

DNA barcodes, expanded distribution, and redescription of *Apanteles hemara* Nixon, 1965 (Hymenoptera, Braconidae, Microgastrinae), a potential biocontrol species against amaranth leaf-webbers in Africa

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Abstract

The microgastrine parasitoid wasp *Apanteles hemara* Nixon, 1965, is currently being considered as a potential biocontrol agent of amaranth leaf-webber pests in Africa. To facilitate future research and identification of the species, we characterize it from an integrative taxonomy perspective by providing a comprehensive morphological redescription, extensive illustrations (including the first images of the holotype), DNA barcodes, wasp biology, host data (Choreutidae and Crambidae caterpillars), and updated geographical distribution of the species (including eight new country records). Despite a wide distribution across four major biogeographical regions (mostly within the Old World tropics), the species seems to be relatively uniform from a molecular and morphological perspective, based on studied specimens from Africa and Asia.

Keywords

Microgastrinae, *Amaranthus*, biocontrol, DNA barcode, geographical distribution, Africa

Introduction

Apanteles hemara was described more than 50 years ago (Nixon 1965), and even at that time it was considered to have a wide distribution. The species has been recorded from many countries: Australia, Bulgaria, Canary Islands, Cape Verde Islands, China, Cyprus, France, Greece, India, Iran, Israel, Italy, Madeira Islands, Mauritius, mainland Portugal, Russia, Senegal, South Africa, mainland Spain, Turkey, Vietnam, and the former Yugoslavia (Austin 1992, Papp 1988, 2007, 2012, Long and Belokobylskij 2003, Shaw 2012, Kedar and Kumaranag 2013, Madl and van Achterberg 2014, Yu et al. 2016).

The host species attacked by *A. hemara* are also varied: it has been considered a regular solitary parasitoid of *Tebenna micalis* (Mann, 1857) (Choreutidae), wherever both species occur (Shaw 2012), but it is also recorded from several species of Crambidae: *Cnaphalocrocis trapezalis* (Guenée, 1854), *Herpetogramma stultalis* (Walker, 1859), *Hydriris ornatalis* (Duponchel, 1832), *Omiodes indicata* (Fabricius, 1775), *Spoladea recurvalis* (Fabricius, 1775) and *Udea ferrugalis* (Hübner, 1796) (Long and Belokobylskij 2004, Papp 2012, Madl and van Achterberg 2014, Yu et al. 2016). The wasp may play some role in the biological control of caterpillars of *S. recurvalis* attacking amaranth crops in India, although the parasitism rate is low (Peter and Balasubramanian 1984, Velmurugan et al. 2006, Arivudainambi et al. 2010).

Due to the wide geographical distribution and host associations, the species was described four times under different names, and it currently has three synonyms (see below for more details on that).

In order to better characterize the species, this paper provides the first molecular information for *Apanteles hemara* (DNA barcodes), expands the known distribution to an additional eight countries, and presents the first color pictures of the species, including the holotype.

Methods

We studied specimens from the California Academy of Sciences (CAS), Canadian National Collection of Insects, Ottawa (CNC), the International Centre of Insect Physiology and Ecology, Nairobi, Kenya (ICIPE), and Naturalis Biodiversity Center, Leiden, Netherlands (RMNH).

Specimens from several countries (Kenya, Madagascar, Republic of the Congo, United Arab Emirates, and Yemen) were sampled for DNA barcodes (the 5' region of the cytochrome c oxidase I (CO1) gene, Hebert et al. 2003). DNA extracts were obtained from single legs using a glass fibre protocol (Ivanova et al. 2006). Total genomic DNA was re-suspended in 30 µl of dH₂O, a 658-bp region near the 5' terminus of the CO1 gene was amplified using standard primers (LepF1–LepR1) following established protocols (<http://v4.boldsystems.org/index.php>), and a composite sequence was generated for all successful amplifications. All information for the sequences associated

with each individual specimen can be retrieved from the Barcode of Life Data System (BOLD) (Ratnasingham and Hebert 2007).

The holotype of *A. hemara* was photographed with a Leica DFC450 camera on a Leica M165 C microscope. Other specimens were photographed with a Keyence VHX-1000 Digital Microscope, using a lens with a range of 10–130 ×. Multiple images were taken of a structure through the focal plane and then combined to produce a single in-focus image. For the Leica camera, the Zerene Stacker program (<http://zerenesystems.com/cms/stacker>) was used; software associated with the Keyence System produced focused images taken with that camera. Plates were prepared using Microsoft PowerPoint 2010.

A map with the distribution of the species was generated using SimpleMapp (Shorthouse 2010).

Results

Apanteles hemara Nixon, 1965

Apanteles hemara Nixon, 1965. Original description Nixon (1965: 58).

Apanteles caboverdensis Hedqvist, 1965. Synonymized by Papp (2003).

Apanteles proalastor Hedqvist, 1965. Synonymized by Papp (2003).

Apanteles bulgaricus Balevski & Tobias, 1980. Synonymized by Papp (1988).

Holotype. INDIA, Dehradun. Female (deposited in the Natural History Museum, London), examined.

Other material examined. **DEMOCRATIC REPUBLIC OF THE CONGO:** Iboubikro, Lesio-Loun Pk.; Pool, -3.2699 15.4711, 340mm, 25.xi.2008, coll. Braet & Sharkey, voucher code: CNCH2805 (1 specimen, CNC); **KENYA:** Eastern Province, Ukasi, Base of Ukasi Hill, -0.8210 38.5440, 613mm, Malaise trap, 11.xii.2012, coll. R. Copeland, voucher codes: 14703BraC10, 14703BraC12 (2 specimens, R. Copeland personal collection); Yatta, -1.2304 37.4579, 1184mm, reared, coll. ICIPE, voucher codes: CNC507541, CNC507542, CNC507543, CNC507544, CNC507545, CNC507546 (6 specimens, CNC); **MADAGASCAR:** Fianarantsoa, Parc National Ranomafana, Radio tower at forest edge, -21.251 47.412, 1130mm, 2.xii.2006, coll. Irwin & Harin'Hala, voucher codes: CASENT2163191, CNC666410 (2 specimens, CAS); -21.2510 47.4120, 1130mm, 7.ix.2006, coll. Irwin & Harin'Hala, voucher codes: CASENT2163390, CNC661376 (2 specimens, CAS); **OMAN:** Salalah, 17.00 54.06, 20m, 24.iv.1992, coll. M.D. Gallagher, voucher codes: CNC661377, CNC666409 (2 specimens, CNC); **UNITED ARAB EMIRATES:** Abu Dhabi, Al-Ajban, 24.6000 55.0167, Malaise trap, 7–28.xii.2006, coll. A. van Harten, voucher code: CNC666414 (1 specimen, CNC); Fujairah, Fujairah, 25.1333 56.350000, light trap, 13–29.xi.2005, coll. A. van Harten, voucher code: CNC666415 (1 specimen, CNC); NARC near Sweihan, 24.4 55.433, 20.iv.2005, coll. A. v. Harten, vouch-

er code: CNCH2681 (1 specimen, CNC); Sharjah, Sharjah Desert Park, 25.2833 55.7000, 21–29.iii.2005, coll. A. van Harten, voucher code: CNC666413 (1 specimen, CNC); 25.283 55.7, 21.iii.2005, coll. A. v. Harten, voucher code: CNCH2674 (1 specimen CNC); 29.iii.2005, coll. A. v. Harten, voucher code: CNCH2675 (1 specimen CNC); 9.iii.2005, coll. A. v. Harten, voucher code: CNCH2678 (1 specimen CNC); **YEMEN**: 12 km NW of Manakhah, Malaise trap, 15.ix–22.x.2003, coll. A. van Harten, voucher code: CNC666412 (1 specimen, CNC); Al Kowd, light trap, vii.2000, coll. A. van Harten & S. Al Haruri, voucher code: CNC666411 (1 specimen, CNC); Al Lahima, 15.xii.2000, coll. A. v. Harten & Hager, voucher codes: CNCH2690, CNCH2718, CNCH2723 (3 specimens, CNC); Malaise trap, 9.iv–5.vi.2001, coll. A. V. Harten, voucher code: CNC666406 (1 specimen, CNC); Al Mahrah, Al Ghaydah, 12–14.xi.1997, coll. A. Sallam & S. Ba Angood, voucher code: CNC666405 (1 specimen, CNC); Sana'a, Malaise trap, ii–iii.1998, coll. A. van Harten, voucher code: CNC666408 (1 specimen, CNC); Seyun, light trap, xi.2002, coll. A.V. Harten, voucher code: CNC661375 (1 specimen, CNC); Ta'izz, light trap, 3–24.i.1999, coll. A.V. Harten & M. Mahyoub, voucher code: CNC666407 (1 specimen, CNC); no specimen data, voucher code: WAM 0209 (1 specimen CNC).

Distribution. Afrotropical, Australian, Oriental and Palearctic regions (Fig. 1). The species is widespread in the Old World tropics, especially Africa. We record here the presence of *Apanteles hemara* in eight additional countries. The new distribution records for Kenya, Madagascar, Republic of the Congo, United Arab Emirates, and Yemen are based on examined specimens from the CAS, CNC, ICIPE and RMNH collections. Additionally, three other countries (Egypt, Pakistan, and Saudi Arabia) are recorded based on BOLD records whose sequences match sequences of the species, although those specimens were not available to us for study. The new data expands the species distribution across mainland Africa (where it was already known from a few countries), to Madagascar and the Arabian Peninsula (where it had not been recorded before). Based on the specimens we examined, the species has been collected throughout the entire year.

Diagnosis. *Apanteles hemara* can be recognized by having antenna slightly shorter than body length, with flagellomere 14 length $1.3\text{--}1.6 \times$ its width; vein R1 about four times as long as distance between ends of veins R1 and 3RS; hind legs with black coxa, yellow trochanter and trochantellus, brown metafemur, metatibia yellow on anterior $0.5\text{--}0.6$ and brown on posterior $0.4\text{--}0.5$, metatibial spurs white, and metatarsus brown; propodeum mostly smooth, but with entire areola entirely defined by strong carinae; tergites 1 and 2 with strong, longitudinal striation; and ovipositor sheaths shorter than metatibia ($0.7\text{--}0.9 \times$). *Apanteles hemara* belongs to the *ater* species group (sensu Nixon 1965), which unfortunately comprises “many aggregates of species that are not closely related but merge into one another through transitional forms” (Nixon 1965: 25). The world species were keyed out by Nixon (1965), but many more species have been described since, and thus that paper is now outdated. Updates are available for species of the *ater* group from Europe (Papp 1980), the former Soviet Union (Tobias 1986), China (Chen and Song 2004) and Mesoamerica (Fernandez-Triana et al. 2014) but unfortunately there is no updated key to world species.

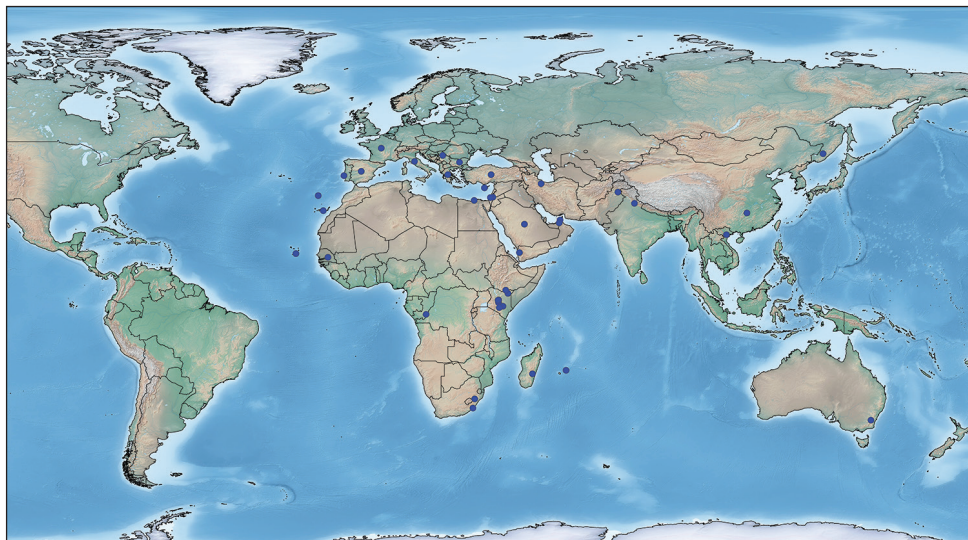


Figure 1. Worldwide distribution of *Apanteles hemara*.

Redescription. Body color mostly black, tergites 3+ dark brown, laterotergites and sternites 3+ light yellow-brown. Head black, except for dark orange-brown labrum, light yellow-white palpi and dark brown antennae. Front and middle legs yellow (except for coxae and mesofemur light yellow-brown to brown); hind leg with black coxa, yellow trochanter and trochantellus, brown metafemur, metatibia yellow on anterior 0.5–0.6 and brown on posterior 0.4–0.5, metatibial spurs white, and metatarsus brown. Wings with most veins transparent or white, except for brown veins R1, r, 2RS and M; pterostigma mostly brown, with a very small white spot at base. Anteromesoscutum and scutellar disc with relatively coarse and dense punctures (distance between punctures smaller than diameter of individual puncture). Propodeum mostly smooth, with areola entirely defined by strong carinae. Tergites 1 and 2 with strong, longitudinal striation, contrasting with remaining tergites which are smooth.

