Univerzita Hradec Králové

Přírodovědecká fakulta

Katedra biologie

Hálky zelenušek rodu *Lipara* (Diptera: Chloropidae) jako hnízdiště žahadlových blanokřídlých (Hymenoptera: Aculeata)

Disertační práce

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Studijní program: Biologie D

Studijní obor: Aplikovaná biologie a ekologie (DR-BI)

Vedoucí práce: Doc. Mgr. Petr Bogusch, Ph.D.

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Zadání dizertační práce

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Název práce:	Hálky zelenušek rodu *Lipara* (Diptera: Chloropidae) jako hnízdiště žahadlových blanokřídlých (Hymenoptera: Aculeata)
Název práce v AJ:	Cigar galls of *Lipara* (Diptera: Chloropidae) flies as nest sites of aculeate Hymenoptera (Hymenoptera: Aculeata)
Cíl a metody práce:	Cílem práce je studium žahadlových blanokřídlých hnízdících v hálkách zelenušek rodu *Lipara* na rákosu obecném. V rámci tohoto projektu budou zpracovány seznamy druhů využívajících tyto hálky jako hnízdní dutiny se zaměřením na specializaci konkrétních druhů na hálky a různé typy mokřadů. Proběhne i studium morfologie hnízd a maturních larev jednotlivých druhů, závislosti druhů na znečištění prostředí, a marginálně i výzkum dalších bezobratlých, kteří využívají hálky zelenušek. Kromě toho se bude práce týkat i parazitoidů zelenušek a blanokřídlých v hálkách a jejich vazbou na hostitele.
Garantující pracoviště:	katedra biologie Přírodovědecké fakulty UHK
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Oponent:	prof. RNDr. Zdeněk Laštůvka, CSc.
Oponent:	Mgr. Jakub Straka, Ph.D.
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Prohlášení:

Prohlašuji, že jsem dizertační práci vypracovala samostatně a že jsem v seznamu použité literatury uvedla všechny prameny, z kterých jsem vycházela.

V Hradci Králové dne 7. 4. 2017 Mgr. Alena Astapenková

Poděkování:

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Anotace:

ASTAPENKOVÁ, A. *Hálky zelenušek rodu Lipara (Diptera: Chloropidae) jako hnízdiště žahadlových blanokřídlých (Hymenoptera: Aculeata)*. Hradec Králové, 2017. Dizertační práce na Přírodovědecké fakultě Univerzity Hradec Králové. Vedoucí dizertační práce Petr Bogusch, 33 s.

Cílem této práce bylo studium žahadlových blanokřídlých hnízdících v hálkách zelenušek rodu *Lipara* na rákosu obecném. Tímto výzkumem se zabýváme od roku 2013 dosud. V rámci tohoto projektu byly zpracovány seznamy druhů využívajících tyto hálky jako hnízdní dutiny se zaměřením na specializaci konkrétních druhů na hálky a různé typy mokřadů (např. přirozené rákosiny v mokřadních rezervacích versus rákosiny na post-industriálních stanovištích). Celkem bylo zaznamenáno 46 druhů žahadlových blanokřídlých asociovaných s rákosovými hálky, z nichž 37 druhů využívá tyto hálky k hnízdění, dále bylo zjištěno šest parazitoidů z čeledi Chrysididae a tři kukaččí včely rodu *Stelis*. Nejdominantnějším zaznamenaným druhem v hálkách byl stopčík *Pemphredon fabricii*, který se na hnízdění v hálkách specializuje. U tohoto druhu byl popsán nový typ progresivního krmení larev a byly zjištěny hlavní druhy plísní, které způsobují úhyn larev tohoto i dalších druhů. Dále byla popsaná struktura hnízd u 13 druhů žahadlových blanokřídlých a byl zhotoven popis 16 maturních larev žahadlových blanokřídlých a také larvy parazitické černule *Thyridanthrax fenestratus*. Byl sestaven seznam druhů bezobratlých živočichů, zejména hmyzu, kteří využívají rákosové hálky jako zdroj potravy, hnízdiště, úkryt či jako místo pro přečkání nepříznivých podmínek.

Klíčová slova:

diverzita, bioindikace, maskonoska *Hylaeus pectoralis*, hnízdní kleptoparazit, *Penicillium buchwaldii*

Annotation:

ASTAPENKOVÁ, A. *Cigar galls of Lipara (Diptera: Chloropidae) flies as nest sites of aculeate Hymenoptera (Hymenoptera: Aculeata)*. Hradec Králové 2017. Dissertation at Faculty of Science University of Hradec Králové. Dissertation Supervisor Petr Bogusch, 33 p.

The aim of this study were aculeate Hymenoptera nesting in cigar galls of *Lipara* flies on common reed. We are working on this project from the year 2013 till now. Within this project, list of all species using these galls as nest cavities was prepared and specialization of every species was evaluated. Also the difference between inhabiting of natural reed beds in wetland reservation versus reed beds in post-industrial sites was evaluated. Forty six species of Hymenoptera: Aculeata are known to be associated with reed galls, of which 37 make their nests there, and the other are six parasitoids of the family Chrysididae and three cuckoo bees of the genus *Stelis*. The most dominant species recorded was crabronid wasp *Pemphredon fabricii*, the specialist on nesting in reed galls. We have described new type of progressive provisioning as typical parental behaviour of this species and also the main fungal species with significant negative effect on its brood. The nest structure of 13 species was recorded and also the description of mature larvae of 16 species and one parasitic fly *Thyridanthrax fenestratus* was described. The list of all invertebrate species, particularly the insect species, using the cigar galls for food source, nest site, shelter or place for overwintering was compiled.

Key words:

diversity, bioindication, *Hylaeus pectoralis*, nest cleptoparasite, *Penicillium buchwaldii*

OBSAH

3.5 ASTAPENKOVÁ A., HENEBERG P. & BOGUSCH P. 2017: Larvae and Nests of Aculeata Hymenoptera (Hymenoptera: Aculeata) Nesting in Reed Galls Induced by *Lipara* spp. (Diptera: Chloropidae) with a Review of Species Recorded. Part II. *PLoS ONE* **12**: e0169592.

3.6 BOGUSCH P., HAVELKA J., ASTAPENKOVÁ A. & HENEBERG P. 2017: New type of progressive provisioning as a characteristic parental behavior of the crabronid wasp Pemphredon fabricii (Hymenoptera: Crabronidae). *Ethology*, *Ecology & Evolution*, accepted.

1 ÚVOD

Úvod k této dizertační práci obsahuje rešerši o druzích bezobratlých v rákosových hálkách zelenušek rodu *Lipara* se zaměřením na žahadlové blanokřídlé, kteří tyto hálky využívají k hnízdění. Úvodní rešerše je vyčerpávají, ale nejsou v ní podrobně opakovány detailní informace, které jsou obsažené v přiložených článcích.

Teoretická část zahrnuje 6 podkapitol. První kapitola shrnuje základní a obecné informace o hálkách, jejich charakteru a původcích, se zaměřením na hmyz.

Ve druhé kapitole jsou uvedeny druhy zelenušek vyskytující se na našem území, a jsou komentovány rozdíly mezi dospělci a rozdíly mezi hálkami, které tyto zelenušky vytvářejí. Je zmíněno, které z hálek jsou vhodné pro hnízdění žahadlových blanokřídlých.

Další kapitola shrnuje využívání rákosových hálek bezobratlými živočichy, zejména hmyzem. Hálky slouží jako zdroj potravy, úkryt, hnízdiště, místo pro přezimování či přečkání nepříznivých podmínek. Hálkoví inkvilíni slouží jako hostitelé pro parazitoidy či hnízdní kleptoparazity. Tato kapitola přináší i informace o nejpočetnějších druzích bezobratlých, které v hálkách najdeme.

Další kapitola představuje všechny druhy žahadlových blanokřídlých, kteří hálky využívají k hnízdění či k parazitaci. Tyto druhy jsou rozděleny na specialisty na rákosové hálky, mokřadní druhy, které rákosové hálky využívají pouze příležitostně, a všudypřítomné druhy, které hnízdí v rozličných typech dutin včetně rákosových hálek. V kapitole jsou uvedeny i některé zajímavosti z výsledků našich průzkumů.

V poslední kapitole je uveden význam těchto druhů využívajících rákosové hálky v ochraně přírody. V rámci našeho výzkumu se věnujeme ochranářským aspektům, které souvisí s mokřadními druhy žahadlových blanokřídlých, proto je zde tato kapitola zařazená.

K práci je přiloženo šest článků publikovaných v časopisech s impakt faktorem, z toho pět vyšlých a jeden přijatý (accepted). Článek Heneberg et al. (2014): Biological Conservation zahrnuje výsledky sběru hálek z 15 přirozených a 15 post-industriálních stanovišť, které byly umístěny do speciálních líhňových pytlů, vynalezených k tomuto studiu. V letním období byly na studované lokality umístěny barevné misky. Tímto

výzkumem bylo zjištěno 183 druhů včel a vos vázaných na rákosiny a rákosové hálky, z nichž 14 druhů lze považovat za hálkové inkvilíny. Výsledky studie ukazují na to, že přirozené rybniční rákosiny jsou druhově chudší, jak co se týká počtu druhů hnízdících v hálkách, tak počtu druhů celkově. Najdeme zde však některé specializované mokřadní druhy, které jsou na post-industriálních stanovištích velmi vzácné nebo tam chybí.

Článek Bogusch et al. (2015): PLoS ONE přináší výsledky studia 6 018 rákosových hálek, které byly získány v zimním období z 34 lokalit šesti zemí střední Evropy. Na každé lokalitě bylo sebráno 500 hálek, z nichž 200 bylo podélně rozstřiženo, a zbytek se ponechal vylíhnutí. Uvádíme podrobné popisy hnízd sedmi dominantních druhů a popisy maturních larev u čtyř druhů žahadlových blanokřídlých a dvou parazitoidů z rákosových hálek.

Článek Heneberg et al. (2016): Journal of Invertebrate Pathology shrnuje výsledky studia 5 200 jedinců (12 druhů) maturních larev žahadlových blanokřídlých nebo jejich parazitoidů z rákosových hálek sebraných na 34 lokalitách střední Evropy. Zaznamenali jsme plísňové porosty na exoskeletonu 83 larev a kukel. Určili jsme tři druhy plísní: *Penicillium buchwaldii* (72 % případů), *Aspergillus pseudoglaucus* (22 %) a *Penicillium quebecense* (6 %), které mají negativní dopad na larvy studovaných druhů žahadlových blanokřídlých. U všech těchto druhů nebylo zatím popsáno entomopatogenní chování, naše studie je tedy první, která to prokázala.

Článek Bogusch et al. (2016): Biodiversity Conservation rozlišuje možnosti využití rákosových hálek bezobratlými živočichy, zejména hmyzem. Analyzovali jsme společenství bezobratlých z 17 791 hálek zelenušek rodu *Lipara* získaných v zimních měsících na 30 lokalitách České republiky (15 přirozených rákosin a 15 post-industriálních). Zaznamenali jsme 18 druhů z červeného seznamu a čtyři nové druhy pro Českou republiku. V hálkách byli zaznamenáni především pavouci, ploštice, brouci, blanokřídlí a dvoukřídlí, ale v malém množství i zástupci chvostoskoků a některých dalších hmyzích řádů. Výsledky ukazují podobné jako článek Heneberg et al. (2014) pro žahadlové blanokřídlé, přirozená stanoviště jsou druhově chudší, ale obohacená o specializované, citlivé druhy.

Článek Astapenková et al. (2017): PLoS ONE shrnuje výsledky studia 20 704 rákosových hálek, ze kterých 9 446 bylo podélně rozstřiženo a zbylých 11 258 hálek

bylo ponecháno vylíhnutí ve speciálních líhňových pytlích v laboratorních podmínkách. Zaznamenali jsme osm druhů žahadlových blanokřídlých, kteří dosud nebyli z rákosových hálek známí. Přinášíme popisy hnízd sedmi druhů a morfologii maturních larev osmi druhů žahadlových blanokřídlých, dvou parazitoidů a jednoho hnízdního kleptoparazita. Studie přináší souhrn všech druhů žahadlových blanokřídlých známých z rákosových hálek, a předběžné rozdělení druhů hnízdících v hálkách podle preferencí.

Článek Bogusch et al. (2017): Ethology, Ecology & Evolution přináší nové informace o nejdominantnější kutilce *Pemphredon fabricii* hnízdící v rákosových hálkách zelenušek rodu *Lipara*. Je zjištěno, že tato kutilka má dvě generace za rok, je podrobně popsáno chování hnízdící samice, která v hálce nevytváří plodové komůrky namísto toho hálkovou dutinu vyplní paralyzovanými mšicemi. Je popsaný nový typ progresivního krmení larev u tohoto druhu, a také analyzováno spektrum druhů mšic, kterým jsou krmeny larvy. Studie přináší informace o všech známých parazitech a predátorech larev této kutilky.

2 TEORETICKÁ ČÁST

2.1 Hálky a hálkotvorné organizmy

Hálky (cecidie) jsou definované jako patologicky vyvinuté buňky, pletiva nebo orgány rostliny, které vznikly hypertrofií (zvětšením buněčné velikosti) anebo hyperplazií (zmnožením počtu buněk) jako výsledek stimulace cizího organizmu (Gullan & Cranston 2010). Habitus hálky je specifický pro původce. Hálky mohou být indukované viry, bakteriemi, houbami, hlísticemi, roztoči, avšak nejčastěji hmyzem (Gullan & Cranston 2010, Skuhravá & Skuhravý 2010).

Hálky jsou atypické rostlinné novotvary, které slouží jako zdroj potravy, úkryt a ochrana pro hálkotvorný hmyz (Shorthouse et al. 2005, Křístek & Urban 2013). Hálkotvorný hmyz pomocí chemických stimulů mění rostlinnou morfologii a fyziologii ke svému prospěchu (Sopow et al. 2003). Podněcuje buňky rostlinného pletiva k množení a napadené buňky zvětšují svůj objem (Skuhravá & Skuhravý 2010). Vznik a charakter hálky je rozličný u různých původců. Hálky vytvořené žlabatkou růžovou (*Diplolepis rosae*) vyrůstají nejčastěji na listu, na řapíku nebo na mladém výhonu, jsou porostlé dlouhými, bohatě rozvětvenými zelenavými, červenavými nebo žlutavými chlupy. Z hálky často vyčnívají nezměněné nebo jen mírně pozměněné listy (Zahradník 1987). Formování hálky vytvořené zelenuškou *Lipara lucens* začíná žírem larvy na zbytcích listů, mimo vlastní vyvíjející se hálku. Během růstu hálky je zastaveno prodlužování internodů. Vnitřní parenchym hálky slouží jako vyživující pletivo pro larvu. Pletivo se cylindricky obtočí okolo hálkové dutiny, která se trojnásobně zvětší. Když je hálka kompletní, larva se prokouše skrz růstový vrchol a vstoupí do hálkové dutiny, kde se živí nutritivním pletivem. V tento moment začíná sklerenchymatický proces za vzniku extrémně tvrdé hálky (Nartshuk & Andersson 2013). Tvrdá sklerenchymatická pletiva chrání larvu před predátory a parazitoidy (Vavřenová 2015). Výsledná hálka brání rostlině v kvetení (Else 1995).

Hálkotvorné organizmy tvoří hálky na všech částech rostlin: na kořenech, stoncích, listech, květních i listových pupenech, květenstvích, plodech i semenech. Hálkotvorný hmyz vyhledává hlavně mladé rostliny a růstové vrcholy, jejichž pletiva jsou měkká a vhodná pro proděravění kladélkem při kladení vajíček, nebo jsou snadno prostupné pro právě vylíhlé larvy (Skuhravá & Skuhravý 2010).

Hálkotvorné organizmy mohou být považovány za „inženýry mikrohabitatu“, protože jimi vytvořené hálky jsou vlastně mikrohabitatem, který mohou využívat býložravé nebo všežravé organizmy, které se přímo neživí původci hálek. Takové organizmy, které cílí na pletiva hálek raději než na původce hálek, žijí uvnitř hálek a živí se jimi, obecně nazýváme inkvilíny (Sanver & Hawkins 2000). Opuštěné hálky jsou dále využívány jako úkryt, hnízdní dutina nebo dutina ke kuklení dalšími bezobratlými živočichy, zejména hmyzem (Bogusch et al. 2016).

Na celém světě nalezneme přibližně 13 000 hmyzích druhů vytvářejících hálky na rostlinách (Sanver & Hawkins 2000, Stone & Schönrogge 2003, Shorthouse et al. 2005), v Evropě pak 5 000–6 000 hmyzích druhů tvořících hálky, z pěti řádů: dvoukřídlí (Diptera), blanokřídlí (Hymenoptera), stejnokřídlí (Hemiptera), brouci (Coleoptera) a motýli (Lepidoptera) (Skuhravá & Skuhravý 2010).

Z blanokřídlého hmyzu jsou to především žlabatky (Cynipidae), které vytvářejí jedny z vizuálně nejnápadnějších a konstrukčně nejsložitějších hálek na rostlinách. Mezi běžné a známé druhy patří například žlabatka růžová (*Diplolepis rosae*) vytvářející nápadné hálky na růžích (*Rosa*) a žlabatka listová (*Cynips quercusfolii*), která vytváří tzv. duběnky na listech dubu (*Quercus*) (Csóka et al. 2005, Hayward & Stone 2005). Na větvích vytváří hálky třeba žlabatka dubová (*Andricus kollari*), na růstových vrcholcích žlabatka bezkřídlá (*Biorhiza pallida*). Z dvoukřídlého hmyzu jsou významnými původci hálek bejlomorky (Cecidomyiidae). Mezi jedny z nejznámějších hálek vytvořených na listech stromů patří špičaté červené hálky na buku lesním (*Fagus sylvatica*), jejichž původcem je bejlomorka buková (*Mikiola fagi*) (Kampichler & Teschner 2002). Ze stejnokřídlého hmyzu jsou známé hálky dutilek (*Pemphigus*) na listech topolů (*Populus*) a hálky korovnic (*Adelges*) na větvích smrku (*Picea*). Drobná mšice révokaz (*Viteus vitifoliae*) je původcem hálek na listech a kořenech révy vinné (*Vitis vinifera*). Z roztočů jsou známými původci hálek vlnovníci (Eriophyidae), kteří vytvářejí hálky na listech stromů a keřů (Skuhravá & Skuhravý 2010).

2.2 Zelenušky rodu *Lipara* Meigen, 1830

Vědě je známo 11 druhů zelenušek rodu *Lipara* Meigen, 1830. Jedná se o monofágní druhy, které se vyvíjejí všechny na rákosu obecném (*Phragmites australis*) (Grochowska 2006a, 2006b, 2007, 2013). V České republice se vyskytují čtyři druhy zelenušek, a to *Lipara lucens* Meigen, 1830, *Lipara pullitarsis* Doskočil & Chvála 1971, *Lipara rufitarsis* Loew, 1858 a *Lipara similis* Schiner, 1854 (Kubík 2006, Heneberg et al. 2014). Pátým a posledním evropským druhem zelenušky je *Lipara baltica* Karps, 1978 (Dely-Draskovits et al. 1994), vyskytující se například v Lotyšsku (Karpa 2001).

Samice zelenušek rodu *Lipara* kladou vajíčka na rákosové výhonky, na kterých se vylíhne první larvální instar, který se živí na nově vznikajících listech. Larvy zelenušek vstupují do hálky v momentu, kdy je kompletně vytvořená. Larvy zelenušek *Lipara lucens* a *L. rufitarsis* se prokousají skrz růstový vrchol a dokončují svůj životní cyklus uvnitř hálky, zatímco larvy zelenušky *L. pullitarsis* nikdy neprocházejí skrz růstový vrchol a mohou být nalezeny mezi zbytky listů na povrchu hálky (De Bruyn 1994). Zelenušky *Lipara lucens* a *L. rufitarsis* se specializují na tenčí stébla rákosu o průměru ≤4,5 mm, zatímco *L. pullitarsis* a *L. similis* preferují silnější stébla o průměru 5-7 mm (Tscharntke 1992). Rostliny rákosu, které trpí nedostatkem živin, toxickým znečištěním, fragmentací stanoviště, nebo umístěním na sušším okraji rákosiny, hostí všechny čtyři druhy zelenušek rodu *Lipara*, zatímco rozlehlé rákosiny, zejména v místech, kde rákos roste z vody, hostí převážně druhy *L. pullitarsis* a *L. similis* (Tscharntke 1992).

Dospělci zelenušky *Lipara lucens* jsou velké a robustní druhy. Jedná se o největší druh tohoto rodu, měří 5.5-8 mm. Mají tmavé nohy a černé tělo, které je hustě pokryto dlouhými nažloutlými chlupy uspořádanými na scutu v podélné pruhy. Tykadla mají nažloutlé bazální články (Nartshuk & Andersson 2013). Maturní larvy jsou 6.9-12 mm dlouhé, mléčně bílé nebo světle nažloutlé, a živí se měkkými parenchymatickými pletivy v internodech hálky pod růstovým vrcholem (Häffliger 2007, Grochowska 2013). V hálce je přítomna vždy pouze jedna larva (Kubík 2006). Larvy způsobují zkracování a zesilování nově se vyvíjejících internodů. Výsledná hálka se obvykle skládá z 10 až 13 zkrácených a silně lignifikovaných internodů. Na Obr. 1 je vidět patrný rozdíl mezi novou a rok starou hálkou. Plně vyvinutá maturní larva na konci léta vstupuje do diapauzy a následně přezimuje. Následující jaro se larva kuklí. Dospělci se líhnou od konce května do začátku června (Nartshuk & Andersson 2013).

Obr. 1. Hálky zelenušky *Lipara lucens*: a - nová hálka, b - stará hálka.

Dospělci zelenušky *Lipara pullitarsis* jsou černé, lesklé, robustní druhy, ale znatelně menší než zelenušky *L. lucens*. Scutum mají rovnoměrně a hustě pokryto nažloutlými chlupy, neuspořádanými v pruhy. Tykadla jsou černá, bazální články jsou někdy hnědé. Obličejová maska je širší než u druhu *L. lucens*. Nohy jsou černé se žlutými bázemi chodidel (Nartshuk & Andersson 2013). Maturní larvy jsou 6-9 mm dlouhé, světle nažloutlé. Živí se vždy nad růstovým vrcholem, zejména na silnějších stéblech (v průměru větších než 4 mm). Zelenuška *L. pullitarsis* vytváří měkké hálky, které neobsahují ztvrdlou hálkovou dutinu (Nartshuk & Andersson 2013). Růstový vrchol není prokousán (Grochowska 2006b, Häffliger 2007). V každé hálce se nachází pouze jedna larva (Kubík 2006). Larvy způsobují zkracování a zesilování nově se vyvíjejících internodů. Výsledná hálka se skládá z pěti až šesti zkrácených internodů, ve kterých larvy neminují (Häffliger 2007).

Dospělci zelenušky *Lipara rufitarsis* jsou černé, lesklé, robustní druhy, ale znatelně menší než zelenušky *L. lucens*. Scutum mají rovnoměrně a hustě pokryto bělavými chlupy, neuspořádanými v pruhy. Tykadla jsou žlutá, bazální články jsou někdy hnědé. Obličejová maska je rovnější než u druhu *L. pullitarsis*. Nohy jsou černé se zářivě žlutými chodidly (Nartshuk & Andersson 2013). Maturní larvy jsou 6-9 mm dlouhé, světle nažloutlé. Larvy se živí v hálkových internodech pod růstovým vrcholem, zejména v tenčích stéblech (Grochowska 2007, Häffliger 2007). V každé hálce se nachází pouze jedna larva (Kubík 2006). Larvy způsobují zkracování a zesilování nově se vyvíjejících internodů. Výsledná hálka je často zploštělá a skládá se z pěti až šesti zkrácených internodů. Vytvořené hálky tímto druhem jsou většinou užší oproti hálkám vytvořeným zelenuškou *L. lucens*, ale někdy jsou od sebe k nerozeznání (Nartshuk & Andersson 2013). Tento druh je spojován s nově vzniklými rákosinami (Bogusch et al. 2016).

Dospělci zelenušky *Lipara similis* jsou velmi podobní zelenušce *Lipara lucens*. Na rozdíl od *L. lucens* má tento druh scutum pokryto bělavými až stříbřitými chlupy uspořádanými do zřetelných podlouhlých pruhů, což vytváří rýhovaný vzhled. Jedná se o nejmenší druh, měří 3.3-4.6 mm (Chvála et al. 1974). Maturní larvy zelenušky *Lipara similis* jsou 5.5-10 mm dlouhé, lesklé, světle nažloutlé, s tmavými sklerotizovanými konci, jsou vždy nad růstovým vrcholem (Grochowska 2006a, Häffliger 2007). Ve štíhlé a drobné hálce je přítomna vždy jen jedna larva (Kubík 2006). Larvy způsobují zkracování pouze dvou až čtyř internodů, ve kterých larvy nikdy neminují. Kvůli žíru larev je nejvyšší list zkrácený a odumírá a jeden až dva vrchní listy jsou roztřepené. Výsledná hálka není ztlustlá a skládá se ze dvou zkrácených internodů, dvě nejvyšší listová pouzdra vytváří dutinu nad růstovým vrcholem, kde se larva živí (Häffliger 2007). Tento druh je spojován s dobře vyvinutými rákosinami, které jsou často více eutrofní a dovolují tak vzniku vyšších a silnějších stébel rákosu obecného (Bogusch et al. 2016).

V rákosině se můžeme také setkat s hálkami, které jsou vytvořeny jinými organismy, jako jsou další druhy zelenušek jiných rodů nebo například viry, viz Obr. 2. Jimi vytvořené hálky ovšem nejsou vhodné pro hnízdění žahadlových blanokřídlých, kvůli malým rozměrům, neztlustlému charakteru a absenci dutiny uvnitř.

Obr. 2. Hálky na rákosu obecném vytvořené viry.

2.3 Hálky jako úkryt či hnízdiště

Monotypické rákosiny slouží jako důležitý biotop pro mnoho ohrožených obratlovců a hostí rozmanitá společenstva bezobratlých živočichů (Schmidt et al. 2005, Bogusch et al. 2016). Členovci využívají rákos obecný jako zdroj potravy (minující druhy v aerenchymu, druhy živící se listy a pylem) nebo také jako hnízdiště, úkryt či místo pro přezimování (druhy minující ve stéblech, producenti hálek, inkvilíni) (Bogusch et al. 2016).

Hálky vytvořené zelenuškami rodu *Lipara* využívá rozmanité spektrum bezobratlých živočichů. Larvy zelenušek slouží jako hostitelé pro parazitoidy, jako jsou například dva druhy specializovaných lumčíků *Polemochartus liparae* a *Polemochartus melas* nebo lumek *Exeristes arundinis*. Specialistou je i chalcidka ze skupiny krásenek *Stenomalina liparae* (De Bruyn 1994, Nartshuk 2006), v hálkách jsou pak velmi početné chalcidky čeledi Eulophidae *Aprostocetus orithyia* a *Tetrastichus legionarius* a drobný druh čeledi Aphelinidae *Centrodora amoena* (Bogusch et al. 2016). Bogusch et al. (2016) uvádějí, že se z bezobratlých živočichů v hálkách vyskytují zástupci skupin Arachnida, Collembola, z hmyzu zástupci řádů Dermaptera, Psocoptera, Thysanoptera, Hemiptera, Raphidioptera, Neuroptera, Coleoptera, Diptera, Lepidoptera a Hymenoptera.

Pavouci využívají hálky jako lovecké úkryty, některé druhy si staví lapací sítě v rákosových dutinách i hálkách. Dominantními druhy pavouků v hálkách jsou zápředník *Clubiona phragmitis*, křižák *Singa nitidula* a skákavka *Synageles venator* (Bogusch et al. 2016). Zápředník *Clubiona phragmitis* je úzce vázán na rákosiny, kde žije a loví svou kořist, mšice, na stéblech rákosu. V ČR byl zaznamenán jen vzácně v rákosinách a také v hálkách (Hendrickx et al. 2004, Bogusch et al. 2016). Některé druhy pavouků, jako například zmíněný zápředník *Clubiona phragmitis*, umisťují na rákos své kokony (Schmidt et al. 2005). Vnitřní prostory hálek využívá štírek *Dactylochelifer latreillii*, který vyhledává v hálkách svou kořist (A. Astapenková a kolektiv, nepublikovaná data).

