental abnormality, resulting in chimaeric individuals, which combine patches of both genetically female- and male-type tissues (Narita et al. 2010). Their generation is thought to be associated with the sex-determination systems, and study of this phenomenon broadens our current knowledge of the evolutionary mechanisms driving the diverse sex determination and differentiation systems (Narita et al. 2010; Yang and Abouheif 2011; Dobata et al. 2012; Aamidor et al. 2018).

Among Hymenoptera, females predominantly develop from fertilized diploid eggs that have biparentally inherited genomes, while males develop from haploid unfertilized eggs that contain only the maternally inherited genome (Cook 1993). The single-locus complementary sex determination (sl-CSD) mechanism, based on the complementary sex determiner (*csd*) gene, is well explored and understood, though a few other mechanisms are also known (Heimpel and de Boer 2008).

Gynandromorphs are thought to arise in a number of different ways, of which the most possible cytogenetic mechanisms are either fertilization of only one maternal nucleus in a bi-nucleate egg, or a fusion of two sperm nuclei in a mono-nucleate egg. A combination of these events is also possible (Michez et al. 2009; Narita et al. 2010; Aamidor et al. 2018). The resulting gynandromorph individuals are likely to comprise diploid female tissues of either biparental or paternal (androgenic) origin and haploid male tissues of either maternal (gynogenetic) or paternal origin. Impaternate gynandromorphs arising from unfertilized eggs may also occur, when either one of the two meiotic spindles of the second meiotic division fails to segregate a heterozygous nucleus, or two of the four nuclear products of meiosis fuse to form a heterozygous for the *csd* gene alleles diploid nucleus (Cooper 1959). This nucleus and one of the two haploid meiotic products are believed to undergo cleavage producing a wholly gynogenetic embryo with female-determined diploid nuclei and male-determined haploid nuclei. Such aberrant meiosis, leading to a gynandromorphy, has been thought to be induced by *Wolbachia* (Alphaproteobacteria) or possibly other endosymbionts (Narita et al. 2010). In addition, chromosomal aberrations, eliminations or differences in sl-CSD expression within embryonic tissues during embryogenesis have been proposed to be responsible for the generation of gynandromorphs (Michez et al. 2009). The occurrence of gynandromorphism might be linked with an inbreeding depression (Kinomura and Yamauchi 1994), higher or lower than normal temperatures (Pereira et al. 2004; Kamping et al. 2007; Gjershaug et al. 2016) or fluctuations of other specific environmental factors (Fateryga et al. 2011).

Although rare, gynandromorphs have been regarded as more or less randomly distributed among ants, wasps and bees, probably occurring in most species of Hymenoptera (Cooper 1959). Overall, gynandromorphs are known from at least 29 hymenopteran families (Rosa and Zettel 2018). In some of these, gynandromorphs have been extensively explored and described, e.g. in bees (Wcislo et al. 2004; Michez et al. 2009; Fateryga et al. 2011; Hinojosa-Díaz et al. 2012; Lucia and González 2013; Suzuki et al. 2015) and ants (Jones and Phillips 1985; Kinomura and Yamauchi 1994; Heinze and

Trenkle 1997; Yoshizawa et al. 2009; Gjershaug et al. 2016). Among other Hymenoptera, this phenomenon has been documented less frequently, e.g. in Diprionidae (Martini et al. 1999), Chrysididae (Rosa and Zettel 2018; Rosa 2019) and Eumeninae (Cooper 1959; Turrisi and Borsato 2008). Based on the external morphology, the distribution of male and female characteristics in gynandromorphs can be most commonly categorized into three types: axial (anterior-posterior), bilateral (left-right) or mosaic (patchily distributed) (Wcislo et al. 2004; Kamping et al. 2007; Michez et al. 2009). Most studies have focused on the description of the morphology of the gynandromorphs, while aspects of their reproductive behaviours have remained largely unexplored.

A long-standing problem in the evolutionary biology of gynandromorphs concerns the questions of to what extent do their behaviours deviate from the norm, or what gynandromorphs could reveal about the systems controlling sex-specific behaviours (O'Dell and Kaiser 1997; Hinojosa-Díaz et al. 2012). Developmental biologists and geneticists have long been interested in system analysis of the behaviour using gynandromorphs. An example is the use of gynandromorphs of the parasitic wasp *Habrobracon* as a tool to locate the anatomical foci in the nervous system determining sex-specific steps in their reproductive behaviour (Whiting 1932). Within the social Hymenoptera, it has been revealed that gynandromorphs with various patches of female- and male-type tissues demonstrate diverse levels of abnormalities in sex-specific behaviour (Yoshizawa et al. 2009; Ugajin et al. 2016; Matsuo et al. 2018). Within the solitary Hymenoptera, one study has described the sexual behaviour of a gynandromorphic specimen of *Osmia* (Megachilidae) (Sampson et al. 2010). Yet, the sexual behaviour of gynandromorphs in the solitary vespid wasps has not been investigated.

The objectives of this study were: (1) a description of a gynandromorphic specimen of a solitary predatory wasp *Discoelius dufourii* Lepeletier 1841 (Hymenoptera: Vespidae: Zethinae); (2) an investigation of the behavioural response of the gynandromorph to a conspecific female, comparing it with the behavioural interactions between normal males and females of *D. dufourii* and a related zethine wasp *D. zonalis* Panzer 1801. Based on the nest cell parameters of *D. dufourii* and *D. zonalis*, we also estimated the fertilization probability of the egg that developed into the gynandromorphic specimen.

Materials and methods

Study species

Discoelius dufourii and *D. zonalis* are the only European representatives of the predominantly tropical subfamily Zethinae (Vespidae). In northern Europe, both species are uncommon, they are typical of woodlands in a semi-natural landscape (Budrys et al. 2010).