Body measurements (in mm) and ratios. Body length: 2.50–3.20, fore wing length: 2.50–3.00, ovipositor sheaths: 0.62–0.84, metafemur: 0.65–0.77, metatibia length: 0.87–0.95, tergite 1 0.36–0.49. Length of flagellomeres: 1st (0.18–0.22), 2nd (0.18–0.22), 3rd (0.17–0.21), 14th (0.08–0.10), 15th (0.08–0.10) and 16th (0.12). Length/width of flagellomere 2: 2.75–3.14; length/width of flagellomere 14: 1.28–1.60. Head height/width: 0.82–0.88; head slightly narrowing towards mandibles, width at clypeus base 0.88–0.88 × head width at antennal base. Malar line 1.12–1.50 × mandibular base. Ocular ocellar line 1.67–2.00 × posterior ocellus diameter; interocellar distance 1.71–2.17 × posterior ocellus diameter. Scutellar disc length 1.09–1.15 × width at base. Maximum height of mesoscutellum lunules 0.42–0.56 × maximum height of lateral face of mesoscutellum. Tergite 1 widening from anterior margin to two thirds of tergite length, then slightly narrowing towards posterior margin; tergite 1 length

1.64–2.09 × tergite width at posterior margin; tergite widths (at anterior margin/maximum width/posterior margin): 0.19–0.25/0.27–0.30/0.22–0.25. Tergite 2 width at posterior margin 2.31–2.64 × length medially. Tergite 2 length medially 0.48–0.64 × tergite 3 length medially. Metafemur length 3.25–3.67 × metafemur width. Pterostigma length 2.55–2.86 its width. Vein R1 length 1.15–1.27 × pterostigma length. Vein r length 1.82–2.20 × vein 2RS length.

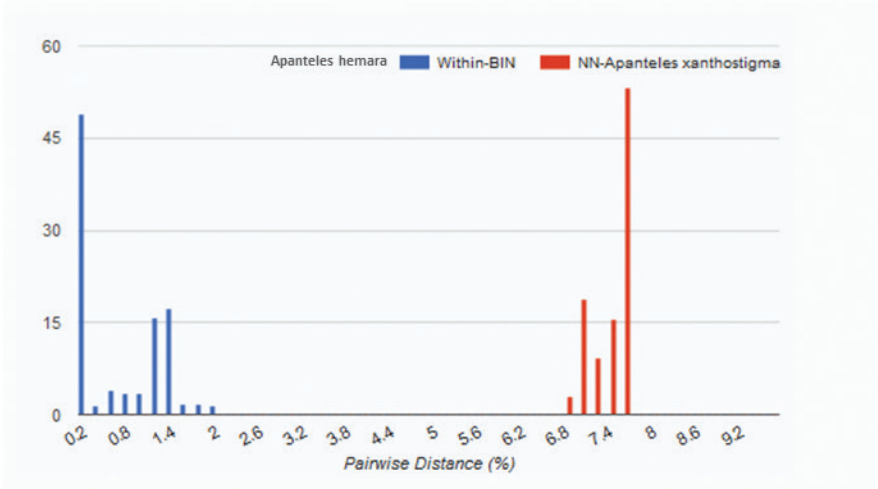
Variation. Despite the widespread distribution of the species across four major biogeographical regions (mostly Old World tropics), the specimens we examined were very similar morphologically (Figs 4–8), with only minor variation in the color of the hind legs and tergites 3+ of the metasoma. However, we could not examine specimens from the Australian or the Palaearctic regions, which might be more variable than the specimens from Africa and Asia we studied.

Biology. Solitary parasitoid (Fig. 4E); over 41,280 parasitism cases were observed by us under laboratory condition at ICIPE in Kenya during a three years study. Hosts: Choreutidae, *Tebenna micalis*; Crambidae, *Cnaphalocrocis trapezalis*, *Herpetogramma stultalis*, *Hydriris ornatalis*, *Omiodes indicatae*, *Spoladea recurvalis*, *Udea ferrugalis*. For additional details see Comments below.

DNA barcodes. A total of 17 DNA barcodes were obtained from the specimens we studied. All sequences but one were over 600 base pairs (bp) long, with most representing full barcodes (658 bp). Additional sequences representing the species are found in BOLD, but we could not examine those specimens because they belong to projects that are not public yet. Overall, there are currently 32 sequences belonging to *A. hemara* in BOLD, 24 of them being public records and 19 being barcode compliant (Fig. 2). The species has been assigned the BIN number BOLD:AAB1927 (for the concepts of ‘BIN’ and ‘barcode compliant’ see Ratnasingham and Hebert 2007). The DNA barcode intraspecific variation (p-distance) for *A. hemara* averaged 0.61% (4 bp), with a maximum of 1.91% (12 bp), but more than half of the analyzed sequences differed by only 0.2–0.4 % (1–2 bp). There were six different haplotypes recognized among the barcoded specimens. From a DNA barcode perspective, the nearest species in BOLD is *Apanteles xanthostigma* (Haliday, 1834), which differs from *A. hemara* by 6.93% (45 bp) (Figs 2, 3).

Comments. In Kenya, the parasitoid was collected during outbreaks of two amaranth leaf-webber species, *Spoladea recurvalis* and *Udea ferrugalis* on two species of amaranth, *Amaranthus cruentus* L. and *A. dubius* L. It was successfully reared under laboratory conditions on both amaranth leaf-webber species at ICIPE. However, it failed to attack the leafworm species *Spodoptera littoralis* (Boisduval, 1833) and *S. exigua* (Hübner, 1808) (Noctuidae). During population dynamics studies carried out under field conditions at high and mid altitude levels in Central Kenya, field parasitism rates on *S. recurvalis* as low as 3% were obtained during outbreak periods while parasitism rates as high as 25 to 75% were achieved outside outbreak periods. These observations prompted studies for potential augmentative biological control strategies for early interventions aiming at preventing or reducing outbreaks of the amaranth leaf-webbers in farmers’ fields. Under laboratory conditions (25 ± 2°C, 60 ± 10% RH and 12L:12D

DISTANCE DISTRIBUTION



Barcode Index Number Registry For BOLD:AAB1927

[Go to public records in this BIN](#)

BIN DETAILS

BIN URI:	BOLD:AAB1927	Average Distance:	0.61% (p-dist)
DOI:	REQUEST DOI	Maximum Distance:	1.91% (p-dist)
Member Count:	32 [24 Public]	Distance to Nearest Neighbor:	6.93% (p-dist)
Barcode Compliant Members:	19		
Founding Record:			

NEAREST NEIGHBOR (NN) DETAILS

Nearest BIN URI:	BOLD:AAB1922	Average Distance:	0.5% (p-dist)
Member Count:	28	Maximum Distance:	1.54% (p-dist)
Nearest Member:	NOFIG607-15	Distance Variance:	0.17% (p-dist)
Nearest Member Taxonomy:	Arthropoda, Insecta, Hymenoptera, Braconidae, Microgastrinae, Apanteles, Apanteles xanthostigma		

Figure 2. Details of the Barcode Index Number Registry for *Apanteles hemara*, data from BOLD (<http://v4.boldsystems.org/>).

photoperiod), *A. hemara* demonstrated high performance on *S. recurvalis* and *U. ferrugalis* both through high direct parasitism rates and significant non-reproductive mortalities caused to the hosts. The parasitized caterpillars can easily be distinguished from

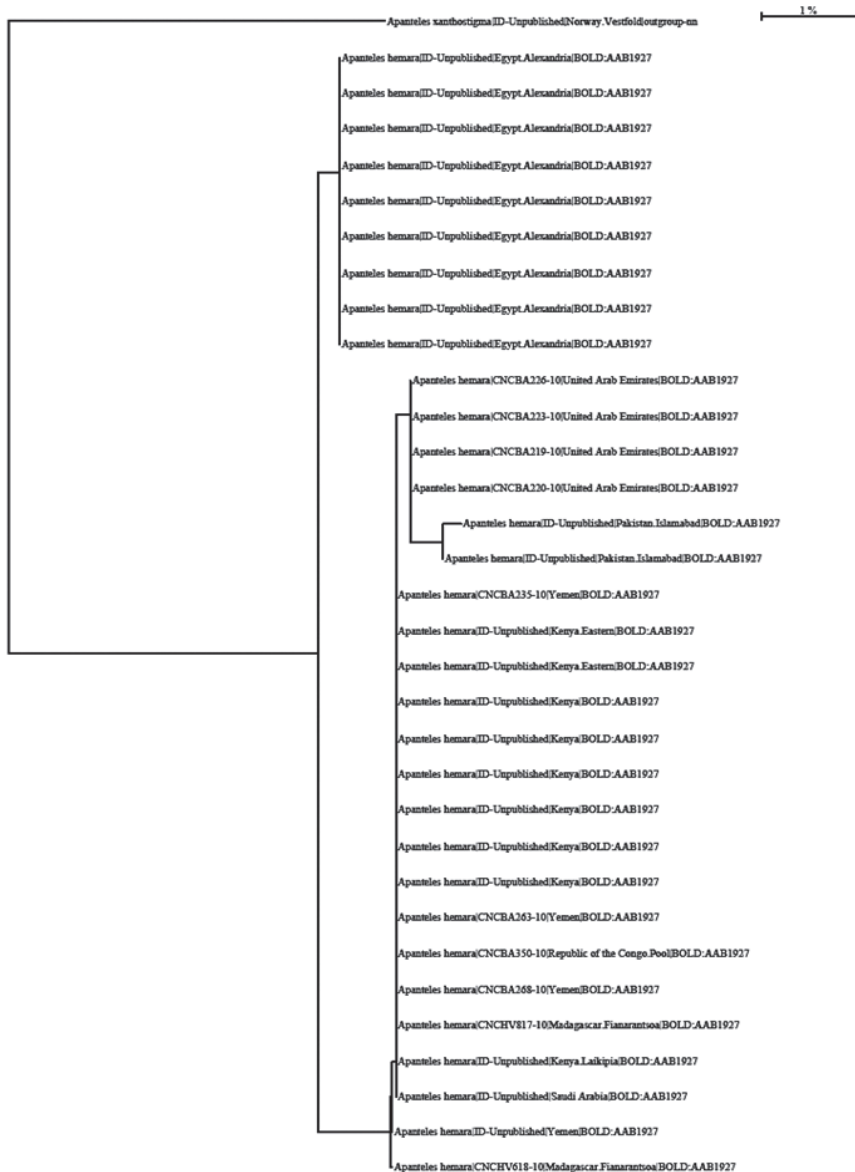


Figure 3. Neighbour Joining tree (K2P) of all known DNA barcoding sequences of *Apanteles hemara* and a representative of the closer species (*Apanteles xanthostigma*) in the BOLD database (as of April 2017).

non-parasitized ones within the first two days after the parasitoid's oviposition in the larva, through a significant reduction in feeding, movement and the lack of windowing on the leaf epidermis. Subsequently their growth rate is reduced and within four days after the parasitoid's oviposition, parasitized caterpillars are considerably smaller in size than their non-parasitized counterparts, turn creamish and will all die whether

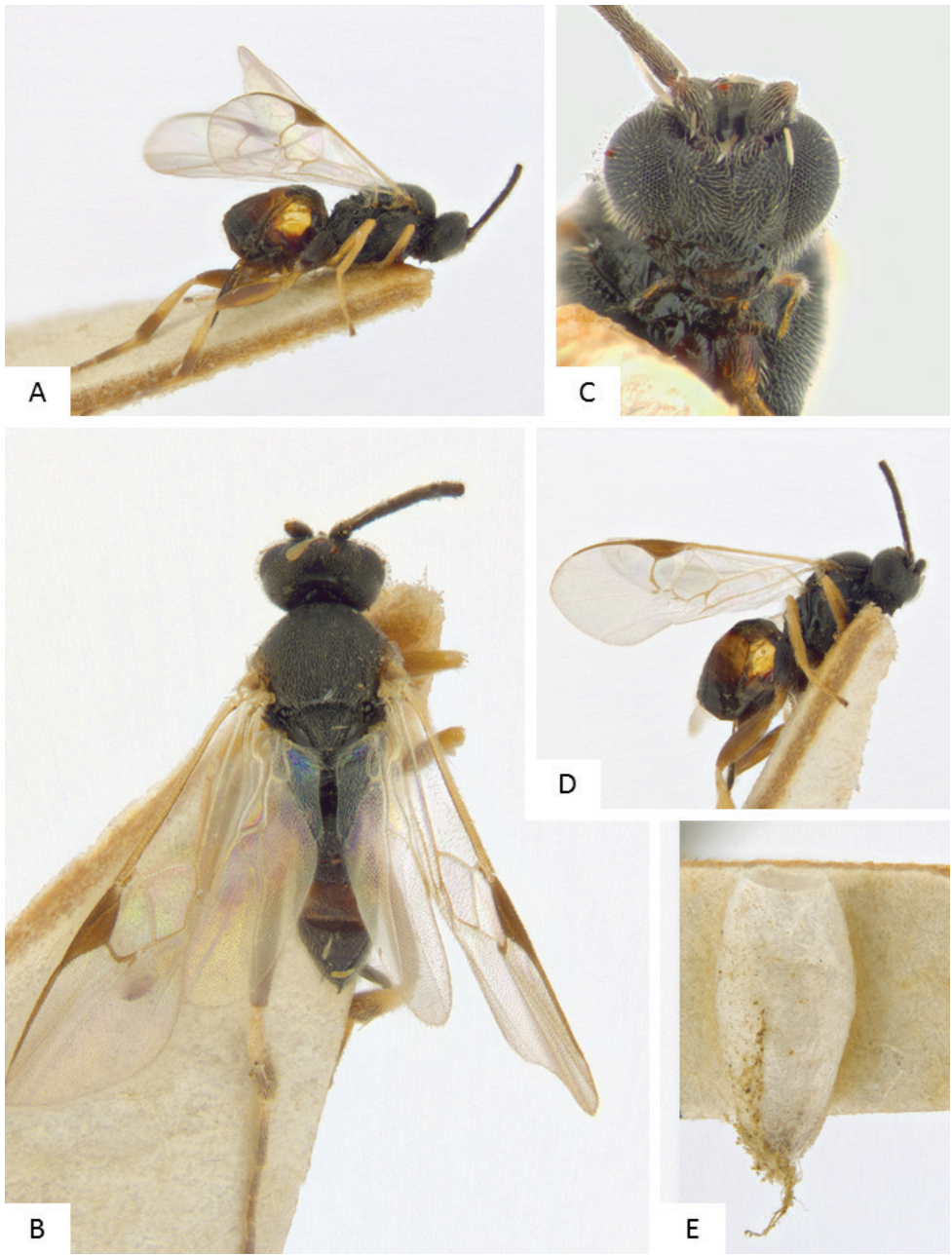


Figure 4. *Apanteles hemara*, female holotype from India.

the parasitoid successfully emerged or not. The developmental times are 12 and 13 days for male and female parasitoids respectively. *Apanteles hemara* is currently being considered for a conservative and augmentative biological control program against the amaranth leaf-webbers in Africa.