Velmi početnou skupinou živočichů jsou ti, kteří hálky využívají jen jako úkryt k přečkávání nepříznivého počasí, nebo častěji k hibernaci. Tak se uvnitř hálek v zimním období vyskytují některé druhy ploštic, z nichž nejpočetnější je ploštička *Ischnodemus sabuleti* (Bogusch et al. 2016). Tento druh vyhledává osluněné, suché i vlhké biotopy, a nejčastěji ji nalezneme v porostech třtiny (*Calamagrostis*), zblochanu (*Glyceria*),

chrastice (*Phalaris*), rákosu (*Phragmites*) a orobince (*Typha*) (Friess et al. 2013). Přezimující druhy brouků mají k rákosu bližší vztah než ploštice – kohoutek *Oulema melanopus* se živí mladými výhonky rákosu a klade na ně vajíčka (Tscharntke & Greiler 1995), slunéčko *Coccidula scutellata* a druh čeledi Scyrtidae *Cyphon laevipennis* vyhledávají na rákosu mšice (Schmidt et al. 2005). Naprostá většina brouků vyskytujících se v rákosových hálkách jsou predátoři, kromě nejpočetnějších již uvedených druhů najdeme v hálkách často střevlíčky rodů *Dromias*, *Demetrias*, *Pseudodromias* a dalších, bradavičníky *Anthocomus coccineus* a *Malachius aeneus* včetně jejich larev, a mravencovníka *Cordicollis gracilis* (Bogusch et al. 2017).

Mezi organizmy žírem vázané na rákos a vyskytující se v hálkách patří jednak četné bejlomorky (Cecidomyiidae), dále pak motýli. Z této skupiny lze jmenovat zavíječe *Brachmia inornatella*, jehož housenky se živí na rákosu obecném (Šumpich & Konvička 2012), housenky zaznamenané píďalky černoproužky *Boudinotiana notha* se vyvíjejí na topolech a vrbách a hálky využívají příležitostně jako dutiny ke kuklení (Šumpich et al. 2014). Další zaznamenané druhy také nejsou na hálky zelenušek potravně vázané. Stejná situace je u zaznamenaných druhů širopasých blanokřídlých. Byli zaznamenáni především pilatkovití (Tenthredinidae). Larvy (housenice) některých druhů této skupiny využívají rákos obecný ke kuklení (Bogusch et al. 2016).

Druhové složení společenstev členovců spojovaných s hálkami zelenušek rodu *Lipara* se silně liší mezi stanovišti přírodě blízkými a post-industriálními, oba typy stanovišť hostí rozmanitá společenstva druhů (Bogusch et al. 2016). Na přírodě blízkých stanovištích se vyskytují častěji stenoekní druhy vázané na rákos, mokřady, rybníky apod., často se jedná o druhy z červeného seznamu. Naopak na post-industriálních stanovištích najdeme více druhů, ale řada z nich jsou druhy nespecializované, obývající řadu stanovišť. Jsou však i výjimky, jako např. bodulka *Belomicrus italicus* Costa, 1866, šíronožka *Crabro scutellatus* (Scheven, 1781) nebo stopčík *Mimumesa littoralis* (Bondroit, 1934), což jsou vzácné druhy, které se podařilo najít jen na post-industriálních stanovištích. Heneberg et al. (2014) uvažují, že vyšší diverzita druhů na post-industriálních stanovištích je zapříčiněna přítomností nezarostlého písčitého podloží pod rákosinou. Řada druhů mizejících z kulturní krajiny a z dlouhodobě existujících rákosin v okolí řek, potoků a rybníků nachází svá refugia v rákosinách

vyskytující se nově na exponovaném volném podloží post-industriálních stanovišť (Heneberg et al. 2014, Bogusch et al. 2016).

2.4 Žahadloví blanokřídlí v rákosových hálkách

Některé druhy kutilek a samotářských vos hnízdí v duběnkách vytvořených žlabatkou *Andricus kollari*, a to konkrétně kutilka *Pemphredon austriaca* (Kohl, 1888) a některé další druhy tohoto rodu, a také hrnčířka *Stenodynerus chevrieranus* (Saussure, 1856) (Blommers 2008, Macek et al. 2010).

Žahadloví blanokřídlí hnízdící v rákosových hálkách nejčastěji preferují rok staré či starší hálky vytvořené zelenuškou *Lipara lucens* (Dely-Draskovits et al. 1994, Westrich 2008, Bogusch et al. 2015, Heneberg et al., submitted). Tyto hálky jsou nejhojnější v ekotonech rákosin, v přilehlých rašeliništích nebo loukách, a jsou vytvářeny na rostlinách rákosu rostoucích na suché půdě, nikoliv z vody (Heneberg et al., submitted).

Druhy žahadlových blanokřídlých, využívající k hnízdění hálky zelenušek, náleží do různých čeledí. Tato ekologická skupina je velmi heterogenní, zahrnuje specialisty na rákosové hálky, mokřadní druhy, které rákosové hálky využívají pouze příležitostně, a všudypřítomné druhy, které hnízdí v rozličných typech dutin včetně rákosových hálek (Astapenková et al. 2017). Čtyři výše zmíněné druhy zelenušek rodu *Lipara* a jejich blanokřídlí inkvilíni jsou rozšířeni v mnoha zemích (Nartshuk & Andersson 2013).

Celkově je známo 46 druhů žahadlových blanokřídlých, kteří jsou asociováni s rákosovými hálkami, z nichž 37 druhů využívá rákosové hálky k hnízdění, dále bylo zjištěno šest parazitoidů z čeledi Chrysididae a tři kukaččí včely rodu *Stelis* Panzer, 1806, viz Tab. 1. Ze zjištěných hnízdních kleptoparazitů – smutěnek, lze jmenovat tyto druhy: *Stelis punctulatissima* (Kirby, 1802) a *Stelis ornatula* (Klug, 1862) parazitující u zednice *Hoplitis leucomelana* (Kirby, 1802) a smutěnku *Stelis breviuscula* (Nylander, 1848) parazitující u dřevobytky *Heriades rubicola* Pérez, 1890. Nejpočetnějším druhem parazitoidů z čeledi Chrysididae je zlatěnka *Trichrysis cyanea* (Linnaeus, 1761), která parazituje u blanokřídlých, kteří loví pavouky a hnízdí v dutinách, v hálkách u kutilek *Trypoxylon deceptorium* Antropov, 1991 a *Trypoxylon minus* Beaumont, 1945 (Westrich 2008, Heneberg et al. 2014, Bogusch et al. 2015, Astapenková et al. 2017). V České republice bylo zaznamenáno 24 druhů žahadlových blanokřídlých hnízdících či parazitujících v rákosových hálkách rostoucích v rákosinách říčních niv, rybníků

a na post-industriálních stanovištích (Heneberg et al. 2014, Bogusch et al. 2015, Astapenková et al. 2017).

Tab. 1. Přehled všech zaznamenaných žahadlových blanokřídlých hnízdících nebo parazitujících v rákosových hálkách. Hvězdičkou označené druhy jsou parazitické. Převzato a upraveno podle Astapenkové et al. (2017).

Čeleď / Druh
Chrysididae – zlatěnkovití
Chrysis angustula Schenck, 1856 *
Chrysis rutilans Olivier, 1790 *
Holopyga fastuosa generosa Förster, 1853 *
Pseudomalus auratus (Linnaeus, 1761) *
Trichrysis cyanea (Linnaeus, 1761) *
Trichrysis pumilionis Linsenmaier, 1987 *
Formicidae – mravencovití
Dolichoderus quadripunctatus (Linnaeus, 1771)
Temnothorax spp. (Mayr, 1861)
Vespidae – vosovití
Stenodynerus chevrieranus (Saussure, 1855)
Stenodynerus clypeopictus (Kostylev, 1940)
Stenodynerus xanthomelas (Herrich-Schaeffer, 1839)
Symmorphus bifasciatus (Linnaeus, 1761)
Symmorphus fuscipes (Herrich-Schaeffer, 1839)
Crabronidae – kutíkovití
Ectemnius confinis (Walker, 1871)
Nitela spinolae Latreille, 1809
Passaloecus clypealis Faester, 1947
Passaloecus corniger Shuckard, 1837
Passaloecus gracilis (Curtis, 1834)
Passaloecus singularis Dahlbom, 1844
Pemphredon fabricii (Müller, 1911)
Pemphredon inornata Say, 1824
Pemphredon lethifer (Shuckard, 1837)
Pemphredon rugifer (Dahlbom, 1844)
Pemphredon wesmaeli (Morawitz, 1864)
Rhopalum clavipes (Linnaeus, 1758)
Rhopalum gracile Wesmael, 1852
Trypoxylon attenuatum Smith, 1851
Trypoxylon deceptorium Antropov, 1991
Trypoxylon figulus (Linnaeus, 1758)
Trypoxylon minus Beaumont, 1945
Megachilidae – čalounicovití
Chelostoma campanularum (Kirby, 1802)
Heriades rubicola Pérez, 1890
Hoplitis leucomelana (Kirby, 1802)
Megachile centuncularis (Linnaeus, 1758)
Megachile versicolor Smith, 1844
Pseudoanthidium lituratum (Panzer, 1801)
Pseudoanthidium tenellum (Mocsáry, 1881)
Stelis breviuscula (Nylander, 1848) *
Stelis ornatula (Klug, 1807) *
Stelis punctulatissima (Kirby, 1802) *
Colletidae – hedvábnicovití
Hylaeus communis Nylander, 1852
Hylaeus confusus Nylander, 1852
Hylaeus gracilicornis (Morawitz, 1867)
Hylaeus incongruus Förster, 1871
Hylaeus moricei (Friese, 1898)
Hylaeus pectoralis Förster, 1871

Žahadlové blanokřídlé asociované s rákosovými hálkami zelenušek rodu *Lipara*, můžeme rozlišit do tří hlavních skupin (dle Astapenkové et al. 2017 a Bogusche et al., submitted).

1. Druhy preferující rákosové hálky, specializované na hnízdění v hálkách. Tato skupina zahrnuje stopčíka *Pemphredon fabricii* (Müller, 1911), maskonosku *Hylaeus pectoralis* Förster, 1871 a pravděpodobně také hrnčířku *Stenodynerus clypeopictus* (Kostylev, 1940). První zmíněný druh hnízdí také v dutých stéblech rákosu, hálky však preferuje a je v nich řádově početnější (Bogusch et al. 2017).

2. Druhy hnízdící v rákosových stéblech nebo v jiných typech dutin, které často hnízdí v rákosových hálkách. Tato skupina je reprezentovaná kutilkou *Trypoxylon deceptorium*, maskonoskou *Hylaeus moricei* (Friese, 1898), dřevobytkou *Heriades rubicola* a kutilkou *Passaloecus clypealis* Faester, 1947. Jedná se o druhy vázané na rákosiny a mokřadní stanoviště, které hnízdí v různých typech dutin. Stébla rákosu jsou zřejmě nejčastějšími hnízdními dutinami, ale hálky zelenušek jsou těmito druhy také běžně využívány.

3. Druhy hnízdící v různých typech dutin a náhodně nebo velmi vzácně hnízdící také v rákosových hálkách. Tato druhově nejpočetnější skupina zahrnuje všechny ostatní druhy nalezené v rákosových hálkách. Některé z těchto druhů jsou v rákosových hálkách běžné, a to proto, že tyto druhy tvoří velmi početné populace, i když pouze malé procento využívá rákosové hálky k hnízdění. Z početných druhů lze uvést hrnčířku *Symmorphus bifasciatus* (Linnaeus, 1761), kutilku *Trypoxylon minus* a zednici *Hoplitis leucomelana*.

První dvě skupiny jsou citlivé ke změnám v okolí rákosin a k četnostem disturbancí rákosin, proto mohou být využívány jako bioindikátory dobře zachovalých rákosin v intenzivně kultivované krajině (Astapenková et al. 2017).

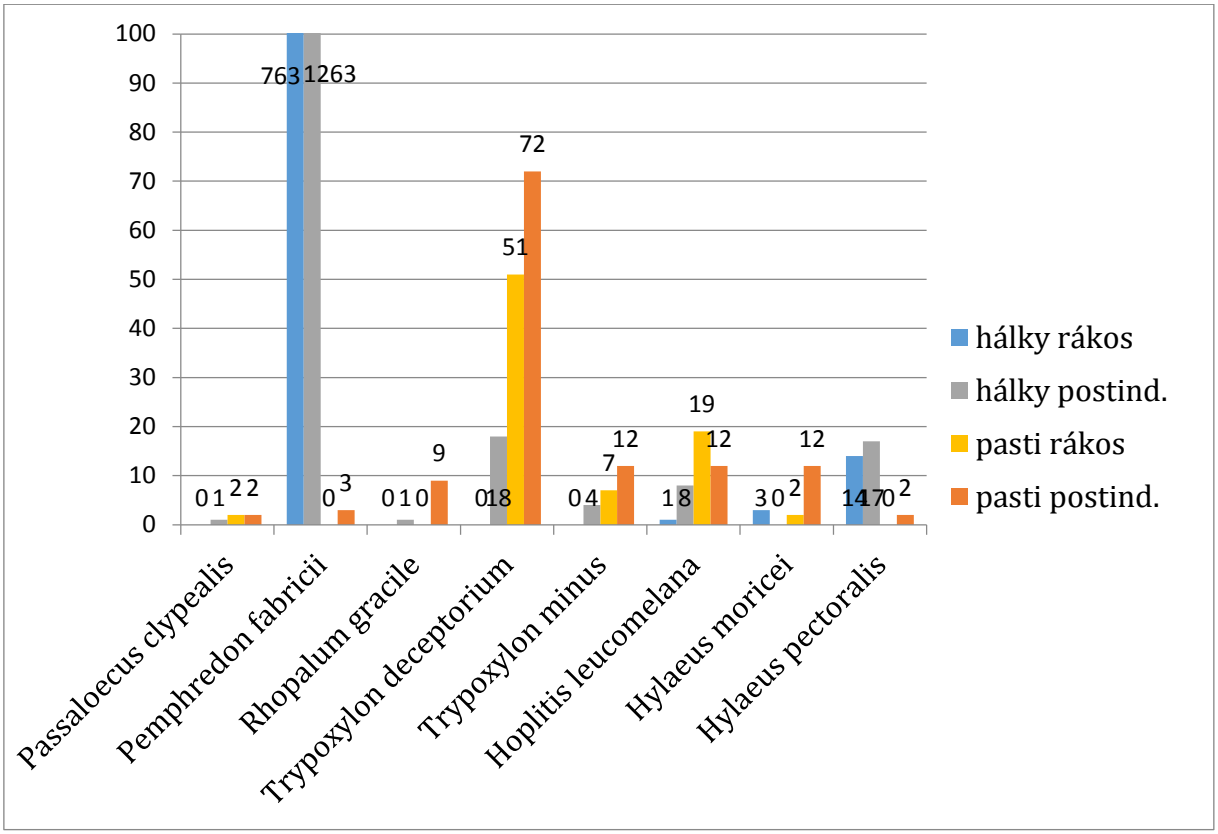
Ve společenstvech žahadlových blanokřídlých v rákosových hálkách na přírodě blízkých stanovištích i post-industriálních stanovištích vysoce dominuje stopčík *Pemphredon fabricii*, který zahrnuje více než 90 % všech jedinců žahadlových blanokřídlých nalezených uvnitř rákosových hálek (Bogusch et al. 2015), viz Obr. 3. Tento druh hnízdí v hálkách zelenušek *Lipara lucens* a *L. rufitarsis* a vytváří dvě generace za rok (Blösch 2000, Bogusch et al. 2017). *Pemphredon fabricii* se živí mšicemi (Blösch

2000, Westrich 2008, Macek et al. 2010, Bogusch et al. 2017), hnízdící samice nosí do hnízda druhy mšic, které jsou v okolí hálky početné. Nejčastější potravou je tak mšice švestková (*Hyalopterus pruni*), jejíž nepohlavní letní generace se vyvíjí na rákosu (Bogusch et al. 2017). Samice této kutilky nevytváří uvnitř hálky oddělené komůrky, namísto toho vyplní hálkovou dutinu paralyzovanými mšicemi a na některé z nich naklade vajíčko. Larvy posledního instaru se uvnitř hálky seřadí od největší na bázi po nejmenší na vrcholu hálky. Do některých hnízd přináší hnízdící samice nejmenším larvám čerstvé mšice. Tento typ progresivního krmení představuje nově objevený a popsaný typ progresivního krmení u hmyzu (Bogusch et al. 2017). Zajímavé je i to, že zbarvení larev je různé (nejčastěji sytě žluté, ale i bílé, světle žluté, oranžové až růžovofialové) a závisí zřejmě na druhu mšice, jímž je larva krmena (Wolf 1988). Larvy eudominantní kutilky *Pemphredon fabricii* byly v některých případech zasaženy plísněmi, konkrétně druhy *Penicillium buchwaldii*, *Aspergillus pseudoglaucus* a *Penicillium quebecense* (Heneberg et al. 2016).

Maskonoska *Hylaeus pectoralis* je druhým nejpočetnějším druhem nalezeným v rákosových hálkách, který hnízdí téměř výhradně v hálkách zelenušky *Lipara lucens* (Else 1995, Macek et al. 2010, Bogusch et al., submitted). Jedná se o typický mokřadní druh, který preferuje dobře zachované biotopy s mokřadními loukami plnými kvetoucích rostlin (Else 2012). V České republice je považovaný za kriticky ohrožený druh (Farkač et al. 2005). *H. pectoralis*, který je známý jen z tří lokalit České republiky, jsme zaznamenali na mnoha lokalitách naší země a není tedy tak vzácný, jak se předpokládalo. Má však významné bioindikační vlastnosti a vyskytuje se na stanovištích, která jsou velmi cenná z hlediska ochrany přírody. Častými zjištěnými parazitoidy tohoto druhu jsou srpušky *Gasteruption phragmiticola* Saure, 2006 a *Gasteruption assectator* (Linnaeus, 1758).

Třetím běžně zastoupeným druhem je kutilka *Trypoxylon deceptorium*, druh vázaný na mokřady, hnízdící v rákosových hálkách a jiných dutinách, v některých případech je početnější než *Hylaeus pectoralis*, především na křovinatých rákosinách a post-industriálních stanovištích (Bogusch et al. 2015, submitted). Tento teplomilný druh loví pavouky jako potravu pro své larvy. Všechny ostatní druhy žahadlových blanokřídlých jsou obvykle velmi slabě zastoupeny většinou jedním nebo několika hnízdy na studované lokalitě. Výjimkou je dřevobytka *Heriades rubicola*, která je relativně

častým druhem v rákosových hálkách v jižních částech střední Evropy a v posledních létech se výrazně šíří i na jižní Moravě a byla poprvé zaznamenána i na území Čech (Astapenková et al. 2017).



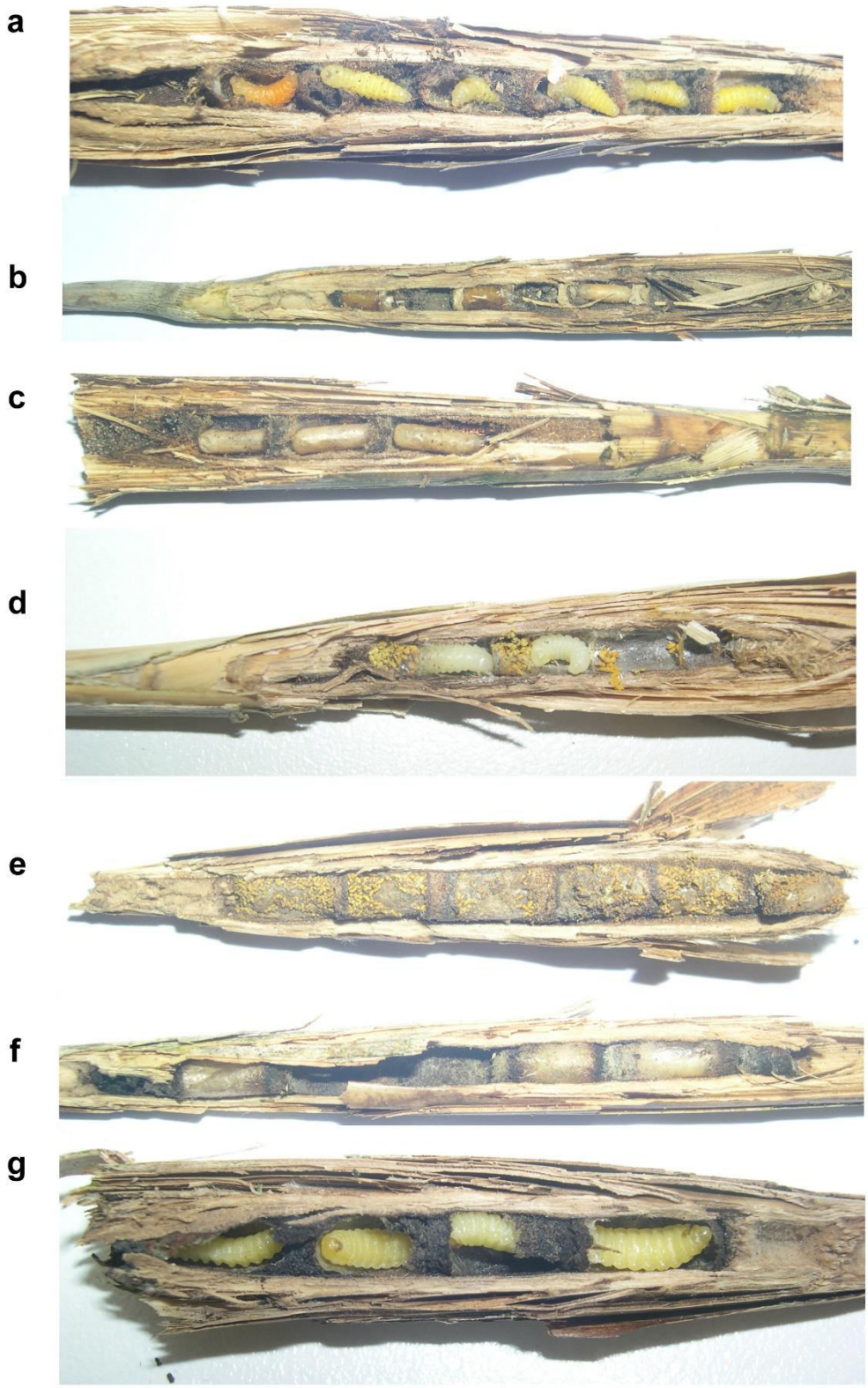
Obr. 3. Počty jedinců významných druhů žahadlových blanokřídlých v rákosových hálkách a v barevných pastech na přírodních a post-industriálních stanovištích (data z let 2013-2014).

Zajímavá je i struktura hnízd žahadlových blanokřídlých hnízdících v rákosových hálkách. Například hnízda nejdominantnější kutilky stopčíka *Pemphredon fabricii* mají velmi variabilní počet hnízdních komůrek (1-12), které často expandují až do měkké části hálky. Hnízdo přiléhá těsně na vnitřní povrch hálky. Typické jsou sytě žlutě nebo oranžově zbarvené larvy.

Hnízdo kutilky *Trypoxylon deceptorium* obvykle obsahuje jednu až dvě velké hnízdní komůrky. Velmi křehké larvy jsou v dlouhých tenkých kokonech užších než hálka. Nerozeznatelně vypadá hnízdo druhu *T. minus*. Pokud je přítomná parazitická zlatěnka *Trichrysis cyanea*, prozradí se rezavě hnědým trychtýřovitým kokonem.

Hnízdo maskonosky *Hylaeus pectoralis* obvykle nevyplňuje celou hálkovou dutinu, často byly zaznamenány prázdné hnízdní komůrky, které zřejmě slouží jako ochrana proti parazitoidům. Hnízda tohoto druhu jsou charakteristická přítomností celofánové vrstvy mezi hnízdními komůrkami a také na jejich povrchu. Mezi komůrkami a na konci hnízda jsou prostory vyplněné rozžvýkanými suchými listy rákosu. Larvy jsou bíle zbarvené.

Hnízdní komůrky dřevobytky *Heriades rubicola* jsou v hnízdě nahloučené jedna za druhou a od sebe jsou oddělené velmi slabými přepážkami. Na povrchu kokonů s larvami je často trus, který plesniví. Larvy hnízdních parazitů tohoto druhu – smutěnek, jsou umístěné v hnědavých oválných kokonech se špičkou (Bogusch et al. 2015, Astapenková et al. 2017). Hnízda vybraných druhů žahadlových blanokřídlých z rákosových hálek jsou prezentovaná na Obr. 4.



Obr. 4. Hnízda vybraných druhů žahadlových blanokřídlých z rákosových hálek: a – *Pemphredon fabricii*, b – *Trypoxylon deceptorium* se dvěma zlatěnkami *Trichrysis cyanea*, c – *Trypoxylon minus*, d – *Hylaeus pectoralis*, e – *Heriades rubicola*, f – *Hoplitis leucomelana*, g – *Symmorphus bifasciatus*.

2.5 Bioindikace žahadlových blanokřídlých v rákosových hálkách

Některé druhy žahadlových blanokřídlých asociované s rákosovými hálkami můžou být považovány za bioindikátory, konkrétně především maskonoska *Hylaeus pectoralis* a hrnčířka *Stenodynerus clypeopictus*. Tyto druhy jsou obecně velmi vzácné a tvoří málo početné populace na svých stanovištích. Tyto vzácné druhy jsou specializované na specifická stanoviště, jako jsou rákosiny napojené na mokřadní louky s hojností kvetoucích rostlin (*H. pectoralis*) a slané rákosové mokřady (*S. clypeopictus*) (Astapenková et al. 2017). Rákosiny s návazností na mokřadní louky jsou často disturbované sečí rákosu. Častý management rákosin v maloplošných zvláště chráněných územích je založen na jejich kosení, které je prováděno jednou či dvakrát za rok (Heneberg et al., submitted). Tento management velmi často zahrnuje kosení okrajů rákosin, který vede k zabránění expanzi rákosu dále do navazujících porostů. Okraje rákosin a rozvolněný porost rákosu v navazujících mokřadních loukách jsou však vysoce důležité biotopy pro hnízdění žahadlových blanokřídlých v hálkách zelenušek rodu *Lipara*, protože právě na oslabeném okrajovém rákosí se nachází nejvíce hálek. Tato stanoviště pak žahadloví blanokřídlí obsazují nejčastěji a, jejich výskyt dále od kraje rákosiny klesá, uvnitř husté rákosiny je velmi sporadický, i v případě, že jsou zde přítomné hálky.

Studium druhů hnízdících v rákosových hálkách tak může být důležité při ochranářských průzkumech navrhovaných mokřadních či rákosních chráněných území a při podobných činnostech v již stávajících chráněných území (jak se již v současnosti v praxi ukazuje). Výskyt některých druhů, např. maskonosky *Hylaeus pectoralis*, lze prokázat nejlépe sběrem a studiem hálek v zimním období. Navíc naše studie prokázaly, že z druhů hnízdících v hálkách je řada zařazených v červeném seznamu, a proto jsou hálky zelenušek důležitým mikrohabitatem a rákosiny důležitým stanovištěm výskytu vzácných druhů zasluhujících ochranu. To platí i pro ostatní druhy hmyzu a bezobratlých na hálky vázané nebo se v nich jen občas vyskytující, přičemž ty nejvýznamnější jsou na výskyt v hálkách většinou specializované (Heneberg et al. 2014, Bogusch et al. 2016).

2.6 Literatura

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3 PŘÍLOHY

3.1 HENEBERG P., BOGUSCH P. & ASTAPENKOVÁ A. 2014: Reed galls serve as an underestimated but critically important resource for an assemblage of aculeate hymenopterans. *Biological Conservation* **172**: 146-154.