Females of these univoltine cavity-nesting (xylicolous) mass-provisioning solitary predatory wasps build their nests in pre-existing tubular cavities, e.g. the exit holes of xylophagous beetles. The nest consists of a row of brood cells, built starting with the deepest one and separated by diaphragms made of fragmented and partly crushed leaves. The egg

is attached to the upper internal surface of the brood cell by a thin stalk. After oviposition, the wasp supplies the brood cell with several paralyzed caterpillars or sawfly larvae.

As in other aculeate Hymenoptera, females of *D. dufourii* and *D. zonalis* predetermine both the sex (by the arrhenotokous haplodiploidy) as well as the body size (by the amount of cell provision) of their offspring. Similarly to most Hymenoptera, females of *D. dufourii* and *D. zonalis* are the larger sex (Budrienė and Budrys 2007). Commonly the mother wasp oviposits the fertilized eggs developing into females in the innermost brood cells of the nest, while the unfertilized male eggs are placed in the cells closer to the entrance of the nesting cavity.

Material

Wasp nests for the study were obtained using small trap-nests: 20–35 internode fragments of dry reed (*Phragmites australis*) stems wrapped into polyethylene or Tetrapack cartons and fixed using sticky tape (Budrys et al. 2010). Most of the material was collected in Alytus (54°32'N, 24°03'E), Anykščiai (55°24'N, 25°16'E), Molėtai (55°08'N, 25°16'E), Pasvalys (55°56'N, 24°17'E), Varėna (54°00'N, 24°25'E) and Vilnius (54°50'N, 24°58'E) districts, Lithuania, in the years 1998–2018. Some material was collected using the same method in Hirya, Belarus (54°39'N, 26°12'E), Koeru, Estonia (58°59'N, 26°04'E), Pychowice, Poland (50°01'N, 19°53'E), Tázlár, Hungary (46°31'N, 19°31'E) and Livenza, Italy (46°02'N, 12°30'E) in the years 2007–2013. After exposure, the trap-nests were collected and the reed stems with nests dissected. The diapausing prepupae were individually placed in plastic containers for hibernation in a climatic chamber at +4 °C. After reactivation, the containers were kept at +25 °C until the adults hatched. After eclosion, the wasps were kept individually in plastic cages (6 cm height × 5 cm diameter) at an ambient temperature (+22–26 °C) with honey solution and water available. In total, 677 nests of the two *Discoelius* species with 2722 brood cells were obtained and studied in 1996–2019. Of these, the sex of the brood could be established in 509 nest cells of *D. dufourii* (235 females, 273 males and 1 gynandromorph) and 1557 nest cells of *D. zonalis* (516 females and 1041 male).

The single gynandromorph specimen of *D. dufourii* was reared from a nest in a reed stem bundle trap-nest exposed from 31 May 1998 to 30 June 1998 on an old wooden building in Varnupys, Lithuania (55°24'N, 25°16'E) (Budrienė 2003). The nest with prepupae was reactivated in a refrigerator from 05 Nov. 1998 to 28 Mar. 1999, with the imago eclosing on 04 May 1999. The specimen was used for a mating experiment on 15 May 1999, when a conspecific female specimen emerged. Afterwards, the gynandromorph specimen was pinned and deposited in the collection of the Nature Research Centre, Vilnius, Lithuania.

Morphology

The external morphological characters of the *D. dufourii* gynandromorph were studied and measurements were taken using a Nikon SMZ800 binocular microscope with an

ocular micrometer (www.nikon.com). Images were obtained using a digital camera Nikon DS-Fi2 connected to the microscope. The images were stacked using software CombineZP by Alan Hadley (alan-hadley.software.informer.com).

Mating behaviour

Mating trials were conducted in 1999–2008, applying a no-choice mating design in which the subjects were presented with a single option, having to choose between this option or nothing (Dougherty 2020). Following the mating protocol described in Budrienė and Budrys (2007), the pairings were observed on a white paper arena under a bell-glass (22 cm height × 22 cm diameter). A portion of the pairing experiments took place on a smaller white paper arena under a transparent plastic cage (12 cm height × 10 cm diameter). Pairing arenas were exposed to daylight, with additional artificial illumination, at a temperature of +27–29 °C in a climatic chamber. A single virgin female was let into the arena and the cage with a single male was placed nearby for a one minute habituation. Afterwards, the male was introduced into the arena. In order to prevent the wasps from responding to odour cues of previous pairings, the arena paper was replaced each time. Observations lasted 30 min, or until copulation or mounting had finished. The behavioural interactions between a normal female and the gynandromorph of *D. dufourii* were observed over 37 min. Unfortunately, it was a single observation of the specimen's behaviour due to the unavailability of other conspecific specimens during the life time of the gynandromorph.

Among the previously identified and described sex-specific behavioural elements (Budrienė and Budrys 2004), the following elements were recorded in the mating experiments with *Discoelius*:

aggression	moving towards the other sex with opened mandibles, sometimes followed by biting;
approaching	walking or flying towards the other sex;
jumping	pouncing flight at short distance;
mate body antennation	moving towards the other sex followed by touching it with the tips of the antennae;
plopping	short flight with deliberate falling on the surface with a plopping sound;
substrate antennation	inspection of the substrate with antennae.

The probability of occurrence of a behavioural element was estimated as the proportion of the experiments where the element was observed at least once. The frequency was estimated as the number of the occurrences of the behavioural element divided by the duration of the mating phase in minutes. We recorded the duration of the following phases of mating: premounting (the period from the start of an experiment until the male mounts the female), mounting (the male taking a parallel position on the dorsum of the female) and postmounting (the male continuing copulation after

released leg-hold, laying or hanging behind the female). In the cases in which the male did not succeed in mounting the female, the duration of the whole experiment was considered as the duration of the premounting phase. Since the studied gynandromorph did not succeed in mounting, the frequency of its behavioural elements was compared with the respective frequencies during the premounting phase of the mating in the experiments with normal males.