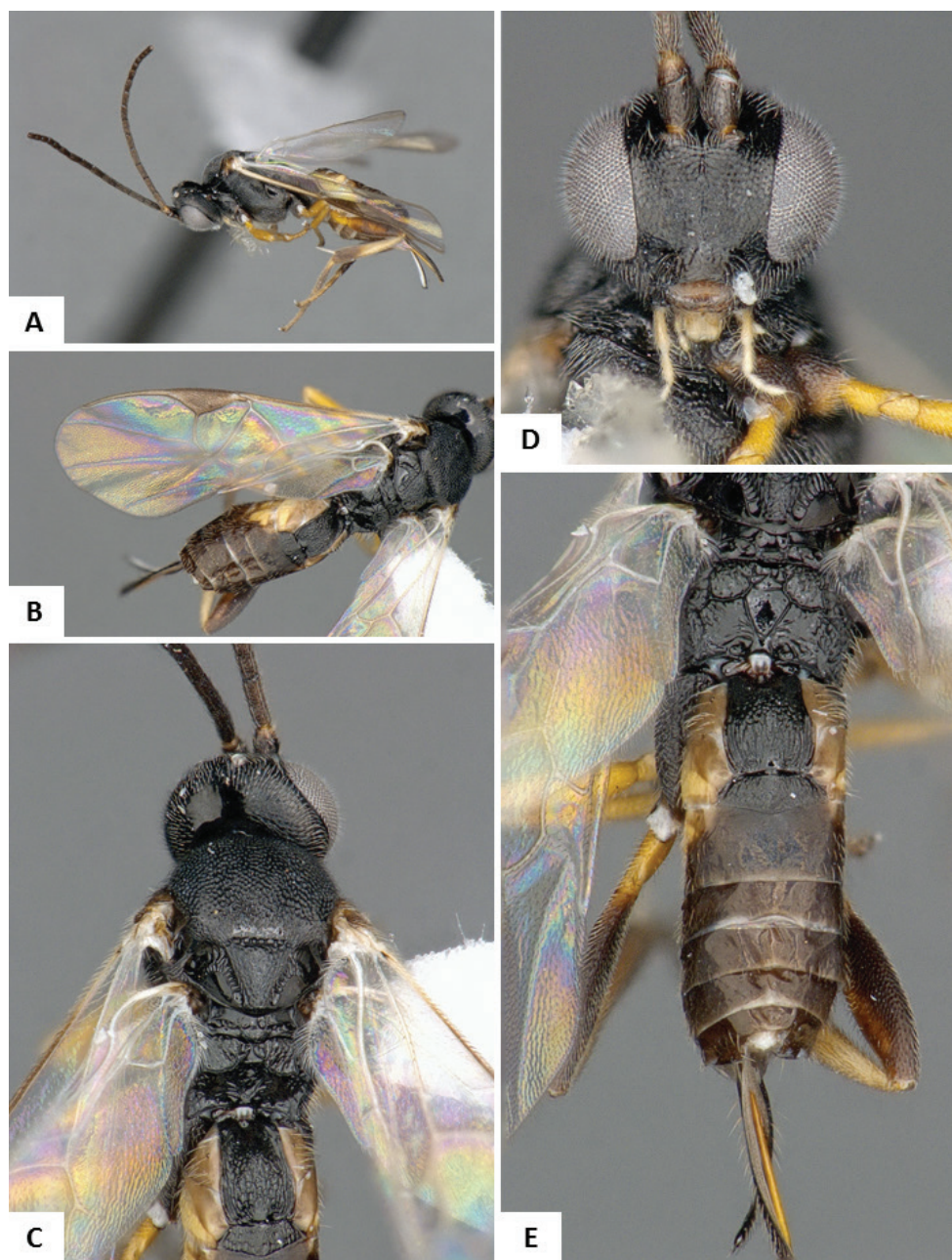


Figure 5. *Apanteles hemara* female specimen from Kenya (Voucher code: CNC507541).

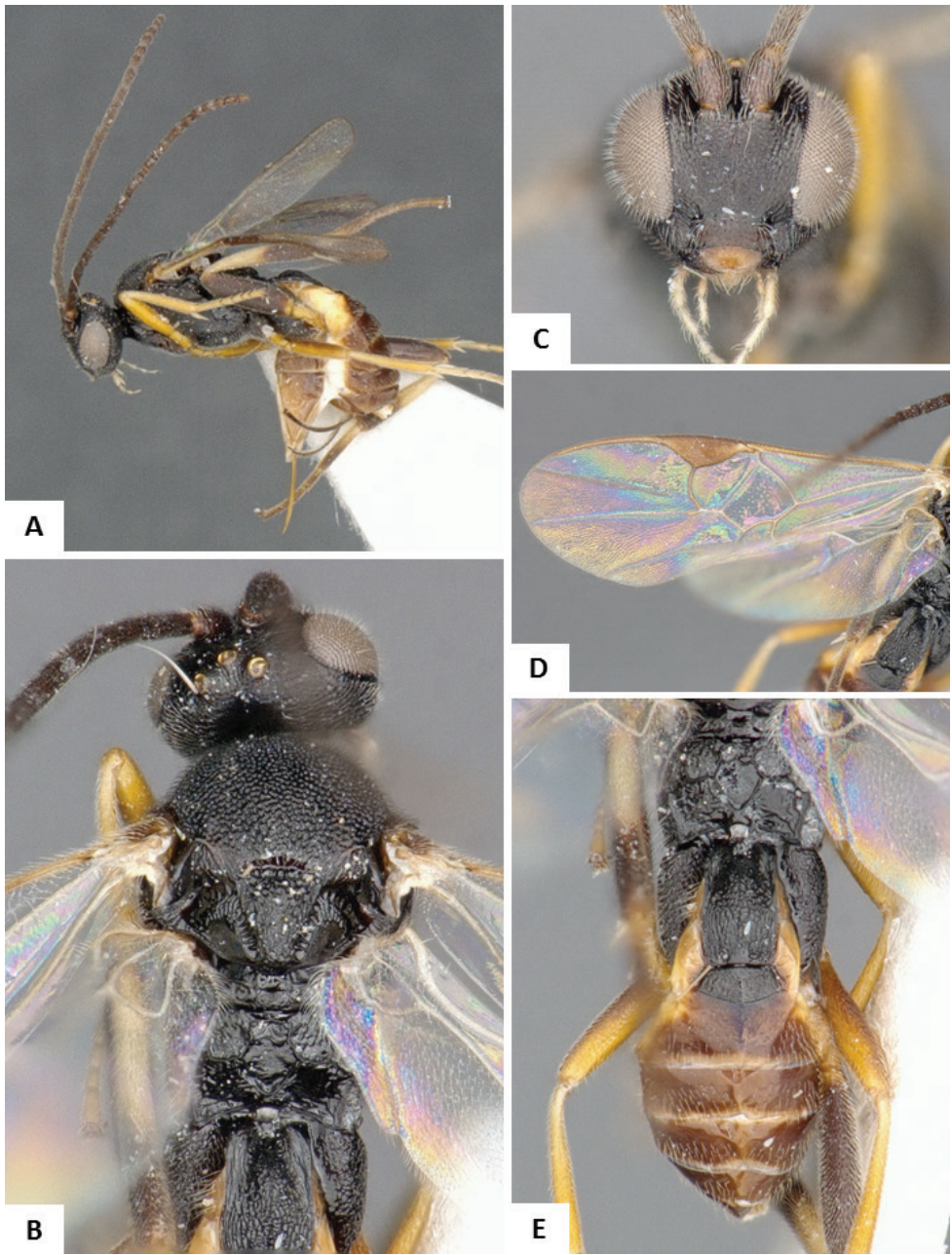


Figure 6. *Apanteles hemara* female specimen from Yemen (Voucher code: CNC661375).

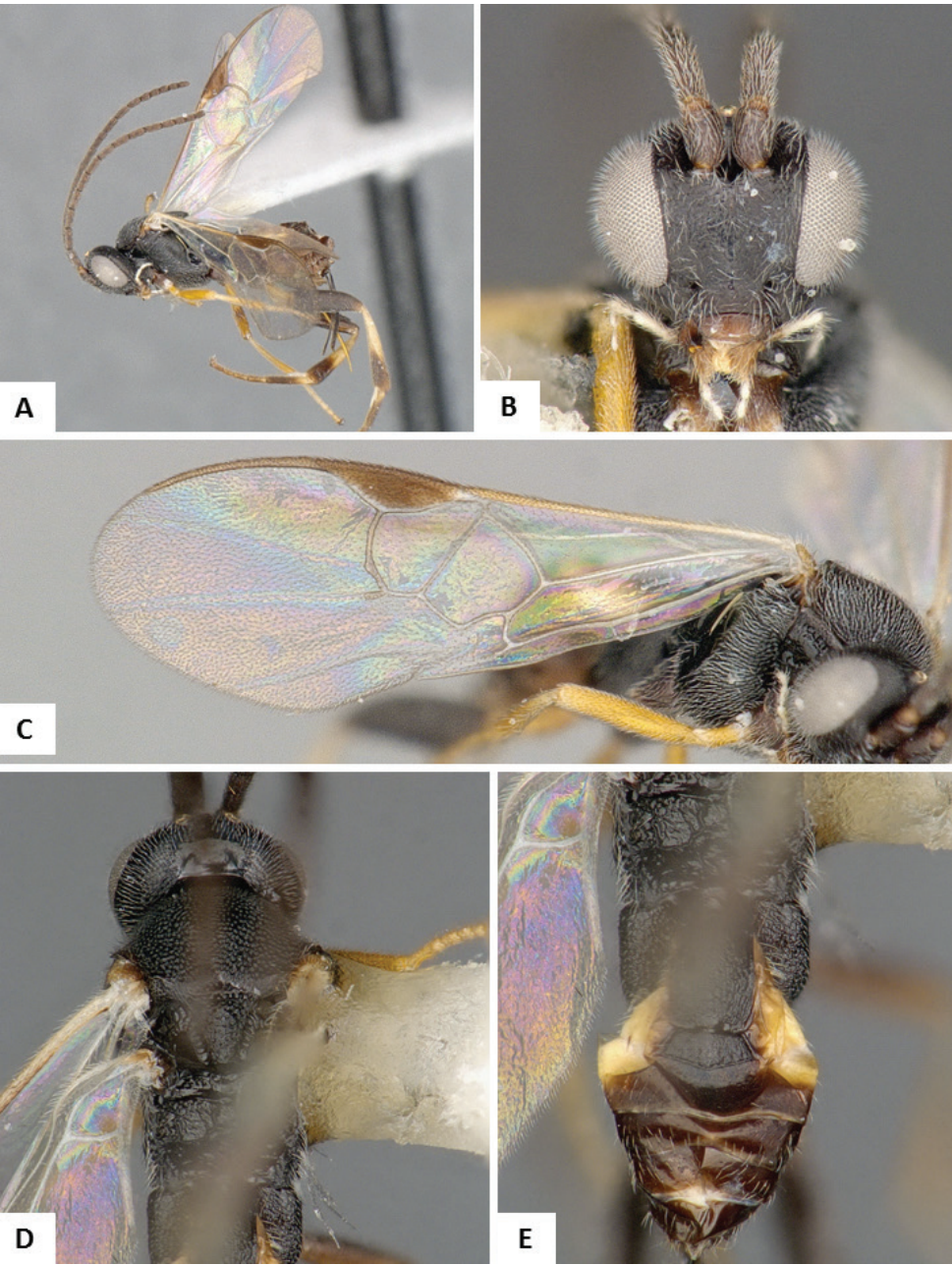


Figure 7. *Apanteles hemara* female specimen from Madagascar (Voucher code: CNC661376).