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Reed galls serve as an underestimated but critically important resource for an assemblage of aculeate hymenopterans

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Pemphredon fabricii

ABSTRACT

Common reed (*Phragmites australis*) beds are frequently considered as aggressive and invasive, being subject to numerous conservation management efforts aimed at their eradication by repeated mowing or more aggressive measures. However, the reed beds are associated with a specific community of reed bed specialists, represented typically by various bird flagship species or by *Lipara* flies. We show here that the reed beds and particularly the reed galls induced by *Lipara* flies provide unique habitat serving at least 183 bee and wasp species (amounting to 13.6% of the total bee and wasp species known to occur in the Czech Republic, throughout which the sampling sites were located). The reed galls themselves were found to host 13 species of bees and wasps, five of them red-listed, and some of them considered as reed bed specialists. *Pemphredon fabricii* and *Hylaeus pectoralis* were the dominant reed gall aculeate hymenopteran inquilines. *Hylaeus moricei*, *Passaloecus clypealis*, *Rhopalum gracile* and *Trypoxylon deceptorium* were identified as species tightly bound to the presence of reed galls. Among the other species detected was also one previously considered as regionally extinct (*Nysson quadriguttatus*), nine were critically endangered, 11 were endangered, and 19 were considered as vulnerable. The species found displayed specific habitat requirements, often requiring not only the presence of reed, but also the presence of loose sandy bedrock below the reed bed. These species, which have nearly disappeared from the surrounding cultural landscape, found their surprising refuge in reed beds occurring newly on the loose bedrock of (post)industrial sites, including gravel-sandpits, ash ponds and tailing ponds. The data obtained challenge the common view of the expanding reed beds as a threat to biodiversity, and highlight the importance of reed beds, particularly those of the oligotrophic nature, for effective conservation of the aculeate hymenopteran reed gall inquilines.

1. Introduction

Galls represent discrete microhabitats that support relatively closed communities of specialized inhabitants (Stone and Schönrogge, 2003). While the diversity among gallers is relatively well understood, with an estimated 13,000 species distributed across several insect groups, much more limited is the information on the complex network involving natural enemies of the gallers (predators and parasitoids) and especially on the gall tissue inquilines (organisms utilizing the gall tissue instead of directly feeding on the galling insect) (Sanver and Hawkins, 2000). Despite being poorly understood, Sanver and Hawkins (2000) proposed that gall inquilines represent a very common and perhaps key component of gall assemblages.

In this current study we addressed the value of common reed (*Phragmites australis*, Poaceae) and reed galls as a potentially underestimated but critically important resource for the community of specialized aculeate hymenopteran (Hymenoptera: Aculeata) inquilines. Reed stands are commonly considered as aggressive and invasive (e.g., Tewksbury et al., 2002), exploited commercially in some regions (e.g., Danube delta), and facing numerous conservation management efforts aiming to eradicate the reed stands by repeated mowing or other types of control measures (Schmidt et al., 2005; Derr, 2008; Mamolos et al., 2011; Martin and Blossey, 2013). Contrary to that, in some regions, reed beds are considered as receding (van der Putten, 1997) habitats of conservation interest (Valkama et al., 2008; Poulin et al., 2010; Horiuchi et al., 2011). Recently, reed beds are occasionally formed *de novo* in order to reduce the nutrient content in waste waters or

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to remove pollutants (Athen and Tscharntke, 1999; Tian et al., 2009).

The reed stands serve as an exclusive habitat for four monophagous and univoltine species of the genus *Lipara* (Diptera: Chloropidae). *Lipara* flies emerge in late spring and oviposit on reed shoots where the larvae induce gall formation; a process which takes place throughout the whole summer. In late summer, the larvae relocate to the parenchymatous pith of the galls, where they eat out a chamber and hibernate (Mook, 1967). The four *Lipara* species occurring in Central Europe differ slightly in resource utilization. While *L. lucens* and *L. rufitarsis* specialize on shoots with a basal shoot diameter ⩽4.5 mm, *L. pullitarsis* and *L. similis* occur in reed stems of any width but preferring those which are 5–7 mm wide (Tscharntke, 1992, 1994). While reed shoots suffering from water deficiency, toxic pollution, habitat fragmentation, or simply from being positioned at the edge of reed beds, are suggested to host all the four *Lipara* species, large habitats and specifically the permanently wet reed beds standing in water host predominately the generalists *L. pullitarsis* and *L. similis* (Tscharntke, 1992).

While the biology and ecology of *Lipara* flies and of their parasitoids has been addressed extensively throughout their distribution range [cf. Dely-Draskovits et al. (1994) for a detailed list of references], reports on the assemblages of hymenopteran *Lipara* gall inquilines are scarce, limited predominately to incidental findings, assumptions lacking support by any experimental data, local or small-scale studies, or simply to captures of the gall inquilines in the reed beds or in their surroundings (e.g., Dely-Draskovits et al., 1994; Kopf and Schiestl, 2000; Bogusch, 2007; Lee and Scott, 2007). Tolerance of reed gall hymenopteran inquilines to flooding was addressed by Westrich (2008), who also reported the species spectrum of aculeate hymenopterans reared from 294 galls collected in the vicinity of German Konstanz.

In this paper, we address the conservation value of reed galls (and reed beds in general) as an underestimated but critically important resource for the community of aculeate hymenopterans. We compare the data obtained using two independent methods: by rearing of reed gall inquilines from extensive set of reed galls collected, and by the non-selective method based on Moericke traps exposure. We assess the data from near-natural sites as well as from industrial and postindustrial sites. Thus, we perform the first comprehensive survey of aculeate hymenopterans utilizing reed galls correlated with various site-specific variables, providing the evidence to re-assess the case for the conservation of reed beds in European wetlands.

2. Materials and methods

2.1. Study area

The study was carried out in the Czech Republic (Central Europe, 48°39′–50°59′N, 12°19′–18°29′E). Sampling sites were selected to cover four major areas with extensive pisciculture industry or with major remnants of reed beds in the floodplains of rivers and streams. Sedimentary deposits within the study area are extensively exploited by coal and industrial minerals extraction activities, comprising of 10.3% of world kaolin production, 4.2% of brown coal production, 2.0% of feldspar production, 1.8% of diatomite production, 1.3% of quartz sands production, and 1.3% of bentonite production (Starý et al., 2011).

We selected 15 sampling sites in near-natural habitats (reed beds in the littoral of medieval fishponds or those along rivers and streams), representing reed beds spanning 0.2–480 ha and occurring within the altitude range 163–452 m a.s.l. (Fig. 1, black dots). Another 15 sampling sites were located in reed beds in industrial and post-industrial habitats formed between the years 1922 and 2010 (gravel-sandpits, tailing ponds, stone quarries, colliery dumps and reclaimed open-cast mines), again representing a broad range of reed bed surface areas (0.2–19 ha) and occurring within the altitude range 157–467 m a.s.l. (Fig. 1, grey dots). Since this is the first study comparing aculeate hymenopteran assemblages associated with the reed galls in (post)industrial and near-natural habitats in extenso, the selected sampling sites were chosen to represent the whole spectrum of reed beds present throughout the study area. A detailed list of the sampling sites is provided in Supplementary Table A.1 and their characteristics are provided in Table 1.

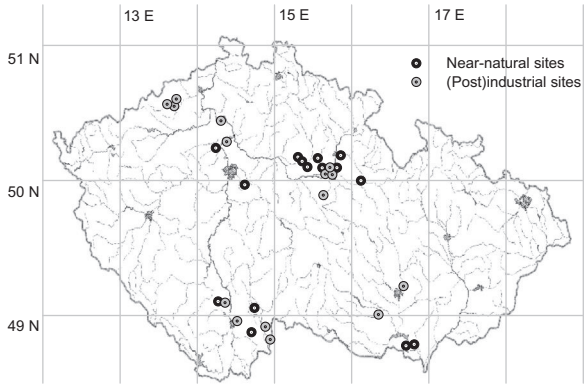

Fig. 1. Location of study sites in the Czech Republic. Black dots represent those located to near-natural habitats (reed bed at the banks of rivers or streams, or in the littoral of medieval fishponds). Gray dots represent those located to (post)industrial habitats (active or disused gravel-sandpits, ash ponds and ash deposits, tailing ponds, foreland of opencast mines, quarries and colliery dumps).

The study sites included those in agrarian lowlands (<250 m a.s.l., mean annual temperature 8.0–9.3 °C, mean annual precipitation 480–550 mm) and also those in mostly woodland uplands (>250 m a.s.l., mean annual temperature 6.7–7.9 °C, mean annual precipitation 551–780 mm). This study was based on a space-for-time substitution paradigm (Pickett, 1989). The sampling sites ranged from those formed very recently (in 2010) to those which were probably already present before the onset of any extensive human activities in the study area (river- and stream-associated reed beds). The area of reed beds was measured from recent aerial photographs of the sampling sites which are publicly available from http://www.mapy.cz [cited as 28 Nov 2013]). To age the sites and to assess their continuity over time, we checked also aerial photographs taken in years 2004–2006 and 2002–2003 (available from http://www.mapy.cz [cited as 28 Nov 2013]) and in 1952–1954 (available from http://kontaminace.cenia.cz [cited as 28 Nov 2013]). The information on water bodies presence in the past was based on the 3rd Military survey initiated by Franz Joseph I. of Austria in 1876–1880 (available from http://kontaminace.cenia.cz [cited as 28 Nov 2013]) and based on the 2nd Military survey initiated by Franz I. of Austria in 1836-1852 (available from http://www.mapy.cz [cited as 28 Nov 2013]) (Table 1).

2.2. Sampling of reed galls

At each of the sampling sites listed in Supplementary Table A.1, 300–1000 deformed reed shoots were cut right under the gall. Their protruding leaves were also cut out in order to fit them to the rearing bags. At each site, the galls induced by *Lipara* spp. were selected randomly, regardless of their position, size or age, reflecting their variation at each sampling site. The galls were sampled between 12 January and 16 March 2013. Collected galls were either moved immediately to the rearing bags or were kept frozen for several days or weeks until placed in the rearing bags. The rearing bags, designed as emergence traps, consisted of white nonwo-

Table 1
Parameters of sampling sites utilized for collection of reed galls in late winter 2013 as well as for control captures by Moericke traps in July 2013. Indicated are the altitude, reed bed area [ha], water surface area [ha], year when the habitat was formed (this may represent the year of foundation of the water body, the year of cessation of aggregates production at the respective site, or the year of last substantial landscaping operations), and data on the continuity of the habitat retrieved from publicly available map sources. *Abbreviations used:* N/A = not applicable, N/D = not detected (study site I/12 is located outside of the Bohemian crown lands mapped during the 2nd Military survey).

No.	Altitude [m a.s.l.]	Reed bed area [ha]	Water surface area [ha]	Year when the habitat was formed	Continuity of reed bed presence [%]		Continuity of water bodies presence [%]	
					2003	1950s	1870s	1836-1852
Near-natural habitats								
N/1	374	0.2	N/A (stream)	N/A (stream)	100	100	100	100
N/2	254	9.0	6.0	1989 (pond restoration)	100	0	0	0
N/3	228	20.0	37.5	1467 (pond foundation)	100	100	100	100
N/4	232	6.0	23.1	Mid 16th Century (pond foundation)	100	20	100	100
N/5	202	70.0	173.0	1492–1497 (pond foundation)	100	25	100	100
N/6	215	7.0	41.4	≪1836 (pond foundation)	100	100	100	100
N/7	219	63.0	85.2	1480 (first written mention)	100	100	100	100
N/8	222	22.0	6.0	1496 (Velká Čeperka foundation)	100	100	100	100
N/9	239	0.5	2.2	1905 (pond restoration)	100	100	0	100
N/10	452	480.0	N/A (river)	N/A (river)	100	30	100	100
N/11	402	3.5	42.5	1510 (pond foundation)	100	100	100	100
N/12	421	150.0	317.0	1505 (pond foundation)	100	100	100	100
N/13	163	10.2	86.0	First half od 15th Century (pond foundation)	100	100	100	100
N/14	175	180.0	288.0	1414–1417 (pond foundation)	100	100	100	100
N/15	197	11.0	N/A (stream)	N/A (stream)	100	100	100	100
Industrial and postindustrial habitats								
I/1	409	10.0	156.0	1922 (ash deposit foundation)	100	10	0	0
I/2	164	0.3	17.0	1998 (quarrying termination)	20	0	0	0
I/3	432	18.0	12.0	1985 (ash pond foundation)	100	0	0	0
I/4	443	0.3	37.0	2007 (extensive landscaping of the study area)	0	0	0	0
I/5	157	2.8	5.2	1994 (ash pond foundation)	10	0	0	0
I/6	271	0.3	0.0	2007 (mine foreland landscaping)	50	0	0	0
I/7	277	1.5	16.0	1998 (pond foundation)	0	0	0	0
I/8	248	0.2	0.2	2004 (pond foundation)	0	0	0	0
I/9	214	19.0	3.0	2nd half of 20th Century (ash pond foundation)	100	0	0	0
I/10	216	6.0	6.0	2nd half of 20th Century (ash pond foundation)	10	0	0	0
I/11	220	0.3	80.0	2004 (quarrying termination)	0	0	0	100
I/12	467	4.0	19.0	1985 (quarrying termination)	100	0	0	N/D
I/13	278	0.3	2.6	1972 (tailing pond foundation)	100	0	0	0
I/14	312	0.6	0.1	1997 (quarrying termination)	100	0	0	0
I/15	414	0.5	4.0	1950s (quarry foundation)	100	0	0	0

ven fabric and were equipped with a plastic bottle filled with a conservation fluid (ethanol or propylene glycol mixed with water and detergent). The rearing bags were stored in a well aired place side-exposed to daylight, at a temperature between 15 and 23 °C. The plastic bottles within the rearing bags were positioned at a side proximal to the window. The rearing bags were sprayed with water several times a week. The aculeate hymenopterans started to emerge approximately four weeks after being placed in the rearing bags. Numbers of newly emerging hymenopterans were regularly monitored until a period of at least two weeks had elapsed with no further emergences within each respective rearing bag, following which the rearing was stopped (in May or June). The total number of reed galls sampled reached 17,791, out of which 8820 (49.6%) were obtained from near-natural habitats, and 8971 (50.4%) were collected from (post)industrial habitats. Number of galls collected at each sampling site and the collection dates are provided in Supplementary Table A.2. The average number of galls collected at a single sampling site reached 593 ± 152. The sampling was performed by Petr Heneberg, Petr Bogusch and Alena Astapenková, the obtained specimens of Aculeata were determined and collected by Petr Bogusch, and specimens of Chrysidoidea were revised by Pavel Tyrner.

2.3. Sampling using Moericke traps

Moericke traps were used to allow comparison of species spectrum obtained by rearing the reed galls with the overall spectrum of aculeate hymenopterans present at the localities examined. Moericke traps have been successfully used for the collection of bees and wasps in a wide range of habitats (cf. Cruz-Sánchez et al., 2011; Vrdoljak and Samways, 2012; Heneberg et al., 2013). The traps were made from round-shaped 570 ml polypropylene containers, 120 mm in upper diameter and 80 mm deep (Obal Centrum, Sezemice, Czech Republic), filled up to the upper quarter with soapy water and salt, the latter acting as a preservative. Four colors of Moericke traps were used at each sampling site to maximize the obtained species spectrum. Among the colors used were white (RAL 9010), yellow (RAL 1021 or 1003), turquoise (RAL 5018 or 5012) and pink (RAL 3014). The collected specimens were temporarily stored in 75% ethanol until pinned for identification. The Moericke traps were exposed between 23 June and 19 July 2013. The sampling periods for each sampling site as well as the number of traps exposed are indicated in Supplementary Table A.2. In summary, the traps were exposed for 2953 trap-days, with 1456 (49.3%) trap-days exposed in near-natural habitats, and 1497 (50.7%) trap-days exposed in (post)industrial habitats. The number of trap-days per sampling site ranged between 63 and 176, reflecting the weather conditions. At each study site, the Moericke traps were placed at the edge of reed beds, where the collection of reed galls has been performed earlier. The sampling was performed by Petr Heneberg, Petr Bogusch and Lukáš Nývlt, the obtained specimens of Aculeata (except of Formicidae) were determined and collected by Petr Bogusch, and certain Pompilidae specimens were revised by Jakub Straka.

2.4. Statistical analyses

Rarefaction curves were computed in PAST v. 2.14 (Hammer et al., 2001) to analyze species diversity and species richness of bees and wasps. The rarefaction algorithm was based on the use of log Gamma function for computing combinatorial terms as described by Krebs (1989). To estimate the species richness, the Chao-1 estimator was calculated (Colwell and Coddington, 1994) using the program available at http://www2.biology.ualberta.ca/jbrzusto/rarefact.php [cited as 28 Nov 2013].

Basic diversity indices were calculated for each of the datasets. These included the total number of species found, the total number of individuals found, dominance (expressed as 1 – Simpson index, where 1 indicates complete domination of the single species, and 0 indicates equal representation of all the taxa), equitability (evenness measure, where Shannon diversity is divided by the logarithm of number of taxa; Shannon index itself reflects entropy, ranging from 0 for communities with only a single taxon to high values for communities with many species, each with only few individuals), Fisher's alpha [parametric diversity measure assuming that the species abundance follows the log series distribution, useful when the ratio of the total number of individuals to the species number exceeds 1.44, and independent of sample size when the number of individuals sampled exceeds 1000 (Hayek and Buzas, 1997)], and Berger-Parker dominance index (number of individuals of the dominant species relativized to the total number of individuals) (Harper, 1999). To compare the diversity indices, a Shannon *t*-test with bias correction term was used (Poole, 1974). Linear and Spearman correlation coefficients and their significance were calculated when indicated. A chi-squared test was used to test the significance of differences in sex ratios and between the particular habitat types. All the calculations were performed in PAST v. 2.14 (Hammer et al., 2001). Data are shown as mean ± SD unless stated otherwise.

3. Results

3.1. Reed gall inquilines

From 17,791 reed galls sampled, we reared 2176 individuals of aculeate hymenopterans. Of them 781 individuals emerged from galls collected at near-natural sites (8.9 individuals per 100 galls collected), and 1395 individuals emerged from galls collected at (post)industrial sites (15.6 individuals per 100 galls collected). The lower frequency of aculeate hymenopterans reared from near-natural sites when compared to the (post)industrial sites was statistically significant ($X^2 = 163.3$, $d_f = 1$, $p < 0.001$).

In summary, we recorded 14 species of aculeate hymenopteran inquilines emerging from the *Lipara* reed galls (Tables 2 and 3). The

Table 2
Total diversity and abundance of aculeate Hymenoptera obtained from collected reed (*Phragmites australis*) galls at near-natural ($n = 15$) and postindustrial ($n = 15$) sites across the Czech Republic. The data are compared with frequency of captures of the same species in Moericke traps exposed in summer at the identical sampling sites. **(A)** The species are classified according to the Czech red list of invertebrates (Farkač et al., 2005): vulnerable (VU), endangered (EN), and critically endangered (CR). **(B)** Diversity indexes: dominance (D), Fisher's alpha, and equitability. **(C)** Results of the Shannon diversity *t*-tests.

(A)

	Status	Reed galls		Moericke traps	
		Near-natural habitats	(Post)industrial habitats	Near-natural habitats	(Post)industrial habitats
Chrysididae					
Chrysis angustula	EN	0	2	0	0
Pseudomalus auratus	–	0	1	0	0
Trichrysis cyanea	–	0	4	0	0
Formicidae					
Dolichoderus quadripunctatus	–	0	73	N/D	N/D
Vespidae					
Symmorphus bifasciatus	–	0	3	0	0
Crabronidae					
Nitela spinolae	–	0	3	0	0
Passaloecus clypealis	VU	0	1	1	5
Pemphredon fabricii	–	763	1260	0	3
Rhopalum gracile	CR	0	1	0	9
Trypoxylon deceptorium	–	0	18	71	52
Trypoxylon minus	–	0	4	7	12
Hoplitis leucomelana	–	1	8	11	19
Colletidae					
Hylaeus moricei	EN	3	0	2	12
Hylaeus pectoralis	CR	14	17	0	2
Total		781	1395	92	114

(B)

Diversity index	Reed galls		Moericke traps	
	Near-natural sites	Postindustrial sites	Near-natural sites	Postindustrial sites
Dominance (D)	0.955	0.819	0.616	0.267
Fisher's alpha	0.551	1.982	1.134	1.961
Equitability	0.090	0.184	0.486	0.786

(C)

Shannon diversity *t*-test	S_1/S_2	$Index_1 \pm var$	$Index_2 \pm var$	*t*	Df	*p* (same)
Reed galls, near-natural vs. postindustrial	4/13	$0.123 \pm 5.8 * 10^{-4}$	$0.466 \pm 1.0 * 10^{-3}$	−8.608	2175.8	≪0.001
Moericke traps, near-natural vs. postindustrial	5/8	$0.760 \pm 1.1 * 10^{-2}$	$1.604 \pm 7.1 * 10^{-3}$	−6.200	184.9	≪0.001
Near-natural sites, reed galls vs Moericke traps	4/5	$0.123 \pm 5.8 * 10^{-4}$	$0.760 \pm 1.1 * 10^{-2}$	−5.832	10.6	≪0.001
Postindustrial sites, reed galls vs Moericke traps	13/8	$0.466 \pm 1.0 * 10^{-3}$	$1.604 \pm 7.1 * 10^{-3}$	−12.602	148.4	≪0.001

Chao-1 species richness estimator (corrected for unseen species in the samples) indicated total species richness of 18.5 ± 5.4 species for the near-natural and (post)industrial reed beds combined. However, the specimens obtained from near-natural habitats were composed of four species only (Chao-1 estimator was not calculated due to the lack of doubletons), while 13 out of a total 14 aculeate hymenopteran inquiline species were recorded in galls from (post)industrial habitats, with Chao-1 equal to 17.5 ± 5.4 species (Fig. 2).

The assemblages at both near-natural and (post)industrial sites were highly dominated by *Pemphredon fabricii*, with the dominance index D reaching 0.955 and 0.819, respectively (Table 2). *Pemphredon fabricii* represented 97.6% and 90.3% of total individuals reared, and was found at 13 out of 15 sampling sites in near-natural habitats, and similarly at 13 out of 15 sampling sites in (post)industrial habitats. The frequency of *P. fabricii* individuals reared differed dramatically between the sampling sites examined (Table 3), with the highest values obtained at the sites I/5 (ash pond Dobříň; 40.9 individuals per 100 galls) and I/14 (quarry Růženin; 39.1 individuals per 100 galls). The variability in the frequency of reared *P. fabricii* individuals did not correlate with any of the variables tested (altitude, reed bed area, water surface area, year when the habitat was formed, and past habitat status at any of the four time intervals tested) as measured by the significance of linear and Spearman correlation coefficients. At both habitat types, the M:F ratio was significantly skewed towards males, reaching 1.52:1 in near-natural habitats ($X^2 = 32.3$, $d_f = 1$, $p < 0.001$), and 1.34:1 in (post)industrial habitats ($X^2 = 27.0$, $d_f = 1$, $p < 0.001$).

The galls hosted a diverse assemblage of aculeate hymenopterans, including seven crabronid species, two species of the Colletidae family, *Symmorphus bifasciatus* of the Vespidae family, the common Formicidae species *Dolichoderus quadripunctatus*, and three species of the cleptoparasitic family Chrysididae (Tables 2 and 3). Among the 14 species reared, two are considered as critically endangered (*Hylaeus pectoralis* and *Rhopalum gracile*), two species are considered as endangered (*Chrysis angustula* and *Hylaeus moricei*), and one is considered as vulnerable (*Passaloecus clypealis*). Since the above species were captured at multiple sampling sites but in low numbers, the analysis of their distribution was performed only when combined with the data obtained from the Moericke traps.

3.2. Moericke traps exposed at the edge of reed stands

Using the early summer Moericke traps captures we obtained 1743 individuals of bees and wasps including, but not limited to, the gall inquilines. Of that 683 individuals (0.47 individuals per trap-day) were collected in near-natural habitats, and 1060 individuals were collected at (post)industrial sites (0.71 individuals per trap-day). The lower frequency of aculeate hymenopterans captured in near-natural habitats when compared to the (post)industrial ones was statistically significant ($X^2 = 71.3$, $d_f = 1$, $p < 0.001$).

In summary, the Moericke trap captures revealed 178 species of bees and wasps (Supplementary Table A.3). The Chao-1 species richness estimator (corrected for unseen species in the samples) indicated total species richness of 240.0 ± 18.8 species in these two types of habitats together. However, the specimens obtained from near-natural habitats were composed of only 98 species, with Chao-1 estimator indicating the presence of 149.9 ± 16.9 species. Contrary to that, we captured 141 species in the (post)industrial habitats, where the Chao-1 estimator suggested the total species richness of bees and wasps active in early summer is 202.1 ± 17.7 species (Fig. 2).

Both the assemblages in the near-natural habitats and at the (post)industrial sites were dominated by generalist species which are also dominant in the surrounding landscape, such as *Apis mellifera*, *Lasioglossum morio* and *Lasioglossum pauxillum*, by the common obligate (*Trypoxylon deceptorium*) and facultative reed bed specialists (*Anoplius nigerrimus*, *Priocnemis fennica*), and by several species limited to either near-natural or (post)industrial habitats only. The near-natural sites were typically colonized by the endangered obligate reed bed specialist *Anoplius caviventris* and facultative reed bed specialist *Chalicodoma ericetorum*. The post-industrial reed beds were typically dominated by the species present exclusively or predominantly in the (post)industrial dataset and nesting in the sand (Fig. 3). Among the dominant species present exclusively in the (post)industrial habitats were *Nysson distinguendus* (nest parasite of *Harpactus elegans*), *Lasioglossum lucidulum* and vulnerable *Tachysphex obscuripennis*. Among the dominant species present prevalently in the (post)industrial habitats were *Lasioglossum politum*, *Lasioglossum sabulosum*, and the cleptoparasite of poorly defined host spectrum of ground nesting bees and wasps *Hedychrum niemelai*. A detailed overview of the species found, including the sampling site, date, and conservation status, are provided in Supplementary Table A.3.

The Moericke trap captures revealed 39 red-listed species, of which 18 red-listed species (46%; 88 individuals) were obtained at near-natural sites, while 29 red-listed species (74%; 148 individuals) were collected at (post)industrial sites. Importantly, the (post)industrial sites hosted *Nysson quadriguttatus* (female captured at a disused ash pond near Rybitví, PU), a species which had previously been thought regionally extinct. Besides that, nine critically endangered species were obtained, only three of them (five individuals) were found at near-natural sites, while seven of them (19 individuals) occurred at the (post)industrial sites. Among those captured in the near-natural habitats were *Ectemnius confinis*, *Tachytes panzeri* and *Systropha planidens*. The (post)industrial sites hosted *Hylaeus pectoralis*, *Belomicrus italicus*, *Ectemnius confinis*, *Mimumesa littoralis*, *Rhopalum gracile* (9 individuals at four sampling sites), *Halictus compressus* and *Sphecodes marginatus* (3 individuals at a single sampling site). We also obtained ten endangered species of bees and wasps, some of which displayed strong populations at multiple sampling sites. Four endangered species (64 individuals) were obtained at the near-natural sites, while nine endangered species (46 individuals) were obtained at the (post)industrial sites. The near-natural habitats hosted *Hylaeus moricei*, *Hylaeus rinki*, *Lasioglossum trichopygum* (12 individuals at a single sampling site) and *Anoplius caviventris* (48 individuals at seven sampling sites). The (post)industrial habitats hosted *Hylaeus moricei* (12 individuals at four sampling sites), *Crabro scutellatus*, *Oxybellus quattuordecimnotatus*, *Lasioglossum quadrinotatulum*, *Lasioglossum trichopygum* (nine individuals at a single sampling site), *Anoplius caviventris* (four individuals at two sampling sites), *Arachnospila ausa*, *Evagetes pectinipes* (three individuals at a single sampling site) and *Priocnemis minuta* (13 individuals at a single sampling site). We also obtained 19 species of bees and wasps considered as vulnerable, 11 of which (19 individuals) were captured in the near-natural habitats, while 12 (82 individuals) were captured in the (post)industrial habitats. The latter group was dominated by *Tachysphex obscuripennis* (36 individuals at four sites) and *Nysson maculosus* (10 individuals at four sites), both these species were completely absent in the vicinity of near-natural reed beds.