Egg fertilization probability

We estimated the probability of whether the gynandromorph had developed from a fertilized or unfertilized egg based on the following four parameters of the brood cell as independent variables: (1) the diameter of the nesting cavity in mm; (2) the depth (distance from the entrance) of the bottom of the brood cell in mm; (3) the serial number of the brood cell, starting from the first (the deepest) one; and (4) the serial number of the brood cell, counting from the last (the outermost) one. The combination of the parameters (3) and (4) reflected the total number of brood cells in a nest. We estimated the significance of these parameters for a female wasp decision to fertilise an egg as a dependent dichotomic variable 1 (mother wasp fertilizes the egg, this thereafter developing into a female brood) versus 0 (mother wasp does not fertilize the egg, this thereafter developing into a male brood), using logit models for both studied *Discoelius* species, *D. dufourii* and *D. zonalis*. We also built discriminant classification functions, separating the brood cells with female and male broods of the two *Discoelius* species, based on the same four cell parameters, and we estimated the posterior probability of egg fertilization.

Statistical methods

To test the significance of sex differences in *D. dufourii* and *D. zonalis* in the probability of the occurrence of the sex-specific behavioural elements, Fisher's exact test was applied. The significance of differences in the frequency of the behavioural elements was assessed using the Wilcoxon signed rank test with continuity correction.

The discriminant equations were built using Statsoft STATISTICA, version 8 (Statsoft, Tulsa, Oklahoma, United States of America). Wilcoxon signed rank test and Fisher exact test were applied using R 4.0.2 (R Core Team 2020).

Results

Morphology of *D. dufourii* gynandromorph

Body length 12.2 mm, right forewing length 9.6 mm, left forewing length 8.9 mm. Most of the right side of the body was female-like, most of the left side of the body was male-like, with the following exceptions: the right side of the frons and clypeus were male, the left side of the frons and clypeus were female (Fig. 1G); the right side of the abdominal

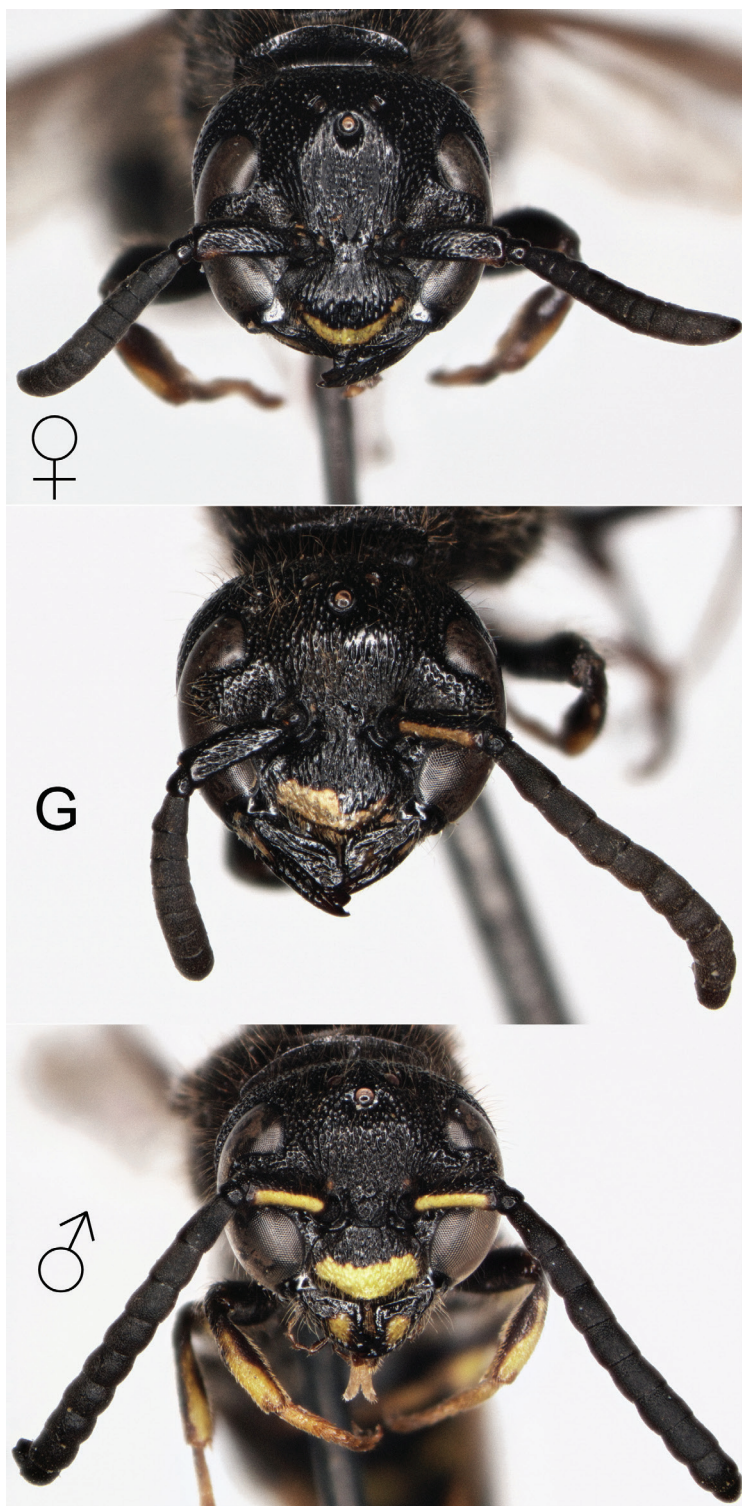


Figure 1. Head of *Discoelius dufourii*, frontal view: ♀ female; G gynandromorph; ♂ male.

segment 8 (7th segment of metasoma) was male-like (Fig. 4G: T8), the left side of the segment was female-like, including three left-side valvules of the sting (Fig. 4G: V1–3); the abdominal segment 9 included two-sided, symmetrical male genitalia.