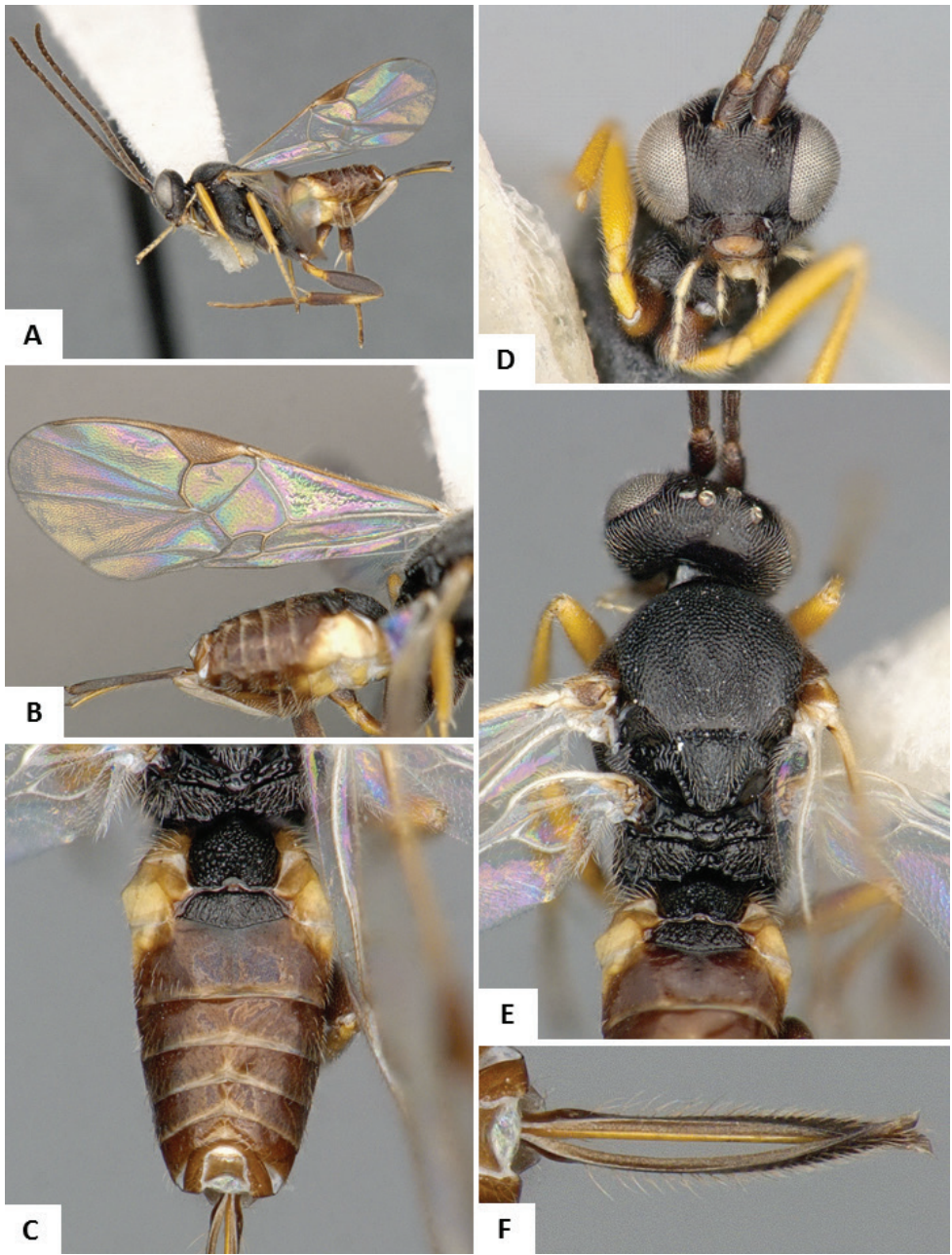


Figure 8. *Apanteles hemara* female specimen from Oman (Voucher code: CNC661377).

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Ichneumonidae (Hymenoptera) associated with xyelid sawflies (Hymenoptera, Xyelidae) in Mexico

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Abstract

Two species of ichneumon wasps (Ichneumonidae), *Gelanes horstmanni* Khalaim, **sp. n.** (Tersilochinae) and *Idiogramma elbakyanae* Khalaim **sp. n.** (Tryphoninae), are described from the pine forest at 2800–2900 m from the State of Tlaxcala in Central Mexico; a third species, *I. comstockii* (Ashmead), is found to occur in the State of Nuevo León in Northeast Mexico. The genera *Gelanes* Horstmann and *Idiogramma* Förster are associated with xyelid sawflies (Xyelidae), and both, as well as the tryphonine tribe Idiogrammatini, are recorded from Mexico for the first time. An identification key to the two *Idiogramma* species occurring in Mexico is provided.

Resumen

Se describen dos especies de Ichneumonidae, *Gelanes horstmanni* Khalaim, **sp. n.** (Tersilochinae) e *Idiogramma elbakyanae* Khalaim **sp. n.** (Tryphoninae), de un bosque de pinos a 2800–2900 msnm en el Estado de Tlaxcala en la zona central de México; una tercera especie, *I. comstockii* (Ashmead), se reporta del Estado de Nuevo León en el noreste de México. Los géneros *Gelanes* Horstmann e *Idiogramma* Förster están asociados con moscas sierra xyelidas (Xyelidae), ambos géneros y la tribu Idiogrammatini de la subfamilia Tryphoninae se reportan para México por primera vez. Se elaboró una clave para la identificación de las dos especies de *Idiogramma* que ocurren en México.

Keywords

Tersilochinae, *Gelanes*, Tryphoninae, *Idiogramma*, Tlaxcala, fauna, new species, taxonomy, parasitoids, key

Introduction

The family Xyelidae Newman is an ancient group of Hymenoptera with the oldest fossil representatives dating back to the Middle or Late Triassic (Gao et al. 2009, Wang et al. 2014). Extant Xyelidae fauna is represented by five genera, of which the most diverse and species rich is *Xyela* Dalman (Taeger et al. 2010).

The genus *Xyela* comprises 48 species occurring predominantly in the Holarctic region, though some species extend with their host plants, *Pinus* spp. (Pinaceae), southwards into northern parts of the Neotropic and Oriental regions (Blank et al. 2013). A small number of imagines of *Xyela* are known from Mexico (Smith 1988; Khalaim, unpublished data), but none has been identified at species level so far. Larvae of *Xyela* live and feed in male cones of pine species (Blank 2002, Blank et al. 2013) but *X. gallicaulis* D.R. Smith causes galls in the vegetative shoots (Yates and Smith 2009). Some European species of *Xyela* were demonstrated to be monophagous or exceptionally oligophagous, being associated with one or a few related *Pinus* species (Blank et al. 2013).

The ichneumonid parasitoid complex of *Xyela* includes a small Palearctic genus, *Xyeloblacus* Achterberg (Braconidae), and two Holarctic genera, *Idiogramma* Förster and *Gelanes* Horstmann (Ichneumonidae) (Achterberg and Altenhofer 1997, Khalaim and Blank 2011, Horstmann 2013). None of these taxa were reported from Mexico hitherto. In addition, the Nearctic *Eurytoma tylodermatidis* Ashmead (Eurytomidae) and *Pteromalus thyridopterigis* (Howard) (Pteromalidae) are known to occur on *Xyela* species (Hetrick 1942, Burdick 1961, Peck 1963, Noyes 1998).

The aim of this work is to describe two new species of *Gelanes* and *Idiogramma* from Central Mexico and record one more Nearctic *Idiogramma* species from Northeast Mexico. An identification key to two Mexican species of *Idiogramma* also will be provided.

Material and methods

A large amount of material of Mexican Ichneumonidae from the Instituto de Biología, Universidad Nacional Autónoma de México, DF, Mexico (further UNAM) was examined. From this material, two undescribed species of Ichneumonidae belonging to the genera *Gelanes* and *Idiogramma* have been recognized. One more Nearctic species associated with *Xyela* was found in the collection of the Universidad Autónoma de Tamaulipas, Cd. Victoria, Mexico (further UAT). Some specimens will be deposited in the Natural History Museum, London, United Kingdom (further BMNH) and the Zoological Institute, Russian Academy of Sciences, St. Petersburg, Russia (further ZISP).

Samples of the following Nearctic species of *Idiogramma* were examined in the Texas A&M University, U.S.A. (further TAMU): *I. comstockii* (Ashmead) (1 male, USA, Maryland), *I. contortae* Townes & Townes (8 females, USA, Wyoming) and *I. longicauda* (Cushman) (1 female, USA, Maryland). Samples of three Palearctic species, *I. alysiina* (Thomson), *I. eurum* Kasparyan and *I. euryops* Förster, were examined in ZISP and the Finnish Museum of Natural History, Helsinki University, Helsinki, Finland.

Morphological terminology generally follows Gauld (1991); nomenclature of *Gelanes* propodeal carinae and areas follows Khalaim (2011). Ocellar index is the shortest distance between the lateral ocellus and the margin of the eye compared with the maximum diameter of the lateral ocellus. Stacks of photographs were taken in the TAMU with a DFC 295 digital camera attached to a Leica stereomicroscope. Composite images with an extended depth of field were created using the Helicon Focus software.

Taxonomy

Gelanes Horstmann, 1981.

Type species. *Thersilochus fuscus* Holmgren, 1860.

Gelanes is a Holarctic genus with 20 species in the Palearctic region (Horstmann 1981; Khalaim 2002, 2017; Khalaim and Blank 2011; Kim et al. 2013; Khalaim and Sheng 2015) and 13 described and many undescribed species in USA and Canada (Horstmann 2013). Some species were reared from *Xyela* spp. in Europe (Khalaim and Blank 2011) and *Xyela* spp. and a species of *Pleroneura* Konow (Xyelidae) in North America (Horstmann 2013). A single species of *Gelanes*, described below, is found in Central Mexico. It represents the first record of *Gelanes* from Mexico.

Gelanes horstmanni Khalaim, sp. n.

<http://zoobank.org/01626456-87CC-4A73-99BF-492D8C8DD836>

Figs 1–5

Comparison. The new species is immediately distinguished from the 13 Nearctic species described by Horstmann (2013) by the combination of the filiform flagellum comprising 25 flagellomeres (Fig. 2) and the ovipositor bearing a deep and narrow dorsal subapical notch (Fig. 5). Only two Nearctic species, *G. incisus* Horstmann and *G. punctipleuris* Horstmann, possess similar ovipositors with a narrow dorsal subapical notch, but in both species the genae are much shorter (0.6–0.7 times as long as eye width) and more strongly constricted behind the eyes, the clavate flagellum comprises only 16–18 flagellomeres, and the apex of the ovipositor bears one or several fine teeth ventrally. In the key to Nearctic species (Horstmann 2013), *G. horstmanni* runs to couplet 14, but does not correspond with either side of the couplet as it has the mesopleuron punctate with smooth interspaces, a weak foveate groove, and an extremely long ovipositor with the sheath being more than 4.5 times as long as the first tergite. *Gelanes horstmanni* also differs from all of its Palearctic congeners by the head strongly prominent behind the eyes in dorsal view (Fig. 3).

Description. *Female.* Body length 5.0 mm. Fore wing length 3.9 mm.

Head prominent, strongly rounded behind eyes in dorsal view (as in Fig. 3); gena 0.85 times as long as eye width. Ocellar index 1.7. Clypeus broad and short, 5.5 times



Figures 1–7. *Gelanes horstmanni* sp. n., holotype female (**1**, **2**, **4**, **5**) and paratype male (**3**). **1** body, lateral view **2** head with antenna and mesosoma, dorso-lateral view **3** head, dorsal view **4** head, antero-ventral view **5** apex of ovipositor, lateral view. *Idiogramma comstockii*, female **6** head, antero-ventral view **7** head, dorso-lateral view.

as broad as long (Fig. 4), flat in lower half, with lower margin almost straight. Mandible with upper tooth subequal in length and size to lower tooth; both teeth with apices widely rounded, probably obliterated because of use (Fig. 4). Malar space almost 0.9

times as long as basal mandibular width. Antennal flagellum (Fig. 2) filiform, long, with 25 flagellomeres; flagellomeres 1–3 rather short, slightly elongate; second flagellomere is the shortest; flagellomeres 4 and following 1.2–1.3 times, subapical flagellomeres almost 1.1 times as long as broad; flagellomeres 4–6 bearing distinct and flagellomere 7 weak finger-shaped structures at apex on outer surface. Face with moderately strong prominence medially. Face and frons distinctly punctate, with smooth interspaces between punctures centrally, finely and densely punctate with granulate and dull interspaces peripherally. Vertex distinctly punctate, smooth between punctures. Gena smooth, upper part with moderately dense and fine punctures, lower part with very fine and sparse punctures. Occipital carina complete. Hypostomal carina completely absent.

Mesoscutum and scutellum with moderately dense fine punctures, polished between punctures. Scutellum with lateral longitudinal carinae present only at extreme base. Notaulus very weak, slightly impressed, with few fine oblique striae somewhat distant from anterolateral margin. Mesopleuron with shallow fine punctures, more or less smooth and weakly shining between punctures centrally, finely granulate peripherally. Foveate groove rather shallow, short, oblique, situated somewhat before centre of mesopleuron, with fine transverse wrinkles. Basal area of propodeum indistinct, moderately broad, rectangular, about 2.5 times as long as broad and 0.4 times as long as apical area. Dorsolateral area with fine, scattered punctures, interspaces between punctures smooth centrally. Distance between propodeal spiracle and pleural carina about as long as diameter of spiracle. Apical area centrally indistinctly punctate with interspaces between punctures more or less smooth, peripherally uneven, flat, widely rounded anteriorly; apical longitudinal carinae well developed, reaching transverse carina anteriorly.

Fore wing with second recurrent vein ($2m-cu$) postfurcal. Intercubitus ($2rs-m$) moderately thick, somewhat longer than abscissa of cubitus between intercubitus and second recurrent vein (abscissa of M between $2rs-m$ and $2m-cu$). First abscissa of radius ($Rs+2r$) straight, distinctly longer than width of pterostigma. First and second sections of radius ($Rs+2r$ and Rs) meeting at right angle. Metacarpus ($R1$) short, running about half way from distal end of Rs and apex of fore wing. Postnervulus ($Cu\&2cu-a$) intercepted distinctly below its middle. Hind wing with nervellus ($Cu1\&cu-a$) vertical.

Legs slender. Hind femur 0.65 times as long as tibia (Fig. 1). Hind basitarsus short, 0.4 times as long as hind tibia. Spurs of hind tibia short and straight. Tarsal claws not pectinate.

First tergite moderately slender, 2.6 times as long as broad posteriorly, dorsally polished, laterally striate before glymma, smooth at base. Glymma deep, situated slightly proximal of centre of first tergite, joining strong furrow to ventral part of postpetiole. Second tergite 1.25 times as long as broad anteriorly. Thyridial depression slightly elongate. Ovipositor very long, evenly upcurved, with a deep dorsal subapical notch, without teeth ventrally (Fig. 5); sheath over 4.5 times as long as first tergite and about 3.0 times as long as hind tibia.

Head, mesosoma and first metasomal segment black. Palpi, mandible (except black teeth), clypeus and tegula yellow. Antennal flagellum black, scape and pedicel brown-

ish black. Pterostigma dark brown. Fore and mid legs brownish yellow, tarsi slightly infusate, mid coxa on outer half dark brown. Hind leg with coxa brownish black, femur predominantly brown (paler apically), trochanters and tibia yellow-brown, tarsus infusate. Metasoma behind first tergite yellow ventrally, brown to dark brown laterally, brownish black dorsally.