Of the species proven by the rearing experiment to utilize the reed galls, we detected both the species of the Colletidae family, six of the total seven crabronid species in the Moericke traps (all but *Nitela spinolae*), but failed to capture any of the cuckoo wasps of the Chrysididae family, or the single Vespidae species reared from the reed galls. We noticed striking differences in species dominance when comparing those reared from reed galls with those captured in Moericke traps. In particular the most numerously reared species, *Pemphredon fabricii*, represented by 2023 individu-

Table 3
Both density and diversity of Hymenoptera occupying the reed (*Phragmites australis*) galls was subject to strong variation between the sampling sites. Below are summarized numbers of males and females obtained from the indicated amount of reed galls collected at each of the particular near-natural ($n = 15$) and postindustrial ($n = 15$) sites across the Czech Republic. The species are classified according to the Czech red list of invertebrates (Farkač et al., 2005): vulnerable (VU), endangered (EN), and critically endangered (CR).

Sampling site (ID, township)		Species:	*Chrysis angustula*	*Pseudomalus auratus*	*Trichrysis cyanea*	*Dolichoderus quadripunctatus*	*Symmorphus bifasciatus*	*Nitela spinolae*	*Passaloecus clypealis*	*Pemphredon fabricii*	*Rhopalum gracile*	*Trypoxylon deceptorium*	*Trypoxylon minus*	*Hoplitis leucomelana*	*Hylaeus moricei*	*Hylaeus pectoralis*
		Status:	*EN*	–	–	–	–	–	*VU*	–	*CR*	–	–	–	*EN*	*CR*
		Number of galls	Number of reared individuals (M/F)													
Near-natural habitats																
N/1	Modletice	733								29/32					1/2	
N/2	Dobříkov	619														
N/3	Kosičky	522								33/5						
N/4	Dlouhopolsko	547								32/17						2/1
N/5	Žehuň	605														
N/6	Žíželice	554								25/10						
N/7	Lázně Bohdaneč	512								9/10						
N/8	Hrobice	516								66/32						
N/9	Nový Hr. Králové	533								6/10						
N/10	Borovany	984								138/72				1/0		1/2
N/11	Nákří	349								14/15						
N/12	Lomnice n. L.	314								30/40						3/1
N/13	Lednice	580								46/24						
N/14	Sedlec	543								26/19						
N/15	Olovnice	909								6/17						0/4
Postindustrial habitats																
I/1	Mydlovary; Olešník	633								58/43		2/1	1/1	0/1		1/0
I/2	Vojkovice	883								31/40		1/4				
I/3	Srubec	756								82/56		0/1				2/1
I/4	Cep	540	0/2	0/1						106/128		2/4	0/1	0/3		
I/5	Dobříň	760						1/2		205/106			0/1			2/4
I/6	Braňany	374								2/1						
I/7	Mariánské Radčice	471								18/9						2/4
I/8	Duchcov	497														0/1
I/9	Rybitví	641			0/3					46/19				0/1		
I/10	Rosice nad Labem	635								13/12						
I/11	Stéblová	542				0/73	3/0			31/9						
I/12	Nová Ves nad Lužnicí	567								9/25						
I/13	Olbramovice	526								2/5						
I/14	Brno	548			1/0				0/1	130/84	1/0	1/2				
I/15	Prachovice	598												0/3		
Total:																
Near-natural habitats		8820	0/0	0/0	0/0	0/0	0/0	0/0	0/0	460/303	0/0	0/0	0/0	1/0	1/2	6/8
(Post)industrial habitats																
Sites		8971	0/2	0/1	1/3	0/73	3/0	1/2	0/1	733/547	1/0	6/12	1/3	0/8	0/0	7/10

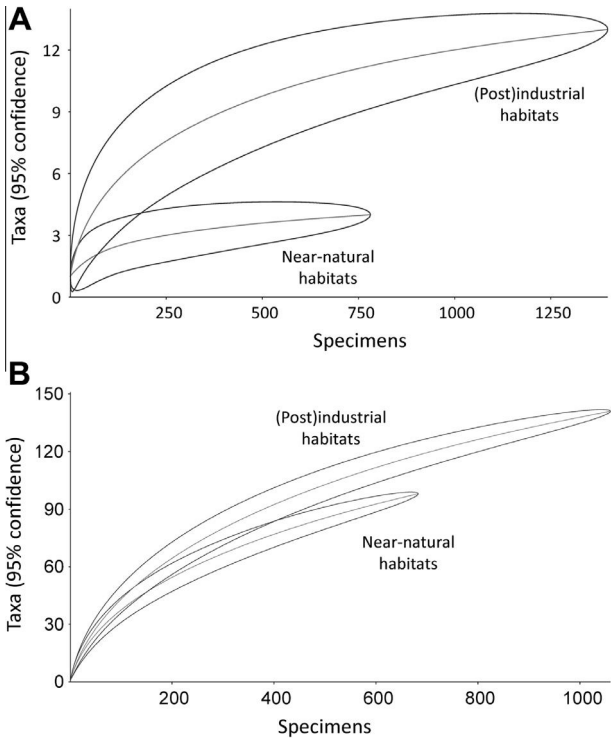

Fig. 2. Expected cumulative number of bee and wasp species as defined by rarefaction curve and associated Chao-1 estimator. Data are shown for specimens reared from reed galls (A) as well as for the specimens captured in Moericke traps exposed at the edge of reed stands (B).

als among the total 2176 hymenopterans reared, was only present in very low numbers in the Moericke trap dataset (just three individuals). Also the critically endangered *Hylaeus pectoralis* was represented by 31 individuals in the rearing experiment, but by only two individuals obtained from the Moericke traps. Contrary to that, the Moericke trap captures revealed higher numbers of the critically endangered *Rhopalum gracile* (1:9 individuals), *Trypoxylon deceptorium* (18 : 123), *Trypoxylon minus* (4:19), *Hoplitis leucomelana* (9 : 30), and endangered *Hylaeus moricei* (3:14) (Table 2).

4. Discussion

Although systematically collected data are limited, repeated incidental findings distinguish two types of reed stem utilization by bees and wasps, both of which were identified in this study. Species of the genera *Pemphredon* and *Spilomena* are considered to actively bite out their nest chambers in soft plant parenchymatous or dead tissues. Contrary to that, numerous other bee and wasp species utilize the galls. Specifically the large spheroid galls induced on oak twigs by *Andricus kollari* are occupied by *Trypoxylon* spp., *Crossocerus* spp., *Pemphredon* spp., *Passaloecus* spp. and *Ancistrocerus trifasciatus*. The galls on pine trees induced by *Retinia resinella* are utilized by the rare species *Ancistrocerus ichneumonideus* (Macek et al., 2010). Species composition of the reed gall inquilines is analyzed in detail in this study, which is the first report attempting to match Moericke trap captures performed at the edge of reed beds with experimental rearing of reed galls, thus allowing an assessment of the importance of reed galls as a potentially limiting nesting resource for assemblages of aculeate hymenopteran inquilines. The Moericke trap captures provide a complex view of the importance of the reed stands, advantaging from low selectivity of the color traps, but suffering from the presence of numerous species of the nearby habitats. Contrary to that, experimental rearing of reed galls provides a unique opportunity to study the specialized community of reed gall inquilines, which are limited in their diversity, but are directly dependent on the complex web offered by reed bed stands and their associated biota.

The 183 bee and wasp species captured at the edge of reed stands or reared directly from the reed galls during this study comprised 13.6% of the total 1,343 species reported from the Czech Republic (Bogusch et al., 2007), which is in strong contrast to the perception of the supposedly low importance of reed stands for aculeate hymenoptera. The reed galls themselves were found to host 13 species of bees and wasps, five of them red-listed, and some of them considered as reed bed specialists.

The species richness of bees and wasps considered as reed gall inquilines was substantially higher than those recorded in the few earlier studies. Dely-Draskovits et al. (1994) found in their extensive dataset of 3893 reed galls collected across a variety of Hungarian sites only three individuals of *Pemphredon fabricii* (determined as *P. lethifer* at that time) and eight undetermined individuals of

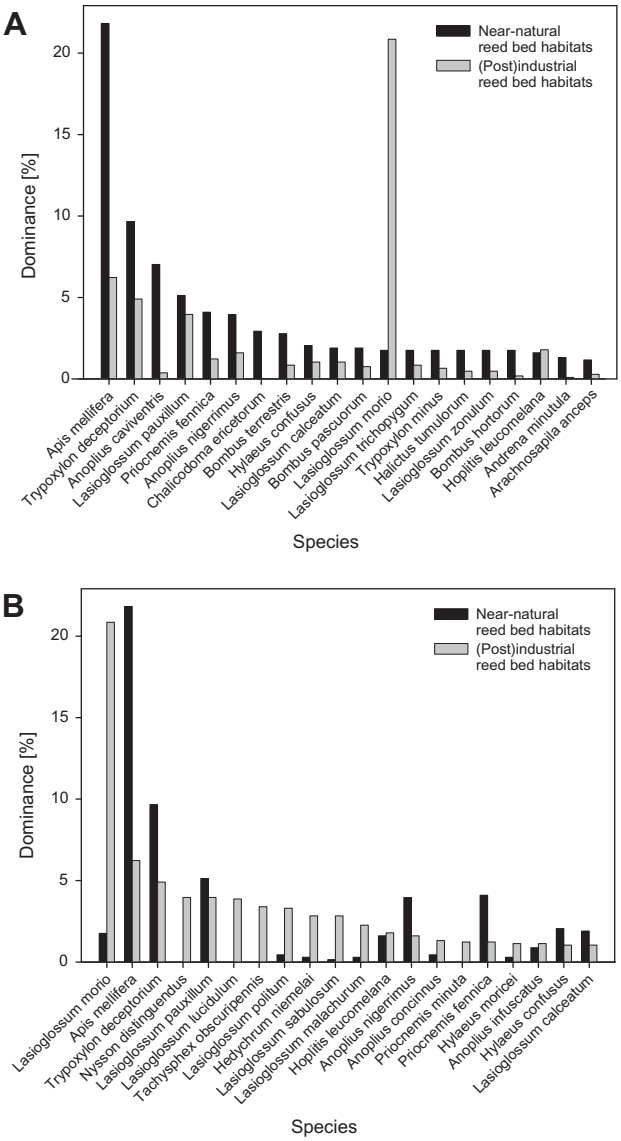

Fig. 3. Overview of dominant species of bees and wasps captured at the edge of reed beds in near-natural and (post)industrial habitats. The species are sorted according to the frequency of their captures at the near-natural sites (A) and at the (post)industrial sites (B). Note very limited overlap of the dominant species among the two datasets analyzed.

Hylaeus sp. Although we also identified *Pemphredon fabricii* and *Hylaeus* sp. as dominant species among our dataset (Table 2), the frequency of reared aculeate hymenoptera was higher than those reported by Dely-Draskovits et al. (1994) by over one order of magnitude (11 individuals out of 3893 galls vs. 2176 individuals out of 17,791 galls). The reasons for the above differences remain enigmatic. Since both these studies utilized sampling across a broad range of sampling sites (which argues against the effects of sampling site properties), the differences probably stem from the efficiency of the rearing technique used.

In another comparable study utilizing the reed gall rearing technique, Westrich (2008) collected 294 reed galls from three sampling sites in the vicinity of German Konstanz, rearing six species of aculeate hymenopterans, including *Hylaeus pectoralis* (129 individuals), *Hoplitis leucomelana* (classified as *Osmia leucomelana* at that time) (single male), *Pemphredon fabricii* (determined as *P. lethifer* at that time) (314 individuals), *Trypoxylon* cf. *deceptorium* (determined as *T. attenuatum* at that time) (two individuals), *Stenodynerus xanthomelas* (single individual) and *Chrysis cyanea* (four individuals). The higher frequency of the aculeate hymenopterans obtained by Westrich (2008) corresponds to his sampling design which selected for >1 year old galls which were examined thoroughly for signs that aculeate hymenopterans had entered (compared to our set of reed galls which was collected non-selectively, depending only on the spectrum of galls available at each sampling site). Similarly to Westrich (2008), we identified *Pemphredon fabricii* as the dominant species, with *Hylaeus pectoralis* as the second most frequently reared species. However, the frequency of *Hylaeus pectoralis* was lower in our samples (1.4% of the individuals reared) than in those obtained by Westrich (28.6% of the individuals reared), which corresponds to its focal occurrence, and to the fact that *H. pectoralis* is considered as critically endangered in the Czech Republic (Farkač et al., 2005), but endangered only in Germany (Westrich et al., 1998) and in the German state Baden-Württemberg (Westrich et al., 2000). *Trichrysis cyanea*, *Trypoxylon deceptorium* and *Hoplitis leucomelana* were present in our dataset as well. The remaining species identified by Westrich (2008), *Stenodynerus xanthomelas*, was absent in our dataset, being considered as critically endangered in the Czech Republic, but more frequently reported from Germany (Schmid-Egger, 2010). Recent reports of *S. xanthomelas* from the Czech Republic are absent; the nearest ones were reported from Southern Slovakia (P. Bogusch, unpubl.). In his study, Westrich (2008) failed to rear a number of inquilines present in our dataset suggesting introduction of some methodological bias when using his very selective method of reed gall collection, which was clearly enriching for *Pemphredon fabricii* and *Hylaeus pectoralis*, but not for the rest of the species spectrum.

Similarly to this study, Westrich (2008) reported a skewed sex ratio in reared *Pemphredon fabricii*, with 226 (72%) of males and only 88 (28%) of females reared. In our study, we obtained the ratio 1193 (58%) : 850 (42%) (Table 3). Westrich (2008) also noticed the similarly skewed sex ratio in *Hylaeus pectoralis*, which we were unable to corroborate due to the low number of *H. pectoralis* individuals reared.

Among the cuckoo wasps identified from the reed galls, all three species found are known to utilize at least occasionally the genera *Trypoxylon*, *Pemphredon*, or *Passaloecus* as their hosts (Macek et al., 2010). Westrich (2008) confirmed *Pseudomalus auratus* as a nest parasite of reed gall nests of *Pemphredon* cf. *fabricii* (1 *P. auratus* per 314 *P. fabricii*) and *Trypoxylon* cf. *deceptorium* (1 *P. auratus* per 2 *T. deceptorium*). Tormos et al. (1996) experimentally confirmed the generalist *Trichrysis cyanea* as a nest parasite with high incidence in the reed and ailanthus stem trap nests formed by *Trypoxylon* cf. *deceptorium* (determined as *T. attenuatum*) (27% incidence), *Trypoxylon figulus* (22% incidence), and *Pemphredon* cf. *fabricii* (determined as *P. lethifera*) (1% incidence).

The distribution of aculeate hymenopterans throughout the Czech Republic is considered as well-known already since early 20th Century. However, using the newly described rearing technique combined with the captures into the Moericke traps across 30 reed bed sampling sites, we substantially enhanced the knowledge on the distribution and abundance of the aculeate hymenopteran reed bed specialists. These include *Hylaeus moricei* (Fig. A.1A), *Hylaeus pectoralis* (Fig. A.1B), *Nysson quadriguttatus* (Fig. A.1C), *Passaloecus clypealis* (Fig. A.1D), *Rhopalum gracile* (Fig. A.1E), and *Trypoxylon deceptorium* (Fig. A.1F), all but *Nysson quadriguttatus* known to be limited to reed beds and with low number of prior records from the Czech Republic. Some of the above species, such as *T. deceptorium*, are newly shown as common across the broad range of reed beds. Many others were found to be limited to reed beds at hygrothermophilous habitats, particularly to those growing under the stress of any kind. We speculate that the presence of highly stressed reed beds at the postindustrial sites causes the higher diversity and abundance of reed gall specialists in the (post)industrial sites when compared to the near-natural habitats.

5. Conclusions

The enormous extent of quarrying activities performed in the Czech Republic provides an attractive basis for studies on the ability of organisms to adjust to the increasingly human-dominated landscape. In this study, we have shown that the reed beds, particularly the reed bed galls, are home to a diverse community of aculeate hymenopteran inquilines, many of which are considered as rare, endangered, and in many cases limited to these sites. These species display specific habitat requirements, often requiring not only the presence of reed, but also the presence of loose sandy bedrock below the reed bed. These species, which nearly disappeared from the surrounding cultural landscape and from the long-time existing reed beds in the vicinity of rivers, streams and medieval fishponds, found their surprising refuge in reed beds occurring newly on the exposed loose bedrock of (post)industrial sites, including gravel-sandpits, ash ponds and tailing ponds. The obtained data challenge the common view of the expanding reed beds as a threat for biodiversity. Interestingly, many rare aculeate hymenopteran inquilines were absent or underrepresented at the Czech near-natural sites when compared to the (post)industrial sites. We assume that reed gall inquilines enriched in the (post)industrial dataset could represent, however, the specialists of river beds growing at the active river terraces, which are freshly formed from sand or gravel-sand. Such habitats are nearly absent in todays' Czech Republic due to extensive river regulation, and differ from the near-natural dataset examined in their oligotrophic nature similar to the reed beds formed *de novo* at the (post)industrial sites.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocon.2014.02.037.

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3.2 BOGUSCH P., ASTAPENKOVÁ A. & HENEBERG P. 2015: Larvae and Nests of Six Aculeate Hymenoptera (Hymenoptera: Aculeata) Nesting in Reed Galls Induced by *Lipara* spp. (Diptera: Chloropidae) with a Review of Species Recorded. *PLoS ONE* **10**: e0130802.

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RESEARCH ARTICLE

Larvae and Nests of Six Aculeate Hymenoptera (Hymenoptera: Aculeata) Nesting in Reed Galls Induced by *Lipara* spp. (Diptera: Chloropidae) with a Review of Species Recorded

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Abstract

Wetland species of aculeate Hymenoptera are poorly known, even though many of them may serve as diagnostic or flagship species in nature conservation. Here we examined 6,018 galls induced ≥1 year prior their collection by the chloropid flies *Lipara* spp. The galls were collected at 34 sites in Central Europe. We examined 1,389 nests (4,513 individuals) of nine species, part of which were parasitized by one dipteran and two chrysidid parasitoid species. We describe the nests of seven dominant species and larvae of four species (*Pemphredon fabricii*, *Trypoxylon deceptorium*, *Hoplitis leucomelana*and *Hylaeus pectoralis*) and two parasitoids (*Trichrysis cyanea* and *Thyridanthrax fenestratus*, both in nests of *Pemphredon fabricii*and *Trypoxylon deceptorium*). All the species, but *H*. *pectoralis*, preferred robust galls at very thin stalks (induced typically by *Lipara lucens*) over the narrow galls on thick stalks. The larvae of *P*. *fabricii* and *T*. *deceptorium*resembled strongly their sibling species (*Pemphredon lethifer*and *Trypoxylon attenuatum* sensu lato, respectively). The larvae of *T*. *fenestratus* showed features different from those previously described. By hatching set of another 10,583 galls induced by *Lipara* spp. ≥1 year prior their collection, we obtained 4,469 individuals of 14 nesting hymenopteran species, two cleptoparasites, three chrysidid and one dipteran parasitoid. Of these species, four new nesting species have been recorded for the first time in galls induced by *Lipara* spp.: *Chelostoma campanularum*, *Heriades rubicola*, *Pseudoanthidium lituratum* and *Hylaeus incongruus*. We also provide first records of their nest cleptoparasites *Stelis breviuscula* and *Stelis ornatula*, and the parasitoid *Holopyga fastuosa generosa*. *Thyridanthrax fenestratus* formed strong populations in nests of *Pemphredon fabricii* and *Trypoxylon deceptorium*, which are both newly recorded hosts for *T*. *fenestratus*. The descriptions provided here allow for the first time to identify the larvae of the most widespread central European aculeate hymenopteran reed gall specialists.

Introduction

Cavity-nesting Hymenoptera developed a wide range of strategies allowing them to use a broad spectrum of cavities for nesting. Among the Palearctic species, a specific community of bees and wasps make their nests in the galls of chloropid flies. Most frequently, they use the galls induced by *Lipara lucens* (Chloropidae) on common reed *Phragmites australis* (Poaceae) stems [1–3]. Some of these aculeate hymenopteran species, such as the digger wasp *Pemphredon fabricii* (Crabronidae) or the solitary bee *Hylaeus pectoralis* (Colletidae) are specialized for nesting in galls induced by *Lipara* spp. more than a year ago (old galls) [2–3]. However, most of the other aculeate hymenopteran species found in the reed galls are capable to use many different kinds of cavities for their nests such as cut reed, old larval galleries in wood and cavities in old walls. Combined, the reed galls in central and north Europe are confirmed to host altogether 25 species of aculeate Hymenoptera (superfamilies Chrysidoidea, Vespoidea and Apoidea) [2–4] and 3 species of their parasitoids of the family Chrysididae. Some of these species (e.g., *Pemphredon fabricii*) are locally very abundant, whereas many others (such as *Rhopalum gracile* (Crabronidae)) are extremely rare and considered critically endangered or endangered in the regional red-lists [5–9].

The biology, nest structure and larval morphology are known for only few species of the aculeate hymenopteran reed gall nesters. *Pemphredon fabricii* is the most numerous aculeate hymenopteran species in reed galls [2–3]. This species was for a long time considered to be a form or a subspecies of closely related *Pemphredon lethifer*, which is more widespread and ecologically tolerant. Special blunt claws on tarsi of this species work as a very good adaptation for moving in reed, and this species does not occur in any other habitats than reed beds [10–11]. This species was resurrected from the synonymy [12] and its nesting habits were described [13] together with its specificity to reeds, which was later confirmed by [2–3]. Mature larvae of the related species *P. lethifer* were described previously [14–15]. In both cases, the descriptions of the nest and mature larvae were based on the specimens obtained from the hollow stems of *Rubus* spp. (Rosaceae), and thus do not represent the nests and larvae of *P. fabricii*.

Hylaeus pectoralis is a rare bee, occurring only in wetlands. It is specialized for nesting in old *Lipara* galls, reaching only low population densities at any sites of its presence [2–3]. The species is classified as critically endangered (CR) in the Red List of Czech Invertebrates [9]. However, [3] found that this species only escaped the traditional sampling techniques and was present at multiple sites at least throughout the Czech Republic, thus not being as rare as was previously supposed (cf. [16–17]). Its larvae and nests were described [18], and its biology was briefly described from England [19]. Reed galls host also a smaller species of the same genus, *Hylaeus moricei*, which is less specialized and may utilize also other types of cavities. Larva and nest of this species are so far unknown. *Hoplitis leucomelana* (Megachilidae) is another bee nesting frequently in reed galls. It is a generalist and uses many different cavity types for nesting [17, 20]. The larva of this species was also described [21] as well as its nest in the cavity of *Rubus* spp. [20].

Asís et al. described the nesting biology, nest structure and morphology of mature larva of *Trypoxylon attenuatum* sensu lato (Crabronidae) [22]. This digger wasp was previously thought to be a single species, but later it was divided it to six species [23]. Of them, *T. deceptorium* is bound to wetlands and nests in reed galls and cavities. Although [3] found this species as the second most abundant aculeate hymenopteran in reed galls (after *P. fabricii*), they found that *T. attenuatum* is the only *Trypoxylon* spp. occurring in reed galls. So far, there are no data allowing differential morphological diagnosis between the larvae of *T. attenuatum* sensu stricto and *T. deceptorium*. *Trypoxylon attenuatum* sensu lato nests may be parasitized by *Trichrysis cyanea* (Chrysididae), and mature larva of *T. cyanea* and *T. attenuatum* were described by

[22]. Among all the parasitoids and nest cleptoparasites, *T. cyanea* is most abundant in nests of digger wasps in reed galls. The main reason should be that *T. cyanea* displays the least host specificity among European golden wasps, invading nests of plenty bee and wasp species [17, 24–26].

In this study, we examined an extensive set of reed galls and analyzed and broadened the known spectrum of aculeate hymenopteran species associated with the reed galls induced by *Lipara* spp. in the Czech Republic and Slovakia, Central Europe. Using the dataset of individually examined reed galls, we analyzed and described the nesting biology and mature larvae of six species of aculeate Hymenoptera. Particularly important are the first available data on the differences in larval morphology of sibling species *P. fabricii* and *P. lethifer*, as well as *T. attenuatum* and *T. deceptorium*.

Materials and Methods

Study sites and sampling

We have collected galls induced by *Lipara* spp. on common reed at 34 sampling sites located across the Czech Republic (33 sites) and in Slovakia (1 site) (Fig 1), for detailed information see S1 Table. All localities with coordinates are listed in Supporting Information (S1 Table). Study of plants and animals was possible at all localities without any restriction, except the following: Třebeč (Brouskův Mlýn National Nature Reserve (NNR)), permission issued from Blanský les Protected Landscape Area (PLA), personally Jana Janáková, MSc.; Lomnice nad Lužnicí (Velký a Malý Tisý NNR), permission issued from Třeboňsko PLA, personally Dr. Miroslav Hátle; Lednice (Lednické rybníky NNR), permission issued from Pálava PLA, personally Dr. Pavel Dedek; Vonšov (SOOS NNR), permission issued from Slavkovský les PLA, personally Přemysl Tájek; Chvaletice and Trnávka (Power Station Chvaletice fly ash deposits), permission issued

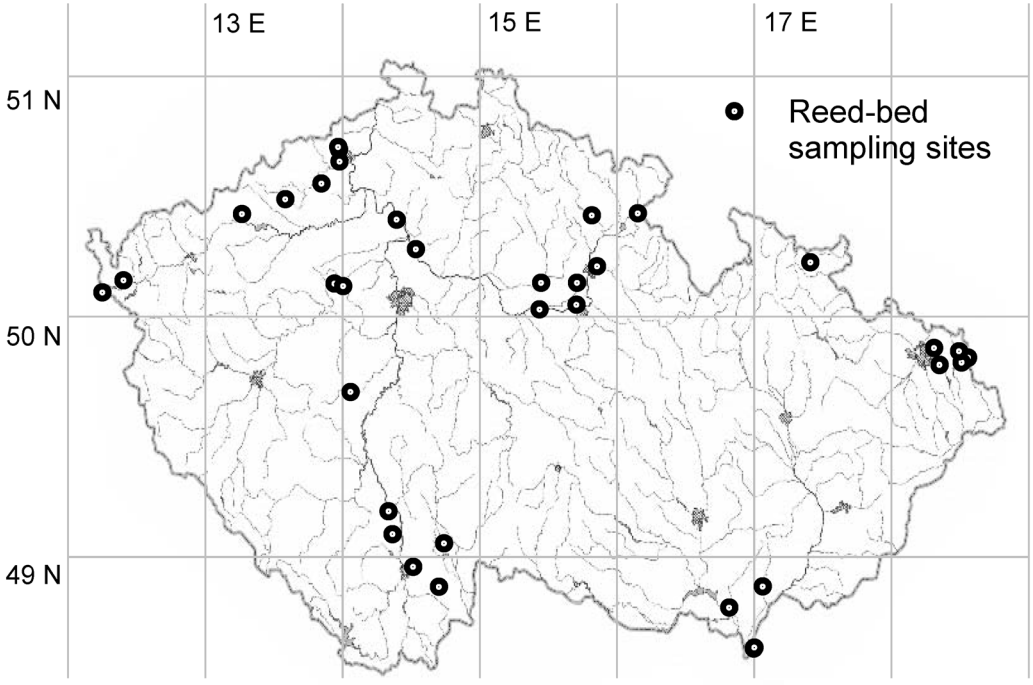

Fig 1. Location of study sites in the Czech Republic and Slovakia.

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from Severní energetická a.s., personally Ing. Karel Polc. The field studies did not involve any animals protected in the Czech Republic or Slovakia and no CITES species.

Only galls older than 1 year (greyish or darker in appearance, usually without leaves and with the apex broken) were collected because of our focus on cavity nesting Hymenoptera (bees and wasps), not on the *Lipara* spp. (inducing the galls) or their parasitoids. We have collected the reed galls in a period from 7 Feb to 23 Mar 2014 and additional material on 4 Sep 2014. In late winter and early spring, the mature larvae are present in their nests, and their rearing is easier than if they would be collected before the hibernation in the autumn months. We collected at least 500 reed galls at each sampling site, of which 200 were longitudinally cut and their contents were analyzed and the rest were allowed to rear. Incidentally collected galls of age <1 year (with *Lipara* spp. or their parasitoids present) were removed from the analyses, thus the total counts of galls analyzed from each site were slightly lower.