The general shape of the head was sub-quadratic, more similar to that of a female than to a more rounded head of a male. The vertex and genae were female-like, relatively long and convex, more shiny and less densely punctate than those of a male; vertex before the occipital carina with a smooth shiny medial longitudinal strip, typical of a female. The face was asymmetrical: the sculpture and colouration of the left side of the frons and clypeus were female-like; those of the right side were male-like (Fig. 1G). The left side of the clypeus was slightly longer, more distinctly striate and with a narrow yellow transverse preapical marking, typical of a female; the right side of the clypeus was shorter, with slightly more distinct punctuation and with a wide yellow preapical marking, typical of a male. The antennae and mandibles were asymmetrical in the opposite way to the face and the clypeus (Fig. 1G). The left antenna was male: the scape with a complete anteroventral yellow strip; flagellum long, with 11 flagellomeres, of which the apical two forming a hook; flagellomeres 6–10 with smooth pale-brown tyloidea. The right antenna was female: the scape with a small apical dark-yellow spot; flagellum short, with 10 flagellomeres. The left mandible was male: the internal edge subrectangular, with nearly straight apical margin; the anterior surface with a dark-yellow patch. The right mandible was female: the internal edge bidentate (in addition to the two apical teeth); anterior surface dark.

The mesosoma was asymmetrical, with a longer female-like right side and a shorter male-like left side (Fig. 2G). The punctate-rugulose sculpture of the scutum and scutellum was more similar to that of a female. The right side of pronotum was longer, with a shorter obtuse right angle of the pronotal carina, typical of female; the left side of pronotum was shorter, with a longer acute left angle of the pronotal carina (dorsal view), typical of male. The right legs were black, except a dark-yellow spot on the fore tibia; fore and mid tarsi are thick, typical of female. The left legs were typical of a male: black with yellow preapical spots on the mid femur and the hind tibia, and with long yellow mark-

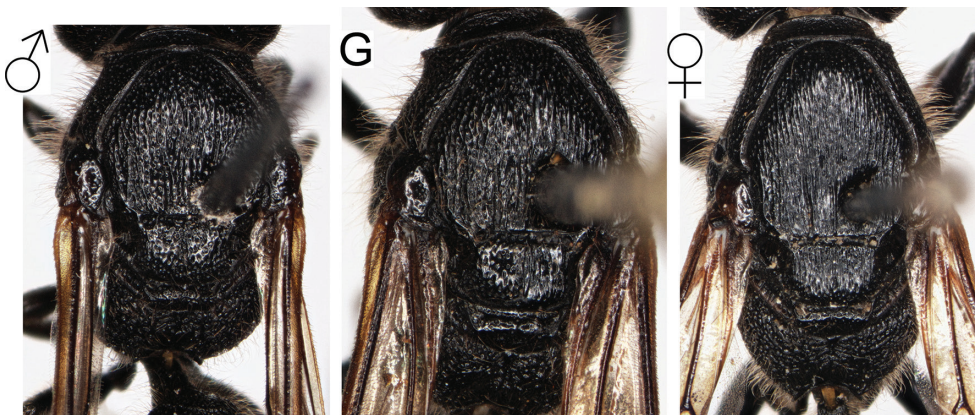


Figure 2. Mesosoma of *Discoelius dufourii*, dorsal view: ♂ male; G gynandromorph; ♀ female.