Male. Flagellum slightly tapered towards apex, with 24–25 flagellomeres. Head rather strongly prominent behind eyes in dorsal view (Fig. 3). Malar space 0.3–0.5 times as long as basal mandibular width. One male with mandibular teeth not obliterated, pointed, subequal in length. Clypeus more or less lenticular, with lower margin weakly convex. Basal area of propodeum narrow, almost as long as apical area. Distance between propodeal spiracle and pleural carina 1.0–2.0 times as long as diameter of spiracle. Tergites 1 and 2 of metasoma more slender. Metasoma posterior to tergite 1 dark brown, subcylindrical. Otherwise similar to female.

Variation. Six males are not included to the type series; they are somewhat smaller, with the gena not prominent behind eyes in dorsal view and the body highly smooth and less punctate.

Etymology. The species name is dedicated to the late Klaus Horstmann, a German expert in Ichneumonidae, in recognition of his contribution to the study of Nearctic Tersilochinae.

Material examined. Holotype female (UNAM), Mexico, Tlaxcala, Nanacamilpa, Ejido Los Búfalos, N 19°28', W 98°35', bosque Pino-Encino (*Pinus* + *Quercus* forest), 2830–2900 m, Malaise trap, 4.IV–3.V.2016, coll. Y. Marquez & A. Contreras.

Paratypes. 3 males (BMNH, UNAM, ZISP), same data as holotype.

Non-type material. 6 males (1 in BMNH, 4 in UNAM, 1 in ZISP), same data as holotype.

Distribution. Central Mexico (Tlaxcala).

Idiogramma Förster, 1869

Type species. *Idiogramma euryops* Förster, 1888.

Idiogramma is a small Holarctic genus with 6 species – 3 in the Palearctic region and 4 in the Nearctic region (Townes et al. 1992), including one species, *I. euryops*, distributed in both continents (Yu et al. 2012). Two species of *Idiogramma* are here recorded from Mexico: *I. comstockii* is recorded from the northeastern State of Nuevo León, and *I. elbakyanae* sp. n. from the State of Tlaxcala in Central Mexico. This is the first record of the genus, as well as its tribe Idiogrammatini, from Mexico.

Key to species of *Idiogramma* occurring in Mexico

- 1 Frons with deep median groove immediately posterior of anterior ocellus (Fig. 7). Mandible not tapering towards apex, as broad basally as apically

- (Fig. 6), in dorsal view with apex strongly turned outwards (Fig. 7). Face and frontal orbits yellow (Figs 6, 7). Ovipositor sheath (in Mexican specimen) 2.4 times as long as hind tibia *I. comstockii* (Ashmead)
- Frons posterior of anterior ocellus weakly convex, without median groove (Figs 10, 11). Mandible strongly tapering towards apex, much broader basally than apically (Fig. 9); in dorsal view convex, unspecialized. Face and frons black, at most face slightly yellowish brown centrally (Figs 9–11). Ovipositor sheath 4.2 times as long as hind tibia (Fig. 8) *I. elbakyanae* sp. n.

***Idiogramma comstockii* (Ashmead, 1895)**

Figs 6, 7

Material examined. 1 female (UAT), Mexico, Nuevo León, San Pedro Garza García, Chipinque, trampa luz negra, 10.IV.1986, coll. O. Cardoso.

Distribution. Canada, USA (south to California, Arizona and Louisiana), North-east Mexico (Nuevo León).

***Idiogramma elbakyanae* Khalaim, sp. n.**

<http://zoobank.org/BAD5B4AC-6C1C-42FC-99D1-BF14A66B971F>

Figs 8–11

Comparison. The new species differs from all Nearctic species by the occipital carina absent dorsally and laterally (Figs 10, 11); the mandible strongly tapering apically (Fig. 9), in dorsal view unspecialized, convex (turned outwards in other species except *I. euryops* – as in Fig. 7); and the entirely black frons (Figs 10, 11).

In addition, *I. elbakyanae* differs from the three species occurring in the Palaearctic region (Kasparyan and Tolkanitz 1999) by its extremely long ovipositor, shorter first tergite, strongly tapered mandibles and dorsally absent occipital carina (in *I. euryops* this carina is sometimes weak or vestigial mediodorsally); it also differs from *I. alysiina* and *I. euryops* by the head, in dorsal view, with long and prominent genae; and from *I. eurum* and *I. euryops* by the fuscous face (yellow in the two other species).

Description. *Female.* Body length 3.8 mm. Fore wing length 3.9 mm.

Head prominent, strongly rounded behind eyes in dorsal view (Fig. 10); gena 1.1 times as long as eye width. Ocellar index 1.8. Clypeus very broad and short, separated from face by deep and sharp groove, with upper margin somewhat convex medially and lower margin slightly concave (Fig. 9), strongly convex in lateral view. Mandible strongly tapered in basal half, 2.6 times broader basally than apically (at level of base of teeth), with raised flanges on lower and upper margins; upper tooth longer than the lower; mandible in dorsal view convex, unspecialized. Malar space very short, upper corner of mandible almost touching eye margin (Fig. 9). Antennal flagellum slender, slightly tapered towards apex, with 23 flagellomeres; flagellomere 1 slightly swollen



Figures 8–11. *Idiogramma elbakyanae* sp. n., holotype female **8** habitus (without antennae and wings), lateral view **9** head, antero-ventral view **10** head, dorsal view **11** head, dorso-lateral view.

basally. Face with rather strong prominence medially, finely punctate with smooth and shining interspaces between punctures. Frons, vertex and genae polished, with scattered fine punctures. Vertex weakly convex, not impressed along midline (Figs 10, 11). Occiput dorsally conspicuously impressed medially (Fig. 11). Occipital carina present ventrally, completely absent laterally and dorsally.

Mesoscutum polished, with scattered fine punctures. Notaulus deep and sharp on anterolateral side of mesoscutum. Epicnemial carina extending somewhat above the level of lower corner of pronotum, not reaching anterior margin of mesopleuron. Me-



Figure 12. Distribution map of *Gelanes* and *Idiogramma* species in Mexico.

sopleuron smooth and shining, with very fine and sparse punctures. Propodeum with median longitudinal carinae distinct, convergent anteriorly; propodeum dorsally with transverse wrinkles between median longitudinal carinae; lateral longitudinal carinae weak but distinct.

First tergite slightly transverse, almost 0.9 times as long as posteriorly broad, in lateral view with upper margin evenly convex; lateromedian carinae distinct in basal half. Second tergite strongly transverse. Ovipositor very long, flexible, at apex with distinct nodus and fine teeth ventrally (Fig. 8); sheath about 4.2 times as long as hind tibia.

Head brownish black to black; lower part of genae and face medially slightly yellowish; clypeus and mandible (except black teeth) yellow; mouthparts yellow with two apical segments of maxillary palp and one apical segment of labial palp fuscous. Antennal flagellum black, slightly paler basally; scape and pedicel brownish black, yellowish ventrally and with extreme apical margin yellow dorsally. Mesosoma entirely brownish black; tegula yellow. Pterostigma brown. Legs predominantly brown; fore coxa yellow, slightly brownish basally; mid coxa pale brown; fore and mid trochanters yellow; fore and mid femora and hind trochanters yellow to brown. Metasomal tergites dark brown, tergites 2 to 7 with hind margin dorsally widely and laterally narrowly emarginate with yellow (Fig. 8).

Male. Very similar to female but metasoma basally more slender, tergite 1 almost twice as long as posteriorly broad, tergite 2 subquadrate and yellow markings on tergites 2–7 generally narrower (especially on tergite 2). Occipital carina sometimes dis-

cernible ventro-laterally. Face sometimes yellowish medially and laterally. Measoma and legs sometimes darker.

Material examined. Holotype female (UNAM), Mexico, Tlaxcala, Nanacamilpa, Ejido Los Búfalos, N 19°28', W 98°35', bosque Pino-Encino (*Pinus* + *Quercus* forest), 2830–2900 m, Malaise trap, 4.IV–3.V.2016, coll. Y. Marquez & A. Contreras.

Paratypes. 4 males (BMNH, UAT, UNAM, ZISP), same data as holotype.

Etymology. The species is named in honour of Alexandra Elbakyan (Kazakhstan/Russia), creator of the web-site Sci-Hub, in recognition of her contribution to making scientific knowledge available for all researchers.

Distribution. Central Mexico (Tlaxcala).

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Influence of natal host and oviposition experience on sex allocation in a solitary egg parasitoid, *Anastatus disparis* (Hymenoptera, Eupelmidae)

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Abstract

Constraints on adaptation are a major topic in evolutionary biology. Sex allocation, in particular the ratio of the sexes, has often been used as a key process for studying constraints on adaptation. *Anastatus disparis* Ruschka (Hymenoptera: Eupelmidae) is a solitary egg parasitoid of gypsy moth, *Lymantria dispar* (L.) (Lepidoptera: Lymantriidae), and several other lepidopteran forest pests. Here, we compared two different sized substitute hosts, the smaller one *Dictyoploca japonica* Moore (Lepidoptera: Saturniidae) and the larger one *Antheraea pernyi* Guerin-Meneville (Lepidoptera: Saturniidae), and investigated the influence of natal host and oviposition experience on sex allocation by *A. disparis*. Results showed that natal host had almost no impact on sex allocation by *A. disparis*, but oviposition experience did influence sex allocation of *A. disparis* on *D. japonica* eggs. This suggests that information females obtain from the environment influences how they allocate sex in their offspring. However, the sex ratios of *A. disparis* emerging from *A. pernyi* eggs were consistent irrespective of oviposition experience of female *A. disparis*. This indicates that the eggs of *A. pernyi* are large enough to maximize female progeny of *A. disparis*.

Keywords

Parasitoid, substitute host, learning, gypsy moth

Introduction

Anastatus dispar Ruschka (Hymenoptera: Eupelmidae) is a solitary egg parasitoid of several noxious lepidopteran forest pests, including the gypsy moth, *Lymantria dispar* (L.) (Lepidoptera: Lymantriidae) (Crossman 1925, Kurir 1944, Yan et al. 1989, Li and Lou 1992, Alalouni et al. 2013, Liu et al. 2017). It can develop three to four generations on *L. dispar* eggs annually in Northern China (Yao and Yan 1994). In our previous research, we found that the offspring of *A. dispar* reared on egg masses of gypsy moth were male-biased and small-bodied (Liu et al. 2017). However, when they were reared on a larger host, *Antheraea pernyi* Guerin-Meneville (Lepidoptera: Saturniidae), the offspring were female-biased and had a larger body size compared with those reared from gypsy moth (Liu et al. 2017).

Sex allocation in haplodiploid arthropods has fitness-related implications and has received much attention with regard to insect parasitoids (West 2009). In particular, an important attribute of parasitoids is that they control the sex ratio of offspring in response to environmental variables (Godfray 1994). In parasitoid wasps, mated females can manipulate the sex-ratio of progeny by controlling fertilization during oviposition. Males develop from unfertilized eggs and are haploid, while females develop from fertilized eggs and are diploid (Heimpel and de Boer 2008). In the case of solitary parasitoids, for which only one individual develops in each host, the theory of host quality-dependent sex allocation has been proposed to explain the sex ratio variation in this group of parasitic Hymenoptera (Charnov 1979, Charnov et al. 1981). This theory states that the pattern of sex allocation is determined depending on male- and female-fitness relationships with host size; either female or male eggs could be allocated to higher quality hosts depending on which sex benefits more from being large (Charnov et al. 1981, Napoleon and King 1999, West and Sheldon 2002). Both theoretical predictions and empirical studies show that the sex (usually female) that benefits most from being larger, should be placed in a larger host for its development (Charnov et al. 1981, King and Lee 1994). The size of an emerging parasitoid is often positively correlated with the size of the natal host, as are other fitness components such as longevity, fecundity, dispersal and patch-finding ability in the field (King and Charnov 1988, King and Lee 1994, Visser 1994, Kazmer and Luck 1995, Bennett and Hoffmann 1998, Ellers et al. 1998, Jervis et al. 2003).

Learning and memory have been demonstrated in a large number of animal species. Hymenopteran parasitoids can learn to recognize particular visual and olfactory stimuli and use them to modify subsequent behaviours (Vet et al. 1995, Vinson 1998). Learning may occur at any phase of the host selection process (Dauphin et al. 2009). Therefore, learning includes 'pre-imaginal learning' at adult emergence (Cortesero and Monge 1994, Gandolfi et al. 2003), 'post-emergence learning' or 'early adult learning' immediately after emergence (Lentz and Kester 2008), and 'ovipositional learning' at oviposition (Turlings et al. 1995). Numerous studies have demonstrated the positive effects of learning on host selection (Vet and Groenewold 1990, Hastings and Godfray 1999, Morris and Fellowes 2002), optimizing foraging efficiency (Vet and Groenewold

1990, Vet et al. 1995, Vinson 1998) and encountering suitable hosts (Papaj and Vet 1990, Baaren and Boivin 1998, Dutton and Dorn 2000).

Trivers and Wilard (1973) suggest that females should adjust the sex of their offspring in response to environmental conditions, and there is considerable empirical evidence for such adjustments and deviations from optimal sex allocation strategies (West and Sheldon 2002, Lewis et al. 2010). As a parasitoid, *A. disparis* can parasitize small body-sized hosts through to large body-sized hosts, with females seeming to prefer larger hosts which produce more female offspring (Liu et al. 2017). However, we do not know whether natal host or oviposition experience influence the offspring sex allocation of adults. For this reason, we devised a series of experiments aiming firstly to determine whether natal host could influence sex allocation in female *A. disparis*, and secondly whether a learning experience, in this case oviposition experience on one host species, influences sex allocation by females during oviposition on a different host species. Two substitute host species of different sizes were evaluated: the smaller was *Dictyoploca japonica* Moore (Lepidoptera: Saturniidae) and the larger was *Antheraea pernyi* Guerin-Meneville (Lepidoptera: Saturniidae).