Data acquisition

In the longitudinally cut reed galls, we have studied the material of the walls separating the brood cells (further termed septa) and closing plug at the top of each nest (further termed closing plug), the structure and number of brood cells, and also the morphology and coloration of larvae and pupae. In the description, first cell means the hind, first-built cell of the nest, and last cell means the nearest cell to the nest entrance. When the larvae were in cocoons, we removed part of the larvae out of the cocoons but left the others inside of them. Of each species, we first tried to rear the adults; only for nests with more than three larvae we conserved some brood for morphological studies. From each hymenopteran nest, all living larvae were taken, placed in Eppendorf 1.5 ml micro-tubes, which were closed with cotton wool, left in the laboratory conditions and reared similarly as was described by [27]. The adults usually hatched within three to four weeks after the pupation and then we fixed them (as well as unreared larvae) in 96% ethanol. We measured the maximum diameter of the reed gall and the diameter of the reed stem just below the reed gall.

The reed galls allowed to rear were placed into rearing sacs as described [3], and allowed to hatch for ten weeks. The reared individuals were fixed in an ethylene glycol solution supplemented with a mixture of ionic and anionic detergents and transferred later to the 96% ethanol.

The obtained material was determined by the first author, and representative specimens (including the nests of each species) are available in the collections of University of Hradec Králové (Hradec Králové, Czech Republic). We used the nomenclature according to [16] (aculeate Hymenoptera), [4] (Chloropidae) and [28] (Bombyliidae).

We documented representative part of the nests using a digital camera (photographs of whole nests) and a special macro-photographing apparatus consisting of a macro-camera attached to a stereo microscope (brood cells, whole larvae and pupae). The documentation included the photographs of nests shared by multiple species of aculeate Hymenoptera and of the parasitized nests. We took photos of both, living larvae and the larvae fixed in Pampel solution (30 parts of distilled water, 15 parts of 96% ethanol, 6 of formaldehyde and 4 parts of glacial acetic acid) as described [29]. The photos of living specimens turned to be more suitable for field identification of the here described species. To describe morphology of the larval specimens, we transferred some larvae (but always only a portion of larvae present in a single nest) into the Pampel solution. As soon as we took the photographs of the whole larvae, we focused on their sclerotized parts. For this purpose, we placed the larvae for 12 hours into 10% solution of hot (60°C) potassium hydroxide to dilute all parts of the body except the integument. Then we colored the integument in 5% Chlorazol Black E for 2 seconds and moved the specimens

into 96% ethanol for conservation. To observe the identification marks, we placed the integument into glycerol and observed separately the head, mouthparts, spiracles and other important parts of the integument under light microscope. We used the same specimens for the study of very small structures such as setae, sensillae or mouthparts. We drew figures of (1) the head with a focus on the clypeus, labrum, maxillae and labium, (2) the mandibles from anterior view, and (3) the spiracles of each larva.

Data analysis

The data are shown as means ± SD unless stated otherwise. We analyzed the occupancy rate of three size categories of reed stems and four size categories of reed galls. To analyze the differences between the observed and expected occupancy rates in the particular size categories of reed galls and reed stems, we used χ^2 tests. The expected frequencies were derived from the frequency of reed galls or stems of the particular diameter within the whole sampled cohort using the following equation:

$$n_{i\ (expected)} = \frac{n_{i\ (observed)}}{\sum_i n_{(observed)}}$$

Where $n_{\mathrm{i\ (expected)}}$ represents the expected frequency, $n_{\mathrm{i\ (observed)}}$ represents the observed frequency of reed galls or stems of the particular diameter within the whole dataset, and $\Sigma_i\ n_{(\mathrm{observed})}$ stands for the total number of reed galls or stems collected and measured. To estimate the completeness of the sampled dataset, we computed the rarefaction curve based on the log Gamma function for computing combinatorial terms in PAST v 2.14. To estimate the species richness in the examined dataset, we calculated the Chao-1 estimator, corrected for unseen species in the dataset in EstimateS 9.1.0. Both calculations included all the adult specimens of aculeate Hymenoptera and their parasitoids obtained in course of this study by hatching the imagines from longitudinally cut galls and those reared directly from the galls.

Results

Aculeate Hymenoptera in longitudinally cut reed galls of *Lipara* spp.

We found 1389 nests of aculeate Hymenoptera in the total 6,018 longitudinally cut reed galls induced by *Lipara* spp. Of them, we identified 1159 nests of nine species of aculeate Hymenoptera, two species of cuckoo wasps and a single parasitic dipteran species (Table 1). The occupancy rate was highly site-specific, ranging from 66.5% (Chvaletice, PU, Czech Rep.) to zero (two sampling sites Cheb and Vonšov, CH, Czech Rep.) despite we used the identical sampling methodology at all the examined sites. The variability found can be explained by the reed gall parameters as described below.

Pemphredon fabricii was the most abundant species of aculeate Hymenoptera, confirmed in 1012 nests (90% of the non-parasitized nests of aculeate Hymenoptera), and identified at all 34 sampling sites where the hymenopteran nests were present in the collected reed galls. Other aculeate hymenopteran species nested in the galls in smaller numbers. The examined dataset included *Trypoxylon deceptorium* (39 nests found at 10 sampling sites), *Hylaeus pectoralis* (27/9), *Hoplitis leucomelana* (10/6), *Symmorphus bifasciatus* (6/5), *Trypoxylon minus* (6/4), *Hylaeus moricei* (3/3), *Passaloecus clypealis* (3/2) and *Hylaeus incongruus* (1/1). The latter species was recorded for the first time nesting in the reed galls induced by *Lipara* spp.

Among the three species of parasitoids of aculeate Hymenoptera found, the most abundant was the chrysidid wasp *Trichrysis cyanea* identified in 16 nests at 7 sampling sites. As its host species, we confirmed *Pemphredon fabricii* (4 nests containing immature individuals of both

Table 1. Number of galls examined and of those containing the brood of species of aculeate Hymenoptera (Vespoidea, Apoidea) and their parasitoids (Hymenoptera: Chrysidoidea; Diptera: Asiloidea).

Species	Number of individuals reared from intact galls	Relative proportion of individuals reared [%]	Number of nests found in longitudinally cut reed galls	Relative proportion of nests found in reed galls [%]
Diptera / Bombyliidae				
Thyridanthrax fenestratus (Fallén, 1814)*	44	0.97	18	1.30
Hymenoptera / Chrysididae				
Chrysis angustula Schenck, 1856*	10	0.22	1	0.07
Holopyga generosa Förster, 1853*	1	0.02	0	0.00
Trichrysis cyanea (Linnaeus, 1761)*	19	0.42	16	1.15
Hymenoptera / Vespidae				
Symmorphus bifasciatus (Linnaeus, 1761)	21	0.47	6	0.43
Hymenoptera / Crabronidae				
Ectemnius confinis (Walker, 1871)	2	0.04	0	0.00
Nitela spinolai Latreille, 1809	3	0.07	0	0.00
Passaloecus clypealis Faester, 1947	11	0.24	3	0.22
Pemphredon fabricii (Müller, 1911)	4205	93.18	1029	74.08
Rhopalum gracile Wesmael, 1852	3	0.07	0	0.00
Trypoxylon deceptorium Antropov, 1991	38	0.84	39	2.81
Trypoxylon minus Beaumont, 1945	6	0.13	6	0.43
Hymenoptera / Megachilidae				
Chelostoma campanularum (Kirby, 1802)	1	0.02	0	0.00
Heriades rubicola Pérez, 1890	3	0.07	0	0.00
Hoplitis leucomelana (Kirby, 1802)	16	0.35	10	0.72
Pseudoanthidium lituratum (Panzer, 1801)	1	0.02	0	0.00
Stelis breviuscula (Nylander, 1848)*	3	0.07	0	0.00
Stelis ornatula (Klug, 1807)*	4	0.09	0	0.00
Hymenoptera / Colletidae				
Hylaeus incongruus Förster, 1871	0	0.00	1	0.07
Hylaeus moricei (Friese, 1898)	17	0.38	3	0.22

(*Continued*)

Table 1. (*Continued*)

Species	Number of individuals reared from intact galls	Relative proportion of individuals reared [%]	Number of nests found in longitudinally cut reed galls	Relative proportion of nests found in reed galls [%]
Hylaeus pectoralis Förster, 1871	105	2.33	27	1.94
Not identified	0	0.00	230	16.56
TOTAL (individuals reared)	**4513**			
TOTAL (nests found)			**1389**	
TOTAL (reed galls examined)	**10583**		**6449**	

We found 20.6% of one year old reed galls positive for the nests of aculeate Hymenoptera. Indicated are the number of individuals hatched in the rearing bags, number of nests positive for each species found as revealed by longitudinal cutting of the reed galls, and their relative proportions within the total datasets. Species marked by asterisks are parasitic.

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species) and *Trypoxylon deceptorium* (1 nest with both species). Additionally, we identified a single nest occupied by *Chrysis angustula* [in a nest of *Pemphredon fabricii* at the sampling site Kamenné Žehrovice (KL, Czech Rep.)]. All the above host-parasitoid associations are new host records (globally). In the nests of *Pemphredon fabricii* at the sampling sites Sekule (SE, Slovakia) and Hodonín (HO, Czech Rep.), we noticed a high parasitation rate by the larvae of bombyliid fly *Thyridanthrax fenestratus*. We identified larvae of this parasitoid in 21 of 89 *P. fabricii* nests (24%) at the sampling site Sekule, and reared the *T. fenestratus* adults from 18 of them (and also reared the adults of the host species from three of these nests). At the sampling site Hodonín, we identified larvae of *T. fenestratus* in 6 of 29 *P. fabricii* nests (21%), but the adults did not hatch from any of them. In September 2014, we recorded another *Thyridanthrax fenestratus* individuals in a set of 200 reed galls collected at the sampling site Sekule, where we also found it in cocoons inside one nest of *Trypoxylon deceptorium* (besides *P. fabricii*).

Besides the nests occupied by a single species and those occupied by the parasitoids (see Fig 2A–2F), we found 12 galls containing immature individuals of two different aculeate hymenopteran species. In all cases, the nests were used sequentially (not at the same time): when one species finished its nest, then the second species started to work on its own nest occupying the rest of the space available in the gall. We did not observe any signs of killed brood. The mixed nests included the following combinations: *Pemphredon fabricii* and *Trypoxylon deceptorium* (9×), *Pemphredon fabricii* and *Hylaeus pectoralis* (2×; Fig 2G), and *Pemphredon fabricii* and *Trypoxylon minus* (1×).

Aculeate Hymenoptera in reared reed galls of *Lipara* spp.

In parallel to the longitudinally cut reed galls, we collected another set of 10,583 reed galls, which were allowed to rear. Their rearing yielded in total 4,469 individuals of 19 species of aculeate Hymenoptera (including 30 individuals of three hymenopteran parasitoid species) and 44 individuals of a single dipteran parasitoid species (Table 1). The species spectrum was similar to that obtained by longitudinal cutting of the collected galls, with some exceptions as specified below. *Pemphredon fabricii* was eudominant among the aculeate hymenopterans with 4,205 adults hatched (94% of the total). The other abundant species included *Hylaeus pectoralis* (91 individuals / 10 sampling sites) and *Trypoxylon deceptorium* (38 individuals / 9 sites). Less

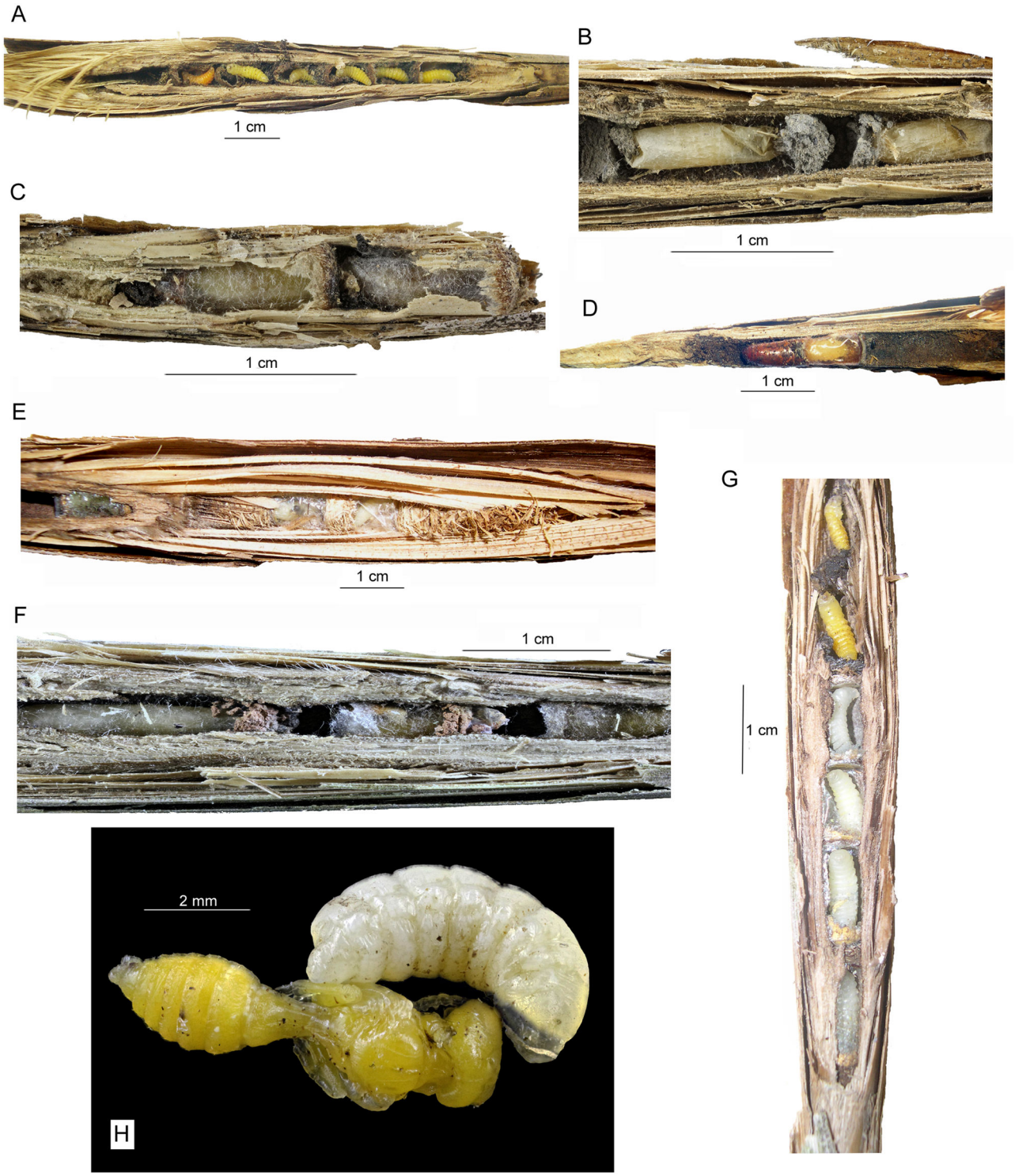

Fig 2. Nests of aculeate Hymenoptera in reed galls of *Lipara* spp. A–*Pemphredon fabricii*, whole nest, B–*Trypoxylon deceptorium*, detail, C–*Hoplitis leucomelana*, detail, D—nest with larva and pupa of *Trichrysis cyanea*, E—*Hylaeus pectoralis*, whole nest, F—parasitized nest of *Trypoxylon deceptorium* with one brood cell of *T. deceptorium* and two brood cells of *Trichrysis cyanea*, G—mixed nest of two species containing four white larvae of *Hylaeus pectoralis* (bottom) and two yellow larvae of *Pemphredon fabricii* (top), H—larva of the bombyliid fly *Thyridanthrax fenestratus* on pupa of *Pemphredon fabricii*. All photos by P. Bogusch.

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than 20 imagines were obtained from the rest of the species found. We reared several species, which were not known as reed gall inquilines so far. Among them were the golden wasp *Holopyga fastuosa generosa* [1 female from Tuchomyšl (UL, Czech Rep.)], the bees *Chelostoma campanularum* [1 female from Dubno (PB, Czech Rep.)], *Heriades rubicola* [3 individuals from Hodonín (HO, Czech Rep.)], *Pseudoanthidium lituratum* [1 male from Hodonín (HO, Czech Rep.)], *Stelis breviuscula* [3 individuals from spoil heap near Srnojedy (PU, Czech Rep.)] and *S. ornatula* [4 specimens: 1 from Stonava (KI), 2 from Stará Pohůrka (CB) and 1 from Tuchomyšl (UL, all Czech Rep.)]. The latter two bee species are cleptoparasitic; *S. breviuscula* parasitizes *Heriades* spp., and *S. ornatula* utilizes *Hoplitis* spp., including *H. leucomelana*, as its host species [17]. We also confirmed the presence of two digger wasp species reported earlier by [3]: *Nitela spinolae* at two ash deposits near Pardubice (PU, Czech Rep.) and *Rhopalum gracile* (3 specimens found in Náchod, NA, Czech Rep.), and another digger wasp species reported earlier by [4]: *Ectemnius confinis* [2 males from Doubrava u Orlové (KI, Czech Rep.)]. Regarding the single dipteran parasitoid of aculeate hymenopterans found, *Thyridanthrax fenestratus*, in total 44 imagines hatched from galls collected at the same sampling sites at which we found it in the longitudinally cut galls (21 and 23 individuals, respectively).

In course of this study, we hatched 6,951 adult imagines of aculeate Hymenoptera and their parasitoids, representing 21 species (Table 1). Rarefaction of the obtained dataset (Fig 3) suggested that we reached high level of completeness. The Chao-1 estimator reached 22.0 ± 1.8 (95% CI 21.1–31.7) species. Combined, the total number of species of aculeate Hymenoptera known from galls induced by *Lipara* spp. in Europe now reached 29, including six hymenopteran parasitoids. In addition, we provided the evidence on the presence of a single dipteran parasitoid species of aculeate Hymenoptera in the examined reed galls (Table 1).

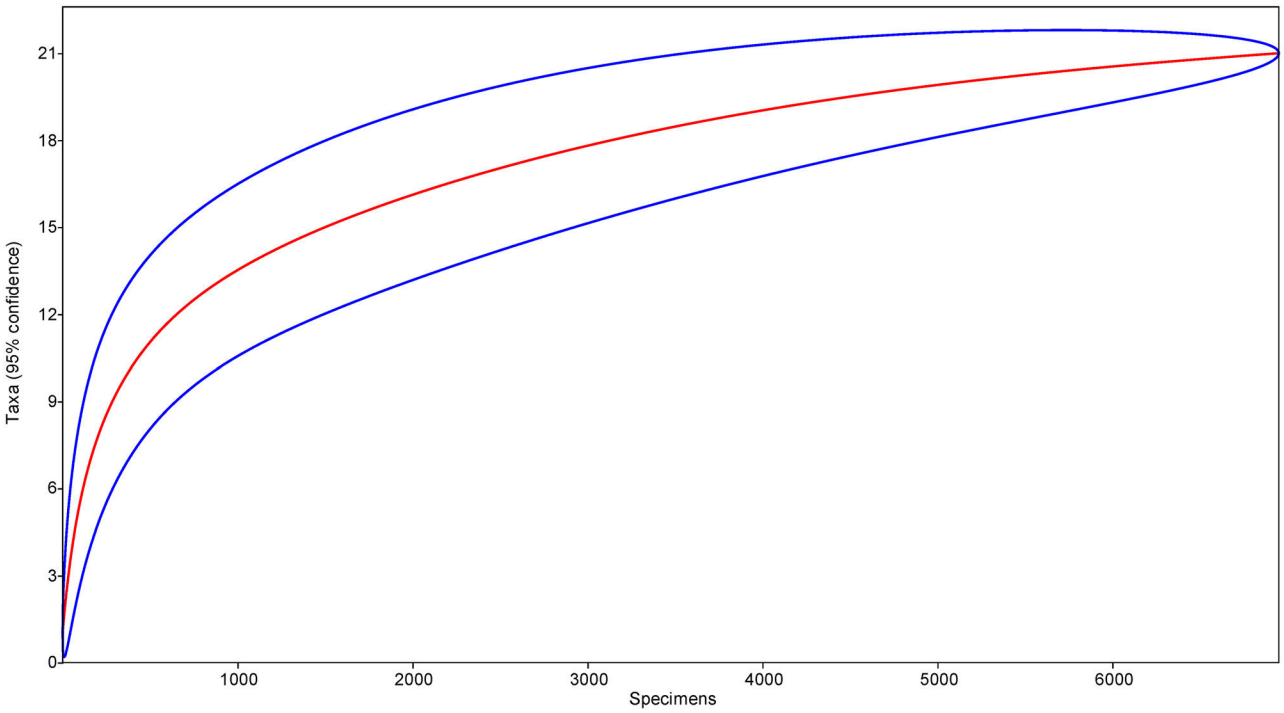

Fig 3. Rarefaction curve of the aculeate Hymenoptera nesting in reed galls and of their parasitoids based on all the hatched adult imagines obtained in course of this study (n = 6,951). Numbers on the X axis indicate the number of individuals hatched (specimens).

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Table 2. Diameter of the reed stems and galls and the number of brood cells in the galls examined and in those containing the brood of species of aculeate Hymenoptera (Vespoidea, Apoidea) and their parasitoids (Hymenoptera: Chrysidoidea; Diptera: Asiloidea).

Species	Number of galls (nests) examined	Diameter of the reed stem [mm]		Diameter of the reed gall [mm]		Number of brood cells per gall	
		Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range
Diptera / Bombyliidae							
Thyridanthrax fenestratus	18	3.4 ± 0.8	2–5	12.1 ± 2.0	9–18	5.6 ± 1.8	2–8
Hymenoptera / Chrysididae							
Chrysis angustula	1	N/A	4	N/A	10	N/A	5
Trichrysis cyanea	16	3.1 ± 1.0	2–5	9.2 ± 1.8	5–13	2.1 ± 1.4	1–5
Hymenoptera / Vespidae							
Symmorphus bifasciatus	6	3.7 ± 0.8	3–5	11.5 ± 3.6	8–16	3.5 ± 2.4	1–7
Hymenoptera / Crabronidae							
Passaloecus clypealis	3	2.3 ± 0.6	2–3	7.3 ± 1.5	6–9	2.3 ± 0.6	2–3
Pemphredon fabricii	1029	3.2 ± 1.0	1–7.5	10.0 ± 2.3	5–25	4.3 ± 1.8	1–12
Trypoxylon deceptorium	39	2.8 ± 0.7	2–5	9.1 ± 2.1	5–15	2.5 ± 1.5	1–6
Trypoxylon minus	6	2.8 ± 0.7	2–4	9.2 ± 1.0	8–10	3.2 ± 1.7	1–6
Hymenoptera / Megachilidae							
Hoplitis leucomelana	10	2.9 ± 0.9	2–4	9.0 ± 2.0	6–12	2.4 ± 1.1	1–4
Hymenoptera / Colletidae							
Hylaeus incongruus	1	N/A	2	N/A	10	N/A	3
Hylaeus moricei	3	3.7 ± 1.2	3–5	7.3 ± 1.5	6–9	4.3 ± 2.3	3–7
Hylaeus pectoralis	27	3.6 ± 1.2	2–6.5	10.7 ± 2.7	8–19	3.7 ± 2.1	1–11
Not identified	230	N/A		N/A		N/A	
Unoccupied	4629	3.7 ± 1.4	1–12	8.7 ± 3.0	2–23	N/A	
TOTAL (reed galls examined)	**6449**						

We examined only the old galls induced by the *Lipara* spp. at least one year prior the sampling.

doi:10.1371/journal.pone.0130802.t002

Structure of nests

We analyzed the nests of six species nesting in reed galls induced by *Lipara* spp. flies (Tables 2 and 3). Of the species found in longitudinally cut galls, we only did not describe the nests of *Passaloecus clypealis* and *Hylaeus incongruus* due to the small number of nests (3 and 1 nests, respectively) in our dataset.

Pemphredon fabricii (Fig 2A). The nests of the most abundant species in our dataset consisted of highly variable numbers of brood cells (range 1 to 12; median 4; mean 4.3 ± 1.8 cells per nest, n = 1029 nests). Brood cells (length 7.9 ± 1.9 mm) were separated from each other by 1–2 mm thick black-colored bars (septa) of unidentified material mixed with silk and larval salivae. This material might consist of the liquid larval feces greased on silk bars between the cells. We made this conclusion because no feces were found in brood cells and defecating younger larvae observed during the summer were extracting black liquid solution (P. Bogusch, unpubl.). Dry plant matter and soil particles were present in this substance, too. In some cases, behind the last cell and/or in front of the first cell, closing plug consisting of small sand grains mixed with very small pieces of plant tissues (leaves, stems of size around 1 mm) was used, but only very sparsely. Every nest had thin bar on the top consisting of the same black matter. The nests of *P. fabricii* were placed in galls induced prevalently at the very thin reed stems (up to 4 mm in diameter, at which 22.5% of galls were occupied. In contrast, *P. fabricii* occupied only 12.6% of galls induced at the reed stems of the diameter 4–5.5 mm, and just 3.7% of galls at thick reed

Table 3. Species-specific occupancy rate of reed galls induced by *Lipara* spp. at least one year prior the sampling.

Species	Number of galls examined	Occupancy rate [%]								
		Diameter of the reed stem [mm]				Diameter of the reed gall [mm]				
		<4	4–5.5	≥6	Significance	<5	5–9.5	10–14.5	>15	Significance
Diptera / Bombyliidae										
Thyridanthrax fenestratus	18	0.35	0.30	0.00	>0.05	0.00	0.06	0.70	0.39	<0.001
Hymenoptera / Chrysididae										
Trichrysis cyanea	16	0.32	0.25	0.00	>0.05	0.00	0.30	0.38	0.00	>0.05
Hymenoptera / Vespidae										
Symmorphus bifasciatus	6	0.09	0.12	0.00	>0.05	0.00	0.09	0.05	0.78	0.005
Hymenoptera / Crabronidae										
Passaloecus clypealis	3	0.09	0.00	0.00	>0.05	0.00	0.09	0.00	0.00	>0.05
Pemphredon fabricii	1029	22.5	12.6	3.7	<0.001	0.00	14.1	23.7	18.8	<0.001
Trypoxylon deceptorium	39	1.07	0.21	0.00	<0.001	0.00	0.72	0.65	0.39	>0.05
Trypoxylon minus	6	0.13	0.08	0.00	>0.05	0.00	0.09	0.14	0.00	>0.05
Hymenoptera / Megachilidae										
Hoplitis leucomelana	10	0.22	0.13	0.00	>0.05	0.00	0.18	0.19	0.00	>0.05
Hymenoptera / Colletidae										
Hylaeus moricei	3	0.06	0.04	0.00	>0.05	0.00	0.09	0.00	0.00	>0.05
Hylaeus pectoralis	27	0.54	0.30	0.61	>0.05	0.00	0.39	0.60	0.39	>0.05
Unoccupied	4629	69.6	82.7	94.7	<0.001	95.4	81.4	67.4	75.0	<0.001
		Number of galls examined								
TOTAL (reed galls examined)	6449	3344	2515	506		281	3416	2381	287	

Total number of galls in each size category and their species-specific occupancy rates are indicated. The *p* values were obtained by χ^2 tests testing the hypothesis that the occupancy rates were equally distributed among the size classes.

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stems of ≥6 mm in diameter (Table 3). However, the galls having less than 5 mm in diameter were completely avoided, and we found the highest occupancy rate in the galls of 10–14.5 mm in diameter (Table 3). Larvae pupated in the spring without any cocoon. Imagines of *P. fabricii* hatched usually before other aculeate hymenopteran species, 10–22 days after the pupation.

Larvae of *Trichrysis cyanea* were well recognizable in the nests of *P. fabricii*, because they pupated in funnel-like fibrous cocoons of red-brownish color (see Fig 2D and 2F). Also the larvae of *Chrysis angustula* pupated in brownish cocoons but their shape was cylindrical.