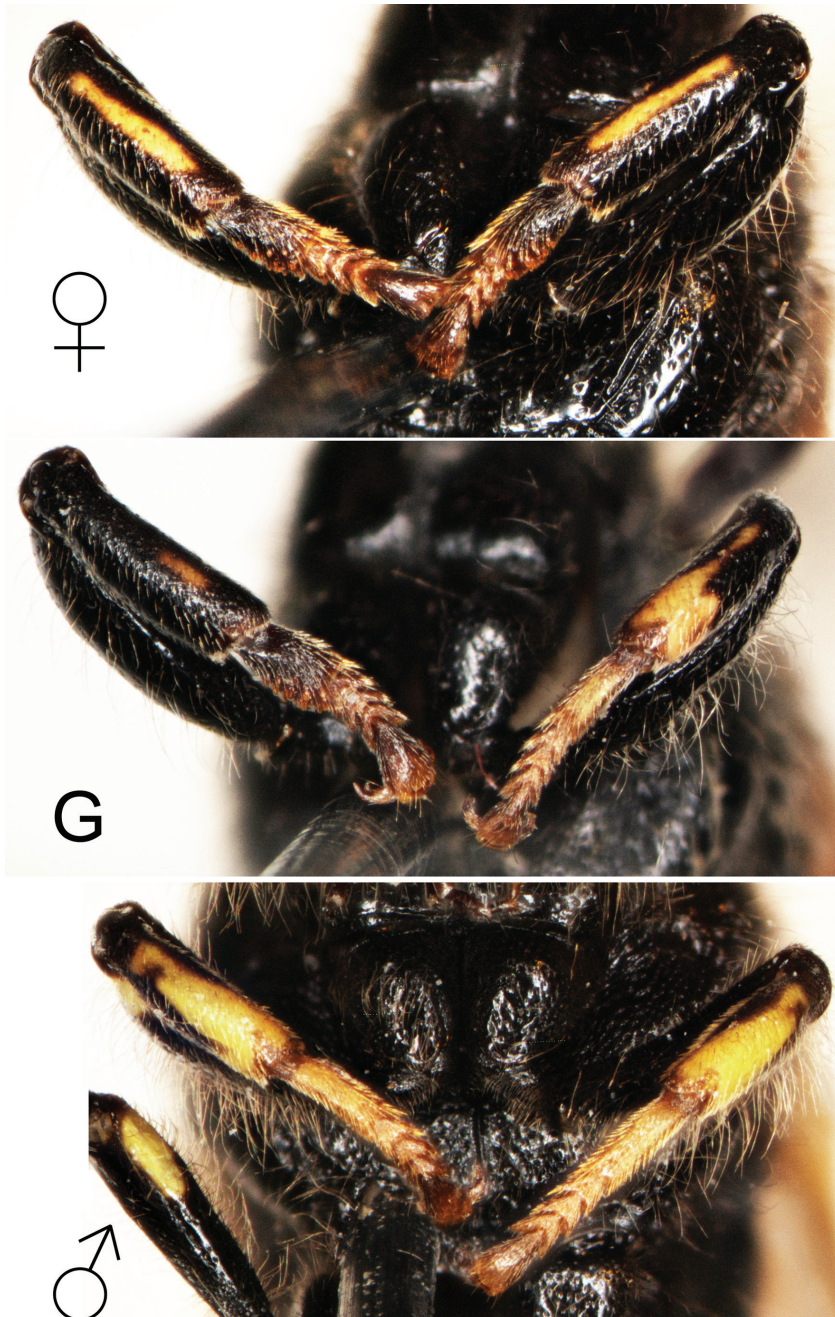


Figure 3. Fore legs of *Discoelius dufourii*, antero-ventral view: ♀ female; **G** gynandromorph; ♂ male.

ings on the fore and mid tibiae. The left fore and mid tarsi were slimmer and longer, and of a paler colouration than the right ones; the right fore basitarsus was 1.4 times as long as wide, the left fore basitarsus was 2.9 times as long as wide (Fig. 3G); the right mid basitarsus was 2.8 times as long as wide, the left mid basitarsus was 4.1 times as long as wide.

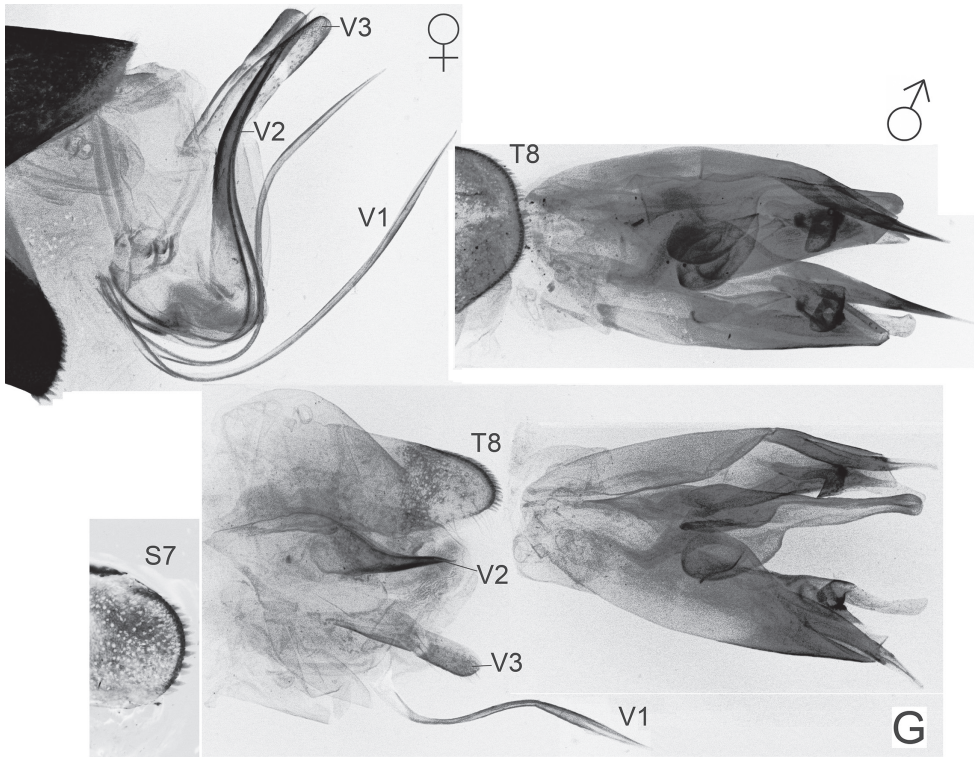


Figure 4. Genitalia of *Discoelius dufourii*, lateral (left side) view: ♀ female; ♂ male; **G** gynandromorph; **T** tergite; **S** sternite; **V** valvula.

The metasoma was nearly symmetrical; an uneven narrow trace of yellow submarginal band on the 3rd tergum, more commonly observed in males, was present on the left side only.

The apical margin of the terminal segment was slightly asymmetrical. The hidden terminal structures included an asymmetrical sclerite with the right side similar to the 7th metasomal (8th abdominal) tergum of a male (Fig. 4G: T8) and the adjacent left side looking like an underdeveloped 2nd valvula of a female sting. The left 1st valvula of the sting was also developed, though its basal structures were a little sclerotised, membranous; posterad of this asymmetrical “half-sting”-like structure, there was a symmetrical male genital capsula present.

The specimen may be considered a predominantly bilateral gynandromorph with half of a female sting and a complete male genital structure.