Material and methods

Insect cultures

Pupae of *Antheraea pernyi* were purchased from a farmer in Qinglong Manchu Autonomous County, Qinhuangdao City, Hebei Province, China; adults that emerged from those pupae were maintained at 25–30 °C for less than two days prior to egg extraction (see below); *Dictyoploca japonica* eggs were provided directly from the Forestry Academy of Liaoning Province, China. Eggs of the two lepidopteran host species for use in experiments were obtained by laparotomizing the adult females' abdomen and removing the eggs; these eggs were maintained at 0 °C prior to use, and for not longer than 60 days (Wang et al. 2014).

An *Anastatus disparis* colony was first established from a population developing in *L. dispar* egg masses collected in Longhua County, Hebei Province (41°31'N, 117°74'E) in March, 2012, and was subsequently maintained on *A. pernyi* eggs in several cylindrical rearing boxes (height: 5 cm, diameter: 5 cm) at 25±0.5°C, RH 60%, 14L:10D. Prior to experiments cohorts of *A. disparis* were also reared on *D. japonica* eggs for three generations to provide the different treatments for the experiments (see below). All adult female *A. disparis* had no experience of hosts or oviposition before experiments began and were fed with honey water (honey: water=4: 6) on cotton balls (Yan et al. 1989). The age of the females selected for the experiments ranged from 3 to 5 days because under standard conditions offspring sex ratio does not fluctuate widely during this period (Liu et al. 2015). All females were placed with males for 24 hours to ensure they were mated before each experiment.

Measurement of egg sizes of different lepidopteran host species

The host egg sizes were determined from their length (Morris and Fellowes 2002), which was measured using a light microscope (Leica M205A, Germany). Thirty replicate eggs were measured for each host species.

Sex allocation by *Anastatus disparis*

One large experiment with six treatments was conducted to answer two primary questions, the first concerning sex-allocation by females offered a choice of different sized hosts (treatments 1 and 2) and the second concerning females offered different sized host sequentially (treatments 3–6; in all cases, they gained oviposition experience when offered the first host which then had the potential to influence their behaviour in relation to the second host offered). All treatments were run at the same time but, to aid interpretation, we describe them below in relation to the question being asked.

Sex allocation by *Anastatus disparis* when offered a choice of eggs from two different lepidopteran host species presented at the same time

This question was answered by comparing between two treatments; specifically, between maternal females that were either reared on *Antheraea pernyi* (treatment 1) or *Dictyoploca japonica* (treatment 2) before being offered a choice of eggs from both lepidopteran species, for oviposition. One maternal adult (3–5 days) reared on either *A. pernyi* or *D. japonica* was introduced into a cylindrical rearing box (height: 5 cm, diameter: 5 cm) containing twenty *A. pernyi* eggs and twenty *D. japonica* eggs at 26 ± 0.5 °C. After 24 hours, the eggs of both host species were collected and placed individually into polyethylene tubes (height: 7.5 cm; diameter: 1 cm) plugged with cotton and incubated at 28 ± 0.5 °C until adult parasitoids emerged. The number and sex of offspring was recorded after eclosion. Since *A. disparis* is haplodiploid, virgin females lay 100% unfertilized eggs, which develop into males, while mated females lay a mixture of unfertilized and fertilized eggs, the latter of which develop into females. Therefore, any replicates resulting in 100% male offspring were assumed to be from unmated maternal adults and were excluded from subsequent statistical analysis. Thirty replicates of maternal adults were tested for each treatment (60 in total).

Sex allocation by *A. disparis* when eggs from two different lepidopteran host species are presented separately and in sequence

This question was answered by comparing amongst four treatments in which maternal females, reared either in *A. pernyi* or *D. japonica*, were offered eggs of one or other of the

Table 1. Number of maternal replicates for each experimental treatment.

Host species on which the maternal parasitoid had been reared	Sequence of parasitism	
	First <i>A. pernyi</i> eggs then <i>D. japonica</i> eggs	First <i>D. japonica</i> eggs then <i>A. pernyi</i> eggs
<i>A. pernyi</i> eggs	73 (treatment 3)	77 (treatment 4)
<i>D. japonica</i> eggs	66 (treatment 5)	64 (treatment 6)

lepidopteran species in sequence, i.e. first *A. pernyi* eggs and then *D. japonica* eggs or *vice versa*: all combinations (i.e. the four treatments, 3-6) and total replicates per treatment combination can be seen in Table 1. Specifically: One maternal adult reared either on *A. pernyi* or *D. japonica*, was introduced into a cylindrical rearing box (height: 5 cm, diameter: 5 cm) containing twenty *A. pernyi* or *D. japonica* eggs (depending on treatment) at 26 ± 0.5 °C. Twenty-four hours later, all of the host eggs were collected and replaced by a new batch of twenty eggs of *A. pernyi* or *D. japonica* (depending on treatment) and incubated for a further 24 hours at 26 ± 0.5 °C. Parasitised host eggs from each treatment were placed individually into polyethylene tubes (height: 7.5 cm; diameter: 1 cm) plugged with cotton and incubated at 28 ± 0.5 °C until adult parasitoids emerged. The number and sex of offspring was recorded after eclosion. Results for maternal adults that had not mated were excluded from the subsequent statistical analysis as described previously.

Statistical analysis

The sex ratio of the parasitoid offspring was represented as the proportion of males (male divided by male+female). Sex ratios of offspring reared from the different host species (for each treatment), and the egg sizes of the two different host species, were compared using independent *T*-tests in the statistical package SPSS version 20, after arcsin (sqrt) transformation of the raw proportion data. For the whole experiment with simultaneous presentation of both host species in treatment 1 and 2, sex ratios and numbers of offspring reared from the different host species were compared by General Linear Model (GLM) with Univariate tests and Generalised Linear Mixed Models (GLMMs). For the effect of oviposition experience (hosts presented with different sequences), results from the whole experiment (treatment 3-6) were analyzed by GLM with Multivariate tests. The confidence interval for all tests was set at 95%.

Results

Sizes of two host species

The eggs of *A. pernyi* (2.94 ± 0.02 mm) were significantly larger than the eggs of *D. japonica* (2.31 ± 0.02 mm; $t=24.44$, $df=58$, $p<0.001$).

Sex allocation by *A. disparis* when offered a choice of eggs from two different lepidopteran host species presented at the same time

When maternal *A. disparis* that had been reared on *A. pernyi* eggs encountered *A. pernyi* and *D. japonica* eggs simultaneously (treatment 1), the proportion of male offspring emerging from *A. pernyi* eggs was $9.35 \pm 1.87\%$ and the proportion emerging from *D. japonica* eggs was $44.53 \pm 8.34\%$ (Fig. 1). When maternal *A. disparis* that had been reared on *D. japonica* eggs encountered *A. pernyi* and *D. japonica* eggs simultaneously (treatment 2), the results were similar: the proportion of male offspring emerging from *A. pernyi* eggs was $10.60 \pm 2.66\%$ and the proportion emerging from *D. japonica* eggs was $30.60 \pm 7.02\%$. GLM with univariate analysis showed that offspring sex ratios of maternal adults from the two host species varied little ($F=1.558$; $df=1, 116$; $P=0.215$), but when females made a choice between the two host species, the sex ratios of offspring from *A. pernyi* eggs and *D. japonica* was significantly different ($F=15.233$; $df=1, 116$; $p<0.001$) (Fig. 1). Analysis by GLMMs also showed that offspring sex ratios of females from the two host species (natal influence) were not significantly different ($p>0.05$), but their offspring differed when emerging from different host eggs (Estimate=0.237, Wald $Z=7.616$, $p<0.001$).

The total number of offspring emerging from *A. pernyi* eggs parasitized by maternal *A. disparis* from host *A. pernyi* or *D. japonica* were 7.27 ± 0.62 and 7.00 ± 0.79 , respectively. The total number of offspring emerging from *D. japonica* eggs parasitized by maternal *A. disparis* from host *A. pernyi* or *D. japonica* were 3.53 ± 0.69 and 2.97 ± 0.55 , respectively. Analysis showed that the total offspring number laid by the two kinds of females varied little ($F=0.387$, $df=1, 116$, $p=0.535$). This was consistent in the treatments where eggs of the two hosts were presented sequentially and so we only report total numbers here. However, there was a significant difference between the two parasitized host species ($F=33.634$, $df=1, 116$, $p<0.001$).

Sex allocation by *A. disparis* when the eggs of two different lepidopteran host species were presented separately and in sequence

When maternal *A. disparis* reared on *A. pernyi* eggs were first offered *A. pernyi* eggs and then *D. japonica* eggs (treatment 3) a significantly higher proportion of male offspring emerged from *D. japonica* eggs ($28.02 \pm 3.20\%$) than *A. pernyi* eggs ($7.65 \pm 0.65\%$; $t=-6.640$, $df=72$, $p<0.001$) (Fig. 2).

When maternal *A. disparis* reared on *D. japonica* eggs were first offered *A. pernyi* eggs and then *D. japonica* eggs (treatment 5) a significantly higher proportion of male offspring emerged from *D. japonica* eggs ($34.37 \pm 3.42\%$) than from *A. pernyi* eggs ($7.57 \pm 1.09\%$; $t=-8.570$, $df=65$, $p<0.001$).

When maternal *A. disparis* reared on *A. pernyi* eggs were first offered *D. japonica* eggs and then *A. pernyi* eggs (treatment 4) there was no significant difference in the proportion of male offspring emerging from *D. japonica* eggs ($10.33 \pm 0.93\%$) com-

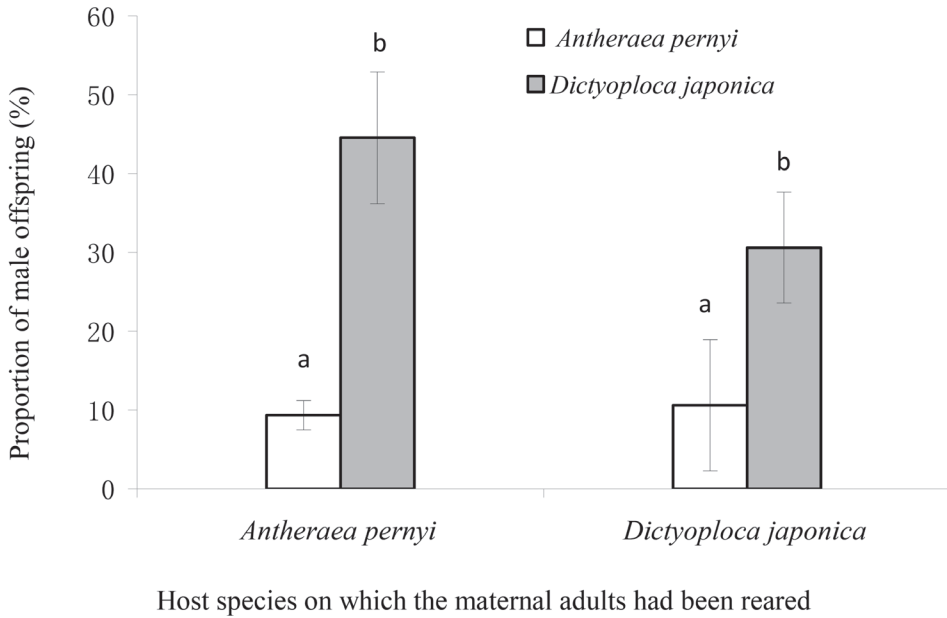


Figure 1. Proportion of male offspring from maternal *A. disparis* when simultaneously presented with eggs of two host species. Bars with different lowercase letters are significantly different from each other from the General Linear Models with Univariate test analysis ($p < 0.001$).

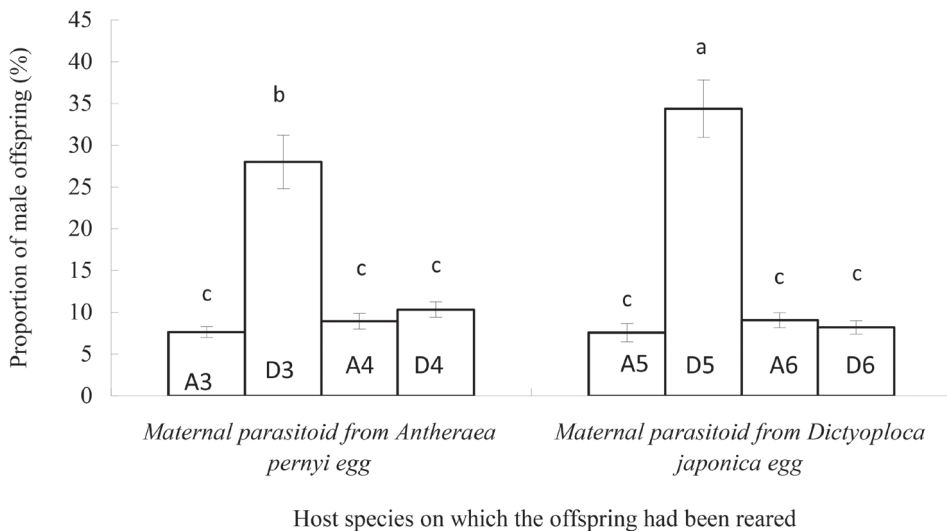


Figure 2. Proportion of male offspring from female *A. disparis* with different prior oviposition experience. Bars with different lowercase letters are significantly different from each other from the General Linear Models with Multivariate tests analysis ($p < 0.001$). A3 and D3 represent host *A. pernyi* eggs and *D. japonica* eggs in treatment 3, respectively; A4 and D4 represent two hosts in treatment 4; A5 and D5 represent two hosts in treatment 5; A6 and D6 represent two hosts in treatment 6.

pared with the proportion emerging from *A. pernyi* eggs ($8.93 \pm 0.93\%$; $t=1.137$, $df=76$, $p>0.05$).

When maternal *A. disparis* reared on *D. japonica* eggs were first offered *D. japonica* eggs and then *A. pernyi* eggs (treatment 6) there was no significant difference in the proportion of male offspring emerging from *A. pernyi* eggs ($9.07 \pm 0.92\%$) compared with the proportion emerging from *D. japonica* eggs ($8.19 \pm 0.80\%$; $t=-0.754$, $df=63$, $p>0.05$).