***Trypoxylon deceptorium* and *T. minus*.** Nests of *T. deceptorium* (Fig 2B) consisted usually of 1–2 brood cells, less frequently up to 6 cells (range 1–6; median 2; mean 2.5 ± 1.5 cell per nest, n = 39 nests). The brood cells were surprisingly large (length 8.3 mm ± 2.0 mm). Larvae were covered in silk cocoons of the light brown non-transparent color, with dark tough bars present in the front part of the cocoons. The pupation took part in the same cocoons. All bars between the brood cells and also the closing plug of the nest were made of mud. At (post)industrial sites (ash dumps of power stations), the grey mud made from the fly ash was used (Fig 2B). We have not found any feces in cocoons but think that black feces were glued in the back side of the cocoons and were recognized as the darkish tough bar. The nests of *T. deceptorium* were placed in galls induced prevalently at the very thin reed stems (up to 4 mm in diameter, at which 1.07% of galls were occupied by this species. In contrast, *T. deceptorium* occupied only 0.21% of galls induced at the reed stems of the diameter 4–5.5 mm, and was absent in galls at thick reed stems of ≥6 mm in diameter (Table 3). However, the galls having less than 5 mm in diameter were completely avoided, and we found the highest occupancy rate in the galls of

5–14.5 mm in diameter (Table 3). The larvae pupated early in the spring and the pupae had the typical *Trypoxylonini*-associated notches in their inner eye margin. Imagines hatched early, similarly to *Pemphredon fabricii*. The larvae of *Trichrysis cyanea* in nests of *T. deceptorium* (see Fig 2F) pupated similarly as in the nests of *P. fabricii*.

Nests of *Trypoxylon minus* displayed the identical structure and were impossible to distinguish from the nests of *T. deceptorium*. We identified six nests of this species (according to the individuals hatched from nests), with 1–4 brood cells (range 1–6; median 3; mean 3.2 ± 1.7 cell per nest, n = 6 nests). The portion of nests with more than two cells was higher than in *T. deceptorium*, but more nests would be needed to check whether these differences are statistically significant. This species had the biggest brood cells, length 8.5 mm ± 1.3 mm. The nests of *T. minus* were absent in galls induced at thick reed stems of ≥6 mm in diameter and in galls with maximum diameter ≤5 mm (Table 3).

Hylaeus pectoralis (Fig 2E). Nests of *H. pectoralis* consisted usually of 1–5 brood cells (range 1–11; median 3; mean 3.7 ± 2.1 cell per nest, n = 27 nests). The nests usually did not comprise the whole gall. All nests of *Hylaeus* species were characteristic by their cellophane-like layers between the brood cells and also on the surface of the brood cells. These layers consist of a secret of female Dufour's glands, which is used as a protection against pathogens. In some nests, the brood cells were separated also by a layer of small particles of reed leaves. The brood cells were 7.8 ± 2.0 mm long. The larvae were present inside the cellophane chambers with a very small bump of feces of nectar and pollen digested. The cavity in the gall behind the first built brood cell was usually filled with small cut parts of reed leaves of the size around 1–3 mm. Similar filling was used between the last built brood cell and closing plug of the nest, which was made of the same small parts of reed leaves but mixed probably with the secret of female's Dufour gland. Bars between the brood cells were of highly variable thickness, from very thin (< 1 mm) to conspicuously thick ones (3–4 mm). The difference in the bar thickness depended on whether the plant material was incorporated in them. In contrast to the other reed gall inquilines, only the nests of *H. pectoralis* (and *P. fabricii*) were present in galls induced at thick reed stems of ≥6 mm in diameter (Table 3). Larvae pupated in brood cells without any cocoon, usually later than those of *P. fabricii* and *T. deceptorium*. Imagines hatched about 1–3 weeks later (after 21–39 days from the pupation) than adults of *P. fabricii*.

Hoplitis leucomelana (Fig 2C). The nests of this species consisted of only 1–4 brood cells (median 2; mean 2.4 ± 1.1 cell per nest, n = 10 nests). The brood cells (length 8.2 ± 1.2 mm) were usually placed at the bottom of the gall cavity, and the rest of the gall was filled with a dry plant particles (usually reed leaves) and soil grains. The part filled by the debris was in some cases longer than the part with brood cells (see Fig 2C). Mature larvae were in brownish, semitransparent silk cocoons with darker back, separated by thin bars of brownish mixture of dry plant particles (probably mixed with soil grains and some other undefined particles). Closing plug of the nest was made of the same substance. The nests of *H. leucomelana* were absent in galls induced at thick reed stems of ≥6 mm in diameter and in galls with maximum diameter ≤5 mm (Table 3). Larvae pupated in their cocoons in similar time (21–35 days) as those of *H. pectoralis*.

Symmorphus bifasciatus. Nests of this species looked very similar to the nests of *Hoplitis leucomelana* (but without remains of pollen) and contained also similar number of cells (range 1–7; median 2.5; mean 3.5 ± 2.4 cell per nest, n = 6 nests). Larvae were in semitransparent cocoons made probably of silk mixed with undefined secret. These cocoons were for the first sight similar to those of *Hoplitis leucomelana* but were more transparent and of yellow or green color. The closing plug was made of chewed substance of soil and dry leaves; similar mixture was found in one nest above the last brood cell. The nests of *S. bifasciatus* were absent in galls induced at thick reed stems of ≥6 mm in diameter and in galls with maximum diameter ≤5 mm (Table 3). Larvae pupated in their cocoons little later than those of *P. fabricii*, first males

emerged with *P. fabricii*, while other imagines reared later (19–37 days after the pupation). The length of brood cells was 7.8 ± 0.8 mm.

Description of mature larvae

We analyzed mature larvae of four species of Hymenoptera: Aculeata nesting in reed galls induced by *Lipara* spp., and two parasitoid species found in their nests (Tables 2 and 3). Below, we provide the descriptions of mature larvae, including the photos of whole larvae (Fig 4) and drawings of main determination characters (Fig 5).

Pemphredon fabricii. Mature larvae of *P. fabricii* were not previously described. The hitherto available descriptions of mature larvae of *P. lethifer* by [14–15] were not based on the material collected from reed stems or reed galls, thus the larvae described as *P. lethifer* sensu lato probably belonged to *P. lethifer* sensu stricto or some other newly established species of the *P. lethifer* complex. Here we provide the first description of mature larva of *P. fabricii*. Of note is that the larva of *P. fabricii* does not differ morphologically from the larva of *P. lethifer* described by the above mentioned authors, which is consistent with very close phylogenetic relationship of *P. fabricii* and *P. lethifer*.

Material: Czech Republic, Bohemia bor., Prunéřov, lignite power station fly ash deposit, 9 Mar 2014, 2 larvae, P. Heneberg lgt.; Slovakia, Slovakia occ., Sekule, terrestric reed bed surrounding little fishponds, 15 Mar 2014, 8 larvae, 4 Sep 2014, 8 larvae, P. Bogusch et A. Astapenková lgt., all P. Bogusch det. et coll.

Body. Length 8.3 ± 1.9 mm (n = 18), maximum width ~2 mm. Color yellow (most common), white or orange, in some cases reddish, or any shades of the above colors (light orange, light yellow, etc.); yellow or orange larvae possess frequently the first body segments (pronotum and part of mesonotum) white (Fig 4A). Posterior parts of segments form on dorsal part transverse welts slightly medially interrupted, continuous on the sides to pleural lobes, which are in some cases weakly developed. These structures are very ill formed on last three abdominal segments and on pronotum. Anus is a transverse slit, the supraanal lobe is produced. Spiracles small, light brown, first pair slightly larger than other nine pairs, atrium very weakly marked with five lines, subatrium unarmed (Fig 5C). Integument smooth, not hairy, only with a few weak setae on the dorsum of each body segment.

Head and mouthparts. Head distinctly visible, not protruded or embedded into the prothorax, slightly narrower than prothorax, width 0.962 ± 0.012 mm, height 0.856 ± 0.02 mm, width: height ratio >1. Pale and unpigmented except for brownish markings at the following positions: anterior and posterior tentorial arms, pleurostomal and hypostomal thickenings, mandibles, entire margin and a medial band on the labrum, latero-basal margin and a subapical ring on the maxilla, a circular ring on the praementum, palpi and galeae (Figs 4B and 5A). Antennal orbits large, circular, with three sensory cones in the membrane. Head with a few punctures, most of them anteriorly positioned from the orbits, clypeus with a broad band of punctures with setae. Labrum bilobed, about three times wider than long, with sclerotized and pigmented marginal and median bands, numerous punctures in the apical half, 14 of them with setae, the other with small sharp projection, several sensilla near the margin, epipharynx with numerous small spinulae. Mandibles (length 0.34 ± 0.009 mm) with four apical teeth, the basal is smallest, two lateral teeth more or less one above the others, only one single lateral basal setae on the mandibles (Fig 5B). Maxillae with some strong lateral setae, palpi stout and blunt with five apical sensillae, galeae smaller. Labial palpi long with four sensillae, spinerettes form a well visible salivary slit, hypopharynx with spinules.

Trypoxylon deceptorium. The species *T. attenuatum* was divided into six separate species [23]. *T. deceptorium* is a thermophilous wetland species, quite common in most of south and

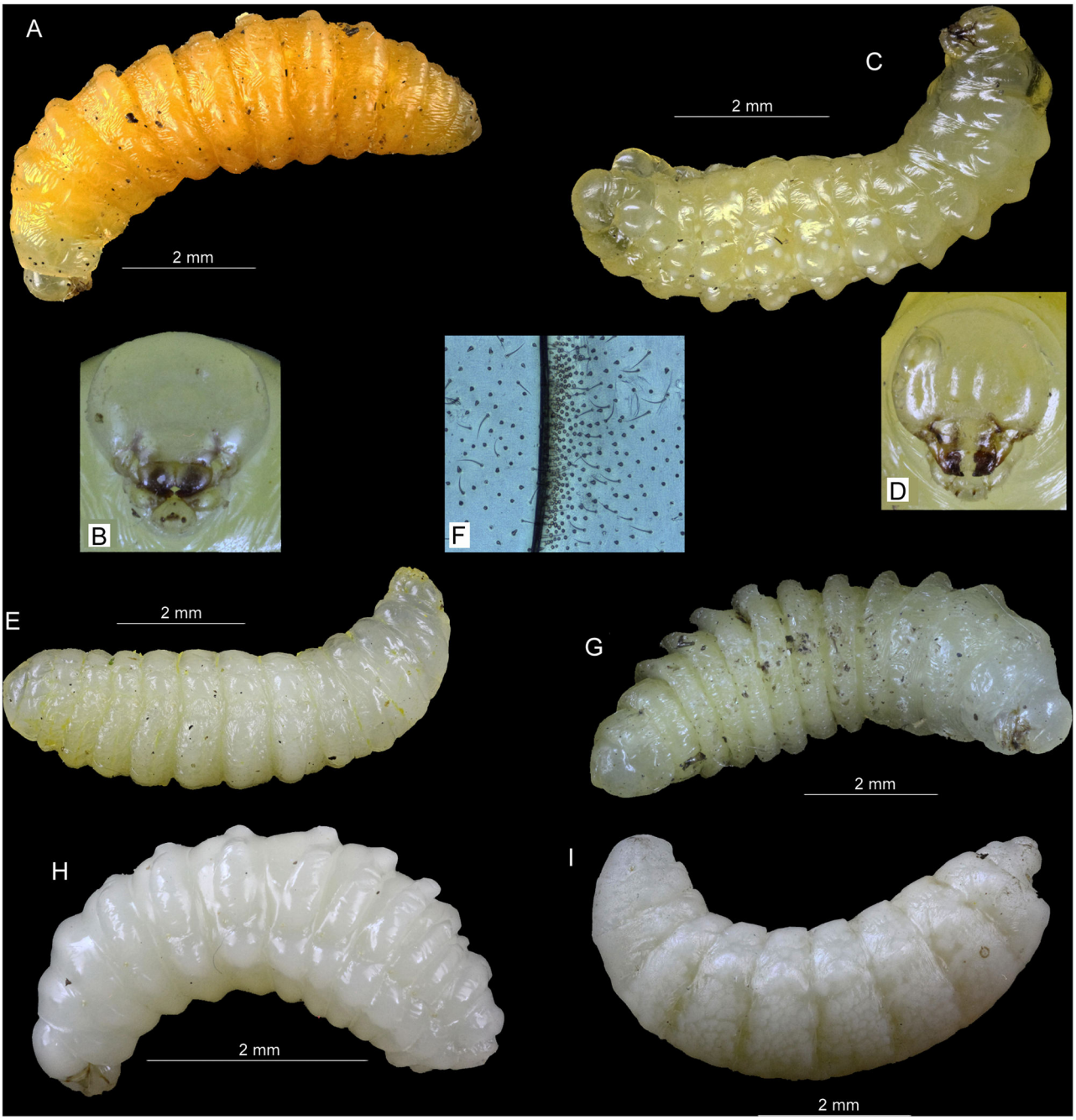

Fig 4. Larvae of aculeate Hymenoptera from nests in reed galls of *Lipara* spp. A—*Pemphredon fabricii*, whole larva, lateral view, B—*Pemphredon fabricii*, head, frontal view, C—*Trypoxylon deceptorium*, whole larva, lateral view, D—*Trypoxylon deceptorium*, head, frontal view, E—*Hoplitis leucomelana*, whole larva, lateral view, F—*Hoplitis leucomelana*, typical structure of setae and sensillae between body segments, G—*Hylaeus pectoralis*, whole larva, lateral view, H—*Trichrysis cyanea*, whole larva, lateral view, I—*Thyridanthrax fenestratus*, whole larva, lateral view. All photos by P. Bogusch.

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central Europe [17]. Asís et al. [22] described mature larva of *T. attenuatum* but the description of the biology suggests that their description could in fact represent *T. deceptorium*. Here we provide the first description of mature larva of *T. deceptorium* identified to the species level according to the current nomenclature. The described larva differs only slightly from that

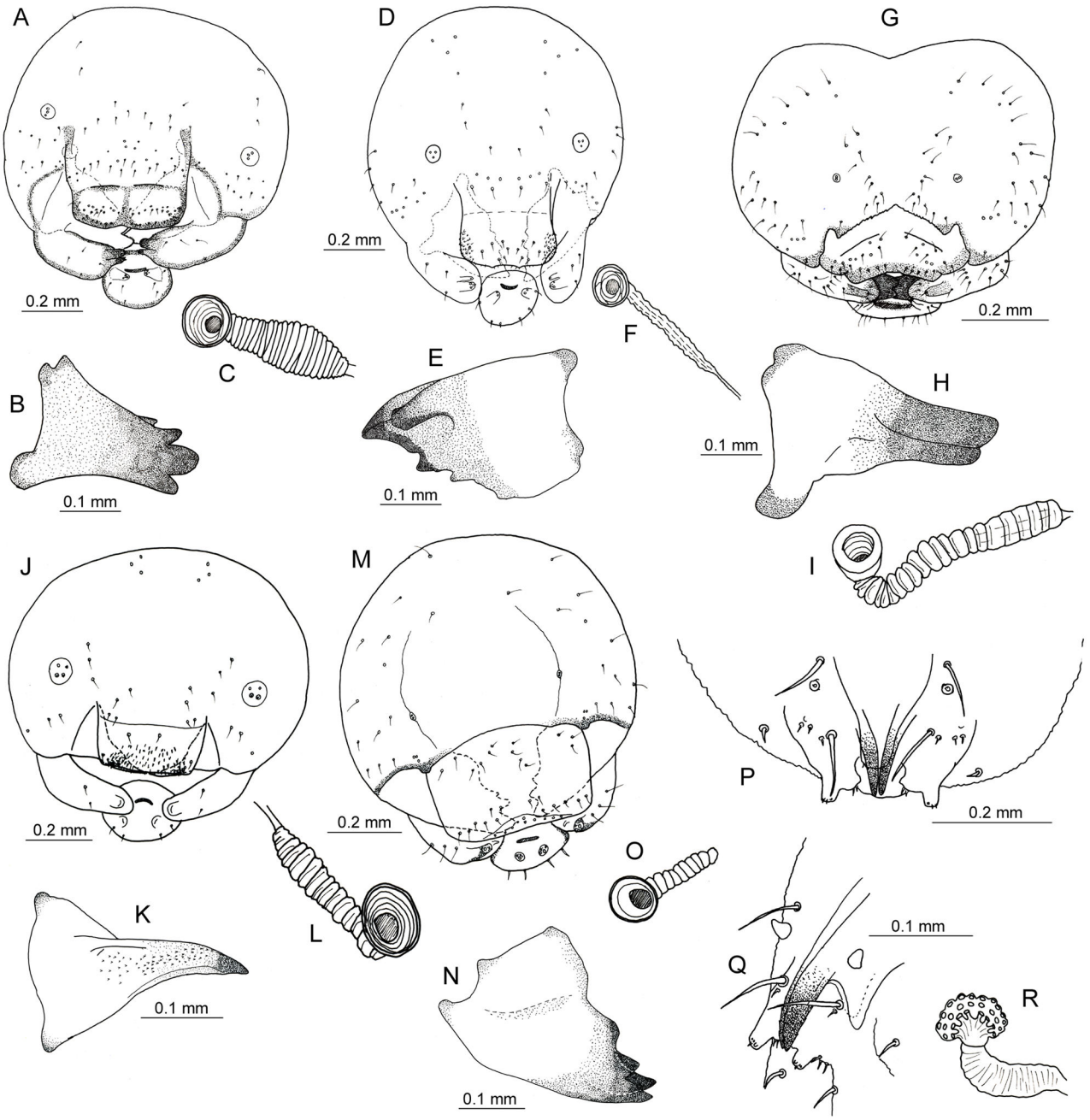

Fig 5. Larval characters. A—C—*Pemphredon fabricii*, A—head, frontal view, B—mandible, C—spiracle, D—F—*Trypoxylon deceptorium*, D—head, frontal view, E—mandible, F—spiracle, G—I—*Hoplitis leucomelana*, G—head, frontal view, H—mandible, I—spiracle, J—L—*Hylaeus pectoralis*, J—head, frontal view, K—mandible, L—spiracle, M—O—*Trichrysis cyanea*, M—head, frontal view, N—mandible, O—spiracle, P—R—*Thyridanthrax fenestratus*, P—mouthparts, frontal view, Q—mouthparts, lateral view, R—spiracle. All drawings by P. Bogusch.

doi:10.1371/journal.pone.0130802.g005

described [22] in the number of mandibular teeth, which could simply represent an unintentional error present in the previously published description.

Material: Czech Republic, Moravia bor., Stonava, wetland at a depression caused by underground black coal mining partially filled with tailings, 7 Feb 2014, 3 larvae, P. Heneberg lgt.,

Slovakia, Slovakia occ., Sekule, terrestric reed bed surrounding little fishponds, 4 Sep 2014, 9 larvae, P. Bogusch lgt., all P. Bogusch det. et coll.

Body. Length 5.5 ± 1.6 mm (n = 12), subcylindrical shape, white or pale yellowish (ochre) colored. Posterior parts of segments with distinct lobes, pleural lobes also well developed, they are weaker on prothorax and most distinct on last abdominal segments, which are also the widest (Fig 4C). Anus ventral, last segment rounded, with two spine-like processes on the sides. Spiracles pale brown, very small, atrium very weakly marked with five lines, subatrium unarmed (Fig 5F). Body integument very thin and smooth, with few setae especially on the dorsum and pleural lobes.

Head. Head well developed, slightly wider than long (width 0.811 ± 0.013 mm, height 0.799 ± 0.01 mm), narrower than prothorax. Pale and unpigmented except the following structures: pleurostomal and hypostomal thickenings, anterior tentorial arms, mandibles, margins of praementum, epipharynx, apical part of laccinia, maxillar palpus and galea, praementum, salivary slit and labial palpus (Figs 4D and 5D). Antennal orbits large, circular, with three sensory cones in the membrane. Head with only a few punctures, most of them bearing setae (three on each side of the head and majority on the labrum). Clypeus with 6 setae and 6 sensillae, rectangular shape, unsclerotized. Labrum unsclerotized with 12 setae and several sensillae in the middle of the apical margin, which forms a very shallow depression, and two small unsharp teeth on each side of this depression. Postero-lateral parts of epipharynx bear plenty of short, sharp tooth-like processes. Mandible (0.269 ± 0.005 mm long) with six teeth, one apical, three lateral, one basal and one on the distal side (Fig 5E). Maxillae well developed with sclerotized palpi bearing three sensillae, which are larger than galeae, lacciniae with a spinulous lobe. Labium with short palpi with one sensilla and salivary slit, all sclerotized. Hypopharynx ill visible, unpigmented, with small spinules.

Trichrysis cyanea. This very common species is a parasitoid in nests of many other aculeate hymenopterans and its larvae were described [22] from the nests of *Trypoxylon attenuatum* s. l. Our description is similar with their and show that also larvae of *T. cyanea* from nests of *Pemphredon fabricii* have the same morphology.

Material: Slovakia, Slovakia occ., Sekule, terrestric reed bed surrounding little fishponds, 4 Sep 2014, 2 larvae, P. Bogusch lgt., det. et coll.

Body. Short and robust, white-colored, length 3.8 and 4.0 mm (n = 2). Shape fusiform with well-developed dorsal posterior lobes reaching the pleurae of the segments. Pleural lobes well developed and forming a line (Fig 4H). Last abdominal segment very short and narrower than other segments. Anus terminal as a transverse slit. Integument with microspinules, especially on dorsal anterior parts of the terga, setae only in very small number. Spiracles pale brown, quite big, atrium very weakly marked with five lines, subatrium unarmed (Fig 5O).

Head. Head quite big but narrower than prothorax, only slightly wider than long (width 0.874–0.899 mm, height 0.825–0.852 mm), most of the head pale, unpigmented. Sclerotized and pigmented are the following structures: pleurostomal and hypostomal thickenings, mandibles, mandibular condyli and genae, apical parts of labrum, maxillae and labium (Fig 5M). Anterior orbits very small, irregularly oval, with two sensory cones in the membrane. Setae in a low number on the whole surface of the head, more very long setae on the sides near mandibular condyli. More setae on apical part of labrum and maxillae. Clypeus rectangular with 18 punctures bearing setae, most of them in basal part. Labrum roundly rectangular with 18 punctures with setae and some sensillae mainly in the basal part. Epipharynx relatively smooth, apically with a row of 14 big sensillae. Mandibles large (length 0.0290–0.291 mm), well sclerotized, with five prominent teeth, without setae (Fig 5N). Maxillae with three prominent setae on lateral part of the laccinia, maxillar palpi short with four sensillae, galeae very narrow.

Labium with short palpi bearing five sensillae (one of them bigger than the other) and long, transverse salivary slit. Hypopharynx rugose and well sclerotized.

Hoplitis leucomelana. The larva of this common bee was described by [21], but the description is insufficient and available only in a poorly available publication. The morphology described fits well the central European specimens of this species.

Material: Czech Republic, Moravia mer., Hodonín env., heating plant slag/ash deposit, 14 Mar 2014, 2 larvae, P. Bogusch et A. Astapenková lgt., Slovakia, Slovakia occ., Sekule, terrestric reed bed surrounding little fishponds, 4 Sep 2014, 4 larvae, P. Bogusch lgt., all P. Bogusch det. et coll.

Body. Robust, white-colored, length 8.1 ± 0.5 mm (n = 6). Shape fusiform with ill developed dorsal posterior lobes on the segments (Fig 4E). Anus terminal as a transverse slit, last abdominal segment rounded. Integument bearing plenty of setae and spinules, especially on lateral parts of tergites (Fig 4F). Very rich concentration of spinulae is near posterior margins of abdominal segments, those ochre or yellowish colored. Spiracles pale brown, smaller, atrium round and widely marked with 7 lines, subatrium unarmed (Fig 5I).

Head. Well-developed but small, wider than long (width 0.788 ± 0.01 mm, height 0.692 ± 0.009 mm), heart shaped, very much narrower than prothorax. Pale, unpigmented, except the following structures: pleurostomal and hypostomal thickenings, anterior tentorial arms, labrum (especially the posterior part), mandible, posterior part of maxillae with galea and palpus, labium with salivary slit and palpus (Fig 5G). Clypeus very weakly sclerotized. Antennal orbits small, circular, unsharply marked, with one sensory cone in the membrane. Head with many punctures bearing setae and with spinules, the concentration of these structures is similar all over the shape. Clypeus V-shaped, very slightly sclerotized, with 10 punctures bearing setae. Labrum triangular with wide concave depression, labral margin thick a pugged with rough structure on the epipharynx. Labrum laterally with 16 punctures with sensillae (8 on each side) and 18 without (9 on each side), several microsensillae on the anterior margin in the middle. Mandible (length 0.243 ± 0.003 mm) with two teeth, sclerotized and without sensillae (Fig 5H). Maxillae with elongated palpus longer than galea with two sensillae on the top, laccinia with 13 punctures with setae on lateral part. Labium with very well developed salivary slit in the middle, palpus elongated with two sensillae. Hypopharynx brownish, sclerotized and rugous.

Hylaeus pectoralis. Janvier [18] described the nest and larva of this species in France. Morphology of here described central European specimens did not differ from the original description but more details and more detailed figures are provided.

Material: Czech Republic, Bohemia mer., Lomnice nad Lužnicí, Velký a Malý Tisý National Natural Reserve, terrestric reed bed surrounding a fishpond, 22 Feb 2014, 3 larvae, Bohemia mer., Třebeč, Brouskův mlýn National Natural Reserve, terrestric reed bed surrounding the river, 21 Feb 2014, 2 larvae, both P. Heneberg lgt., Slovakia, Slovakia occ., Sekule, terrestrial reed bed surrounding little fishponds, 4 Sep 2014, 1 larva, P. Bogusch lgt., all P. Bogusch det. et coll.

Body. White-colored, length 7.3 ± 1.0 mm (n = 6), shape fusiform with dorsal posterior lobes well developed and extending to the pleurae, these structures are missing on prothorax and mesothorax and on last four abdominal segments (Fig 4G). Anus terminal as a transverse slit, last abdominal segment rounded. Integument smooth with only very few setae on dorsal part. Spiracles with narrowly rounded atrium with seven lines, subatrium unarmed (Fig 5L).

Head. Well developed, wider than long (width 0.912 ± 0.014 mm, height 0.822 ± 0.01 mm), transparent, sclerotized only on the following parts: pleurostomal and hypostomal thickenings, mandibles, apical part of clypeus, labium and maxillae only slightly sclerotized (Fig 5J). Orbits round, big, with four sensory cones in the membrane. Head with only few sensillae, most of

them with setae. Clypeus rectangular, wide, with only two sensillae bearing setae on each side. Labrum of similar shape but longer, with punctures bearing very tough setae on the sides and many setae without punctures on the surface, especially in posterior part, and 12 punctures bearing setae in various parts of the labrum. Epipharynx with very rough structure. Mandible (length 0.354 ± 0.016 mm) with one tooth, sharp, with many little spinules on inner lateral side, inner apical part without toothlike processes (Fig 5K). Maxillae elongated and unsharp, maxillar palpi rounded without visible sensillae. Labium with very small round palpi and sclerotized salivary slit in the centre. Hypopharynx well visible and rough.

Thyridanthrax fenestratus. The larva of this common dipteran parasitoid of various digger wasps was described by [30]. However, the hitherto available description is very short and lacks good drawings. We compared the larvae from nests both of *Pemphredon fabricii* and *Trypoxylon deceptorium* and found they look similar to each other.

Material: Slovakia, Slovakia occ., Sekule, terrestric reed bed surrounding little fishponds, 15 Mar 2014, 7 larvae, 4 Sep 2014, 5 larvae, P. Bogusch et A. Astapenková lgt., all P. Bogusch det. et coll.

Body. White-colored with matt appearance, 4.9 ± 0.8 mm long (n = 12). Body segments smooth without processes or lobes, posterior parts only slightly protruding ventrally (Fig 4I). Last abdominal segment narrower with very narrow apical part, which is rounded with circular anus. Spiracles only on the pronotum and posteriorly on the anterior part of last abdominal segment, atrium of abdominal spiracle weakly marked but with several wide lines of rosettes (Fig 5R). Integument strong with rough structure on the whole surface, without any setae. Anterior end of the body wide, head indistinct.

Head unsclerotized except the mouthparts, which are also brownish pigmented. Mandibles prominent, sharplike with three teeth oriented backwards and well visible basal lobe, antennae short but well visible, consisting of two segments. Labrum on dorsal part, trapezoidal, well sclerotized. Maxillae lobe-like formed, with elongated two segmented palpi bearing three (two elongated and one flat) sensillae. Labium small, indistinct. Several setae around the mouthparts present, two on each side very big, prominent (Fig 5P and 5Q).