Behavioural interactions

A typical mating behaviour of *Discoelius dufourii* in experimental conditions consisted of the premounting, mounting and postmounting phases. Mounting occurred in 53 of 59 experiments; it included copulation in 24 observations. The premounting phase (duration 5.83 ± 0.02 minutes, here and hereafter mean \pm SE) included a visual rec-

ognition of the mate and/or chemical recognition of mate traces on the substrate, followed by substrate antennations, with subsequent demonstrations (jumping and plopping), attempts of the male to mount the female and a more or less aggressive rejecting behaviour of the female. In cases of copulation present, the mounting phase included the precopulatory (before insertion of the male genitalia; duration 0.46 ± 0.05 minutes) and the copulatory (duration 0.23 ± 0.08 minutes) sub-phases. When mounted, the male continued the courtship by an antenation of female antennae, presumably spreading a pheromone from the cuticular glands of his tyloidea, present on terminal flagellomeres, along female flagella. The postmounting phase (duration 1.03 ± 0.11 minutes) was observed in 21 of 24 copulations. During the postmounting phase, the female demonstrated a more or less active rejecting behaviour by kicking the male with her hind legs and wriggling her abdomen.

During the mating experiment, the gynandromorph demonstrated a higher intensity of behavioural activities than a female. It approached the female six times. In two instances, the approach was followed by female head and abdomen antennations, while in one instance, it touched the female's body with its mandibles multiple times. In one instance, the gynandromorph attempted to mount the female from flight. The mounting attempt consisted of a brief (approximately 2 sec) grasping of the female's pronotum with forelegs. The female displayed the plopping behaviour, it avoided the gynandromorph by walking away or flying away. In the case when the gynandromorph approached her "face to face", the female reacted with an apparent aggression display. This behaviour started with a motionless position with antennae obliquely straightened forward and to the sides, and a raising of the anterior part of the mesosoma ("alert posture"), and was followed by a biting of the gynandromorph with mandibles.

Of the six behavioural elements, aggression and plopping were prevalent in females of both *D. dufourii* and *D. zonalis*. The probability and frequency of aggression demonstration in females were significantly higher than those in males (Fig. 5d; Table 1). For the plopping, the difference between sexes in frequency was significant in both species, however the probability of occurrence was significantly higher in females of *D. zonalis* only (Fig. 5f; Table 1). The aggression appeared to occur with a greater frequency and a higher probability in both sexes of *D. zonalis* compared to those of *D. dufourii*, sug-

Table 1. The probabilities and frequencies of occurrence of selected sex-specific behavioural elements in *Discoelius dufourii*, *D. zonalis* and the *Discoelius dufourii* gynandromorph. Significant ($p < 0.05$) values in bold; $N = 59$ for *D. dufourii* and $N = 116$ for *D. zonalis*.

Behaviour-relevant element	Sex-specific prevalence	Occurrence in the gynandromorph	Sex differences in frequency, Wilcoxon signed rank test with continuity correction, p value		Sex differences in probability of occurrence, Fisher exact test, p value	
			<i>D. dufourii</i>	<i>D. zonalis</i>	<i>D. dufourii</i>	<i>D. zonalis</i>
approaching	male	yes	0.000	0.001	0.000	0.014
substrate antennation	male	no	0.001	0.000	0.001	0.000
mate body antennation	male	yes	0.174	0.004	0.244	0.003
aggression	female	no	0.003	0.000	0.002	0.000
jumping	male	no	0.560	0.000	0.836	0.006
plopping	female	yes	0.002	0.008	0.432	0.055

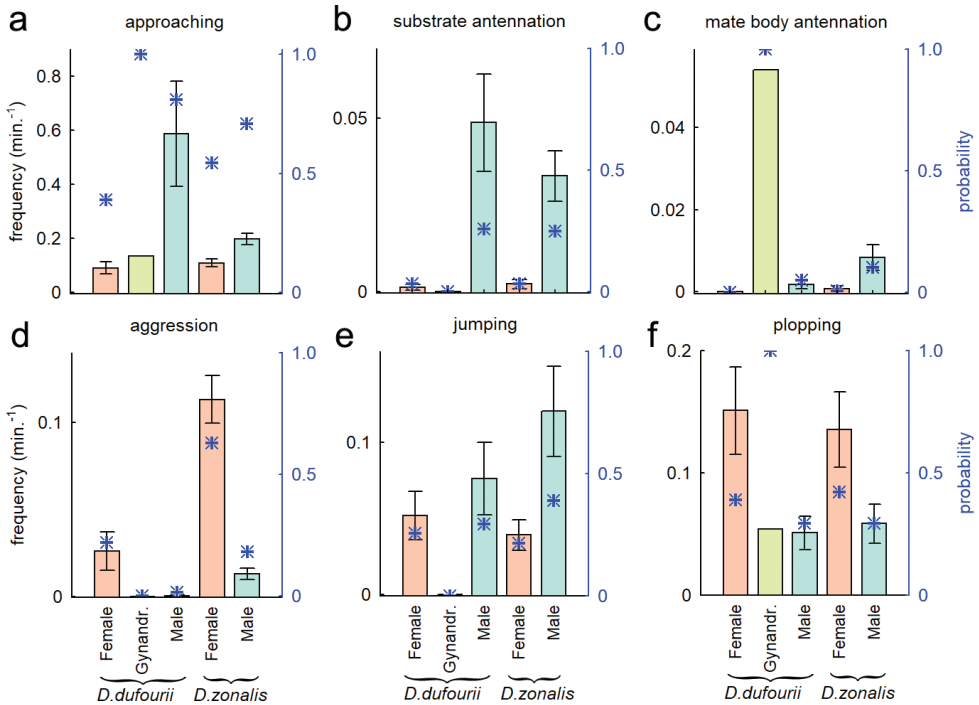


Figure 5. Mean frequency in minutes⁻¹ (bars, left y axis) and probability of presence (blue asterisks, right y axis) of six behavioural elements (a–f) of *Discoelius* females (rose), males (azure) and the gynandromorph (lime green) in mating experiments.

gesting an interspecific difference in the amplitude of this behavioural element. The gynandromorph did not demonstrate any aggression and showed a similar frequency of plopping to the conspecific males, thus behaviourally strikingly resembling a male and contrasting to a female (Fig. 5d, f).