In both treatments in which the maternal adults had been reared on *A. pernyi* eggs (treatments 3 and 4), the proportion of *A. disparis* male offspring emerging from *D. japonica* eggs was significantly higher when the female had previous oviposition experience on *A. pernyi* eggs ($28.02 \pm 3.20\%$; treatment 3), than when they had no previous oviposition experience ($10.33 \pm 0.93\%$; $t=5.02$, $df=148$, $p<0.001$; treatment 4) (Fig. 2). The results were similar for the maternal adults that had been reared on *D. japonica* eggs: a significantly higher percentage of male offspring emerged from *D. japonica* eggs parasitized by females with oviposition experience on *A. pernyi* eggs ($34.37 \pm 3.42\%$; treatment 5), than when they had no previous oviposition experience ($8.19 \pm 0.80\%$; $t=7.46$, $df=128$, $p<0.001$; treatment 6) (Fig. 2).

When treatments 3–6 were put into a 2×2 GLM analysis, ie, the first factor being mother (emerged from *A. pernyi* or *D. japonica*) and the second factor being ‘when experienced’ (first or second order), and the offspring sex ratios of *A. disparis* from *A. pernyi* eggs and *D. japonica* eggs were regarded as two dependent variables, respectively, several interesting results were observed. The first was that the offspring sex ratios were not different across the first and second broods, regardless of host species ($F=0.494$, $df=2$, 275 $p=0.61$), i.e., mothers (natal experience) either from *A. pernyi* or *D. japonica* had little influence on offspring sex ratios. The second was that the offspring sex ratios differed for the *D. japonica* between different orders ($F=6.944$, $df=1$, 276 , $p<0.01$), but were similar for the *A. pernyi* regardless of the oviposition experience of the maternal adult ($F=1.480$, $df=1$, 276 , $p=0.225$). Moreover, host species (natal) and order (oviposition experience) interacted in above treatments ($F=34.835$, $df=2$, 275 , $p<0.001$), and the main contributor was *D. japonica* ($F=62.773$, $df=1$, 276 $p<0.001$).

Discussion

Natal host can influence parasitoid host preference, handling time and sex allocation behaviour. Morris and Fellowes (2002) speculated that, in part, host size may be judged by self-reference by the ovipositing female comparing host size with a component of her own size, such as the time it takes to walk over the surface of the host. However, we found that despite the fact that females emerging from *A. pernyi* were significantly larger than those emerging from *D. japonica* this had no significant effect of their subsequent sex allocation. We suggest that *A. disparis* may, therefore, have no self-reference ability, i.e. it does not judge potential host size by comparing it with its own size.

In theory, the sex (usually female) that benefits most from larger size should be placed in larger hosts (host quality-dependent sex allocation theory) (Charnov and Stephens 1988, King and Lee 1994). We found strong support for this hypothesis as, regardless of whether maternal *A. disparis* had themselves been reared on *A. pernyi* or *D. japonica*, they all preferred to lay more female offspring in relatively larger host species and more male offspring in relatively smaller host species when both host species were supplied simultaneously. The difference in host size could be judged by direct comparison using visual and tactile cues.

We also found that sex allocation in *A. disparis* was affected by oviposition experience. For instance, the proportion of male offspring emerging from *D. japonica* eggs parasitized by females with oviposition experience of *A. pernyi* eggs, was significantly higher than the proportion of males emerging from *D. japonica* eggs parasitized by females that had no oviposition experience. We speculate that females can judge current host size from oviposition experience of previously parasitized hosts. If females have laid eggs in *A. pernyi* eggs, then when they subsequently encounter *D. japonica* eggs, the female would compare the host quality (host size) of the *D. japonica* eggs with its stored oviposition memory of *A. pernyi* eggs (Goubault et al. 2004, Papaj and Prokopy 1989), and determine that the *D. japonica* egg is smaller. If this is the case, then more male offspring would be laid in the *D. japonica* eggs if the host quality-dependent sex allocation theory is correct (Charnov and Stephens 1988, King and Lee 1994). However, we did not find evidence for this when female parasitoids were first offered *D. japonica* eggs and then *A. pernyi* eggs, when the proportion of male offspring emerging from the *A. pernyi* eggs was not significantly different from the proportion of males emerging from *A. pernyi* eggs that had been parasitized by females that had no oviposition experience. Therefore, we supposed that *A. pernyi* eggs were large enough for the parasitoid to achieve maximum female progeny in nature.

In conclusion, sex allocation in *A. disparis* females fitted with the predictions of condition-dependent sex allocation theory in parasitoids (Trivers and Willard 1973, West and Sheldon 2002, Lewis et al. 2010). It also provided a nice test of experience (i.e. context-dependence) in terms of sex allocation: when first presented with eggs of the larger host, females produced a more male-biased clutch on a patch of the second, smaller host eggs; however, when females experienced the small eggs first, the sex ratios did not shift when they moved to larger hosts. This suggests that information females obtain from the environment influences their sex allocation. The asymmetry in response suggests that female-biased sex ratios on the larger host were optimal come what may, whilst if only presented with the smaller host, female biased sex ratios are optimal, but in the presence of (or experience of) larger hosts, then more males are produced in the smaller hosts (Lewis et al. 2010).

Further studies with longer intervals between oviposition on different host species should be performed to evaluate the effects of learning and memory in this species.

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The effect of *Rickia wasmannii* (Ascomycota, Laboulbeniales) on the aggression and boldness of *Myrmica scabrinodis* (Hymenoptera, Formicidae)

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Abstract

The interactions of ectosymbiotic Laboulbeniales (Ascomycota) fungi and their hosts are rather understudied. *Rickia wasmannii* Cavares is a common ant-associated Laboulbeniales species that has been reported in 17 countries of Europe, and frequently infects *Myrmica scabrinodis* Nylander, 1846 (Hymenoptera: Formicidae), a common ant species host, in high density. These make *M. scabrinodis* and *R. wasmannii* appropriate model organisms for studies on fungal host-ectosymbiont interactions. Aggressiveness and boldness of infected and uninfected *M. scabrinodis* workers from northern and eastern Hungary were studied in two laboratory-established behavioural experiments. Infected workers were significantly less aggressive and less bold (i.e. less likely to leave nest shelters) than the uninfected ones. These results suggest that *R. wasmannii* has considerable effects on the behaviour of *M. scabrinodis*. Our study brings an evidence that infection of ants with Laboulbeniales might negatively affect the workers' behaviour. In special, the competitive abilities might be affected most by these fungi, since remaining inside and behaving submissively is not effective behaviour in the case of significant competition for resources among colonies.

Keywords

behaviour, ectoparasite, Laboulbeniomycetes, fungi, infection spreading

Introduction

The ascomycete order Laboulbeniales contains 141 genera and over than 2,100 species of obligatory ectosymbionts (Santamaria et al. 2017). The hosts of these fungi mainly belong to the groups Coleoptera (ca. 80%) and Diptera (ca. 10%) (Weir and Hammond 1997). Most Laboulbeniales fungi exhibit extreme host specificity, with a single host species (stenotopic) or several species in the same genus (eurytopic) (Haelewaters 2012 and references therein), but unrelated insects can also host a single Laboulbeniales species if they share the same microhabitat (De Kesel and Haelewaters 2014, Pfliegler et al. 2016). While such ectosymbiotic associations can have both positive (Konrad et al. 2015) and negative (Benjamin 1971, Gemenio et al. 2004, Nalepa and Weir 2007, Strandberg and Tucker 1974) effects on their hosts, they are sometimes considered to be neutral factors (García et al. 2010, Lapeva-Gjonova and Santamaria 2011, Whisler 1968).

To date, four ant-associated species of Laboulbeniales have been recorded in Europe: *Laboulbenia formicarum* Thaxt. is known in France, Portugal and Spain, parasitizing *Lasius grandis* Forel, 1909, *L. neglectus* van Loon, Boomsma & Andrasfalvy 1990 and *L. niger* (Linnaeus, 1758) (Espadaler and Santamaria 2012, Gómez et al. 2016). *Laboulbenia camponoti* S.W.T. Batra is known in Bulgaria, Spain, Austria, Romania and Italy, on *C. universitatis* Forel, 1890; *C. pilicornis* (Roger, 1859); *C. sylvaticus* (Olivier, 1792) and *C. aethiops* (Latreille, 1798) (Báthori et al. 2014, Espadaler and Santamaria 2012; Gómez et al. 2016). *Rickia lenoirii* Santam. has been reported in Greece, France, Hungary and Romania on *Messor wasmanni* Krausse, 1910 and *M. structor* (Latreille, 1798) (Báthori et al. 2015b, Santamaria and Espadaler 2015). *Rickia wasmannii* Cavara (Fig. 1) is the most widely distributed ant-associated Laboulbeniales in Europe (Santamaria and Espadaler 2015). This species has been reported from England, France, Luxemburg, Germany, the Netherlands, Switzerland, Poland, Italy, Austria, Slovenia, the Czech Republic, Slovakia, Hungary, Romania, Spain, Belgium and Bulgaria, and parasitizes nine *Myrmica* Latreille, 1804 species: *M. gallienii* Bondroit, 1920; *M. rubra* (Linnaeus, 1758); *M. ruginodis* Nylander, 1846; *M. sabuleti* Meinert, 1861; *M. scabrinodis* Nylander, 1846; *M. slovacica* Sadil, 1952; *M. specioidea* Bondroit, 1918; *M. spinosior* Santschi, 1931 and *M. vandeli* Bondroit, 1920 (De Kesel et al. 2016, Haelewaters et al. 2015a, 2015b).

Ant species within the genus *Myrmica* can be found in several different kinds of habitats across European temperate zones, including meadows, steppes, woodlands, forests, and mountainous regions. Colonies of these ants can be either monogynous or polygynous and contain up to a few thousand workers (Radchenko and Elmes 2010, Czechowski et al. 2012). *Myrmica* ants can be hosts to many parasitic organisms from several taxonomic groups including ecto- and endoparasitic fungi and other microbial pathogens, such as: *R. wasmannii*; *Beauveria bassiana* (Bals.-Criv.) Vuill.; *Isaria fumosorosea* Wize; *Metarhizium anisopliae* (Metschn.) Sorokin; *Hirsutella stibelliformis* var. *myrmicarum* H.C. Evans & Groden; *H. subramanianii* var. *myrmicarum* H.C. Evans & J.F. Bischof; *Paraisaria myrmicarum* H.C. Evans, Groden & J.F. Bischof; *Ophiocordyceps myrmicarum* D.R. Simmons & Groden (Radchenko and Elmes 2010, Simmons et al. 2015, Witek et al. 2014). In particular, *M. scabrinodis* is common in Europe



Figure 1. *Myrmica scabrinodis* worker with *Rickia wasmannii* thalli (arrows indicate some thalli).

(Czechowski et al. 2012, Radchenko and Elmes 2010, Seifert 2007), frequently parasitized by *R. wasmannii* (Espadaler and Santamaria 2012) and other (social) parasites (Tartally 2008, Witek et al. 2014). *Myrmica scabrinodis* is a mesothermo- and mesohygrophilic species of different kinds of humid habitats, which is tolerant of soil moisture but avoids expressly xerothermal habitats. The abundance of both *R. wasmannii* and *M. scabrinodis* make them appropriate species for the study of host-Laboulbeniales interactions, as corroborated by a number of recent studies (Báthori et al. 2015a, Csata et al. 2014, Haelewaters et al. 2015a, 2015b, Markó et al. 2016, Witek et al. 2014).

Previous work has shown that different ant-fungal interactions can influence the behaviour of hosts in several ways (e.g., increased grooming and nest cleaning, secretion of antibiotics, pathogen avoidance, dispersal of infected individuals, and the relocation of an entire colony) (e.g., Csata et al. 2014, Roy et al. 2006, Oi and Pereira 1993). Laboulbeniales fungi are often present on the ant body with high thallus densities and the results of recent studies suggest that they could have both positive and negative effects on their hosts and are able to influence their behaviour (Báthori et al. 2015a, Csata et al. 2014, Konrad et al. 2015, Pech and Heneberg 2015). For example, Csata et al. (2014) showed that individuals of *M. scabrinodis* infected by *R. wasmannii* had significantly reduced lifespans under laboratory conditions; heavily infected individuals died significantly faster from starvation and spent more time consuming water than their more lightly parasitized counterparts. Starvation survival of *L. neglectus* workers infected by *L. formicarum* was also significantly decreased (Konrad et al. 2015), while host survival due to *Metarhizium* exposure increased. Ant workers with high thallus densities exhibited significantly longer periods of self-grooming as well as elevated expression of immune genes (Konrad et al. 2015).

To date, it has not been studied whether the boldness and aggressive behaviour differ between infected and uninfected *M. scabrinodis* workers. The behavioural characteristics of different ant workers could be relevant, for example, in terms of competition between infected and uninfected colonies. In this study, we evaluate the boldness and aggressive behaviour of uninfected *M. scabrinodis* workers versus their heavily infected counterparts.

Methods

Ant colony collection and laboratory conditions

We collected *Myrmica scabrinodis* colonies from two different regions in Hungary, including 12 from northern Hungary which comprised six which were infected from a habitat close to Rakaca (i.e., 48°27'N, 20°47'E, 170 m above sea level, a.s.l.) and six that were uninfected from an area close to Aggtelek (i.e., 48°26'N, 20°30'E, 340 m a.s.l.). We collected 12 further colonies from eastern Hungary, including six that were infected from close to Újléta (i.e., 47°26'N, 21°51'E, 120 m a.s.l.) and six that were uninfected from close to Csíkgát (47°25'N, 21°48'E, 110 m a.s.l.). We did not record any sites as part of this study that comprised both infected and uninfected colonies, so a standardisation of experiments per locality by choosing both infected and uninfected colonies from the same sites was unfortunately not possible. However, note that the two paired-sites were in a similar part of Hungary and from similar habitats and elevations, for sake to minimize the environmental effects on the populations. Thus, our total dataset includes 24 colonies, each of which contained fertilized queens and hundreds of workers, larvae, and pupae. To further reduce the effect of different sites, we kept all the ants used for this study for minimum two weeks in artificial lab nests under the same conditions: at $20 \pm 1^\circ\text{C}$ and provided sufficient food resources (i.e., fed with cockroaches twice a week and with a 33% honey water solution *ad libitum*). Plastic boxes treated with Fluon® on their inner walls to prevent ants from escaping were used as formicaria (i.e., length: 16.5 cm; width: 11.5 cm; height: 6 cm); inside these boxes, we created chambers (i.e., length: 5.5 cm; width: 4.5 cm; height: 1 cm) with plaster floors, covered with glass plates. In advance of each experiment, colonies were stored in the laboratory for a minimum of one month for acclimatisation (following Báthori et al. 2015a).