Discussion

Recent research in landscape ecology [31–32] and natural history and taxonomy [27, 33] extensively benefited from the use of trap nests, including the trap nests made from the common reed stems and galls, which are now frequently used to collect solitary cavity-nesting Hymenoptera and their parasitoids. However, the data from the reed stems and galls examined *in situ* (without constructing the trap nest itself) are scarce. Thus, this study improves not only our knowledge on the larvae and nests of the reed gall-associated species, which could be potentially found in such trap nests, but also address the species composition associated with such nesting resources. Combined data ([2–4] and this study) suggest that the assemblage of aculeate Hymenoptera nesting in reed galls induced by *Lipara* flies comprises in Europe at least 29 species of nesting bees and wasps, 2 cleptoparasites of the genus *Stelis* and 4 parasitic golden wasps bound on their nests, including several facultative reed gall inquilines newly identified in this study. The reed galls host a broad spectrum of rare species. Among them are *Passaloecus clypealis*, *Rhopalum gracile*, *Hylaeus moricei* [3] and this study, *Stenodynerus xanthomelas* [2] and *Ectemnius confinis* [4], all nesting in reed galls as well as reed stems. *Stenodynerus xanthomelas*, recorded by [2], is a very rare species of wet meadows and its ecology seems to be similar to *R. gracile* and other species mentioned in this paragraph, but is probably absent in the Czech Republic [16]. Of interest is the record of *Heriades rubicola*. This species is currently expanding to the north and reached the southernmost parts of the Czech Republic only a few years ago.

The first published records in south Moravia date back to the year 2012 [34] but the first finding was made by J. Straka (unpubl.) in Tasovice (ZN, Czech Rep.) already in 2007. Recently, it is increasingly common at sites with the presence of common reed in southernmost Moravia, and here we confirmed that it nests in reed stems and in galls induced by *Lipara* spp.

Only three species found in the reed galls could be considered as common. *Pemphredon fabricii* feeds on reed aphid secrets and does not fly away from the reed beds [11]. It is probably dependent on galls induced by the *Lipara* flies as the only nesting resource [2–3] (determined as *P. lethifer* sensu lato in the reference by Westrich). *Hylaeus pectoralis* prefers reed galls as well but it occurs at a narrower spectrum of sites compared to *P. fabricii* and it is also less abundant at sites of its occurrence. However, by the analysis of the reed galls ([3] and this study), we showed that *H. pectoralis* is more common than was previously thought [3, 16, 35]. Of note is that we have not recorded other wetland species of the genus *Hylaeus* (except *H. moricei*), although *H. rinki* was found in color pan traps at the same sampling sites from where the reed galls were collected [3]. The last abundant species, *Trypoxylon deceptorium*, is a very common wetland specialist, which uses reed stems and perhaps some other cavities in addition to the galls induced by *Lipara* spp. for its nesting. Acceptance of multiple types of cavities by *T. deceptorium* is supported by the results of color pan trapping, which yielded more specimens than rearing the reed galls at sites where both these methods were applied [3]. The digger wasps of the genus *Trypoxylon* forage on flower nectar, so (in contrast to *P. fabricii* feeding mainly on aphid honeydew) its abundance can be effectively determined based on both the above methods. In this study, we found that these species may form mixed nests. In all such nests, brood cells of one species were made first, and then followed by the second, so the nesting females probably did not meet each other.

Most of the newly identified reed gall inquilines use reed galls only occasionally and nest also in other cavity types. Such behavior is characteristic for, e.g., common solitary wasp *Symmorphus bifasciatus*, digger wasps *Nitela spinolae* and *Trypoxylon minus* and bees *Pseudoanthidium lituratum*, *Chelostoma campanularum*, both *Stelis* spp., *Hylaeus incongruus* and *Hoplitis leucomelana*. Nartshuk and Andersson [4] published records on other species, however, without detailed source references. Some of them behave similarly to the above-mentioned species, occur in many kinds of habitats and nest in different cavity types. However, a couple other species published by [4] are unlikely to nest in galls induced by *Lipara* spp. and were probably misidentified. Among them was *Rhopalum clavipes*, which is a species of forests nesting in stems of plants [11] misidentified probably with *R. gracile*, the morphologically similar reed stem & gall specialist [3]. Also the records of other species of *Pemphredon* (*P. inornata*, *P. lethifer*, *P. rugifer* and *P. wesmaeli*), *Passaloecus* (*P. corniger*, *P. gracilis* and *P. singularis*) and *Trypoxylon figulus* are likely based on misidentification. But, contrary to *R. clavipes*, these species could probably occasionally nest in reed galls. In course of this and our previous study [3], we have checked thousands of individuals of *Pemphredon* from reed galls collected at dozens of sampling sites, and all represented *P. fabricii*, not the other species of *P. lethifer* complex.

Reed galls have limited space for nesting but the same situation is in the case of reed stems and most of the other cavities, too. There are also huge differences in the size of reed galls occupied by aculeate hymenopterans, some of them providing more than 15 cm long cavity but the others being shorter than 3 cm, which may in part explain the differences in the number of brood cells found. However, many nests comprised only a part of the cavity in the gall, and we found also nests extending to the soft top of the gall in contrary (and they were not rare). The species-specific size of the nest cells was not related to the size of the gall or larvae: for example mature larvae of *Trypoxylon minus* were shorter and smaller than those of *Hylaeus pectoralis* or *Pemphredon fabricii*, but the length of *T. minus* brood cells was the largest. In this study, we confirmed that most of the species prefer the wider galls but induced at thin stalks over the

other types. Such galls are produced by *Lipara lucens* while other species of *Lipara* cause galls that are much narrower and not so conspicuous [4, 33, 36]. Galls of *L. lucens* have also very tough walls in contrary to the galls of other *Lipara* spp., and thus could serve as a good defensive structure against the predators and parasitoids.

Most of the species analyzed in this study used the reed leaves (cut to pieces or chewed and mixed with some other materials) to construct the septa between brood cells and other parts of the nest (closing plug or interspace fillings). Overall, the nest structures and materials used by the examined species resembled those used by the closely related species [14, 17, 20, 37, 38]. Similarly, the mature larvae were morphologically similar to the related taxa and to the descriptions of representative larvae of the current species complexes. Of note, the original descriptions [14, 15, 18, 21, 22, 37] paid little attention to the chaetotaxy (number and position of setae and sensillae on the body), which, however, should be considered as a good tool allowing the identification of the species.

The mature larva of *Pemphredon fabricii* is very similar to the larva of *P. lethifer*, as described in [14–15]. It shows similar total length, morphology of the body, coloration and the mouthparts, which is actually characteristic for altogether 10 species of the genus *Pemphredon* as described by [14, 37]. According to Janvier [14], the morphology of mandibles displayed the most striking differences between the species analyzed—larvae of all species have four mandibular teeth but their size and position is species-specific. Most prominent differences can be found when comparing the larvae of different subgenera, such as *P. morio* of subgenus *Ceratophorus* (with different size and number of mandibular teeth and also shape of the clypeus) with the subgenus *Cemonus* (*P. lethifer* and *P. fabricii*), which have mandibular teeth of similar size and all located near the apex of the mandible. However, we cannot compare the number of sensillae and setae on the head with other descriptions because there were only few remarks provided by [15] but we assume that the differences are probably minute. There are also small differences in the number of setae (± 1–2) on each part of the head among 10 larvae used for the description in this study. Thus, the main difference between *P. lethifer* and *P. fabricii* larvae consist of their mandibular teeth, where the smallest tooth on the inner side of the mandible is moved more laterally at *P. fabricii* than in *P. lethifer*.

Mature larva of *Trypoxylon deceptorium* corresponds very well to that of *T. attenuatum* described by [22], which was quite surprising. The authors of the description determined identification characters allowing to distinguish between the two subgenera of this genus, *Trypoxylon* and *Trypargilum*, which also allow to compare the larvae of European *Trypoxylon* spp. Because Asís et al. did not distinguish the taxa newly described by [23] from *T. attenuatum* sensu lato, they most likely described larvae *of T. deceptorium* species. Thus, in the future, it will be necessary to re-describe the larvae of *T. attenuatum* sensu stricto as well. Reed galls, however, host only *T. deceptorium*. Larvae of both species are expected to be very similar, because also the adults of both species are very similar and difficult to identify, and differ more ecologically than morphologically. Whereas *T. attenuatum* is a widespread species of various habitats, *T. deceptorium* occurs typically in wetlands and is more common in southern and warmer parts of Central Europe [17]. We found only one difference in the number of mandibular teeth. However, it is possible that [22] only did not notice the smallest tooth or did not mark it as tooth but as a projection or angle. In the nests of *T. deceptorium* and *P. fabricii*, we found the larvae of the parasitoid *Trichrysis cyanea*. The description of the larva of *Trichrysis cyanea* corresponds with that published by the same authors and larvae and imagines of *T. cyanea* from nests of both host species are morphologically similar to each other.

Mature larvae of *Hylaeus pectoralis* corresponded well with the description provided [18]. Yet unexplained is the profound reduction of rough structures at the apex of the larval mandible. We speculate that they could be considered as a result of adaptation for different food but

data from other wetland *Hylaeus* spp. are not available so far. Similar situation is with *Hoplitis leucomelana*. The larva of this small member of the genus differs from the other species in the body size and also in the shape of clypeus and labrum. Most of the described larvae of this genus have two mandibular teeth similarly to *H. leucomelana* and the teeth are usually very similar in size [20].

The mature larva of *Thyridanthrax fenestratus* was already described by [30], and its drawing was published also by [39]. They found that the *T. fenestratus* mandibles are bidentate, with lateral hook. However, these descriptions are very different from the specimens analyzed in this study and differ also from the features of the whole family Bombyliidae. In general, the larvae of the family Bombyliidae have rough body integument and head reduced only to the mouthparts. Their mandibles are sharp and elongated, with three or four opposite spines. Maxillae and labrum look similar, and labrum has five small processes on the margin [39]. We have observed the structures similar to the above features shared with other bee flies, but not those described specifically in the larvae of *T. fenestratus* by the previous authors despite we prepared all mouthparts separately (see Fig 5P and 5Q for anterior and lateral views of the mouthparts). We also confirmed two new hosts of this bee fly, which was previously recorded as a parasitoid of digger wasps of the genera *Ammophila* and *Sphex* [39, 40]. In conclusion, using an extensive dataset of experimentally hatched reed galls, we elucidated the nesting biology and ecology, and provide the descriptions of larvae of aculeate hymenopterans nesting in reed galls and their parasitoids. Obtained data allow for the first time to identify the larvae of the most widespread central European aculeate hymenopteran reed gall specialists.

Supporting Information

S1 Table. List of the study sites. Localities with permission needed are marked by "Y" in the column "Permit".
(DOCX)

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Author Contributions

Conceived and designed the experiments: PB PH. Performed the experiments: PB AA PH. Analyzed the data: PB PH AA. Contributed reagents/materials/analysis tools: PB AA PH. Wrote the paper: PB PH.

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3.3 HENEBERG P., BIZOS J., ČMOKOVÁ A., KOLAŘÍK M., ASTAPENKOVÁ A. & BOGUSCH P. 2016: Assemblage of filamentous fungi associated with aculeate hymenopteran brood in reed galls. *Journal of Invertebrate Pathology* **133**: 95-106.

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Assemblage of filamentous fungi associated with aculeate hymenopteran brood in reed galls

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ABSTRACT

Monotypic stands of common reed and the reed-gall-associated insect assemblages are distributed worldwide. However, fungi associated with these assemblages have not been characterized in detail. Here we examined 5200 individuals (12 species) of immature aculeate hymenopterans or their parasitoids collected at 34 sampling sites in Central Europe. We noticed fungal outgrowth on exoskeletons of 83 (1.60%) larvae and pupae. The most common host was eudominant *Pemphredon fabricii*. However, the less abundant aculeate hymenopteran reed gall inquilines were infected at higher prevalence, these included *Trypoxylon deceptorium*, *Trypoxylon minus*, *Hoplitis leucomelana* and *Hylaeus moricei* (all considered new host records). We identified three fungal species, *Penicillium buchwaldii* (72% of cases), *Aspergillus pseudoglaucus* (22%) and *Penicillium quebecense* (6%). When multibrooded nests were affected, only a part of individuals was infected in 62% of cases. The sampling site-specific infection rate reached up to 13%, thus fungal infections should be considered an important variable driving the abundance of gall inquilines. Infections of generalist host species were more frequent than those of reed gall specialists, suggesting that suboptimal conditions decreased the immunocompetence of non-specialized species, which only occasionally nest in reed galls and feed in reed beds.

1. Introduction

Entomopathogenic fungi are typically found in various lineages of chytrids, zygomycetes and ascomycetes (Samson et al., 1988). Numerous fungal infections affect insect species of economic importance, or may involve zoonotic species as in this study. Their life cycles are usually synchronized with insect host stages and environmental conditions (Shah and Pell, 2003). However, most of the research has been focused to several socially living species of insects or to the synanthropic species, and there is limited knowledge on the contribution of entomopathogens in the regulation of pest populations in agroecosystems and on the delivery of ecosystem services to agricultural production (Altieri, 1999; Gurr et al., 2003; Tscharntke et al., 2005; Meyling and Eilenberg, 2007). Thus, our improved understanding of the ecology of indigenous populations of entomopathogenic fungi is considered a prerequisite for the evaluation of their economic impact and for their consideration as non-market goods (Meyling and Eilenberg, 2007). Here we focus on species associated with cavity-nesting hymenoptera (bees and wasps) making their nests in galls of chloropid flies *Lipara* spp. on common reed *Phragmites australis* stems. Monotypic stands of common reed and the gall-associated assemblages are distributed worldwide. They swiftly colonize newly formed (post)industrial habitats and thus may serve as a ubiquitously available support for the establishment of entomopathogenic fungi. The reed-associated arthropod hosts may utilize reed as a food source (sap suckers, leaf-, pollen-, and phloem-feeding species) or as a nesting resource and shelter (stem borers, gall makers, and gall inquilines). Tewksbury et al. (2002) reported 160 species of reed-associated arthropods in Europe, but they found only 23 species of reed-associated arthropods in North America, where the subsp. *americanus* is native, but subsp. *australis* is considered an alien species.

Aspergilli, Mucorales and Penicillia are considered key fungi associated with honey bees (Gilliam and Prest, 1987; Gilliam et al., 1989; Kirpik et al., 2010). Stonebrood caused predominantly by *Aspergilus flavus* and chalkbrood caused by *Ascosphaera apis* are known as key pathogenic infections contributing to colony losses

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0022-2011/

(Gilliam and Vandenberg, 1997). Interestingly, the resistance of honey bees to obligate parasitic fungi causing chalkbrood is dependent on host genotype, whereas the resistance to facultative parasitic fungi causing stonebrood is negligible (Evison et al., 2013). The epidemiology of stonebrood and chalkbrood is poorly understood, and high number of cases is probably undetected because the diseased brood is thought to be rapidly discarded by worker bees (Foley et al., 2014).

Despite the first record of fungi in aculeate hymenoptera was reported by Franciscan monk José Torrubia already in year 1749 (Samson et al., 1988), vast majority of hitherto performed studies of fungi infesting aculeate hymenoptera addressed nearly exclusively the honey bee *Apis mellifera*, other species of economic importance and ant mounds. Goerzen (1991) studied the microflora associated with healthy adult and larval alfalfa leafcutting bee *Megachile rotundata*, observing increased larval mortality when yeast and bacterial fermentation of provisions was followed by mould overgrowth. Solitary bees *Centris pallida* and *Anthophora* sp. were studied by Gilliam et al. (1984). Filamentous fungi colonizing the exoskeleton of dead *Mellipona subnitida* were reported by Ferraz et al. (2008). Enteric fungi of the paper wasp *Polistes hebraeus* were studied by Fouillaud and Morel (1995). Pathogenicity of selected fungal species to *Vespula vulgaris* and *Vespula germanica* was tested by Glare et al. (1996) and Harris et al. (2000). Strohm and Linsenmair (2001) found that the sphecid wasp *Philanthus triangulum* prolonges the resistance of its paralyzed malaxed prey (bees) to fungal infestation when compared to freeze-killed bees, but did not line their brood cells with any substances, which would prevent contamination by microbes similarly as it was reported from multiple colletid and halictid bees. Other mechanisms may apply, particularly metabolites of symbiotic bacteria of the crabronids and other invertebrate hosts may play an important protective role (Kaltenpoth et al., 2012). The cockroach parasitoid *Ampulex compressa* sanitizes host cuticule and the cocoon with a cocktail of nine antimicrobials, and uses also vaporous isocoumarine (*R*)-(-)-mellein to sanitize the nest by fumigation (Weiss et al., 2014). Also poor composition of the diet of bee larvae was experimentally shown to contribute to the pathogenicity of opportunistic fungi, such as *Aspergillus fumigatus* (Foley et al., 2012). Reliance on proper food supplementation is important to consider not only in relation to the food shortage, but also in relation to co-infections by parasites such as *Nosema ceranae*, which has been confirmed to impose an energetic stress (Mayack and Naug, 2009). Despite passive acquision of fungi from the soil and plant surfaces is usually suggested, Benoit et al. (2004) hypothesized that the mites, such as the honey bee parasitic mite *Varroa destructor* can harbor fungi and bacteria on their cuticle, including the pathogenic *Ascosphaera apis*, and also *Aspergillus* spp. and *Penicillium* spp., and thus may serve as their vectors. Aquino et al. (2013) in turn hypothesized, that aculeate hymenoptera serve as vectors of soil and airborne fungal species, suggesting that these insects should be eradicated in hospitals and other human-associated environments. Some fungal species associated with aculeate hymenopterans are considered symbiotic. Among them are *Amylostereum areolatum* and *Amylostereum chailletii*, which colonize mycangia of woodwasps *Sirex noctilio* and *Sirex nigricornis*. These fungal symbionts are injected during oviposition together with phytotoxic mucus into host pine trees. However, the presence of *Amylostereum* spp. is also considered a necessary pre-condition for a development of the nematode *Deladenus siricidicola*. This nematode serves as a biological control agent of *Sirex* spp., and displays a bicyclic life cycle including mycetophagous free-living and parasitic cycles (Olatinwo et al., 2013).

Insect galls represent stable microhabitats characterized by high humidity and limited air circulation, which is thus favorable for fungal proliferation and both accidental and obligate interactions of the gall insect with fungi (Bissett and Borkent, 1988). An example of such obligate mutualism is the colonization of openings of large *Lasioptera arundinis* galls by the fungus *Radulidium subulatum*. In this association the fungus allows the larva to penetrate into a stem, protects the larva against parasites and allows an easy exit of the imago, whereas the gall midge larvae and adults have structures allowing carrying the fungus, and thus facilitating spread of the fungus, and the larva induces reed gall formation (Yukawa and Rohfritsch, 2005). In non-obligate associations, the fungal assemblages found in galls are typically recruited from local endophytes present in adjacent tissues. Insect galls can affect the species composition of fungal endophytic species, and infecting fungi can negatively affect the fitness or even kill the insect present in the galls (Wilson, 1995; Lawson et al., 2014). It is notable that moulds of the genera *Penicillium* and *Aspergillus*, including the recently described taxa, are known as facultative gall associates, which can be explained by their ability to grow in habitats with limited availability of water. Seifert et al. (2004) reported *Penicillium cecidicola*, *Penicillium glabrum* and *Penicillium paxilli* in galls of Cynipidae species, *Penicillium dendriticum* in galls of unknown origin on *Eucalyptus* leafs, and *Penicillium erythromellis* and *P. pseudostromaticum* in galls induced by *Diplolepis rosae* on *Rosa sitchensis*.

In this study, we have characterized reed gall aculeate hymenopteran inquilines hosting fungal species. The superficial fungal growth was noticed when sampling immature aculeate hymenopterans for the purpose of taxonomic and ecological analyses. The diagnosis was based on the sequencing of multiple DNA loci of both, the primary isolates and the strains transferred to an artificial medium, and based on the phenotyping, which in combination allowed precise identification of the pathogens to the species level. Epidemiological data were provided.

2. Material and methods

2.1. Study area and sampling

The study specimens were collected at 34 sampling sites in the Czech Republic (33 sites) and Slovakia (1 site), Central Europe (48.62–50.71°N; 12.25–18.56°E). Six examined reed beds were located in nature reserves and near-natural habitats, other 28 examined reed beds were at (post)industrial sites. At each of the sampling sites, 200 reed galls were collected between 7 Feb and 23 Mar 2014, with the exception of two sites, where the total number of galls available onsite was less than 200 and thus only the available galls were collected. The chosen sampling period corresponded to the end of high air moisture period in the Czech Republic (September to February). It also reflected the life cycle of host organisms, as all of them survive the winter as larvae in a diapause. Larvae collected during the chosen sampling period can easily develop to adults under laboratory conditions, which is not as trivial for those collected prior the winter onset. Thus, sampling at the end of winter allowed comparing the viability of infected larvae with the uninfected ones.

Only galls older than 1 year (greyish or darker in appearance, usually without leaves and with the apex broken) were collected, because the study focused on aculeate hymenopterans, which preferentially (or, perhaps, exclusively) use the $\geqslant$1 year old galls for their nesting. The collected galls were longitudinally cut, the healthy aculeate hymenoptera were allowed to rear and the specimens with macroscopic signs of fungal infections were collected for further analyses. All the aculeate hymenopteran specimens were identified to a species level. The detailed list of sampling sites

and the description of the sampling technique used were provided in our recent paper (Bogusch et al., 2015) focusing on the morphology of nests and mature larvae of aculeate hymenopterans examined in this study.

2.2. Isolation and cultivation of obtained strains

Part of the mycelium was transferred from each specimen with macroscopically visible fungal infection to the liquid medium. Fungi were cultured stationary for 3–7 days at 21 °C in 50 ml plastic tubes on liquid media containing glucose (4% w/v), sorbitol (2% w/v) and acid casein hydrolysate (3% w/v; all from Sigma–Aldrich, St. Louis, MO), with pH adjusted to 6.5 (Kadlec et al., 1994). Obtained fungi were plated on agar plates containing the identical but solid medium, and the obtained clones were used for DNA isolations, subsequent cultivations, and were stored frozen. All the specimens were transferred successfully. In addition, we isolated the DNA also from the mycelium obtained directly from host specimens, and we also took pictures of the mycelium growing on host specimens. The list of specimens examined, their host species, sampling sites and sampling dates are provided in Table 1.

Representative strains of each obtained species were deposited in the Culture Collection of Fungi (CCF) at the Charles University in Prague, Faculty of Science under the collection numbers CCF 5154–CCF 5157.

2.3. Phenotypic studies

Representative strains of each obtained species (identified by DNA sequencing) were grown on malt extract agar (MEA; malt extract from Oxoid, Basingstoke, UK), Czapek yeast autolysate agar (CYA; yeast extract from Difco Laboratories, Detroit, MI), yeast extract sucrose agar (YESA; yeast extract from Difco Laboratories, Detroit, MI) and Czapek–Dox agar (CZA; Oxoid, Basingstoke, UK) at 20, 25 and 30 °C in the dark and sealed with Parafilm. Agar media were prepared as described by Frisvad and Samson (2004) and Atlas (2010). Representative photographs were taken at 25 °C.

2.4. DNA extraction and amplification

DNA was extracted from three days old colonies using the NucleoSpin Tissue XS kit (Macherey Nagel, Düren, Germany) according to the manufacturer's instructions. Two aliquots of DNA obtained were stored at −20 °C. The extracted DNA was amplified as described (Literák et al., 2013) using the primers targeting four nuclear loci: ITS region, including ITS1, 5.8S rDNA and ITS2, ß-tubulin (*BenA*), calmodulin (*CaM*) and elongation factor 1α (*EF1-α*), and three mitochondrial loci: mtSSU, mtLSU and *CO1*. The primers used are listed in Table 2. The generated DNA fragments were purified using USB Exo-SAP-IT (Affymetrix, Santa Clara, CA) and were subjected to bidirectional Sanger sequencing using ABI 3130 DNA Analyzer (Applied Biosystems, Foster City, CA) or were sent for Sanger sequencing by Macrogen (Seoul, Korea). The resulting consensus DNA sequences were submitted to the GenBank database under the accession numbers KP792130–KP792161 and KT200194–KT200207 (Table 3).

2.5. Identification

We imported the newly generated sequences of mitochondrial and nuclear DNA and the publicly available sequences with the highest similarity as identified by NCBI Blast (as of 4-Jul-2015) into the program MEGA5 and aligned by ClustalW (gap opening penalty 7, gap extension penalty 2 for both pairwise and multiple alignments, DNA weight matrix IUB, transition weight 0.1). We trimmed the aligned sequences, and removed short-length sequences from the alignments; only trimmed sequences were used for further analyses. The barcoding loci *BenA*, *CaM* and ITS, officially adopted for *Aspergillus* and *Penicillium* taxonomy[1], were used for comparison using NCBI Blast similarity search. The trimmed ß-tubulin locus corresponded to nt. 43–349 (307 bp) of EF651917 of *Aspergillus pseudoglaucus* (Table S1). The trimmed calmodulin locus corresponded to nt. 39–360 (321 bp) of EF652007 of *A. pseudoglaucus* (Table S2). The trimmed ITS1 & ITS2 locus corresponded to nt. 8–462 (455 bp) of EF652050 of *A. pseudoglaucus*, which consisted of partial SSU, full-length ITS1, 5.8S rDNA and ITS2, and partial LSU (Table S3). The trimmed mtSSU locus corresponded to nt. 2356–2611 (256 bp) of JN696111 of *Penicillium solitum* (Table S4). The trimmed LSU locus corresponded to nt. 598–1046 (449 bp) of EF652048 of *A. pseudoglaucus* (Table S5). The trimmed *CO1* locus corresponded to nt. 1–521 (521 bp) of EF180200 of *Penicillium concentricum* (Table S6).

Maximum likelihood fits of 24 nucleotide substitution models were performed as described (Řezáč et al., 2014), with all sites used for the analyses, including the gaps. For each model, we calculated the Bayesian information criterion, Akaike information criterion (corrected) and maximum likelihood values. For the *BenA* locus, we analyzed 13 sequences with a total of 341 positions in the final dataset (Table S7). For the *CaM* locus, we analyzed 5 sequences with a total of 321 positions in the final dataset (Table S8). For the ITS1 & ITS2 locus, we analyzed 16 sequences with a total of 500 positions in the final dataset (Table S9). For the SSU locus, we analyzed 24 sequences with a total of 256 positions in the final dataset (Table S10). For the LSU locus, we analyzed 9 sequences with a total of 451 positions in the final dataset (Table S11). For the *CO1* locus, we analyzed 8 sequences with a total of 521 positions in the final dataset (Table S12).

Following determination of the best fit model, we used the respective model to construct a tree. For the *BenA* data, we used Kimura 2-parameter model (Kimura, 1980). Non-uniformity of evolutionary rates among sites was modeled by assuming that a certain fraction of sites were evolutionarily invariable (+I). For the *CaM* data, we used Kimura 2-parameter model (Kimura, 1980). For the ITS1 & ITS2 and SSU data, we used Tamura 3-parameter model (Tamura, 1992). Non-uniformity of evolutionary rates among sites was modeled by using a discrete Gamma distribution (+G) with 5 rate categories. For the LSU data, we used Kimura 2-parameter model (Kimura, 1980). For the *CO1* data, we used Tamura 3-parameter model (Tamura, 1992). We employed the bootstrap procedure at 1,000 replicates. For the tree inference, we used nearest-neighbor-interchange as the maximum likelihood heuristic method of choice, and the initial tree was formed by the neighbor joining algorithm.

We next used the maximum likelihood method to estimate intraspecific evolutionary divergence in the examined fungal species. We calculated the number of base differences per site in the sequences of the *BenA* locus by averaging over all sequence pairs between groups (Distance) ± SE, and employed the bootstrap procedure at 1000 replicates; 287 positions in 13 nucleotide sequences were analyzed. The model used to estimate intraspecific evolutionary divergence was identical with the one used to construct the *BenA* tree but without modeling for non-uniformity by assuming that a certain fraction of sites were evolutionarily invariable, because such modeling was not compatible with the calculation of intraspecific evolutionary divergence by the program used (Tamura et al., 2011).