The other four behavioural elements had significantly higher occurrence probability and frequency in *D. zonalis* males in comparison to females (Fig. 5a–c, e; Table 1). In *D. dufourii*, however, only the difference of approaching and substrate antennation between the sexes was significant (Fig. 5a, b; Table 1). The gynandromorph of *D. dufourii* demonstrated a relatively very high frequency of the mate body antennation, typical of male mating behaviour in *Discoelius* (Fig. 5c). However, it did not perform any substrate antennation and jumping, and it approached the female much less frequently in comparison to the conspecific males (Fig. 5b, e).

Gynandromorph's egg fertilization probability

The analysis demonstrated that all four brood cell parameters, namely the nesting cavity diameter, the depth of the brood cell bottom, the serial number of the cell by building sequence and the serial number of the cell counting from the nest entrance, significantly affected the decision of the *Discoelius* mother wasp about the brood sex.

Table 2. Estimated coefficients of logit models describing the probability of egg fertilization (female brood) in brood cells of *Discoelius dufourii* (model log-likelihood -222.7) and *D. zonalis* (model log-likelihood -677.6).

Brood cell parameter	<i>D. dufourii</i>		<i>D. zonalis</i>	
	Estimate±SE	<i>p</i>	Estimate±SE	<i>p</i>
Intercept	-5.22 ± 0.90	0.000	-6.44 ± 0.57	0.000
Diameter (mm)	0.53 ± 0.19	0.006	0.935 ± 0.097	0.000
Depth of cell bottom (mm)	0.013 ± 0.004	0.000	0.006 ± 0.002	0.008
No of cell from the bottom	-0.378 ± 0.089	0.000	-0.538 ± 0.053	0.000
No of cell from the entrance	0.606 ± 0.086	0.000	0.233 ± 0.035	0.000

Table 3. Coefficients of classification functions for egg fertilization (female brood) in brood cells of *Discoelius dufourii* (correct classification: 79.1%; female brood 75.4%, male brood 82.4%) and *D. zonalis* (correct classification: 79.8%; female brood 61.9%, male brood 88.8%).

Brood cell parameter	<i>D. dufourii</i>			<i>D. zonalis</i>		
	Estimate	<i>F</i>	<i>p</i>	Estimate	<i>F</i>	<i>p</i>
Intercept	-5.632			-7.325		
Diameter (mm)	0.532	7.72	0.006	0.936	111.2	0.000
Depth of cell bottom (mm)	0.0161	18.7	0.000	0.009	17.3	0.000
No of cell from the bottom	-0.356	19.8	0.000	-0.423	110.9	0.000
No of cell from the entrance	0.574	64.2	0.000	0.262	54.1	0.000

We obtained logit models of sufficient predictive power (Table 2) and classification functions well separating brood sex (Table 3) for each species.

The gynandromorph specimen emerged from the 3rd brood cell of a 4-cell linear nest, where the 1st (the deepest, innermost) cell contained a female brood, the 2nd cell contained a dead larva of unknown sex and the 4th cell contained a male brood. According to the logit model (log-likelihood -222.7), the predicted probability of the fertilization of the egg that had developed into the gynandromorph was 0.17 ± 0.22 , 95% CI 0.11–0.24. The posterior classification probability of the gynandromorph's egg fertilization, estimated using discriminant function analysis, was 14.7%. Consequently, the brood cell in which the gynandromorph had developed had to contain an unfertilised egg developing into a male brood with a probability of $83 \pm 22\%$ (logit regression model) or a posterior probability of 85.3% (discriminant function).

Discussion

Morphology

The bilateral gynandromorphic individual of *D. dufourii* described in this paper appears to be the first documented case of gynandromorphy in the subfamily Zethinae. There are documented cases of gynandromorphic individuals in ten species of the related vespid subfamily Eumeninae (Turrisi and Borsato 2008). Based on the head picture, an eleventh recorded eumenine gynandromorph belongs to an undescribed *Ancistrocerus* sp. A. (Buck et al. 2008: fig. C13.4).

The studied gynandromorphic specimen of *D. dufourii* represents a specific case among the gynandromorphs in two aspects. First, the bilateral differentiation of the male and female parts (left side male and right side female) was intervened with an opposite differentiation on the head capsula (but not head appendages), where the left side of the frons and clypeus were female and the right side were male (Fig. 1), as well as on the 7–8th abdominal segments, where the left side with a half-sting was female and the right side was male; the 9th segment was fully male with symmetrical genital capsula (Fig. 4). Such morphology implies a higher probability of tissue exchange between the body sides at the anterior and the posterior end of the developing embryo.

The second aspect that must inspire further research is the high (ca. 80–85%) probability that the gynandromorph had developed from an unfertilised (i.e. male) egg. From the dominating viewpoint, gynandromorphs are considered to arise typically from fertilized eggs, i.e. from female brood cells, in the case of cavity-nesting solitary wasps. The available evidence for the emergence of gynandromorphs with several different parental origins, such as the androgenetic and gynogenetic ones, potentially leading to evolutionary novelties, comes from the studies of social Hymenoptera, emphasizing the range of developmental variants that can potentially lead to an evolutionary novelty. It has been hypothesized that some of these developmental variants, for example androgenetic male production, might share the same underlying cytogenetic mechanism with gynandromorphism (Yoshizawa et al. 2009; Dobata et al. 2012; Aamidor et al. 2018). Our study implies that the gynogenetic origin of a gynandromorph is possible in solitary Hymenoptera as well. Taking into account the much lower fecundity of the solitary wasps and bees compared to social Hymenoptera, such cases, based on the successful development of an egg after a failure in meiotic division of germ cells, must be naturally very rare.