Boldness test

Boldness was tested using individual workers. We assessed their boldness by measuring how long it took for them to leave shelter (based on Gyuris et al. 2011). Prior to the test, 18 melanised workers were randomly selected from each colony (total $n = 432$). These mini test-colonies were fed a Bhatkar diet (Bhatkar and Whitcomb 1970). After 12 hours, single individuals were placed into different shelters in random order, inside new sterilized thin and plugged black plastic tubes (length: 60 mm; diameter: 5 mm) for an acclimatisation period of one minute (Gyuris et al. 2011, Spicer Rice and Silverman 2013) (Fig. 2a). After the removal of the plug, we measured the time that passed before individual ants emerged from the tubes, allowing a maximum waiting time of three minutes (see Báthori et al. 2015a).

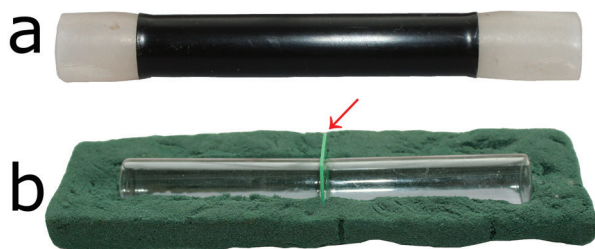


Figure 2. Tubes used for **a** boldness and **b** aggression tests. Arrow indicates the flap which was removed at the beginning of the test.

Aggression test

In this experiment, 120 worker pairs, including one infected and one uninfected melanised worker, were randomly selected from both geographic regions. Worker pairs from the two different geographical regions, northern and eastern Hungary were tested separately, and two facing glass tubes (length: 53 mm; diameter: 15 mm), separated by a thin plastic flap, formed the experimental arena (Fig. 2b). After an acclimatisation period of 1 minute (Spicer Rice and Silverman 2013), the flap was removed so that the infected and uninfected workers could meet. The number of different behaviour patterns was registered for a period of 3 minutes (like in Báthori et al. 2015a). The following categories were used: initiation of aggressive behaviour, antennation, mandibular threats, biting, stinging, autogrooming and allogrooming (see Maák et al. 2014 and references therein). At the end of the experiment, the ants were preserved in a solution of 67.5% ethanol, and the number of fungal thalli was counted for statistical analyses (see below) under a Leica MZ12.5 stereomicroscope at magnifications of 10-160x.

Statistical analyses

Statistical analyses were performed using the R statistical software (ver. 3.0.2, R Core Team 2013). Data was mainly analysed using generalized linear mixed-effects models (GLMM) of the R-package “lme4” (Bates et al. 2014), because these types of models offer a range of ways to handle non-Gaussian error distributions and random effects. In GLMMs of significant correlations we acquired conditional R^2 of the model using the R-package “MuMIn” (Barton 2013).

To assess whether there was significant difference between infected and uninfected workers in terms of their probability to leave shelter, we applied a binomial GLMM, specifying leaving shelter as binary response (1 meaning the individual left shelter), and infection as binary predictor factor (1 meaning the individual was infected). We also tested if infected and uninfected workers showed significant differences in their latency before leaving shelter by fitting a mixed-effects Cox regression model in the R-package

‘coxme’ (Therneau 2016). In this model, time latency before leaving the shelter was defined as follow up time, while the event of leaving was specified as the status indicator event, and infection was a binary predictor factor variable. To test whether the number of thalli on infected workers affected the time latency before leaving shelter, we fitted a log-linked Gamma GLMM, because this model is able to handle Gamma-distributed time data. When fitting this Gamma GLMM, we excluded observations from uninfected workers and defined the number of thalli as predictor, while using time latency before leaving shelter as response variable. In all the models described above, the time-block of measurements and the habitat of origin were specified as random factors.

In order to measure aggressiveness, we calculated an index based on Martin et al. (2009), dividing the number of observed aggressive behaviours (mandible threat, biting, stinging) by the total number of interacting behaviours (mandible threat, biting, stinging, antennae-interaction, and allogrooming). This index represent the proportion of aggressive behaviours in the overall observed and recorded interactive behaviours. When testing whether infection has a significant effect on the proportion of aggressive behaviours, we fitted a binomial GLMM, with infection as the predictor factor, and calculated aggression index as dependent variable. To see if infected or uninfected workers were more likely to initiate aggressive interactions, we used a different binomial GLMM that incorporated infection as a predictor, and initiation (binary variable, 1 meaning that the given ant was the first to perform aggressive behaviour in the given trial) as a dependent variable. In the two models described above, the ID of tested pairs and habitat of origin were specified as random factors. Furthermore, we tested whether, the number of thalli had any effect on the proportion of aggressive behaviour by fitting a binomial GLMM (excluding all uninfected ants) with aggression index as the dependent and the number of thalli as the predictor variable. In this model, habitat of origin was also specified as a random factor.

Results

Boldness

Infected ants did not differ from uninfected ones in their probability of leaving shelter (binomial GLMM: $z = -0.99$; $p = 0.318$). However, infected workers were significantly slower to leave shelter, i.e. showed higher latencies before leaving (mixed-effects Cox-model: $z = -2.13$; $p = 0.033$) (Fig. 3). The number of thalli on infected workers did not affect significantly the time latency before leaving shelter (log-linked Gamma GLMM: $z = -0.33$; $p = 0.744$).

Aggression

Infection alone does not exert a significant effect on the proportion of aggressive behaviours (binomial GLMM: $z = -0.41$; $p = 0.68$). Infected workers were, however,

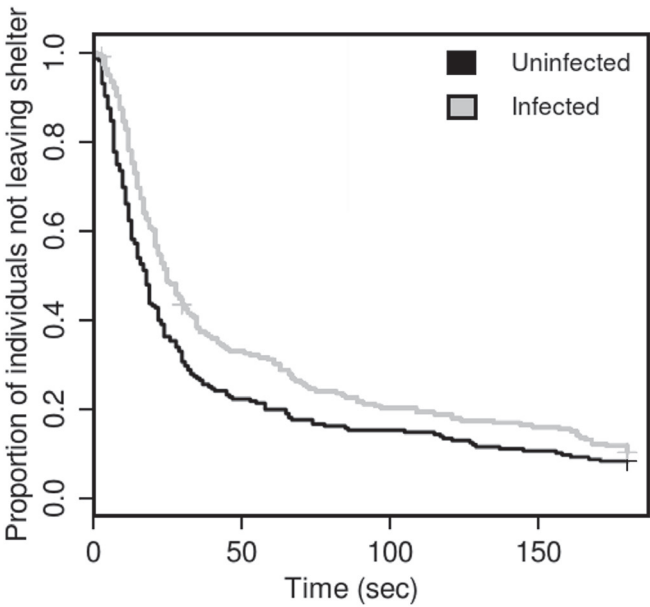


Figure 3. Time latency until leaving shelter in case of uninfected (N=215) and infected (N=215) workers.

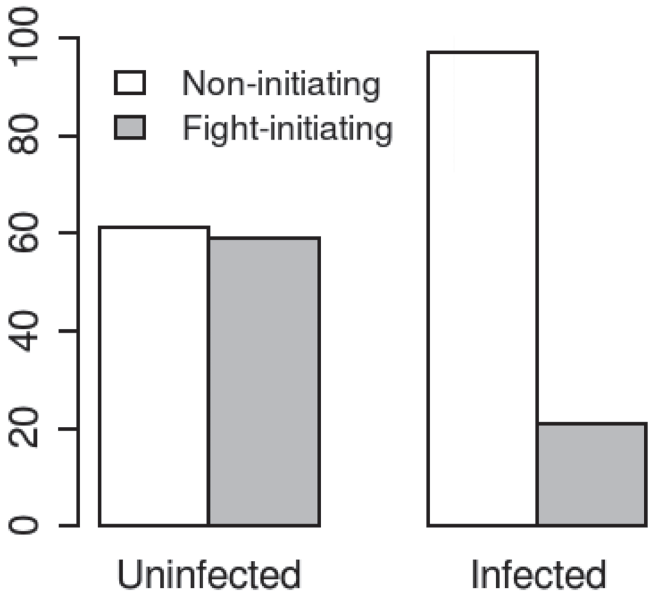


Figure 4. Probability if initiating aggressive behaviour in cases of uninfected (N=119) and infected (N=119) workers.

significantly less likely to initiate aggressive behaviour than uninfected ants (binomial GLMM: $z = -2.91$; $p = 0.004$; conditional $R^2 = 0.23$) (Fig. 4). Interestingly, the number of thalli on infected workers nevertheless seems to exert a mild, but significantly positive effect on the proportion of aggressive behaviours (binomial GLMM: $z = 2.85$; $p = 0.005$; conditional $R^2 = 0.50$).

Discussion

On the basis of our experimental results, *R. wasmannii* seems to have a considerable effect on the behaviour of *M. scabrinodis* workers with regards to boldness and interactions with uninfected workers. Since infection by the fungus appears to be permanent (Haelewaters et al. 2015c, AT and FB pers. observ. on long-term reared laboratorial colonies), this could lead to persistent differences in the ants' behaviour in a given colony (De Kesel et al. 2016). It is worth noting that when a colony is infected, the vast majority of its workers are also infected (Markó et al. 2016; AT and FB, pers. observ.), and the infection therefore could have effects not only on the individual but also on the colonial level. Thus, infection by *R. wasmannii* possibly has long-term effects on the performance of infected individuals and colonies in natural habitats. It should be noted here that we had the possibility to work with populations with either infected or uninfected colonies from slightly different sites (see Methods for details). Thus, it would be important to follow similar experiments on infected and uninfected colonies collected from the same site, if such sites would be recorded in the future to confirm that our results are not affected by habitat-driven differences of maternal colonies, although it is here unlikely. It should be also emphasized that so far all results about negative and positive interactions were reached in the course of laboratory experiments, so additional experiments are needed to conduct with the ant hosts in nature.

We propose the following hypotheses regarding the increased latencies in time of leaving shelter in the case of infected workers: (1) they may have been in poorer condition and physically weak or impaired, e.g. the chances of survival of infected ants were lower under starvation (Konrad et al. 2015) and lab conditions (Csata et al. 2014); and/or (2) the fungus induced the host to remain in the shelter tube for a longer period of time. If the latter were true, this would mean that *R. wasmannii* would induce behavioural changes. It should be noted that Csata et al. (2017) did not found any differences in the locomotion of infected worker specimens compared with the uninfected ones. Thus, the infected ants did not left the tube later because of locomotion but rather because of other behavioural changes (i.e. boldness). The mechanisms causing infected ants to stay longer in the shelter could be of importance, and should be the subject of future study.

On the other hand, *R. wasmannii*, as an ectosymbiotic fungus, could be expected to be capable of spreading not only within but also between ant colonies. If increased worker activity (outside of the nest) would increase the spreading success of the fungus, one may expect infected ants to exhibit a higher degree of out-of-nest activity (Bos et al. 2012, sensu Hughes et al. 2011). Where a host forages more outside and fights, this would have a positive effect on the spread of the fungus compared to the behaviour of a submissive host who stays inside the nest. Furthermore, local competitive disadvantages that influence infected colonies (see: Báthori et al. 2015a, Csata et al. 2014) in a given habitat might be reduced by spreading the infection to local rival colonies. However, our results show that the infected *M. scabrinodis* workers do not behave in these ways. Thus, the intercolonial spread of *R. wasmannii* caused by individual foragers might be rare and occasional. It would need further research to check whether the fungus rather spreads by queens or by

whole colony buddings, a phenomenon that is well-known in *Myrmica* ants (Radchenko and Elmes 2010). Spreading mainly by colony budding would suggest an extreme host specialisation of the fungus on the host ant colonies. Such an extreme host colony specialization is recorded in the case of at least one ant-parasitic species [the social parasite *Microdon mutabilis* (Linnaeus, 1758) hoverfly (Diptera: Syrphidae); see Schönrogge et al. 2006], but since currently no information is available on the genetic relationships among different *R. wasmannii* populations, it is impossible to determine whether or not fungus lineages are similarly adapted genetically to the different host ant colonies (and thus infecting genetically distant host colonies would not be effective). Furthermore, we cannot exclude the possibility that the spreading of the fungus requires the specific microhabitat provided by the ant nest [compare to the ecological specificity of *R. wasmannii*, (Pfliegler et al. 2016)], e.g. because the ascospores of *Rickia* ejected from mature thalli are extremely vulnerable to desiccation outside the ant nest microhabitat. Such hypothetical constraints on the spreading of the infection could actually act in favour of the observed behavioural effects on the host, or at least may account for the lack of negative selection regarding less active workers. In this sense, the poorer general condition of the infected host that results in less active behaviour would mean an advantage for the fungus.

For a long time, there was no knowledge of the effects of ant-associated Laboulbeniales fungi on the behaviour of their hosts, but recent research (Báthori et al. 2015a, Csata et al. 2014, Konrad et al. 2015) has begun to fill this gap. These studies revealed behavioural changes in the ant hosts, such as increased water consumption (Báthori et al. 2015a) and intensified grooming (Csata et al. 2014). Based on our current results, infection might negatively affect also the workers' behaviour linked to competitive abilities, since remaining inside and behaving submissively is not effective when there is significant competition for resources.

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