The studied gynandromorph had a smaller body size than a typical female and demonstrated a distinct size asymmetry, particularly in the appearance of the thorax (Fig. 2). This observation supports the suggestion that the development of tissues of each sex is self-determining (Cooper 1959) and confirms that, at the same amount of provision, the average relative growth rate in female tissues is higher than in male tissues, as has been observed in an eumenine wasp *Symmorphus allobrogus* (Budrienė et al. 2013).

Behaviour

Our mating experiments with *Discoelius* wasps demonstrated that approaching the mate, substrate antennation, mate body antennation and short flights (“jumping”) were male-specific behavioural elements, while aggression and plopping on the surface were female-specific behaviours, with a greater frequency and higher probability of occurrence compared with the opposite sex (Fig. 5, Table 1). The most distinctive sex-specific behavioural traits were female aggression and male substrate antennation (Fig. 5b, d). We observed interspecific differences in aggression levels between *D. dufourii* and *D. zonalis* in both sexes (Fig. 5d). *Discoelius* females possess strong mandibles used to cut and macerate leaf fragments for nest construction or in threat displays. Using their

mandibles, the females can inflict significant damage on their opponents, cutting their appendages, such as legs and antennae (AB, personal observations).

In our mating experiment, the gynandromorph showed no aggression, demonstrated a similar plopping frequency to conspecific males, intensively antennated the female's body (Fig. 5c, d, f) and attempted to mount the female. Despite the relatively low frequency of approaches to the female and the lack of substrate antennation, which are typical of males, we consider the gynandromorph's behavioural displays as predominantly male-specific and its sexual identity as male.

Among insects, gynandromorphs commonly tend to express a unisexual sex-specific behaviour, either like females or like males. The behaviour may correlate with the phenotype of the abdomen; this regularity was observed in an *Osmia ribifloris biedermannii* gynandromorph with a predominantly female body form, which was courted by normal males (Sampson et al. 2010). Other studies have suggested that gynandromorphs' sexual behaviours may be more strongly influenced by the head (brain) rather than by the abdomen (reproductive organs) (Yoshizawa et al. 2009). Under effect of sex-specific *fruitless* (*fru*) gene products, neurones of the insect brain form sexually dimorphic circuits, regulating sexual behaviours such as courtship and aggression (Watanabe 2019). Matsuo et al. (2018) have analyzed the encephalic and antennal expression patterns of the *fru* gene in a *Bombus ignitus* gynandromorph with a bilaterally dimorphic head and thorax (left side-male, right side-female) and a uniformly masculine gaster. This gynandromorph with left-side-biased expression of male-type *fru* transcripts demonstrated approaching and touching behaviour towards virgin queens, though it rarely attempted to copulate. Such behaviour has been interpreted as an outcome of the difficulty in the olfactory-dependent transition from the male-like approaching behaviour to copulation. In our case of the *D. dufourii* gynandromorph, the absence of substrate antennation contrasting with very high estimates of mate body antennation may suggest an incapability of a male-like antennal response to weak chemical cues left on the substrate by the female. However, in closer proximity, this response might be triggered by stronger chemical cues on the cuticle of the female, thus the mate antennation behaviour could be initiated.

Gynandromorphs may express behaviours that resemble normal sex-biased behavioural patterns in the wild as well. These include attraction to scent traps (Mertins and Coppel 1971; Martini et al. 1999; Giangarelli and Sofia 2011; Silveira et al. 2012), foraging for nectar or pollen (Gordh and Gulmahamad 1975; Michez et al. 2009), nesting activities (Michez et al. 2009) and defence (Silveira et al. 2012).

In some cases, gynandromorphs may express conflicting sex-specific behaviours. For instance, male-specific eucalyptol essence collecting combined with the female-specific stinging have been recorded in a gynandromorphic individual of the euglossine bee *Eulaema atleticana* (Silveira et al. 2012). However, Camargo and Gonçalves (2013) urge caution in predicting the sex-specificity of gynandromorph behaviour in context of scent attraction. Krichilsky et al. (2020) have speculated that in a gynandromorphic bee *Megalopta amoena*, a bilaterally split brain was unable to integrate conflicting sex-specific signalling in relation to the circadian rhythm. In our case of the *D. dufourii* gynandromorph, the female-like low intensity of substrate antennation, approaching to the female and jumping and, at the same time, the male-like high intensity of female

body antennation and the attempt to mount the female may be interpreted as a result of conflicting activities of the female and male parts of the brain.

Despite the predominantly male-specific mating behaviour, the gynandromorph of *D. dufourii* was not able to copulate. Opportunities of gynandromorphs to reproduce are usually limited, resulting in unviable egg production by mated individuals (Melcher 1997). However, the oviposition by a virgin gynandromorphic individual of haploid eggs, developing into males (Melcher 1997) and successful copulations of a gynandromorph with a normal queen, producing normal workers and fertile queens (Yoshizawa 2009), may occasionally happen. The ability of gynandromorphs to successful reproduction could have important consequences enabling a generation of evolutionarily novel body traits. Yang and Abouheif (2011) have proposed that mutations related to gynandromorph-like development may facilitate the evolution of new phenotypes. For instance, the deviant expression of male-like traits (e.g. absence of scopa) in a gynandromorphic bee female may be considered an evolutionary novelty associated with the evolution of brood parasitism (Wcislo et al. 2004; Fateryga et al. 2011).

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