[1] International Commission of Penicillium and Aspergillus. Available from: http://www.aspergilluspenicillium.org, cited as 15-Sep-2015.

Table 1
Species, host organisms, sampling sites and sampling dates of specimens of entomopathogenous fungi found in course of this study. ENV = DNA specimens isolated from a fungus growing directly on the host body. ISO = DNA specimens isolated from a fungus cultivated on agar plates. *Co-infection of the same host individual by two fungal species (gall IDs Hodonín 82 and Hodonín 152). Collection numbers of strains deposited in the Culture Collection of Fungi (CCF) at the Charles University in Prague, Faculty of Science are indicated.

Specimen ID		Species	Host	Sampling site, country, gall ID	Coordinates	Date
ENV	ISO					
2226	2242	*Penicillium buchwaldii*	*Pemphredon fabricii*	Sekule, Slovakia, 182	48.62N 17.00E	15-Mar-2014
2227	2243	*Aspergillus pseudoglaucus*	*Pemphredon fabricii*	Hodonín, Czech Republic, 63	48.88N, 17.06E	14-Mar-2014
2228	2244	*Penicillium buchwaldii*	*Pemphredon fabricii*	Darkov, Czech Republic, 120	49.83N, 18.56E	08-Feb-2014
2229	2245	*Penicillium buchwaldii*	*Pemphredon fabricii*	Hodonín, Czech Republic, 47	48.88N, 17.06E	14-Mar-2014
2230	2246 (CCF 5157)	*Penicillium quebecense*	*Pemphredon fabricii*	Sekule, Slovakia, 178	48.62N 17.00E	15-Mar-2014
2231	2247	*Penicillium buchwaldii*	*Pemphredon fabricii*	Hodonín, Czech Republic, 140	48.88N, 17.06E	14-Mar-2014
2232	2248 (CCF 5155)	*Penicillium buchwaldii*	*Hoplitis leucomelana*	Chvaletice, Czech Republic, 186	50.03N, 15.43E	07-Mar-2014
2233	2249 (CCF 5156)	*Penicillium buchwaldii*	*Hylaeus moricei*	Darkov, Czech Republic, 67	49.83N, 18.56E	08-Feb-2014
2234	2250	*Penicillium buchwaldii*	*Trypoxylon minus*	Stará Pohůrka, Czech Republic, 157	48.96N, 14.52E	20-Feb-2014
2235	2251 (CCF 5154)	*Aspergillus pseudoglaucus*	*Pemphredon fabricii*	Darkov, Czech Republic, 7	49.83N, 18.56E	08-Feb-2014
2236	2252	*Penicillium buchwaldii*	*Pemphredon fabricii*	Sekule, Slovakia, 173	48.62N 17.00E	15-Mar-2014
2237	2253	*Penicillium buchwaldii*	*Pemphredon fabricii*	Hodonín, Czech Republic, 56	48.88N, 17.06E	14-Mar-2014
2238	2254	*Penicillium buchwaldii*	*Pemphredon fabricii*	Sekule, Slovakia, 51	48.62N 17.00E	15-Mar-2014
2239	2255	*Penicillium buchwaldii*	*Trypoxylon deceptorium*	Olešník, Czech Republic, 85	49.09N, 14.36E	21-Feb-2014
2240A	2256	*Penicillium buchwaldii*	*Pemphredon fabricii*	Hodonín, Czech Republic, 152*	48.88N, 17.06E	14-Mar-2014
2241A	2257A	*Penicillium buchwaldii*	*Pemphredon fabricii*	Hodonín, Czech Republic, 82*	48.88N, 17.06E	14-Mar-2014
2241B	2257B	*Aspergillus pseudoglaucus*	*Pemphredon fabricii*	Hodonín, Czech Republic, 82*	48.88N, 17.06E	14-Mar-2014
2240B	2258	*Aspergillus pseudoglaucus*	*Pemphredon fabricii*	Hodonín, Czech Republic, 152*	48.88N, 17.06E	14-Mar-2014

Table 2
Primers employed for the amplification and sequencing of nuclear and mitochondrial DNA loci from entomopathogenous fungi found in course of the study.

Locus	Primer name	Sequence	Reference
ITS1 & ITS2	ITS1-F	CTTGGTCATTTAGAGGAAGTAA	Gardes and Bruns (1993)
	5.8SF	GGATCACTCGGCTCRTGNRTCGATGAAG	Medina et al. (2001)
	ITS4	TCCTCCGCTTATTGATATGC	White et al. (1990)
ß-tubulin	Bt2a	GGTAACCAAATCGGTGCTGCTTTC	Glass and Donaldson (1995)
	Bt2b	ACCCTCAGTGTAGTGACCCTTGGC	Glass and Donaldson (1995)
Calmodulin	CF1 M	AGGCCGAYTCTYTGACYGA	Peterson (2008)
	CF4	TTTYTGCATCATRAGYTGGAC	Peterson (2004)
mt SSU	mrSSU2R	CCTTCGTCCTTCAACGTCAG	Zoller et al. (1999)
	MSU7	GTCGAGTTACAGACTACAATCC	Zhou and Stanosz (2001)
mt LSU	5.8SF	GGATCACTCGGCTCRTGNRTCGATGAAG	Medina et al. (2001)
	R635	GGTCCGTGTTTCAAGACGG	Yamada and Kawasaki (1989)
CO1	PenF1	GACAAGAAAGGTGATTTTTATCTTC	Seifert et al. (2007)
	AspR1	GGTAATGATAATAATAATAATACAGCTG	Seifert et al. (2007)

Table 3
Sequences generated newly from the specimens of entomopathogenous fungi found in course of this study. NCBI GenBank accession numbers (KP792130–KP792161 and KT200194–KT200207) are indicated.

Specimen	Locus					
	ITS1, 5.8S rDNA and ITS2	ß-tubulin	Calmodulin	mt SSU	mt LSU	*CO1*
2226				KP792130	KP792156	
2237				KP792131		KP792150
2242				KP792132	KP792158	KP792151
2243	KT200202		KT200199	KP792133	KP792159	
2244				KP792134		
2245				KP792146		
2246	KT200203	KT200194		KP792147		
2247				KP792148		
2248	KT200204	KT200195		KP792135		
2249	KT200205	KT200196		KP792136		
2250				KP792137		KP792152
2251	KT200206	KT200197	KT200200	KP792138		
2252				KP792149		KP792153
2253				KP792139	KP792160	KP792154
2254				KP792140		KP792155
2255				KP792141		
2256				KP792142		
2257A				KP792144		
2257B				KP792145		
2258	KT200207	KT200198	KT200201	KP792143	KP792161	

2.6. Statistical analyses

All aculeate hymenopterans obtained in course of the rearing experiments were examined for macroscopic signs of fungal infections. To estimate the prevalence of infection, we used the statistical software L-Calc, version 1.1 (StemSoft Software, Vancouver, Canada), which estimates the parameters by fitting a generalized linear model with a complementary log–log link and finds the maximum likelihood using Newton–Raphson method. The χ^2 statistics was determined to assess the degree of consistency in the data with a Poisson dose–response relationship. A 5% or less type I error was considered to be statistically significant. A significant χ^2 test occurs when there is inconsistency in the data distribution. Fisher's test was used to calculate the differences in infection rates in species complexes of specialized and ubiquitous hosts. All other calculations were performed in PAST 2.14. Data are shown as mean ± SD unless stated otherwise.

3. Results

3.1. Epidemiology

We examined 6449 reed galls induced by *Lipara* spp., of which 4629 were unoccupied and 1820 (18.2%) were occupied by aculeate hymenopterans or their parasitoids. In these 1820 nests, we recorded in total 5200 immature individuals of aculeate hymenopterans or their parasitoids. Most of them were represented by the eudominant species *Pemphredon fabricii* (91.9% of specimens identified to the species level). The examined reed galls hosted 12 species of aculeate hymenopterans and their parasitoids. Most of the specimens hatched successfully. However, in 230 nests (represented by 532 individuals, 10.2% of the total), there were dead individuals or the immature individuals did not hatch due to unknown reasons; such immature individuals were not identified to a species level.

During the examination of immature individuals identifiable to a species, we noticed fungal outgrowth on the surface of the exoskeleton of 83 (1.60%) of larvae and pupae, which corresponds to the estimated prevalence 1 in 56 (95% CI: 1 in 45–69). We noticed the infections in six independent species of reed gall-associated aculeate hymenopterans and one their parasitoid, with most records (but not the highest prevalence) recorded in the eudominant species *P. fabricii* (Table 4). Within the species complex of *Trypoxylon* spp., the *Lipara*-induced reed gall specialist *Trypoxylon deceptorium* displayed lower infection rates than the closely related species *Trypoxylon minus*, which uses *Lipara*-induced reed galls only occasionally (Fisher's test $p < 0.01$). Similar trend was observed in *Hylaeus* spp. (*Hylaeus pectoralis* vs. *Hylaeus moricei*), but the sample size did not allow sufficient power to prove such relationship in the genus *Hylaeus* too.

We identified the infected individuals at 16 of the 34 examined sampling sites, two of which consisted of near-natural sites (Sekule, Borovany) and other 14 sites were located to fly ash and slag deposits of lignite heating plants and powerplants. The relative number of infected individuals differed dramatically, reaching up to 16 individuals at the Hodonín sampling site (13.1% of live individuals identified to the species level at this sampling site), 15 individuals at Sekule (3.7%), 8 individuals at Darkov (2.5%) and at Chvaletice (1.8%), and less or none at all at other sampling sites. Altogether 39 infected nests were multibrooded; five infected nests were singlebrooded. In 24 cases (62%), only a part of individuals within a multibrooded nest were infected. Such infections consisted in 19 cases of infection of only a single individual within a multibrooded nest, and in 5 cases of infection of multiple (but not all) individuals within a multibrooded nest. In 15 cases (38%), all individuals within a multibrooded nest were infected. We allowed all the infected immature individuals to develop into adults similarly to the uninfected ones, but all the infected individuals died at various immature stages.

We next identified specimens from six sampling sites ($n = 18$ specimens, Table 1) to the species level by combined morphologic and genetical approaches. We recorded the infection by three fun-

Table 4
Epidemiology of the fungi infecting immature reed gall-associated aculeate hymenopterans and their parasitoids. Indicated is the number of galls, nests and individuals of each host species examined, absolute and relative number of live individuals found to be infected and estimated prevalence of macroscopic fungal infections of the exoskeleton.

Species	Number of galls (nests) examined	Number of immature host individuals examined	Absolute/relative [%] number of individuals with macroscopic fungal infection of the exoskeleton	Estimated prevalence (95% CI); χ^2 (Pearson); χ^2 (Deviance)
Diptera/Bombyliidae				
Thyridanthrax fenestratus	18	75	9/12.0	1 in 8 (4–15)
Hymenoptera/Chrysididae				
Chrysis angustula	1	5	0	
Trichrysis cyanea	16	32	0	
Hymenoptera/Vespidae				
Symmorphus bifasciatus	6	20	0	
Hymenoptera/Crabronidae				
Passaloecus clypealis	3	7	0	
Pemphredon fabricii	1029	4289	59/0.33	1 in 72 (56–93)
Trypoxylon deceptorium	39	91	1/1.1	1 in 90 (13–642)
Trypoxylon minus	6	19	4/5.3	1 in 4 (2–11)
Hymenoptera/Megachilidae				
Hoplitis leucomelana	10	24	1/4.1	1 in 23 (3–167)
Hymenoptera/Colletidae				
Hylaeus incongruus	1	3	0	
Hylaeus moricei	3	13	3/23.1	1 in 4 (1–12)
Hylaeus pectoralis	27	90	6/6.7	1 in 14 (7–32)
Not identified	230	532	N/A	
Unoccupied	4629	N/A		
Total	6449	5200	83/1.60	1 in 56 (45–69); 137.6 ($p < 0.001$); 58.4 ($p < 0.001$)

gal species, *Penicillium quebecense*, *Penicillium buchwaldii* and *A. pseudoglaucus*. We found *P. buchwaldii* as the most abundant fungal pathogen associated with the exoskeleton of the live immature individuals of aculeate hymenoptera. We found this species at all the six mould-positive sampling sites from which the fungi were subjected to morphologic and genetical analyses (in total 13 cases, 72%), associated with five host species, namely *P. fabricii* (9 cases), *T. deceptorium*, *T. minus*, *Hoplitis leucomelana* and *H. moricei* (1 case each). We found *A. pseudoglaucus* at two sampling sites, represented by four cases (22%), all hosted by *P. fabricii*; two of the four cases were in fact co-infections of the *P. fabricii* host individual with *A. pseudoglaucus* and *P. buchwaldii*. Last but not least, we identified a single case (6%) of infection by *P. quebecense*, hosted by *P. fabricii*. All the above cases are considered new host records.

3.2. Fungal strain identification

Maximum likelihood analysis of nuclear DNA loci (ß-tubulin, calmodulin and ITS1 & ITS2) revealed the infection by three fungal species. The highest resolution and the best comparative data for differential diagnosis were available for the ß-tubulin locus (Fig. 1A).

The ß-tubulin sequences of specimens 3LF-2251 and 3LF-2258 displayed 100% similarity to *A. pseudoglaucus* (NCBI Acc. No. EF651917; mean distance $d = 0.000 \pm 0.000$), with 100% bootstrap support for *A. pseudoglaucus* over the closely related *Aspergillus tonophilus* and *Aspergillus sloanii* (Fig. 1A). The identification was supported by comparison of obtained *CaM* and ITS sequences with barcodes of the same species (EF652007 and EF651917, respectively; Fig. 1).

The ß-tubulin sequences of specimens 3LF-2248 and 3LF-2249 displayed the highest similarity to *P. buchwaldii* (JX313182; $d = 0.000 \pm 0.000$), with 84% bootstrap support over *Penicillium spathulatum* (Fig. 1A). The identification was supported by comparison of obtained ITS sequences with the barcode of the same species (JX313164, respectively; Fig. 1).

The ß-tubulin sequences of the specimen 3LF-2246 displayed the highest similarity to *P. quebecense* (JN606700; $d = 0.003 \pm 0.003$) as compared to *Penicillium aurantiacobrunneum* and *Penicillium cairnsense* (Fig. 1A). The identification was supported by comparison of obtained ITS sequence with the barcode of the same species (JN617661, respectively; Fig. 1).

We obtained also the sequences of mitochondrial (SSU, LSU and *CO1*) DNA loci for all the three analyzed species, but only LSU was in part sufficiently informative to support the diagnoses because they were not sufficiently informative at the species level (SSU), or did not allow to address this issue due to the lack of publicly available sequences of the three identified species in GenBank (*CO1*, in part LSU and SSU) (Figs. 2). Negligible intraspecific variability in sequences of the six DNA loci tested prevented us from identifying any potential host- or site-specific population structure in examined species.

3.3. Morphologic analyses

3.3.1. Penicillium quebecense

Macromorphology of *P. quebecense* was examined using strain 3LF-2246 (CCF 5157): Colonies on MEA attaining 36–37 mm diameter in 7 d at 25 °C, colony color grayish yellow green (#8F9779) and white (#F2F3F4) in marginal part, finely granular, flat, reverse pale greenish yellow (#EBE8A4). Colonies on CYA attaining 45 mm diameter in 7 d at 25 °C, colony color yellowish white (#F0EAD6), floccose, crateriform, radially sulcate, reverse moderate orange yellow (#E3A857). Colonies on CZA attaining diameter of 30–33 mm after 7 d at 25 °C, floccose, plane, colony and reverse color pale yellow (#F3E5AB) to yellowish white (#F0EAD6). Colonies on YESA attaining 39–42 mm diameter in 7 d at 25 °C, finely granular, flat, radially sulcate in center, colony color pale green (#8DA399), reverse color similar to MEA (Fig. 3A-D). Red (#F91202) (on MEA) and vivid red (#BE0032) (on YESA) diffusible pigments were produced at 30 °C.

3.3.2. Penicillium buchwaldii

Macromorphology of *P. buchwaldii* was examined using strains 3LF-2248 (CCF 5155), 3LF-2249 (CCF 5156) and 3LF-2256: Colonies on MEA attaining 24–29 mm diameter in 7 d at 25 °C, colony color dark bluish green (#004B49) and white (#F2F3F4) in marginal part, finely granular, flat, radially sulcate in center, reverse light orange yellow (#FBC97F). Colonies on CYA attaining 21–28 mm diameter in 7 d at 25 °C, colony color light bluish green (#66ADA4) to very light bluish green (#96DED1), finely granular, crateriform, central part of colony raised or flat, radially sulcate at margins and sulcate to wrinkled centrally, reverse light orange yellow (#FBC97F). Colonies on CZA attaining diameter of 18–21 mm after 7 d at 25 °C, floccose, central part of colony raised, colony color dark bluish green (#004B49) to grayish greenish yellow (#B9B57D) and white (#F2F3F4) in center and marginal part, reverse vivid yellow (#F3C300) to pale yellow (#F3E5AB) in marginal part. Colonies on YESA attaining 19–26 mm diameter in 7 d at 25 °C, crateriform, central part of colony raised or flat, radially or concentrically sulcate, colony and reverse color similar to CYA (Fig. 3E–H). No growth observed at 30 °C on MEA.

3.3.3. Aspergillus pseudoglaucus

Macromorphology of *A. pseudoglaucus* was examined using strains 3LF-2251 (CCF 5154), 3LF-2243 and 3LF-2258: Colonies on MEA attaining 19–22 mm diameter in 7 d at 25 °C, colony color very light yellowish green (#B6E5AF), floccose, convex, reverse light orange yellow (#FBC97F). Colonies on CYA attaining 22–25 mm diameter in 7 d at 25 °C, colony color moderate yellowish green (#679267) to very light bluish green (#96DED1), floccose, central part of colony raised or flat, reverse vivid yellow (#F3C300). Colonies on CZA attaining diameter of 18–23 mm after 7 d at 25 °C, floccose, plane, brilliant greenish yellow (#E9E450), reverse pale yellow (#F3E5AB) to brilliant yellow (#FADA5E). Colonies on YESA attaining 18–24 mm diameter in 7 d at 25 °C, floccose, umbonate, colony color white (#F2F3F4) and moderate yellowish green (#679,267) in marginal part, reverse strong greenish yellow (#BEB72E) to light greenish yellow (#EAE679) (Fig. 3I–L). No growth observed at 30 °C on MEA.

4. Discussion

Reed galls induced by *Lipara* spp. represent a specific but widely distributed microhabitat characterized by high moisture and low air circulation, where the spread of fungi is facilitated by various vectors, including ovipositing females, their mites and possibly also the prey stored abundantly in the brood cells (Benoit et al., 2004; Aquino et al., 2013; Foley et al., 2014). The most, if not all, aculeate hymenopterans defend against the fungi by antifungal compounds, which are produced to protect not only the potential hymenopteran host, but they are also distributed throughout brood cell to protect the prey stored for up to several months before being eaten (Strohm and Linsenmair, 2001; Weiss et al., 2014) and may be produced also by symbiotic prokaryotic and eukaryotic organisms (Flórez et al., 2015). Thus, the environment of the reed gall serves as a highly specific environment, which is hostile to some fungal species, but which provides abundant resources for those resistant to the insect defense mechanisms. In this study, we documented for the first time the species richness, prevalence and host affinity of macroscopic fungal infections

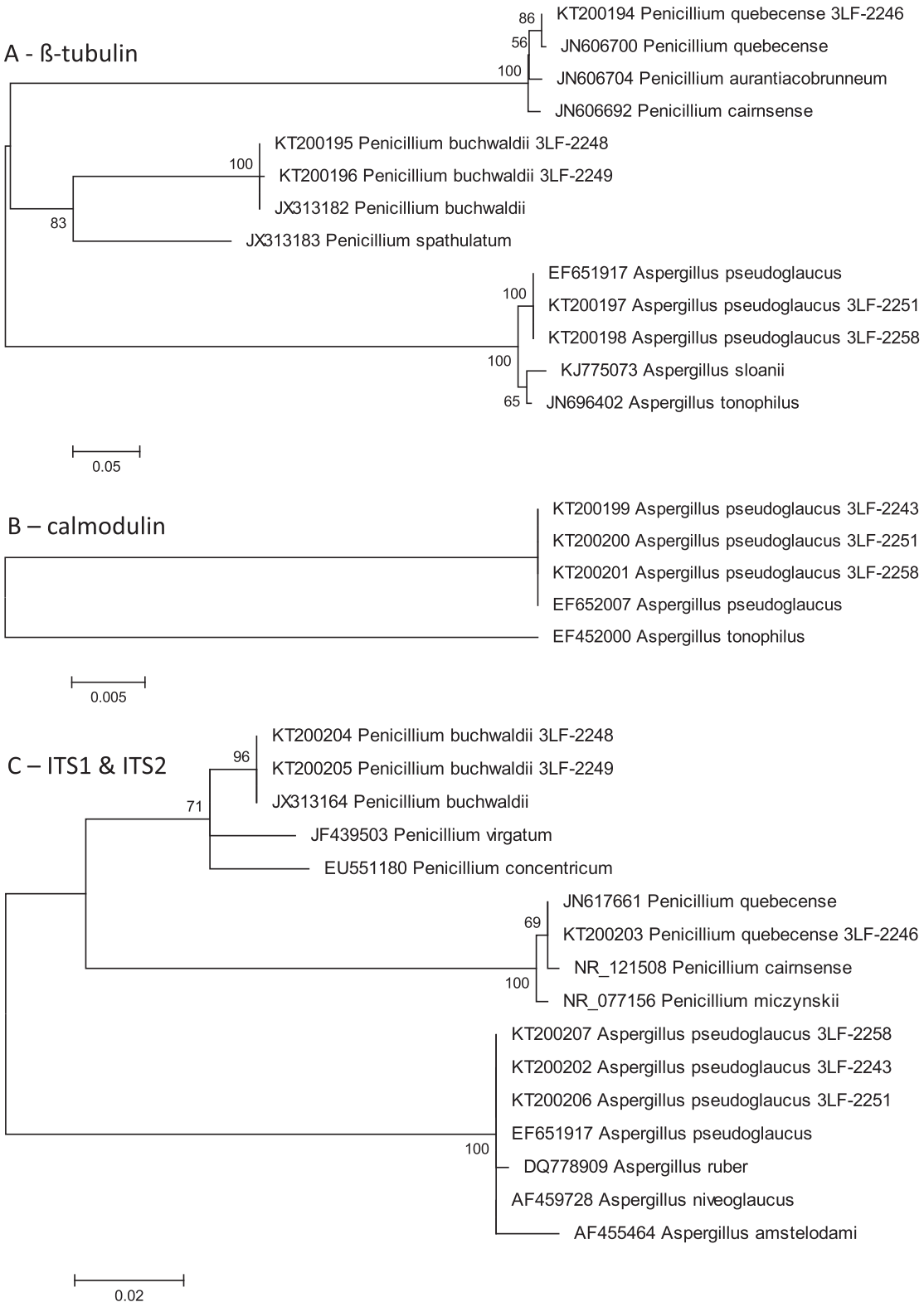


Fig. 1. Maximum likelihood analysis of nuclear DNA loci (ß-tubulin (A), calmodulin (B) and ITS1 & ITS2(C)). Sequences of analyzed specimens are shown together with publicly available sequences with the highest similarity as revealed by NCBI Blast.

of exoskeletons of the brood of aculeate hymenopterans associated with reed galls. The infection rates were highly variable and further research should elucidate the conditions under which massive infestation (we detected up to 13% of live infected brood) occurs. The relatively high abundance of infected but alive larvae may be associated with low temperatures of the reed gall environment experienced throughout the winter period when the galls were collected and analyzed. For example, most of the *Aspergillus* spp. grow

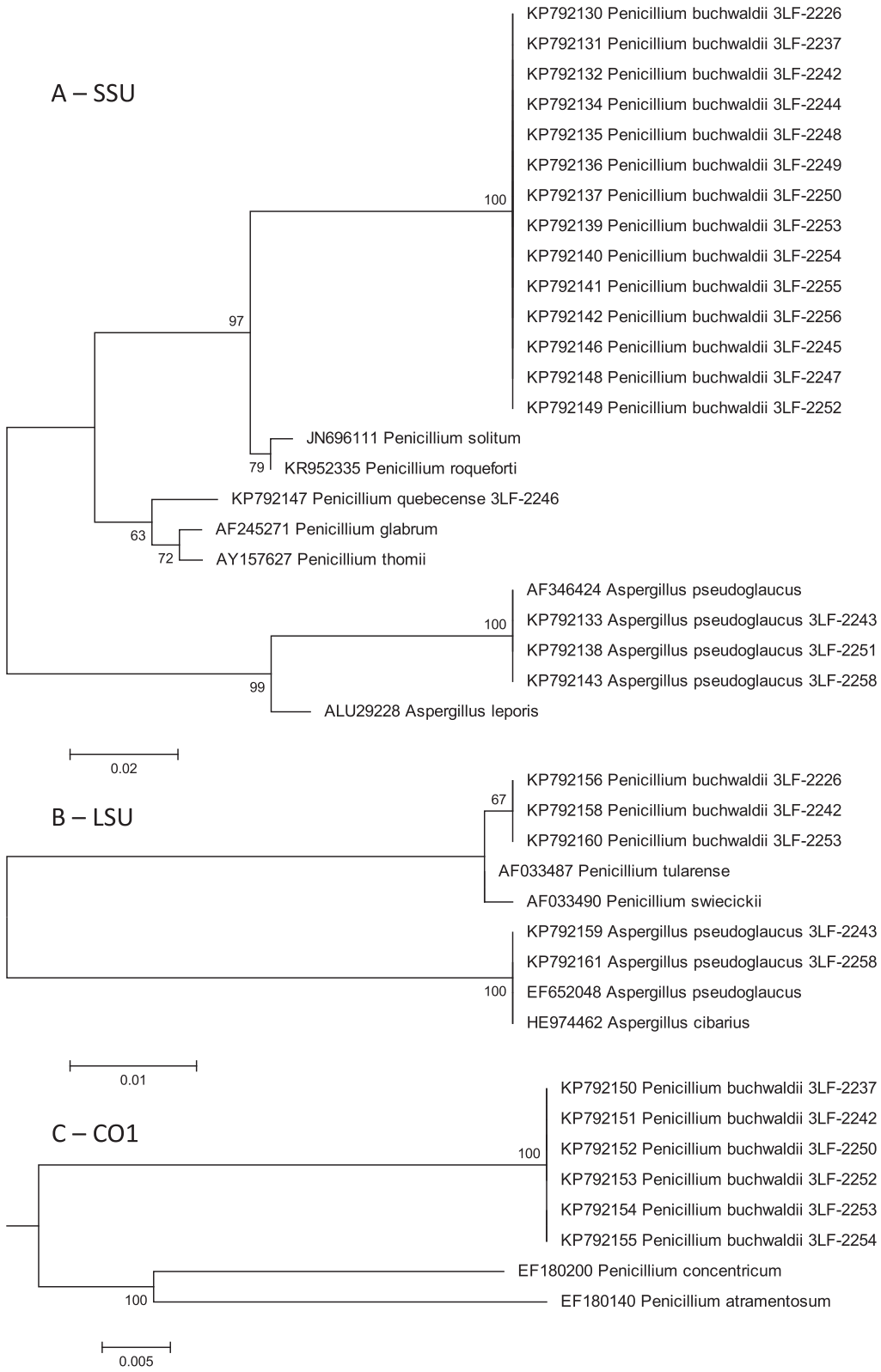


Fig. 2. Maximum likelihood analysis of mitochondrial DNA loci (SSU (A), LSU (B) and *CO1* (C)). Sequences of analyzed specimens are shown together with publicly available sequences with the highest similarity as revealed by NCBI Blast.

in temperatures ranging from 12 °C to 50 °C (Foley et al., 2014), thus negligible growth should be experienced in course of winter months.

In course of this study, we identified three fungal species associated with aculeate hymenopteran larvae (Fig. 4). *P. quebecense* is a little known species of the section *Citrina* characterized by its rapid growth on CYA, pale orange sclerotia, dark red reverse on YES, and CYAS:CYA growth ratio 0.85:1.00 (Houbraken et al., 2011), known to produce the following extrolites: citreoviridin, phoenicin, terrein, SENOE, MIF, MIM, SENGA and alk-770

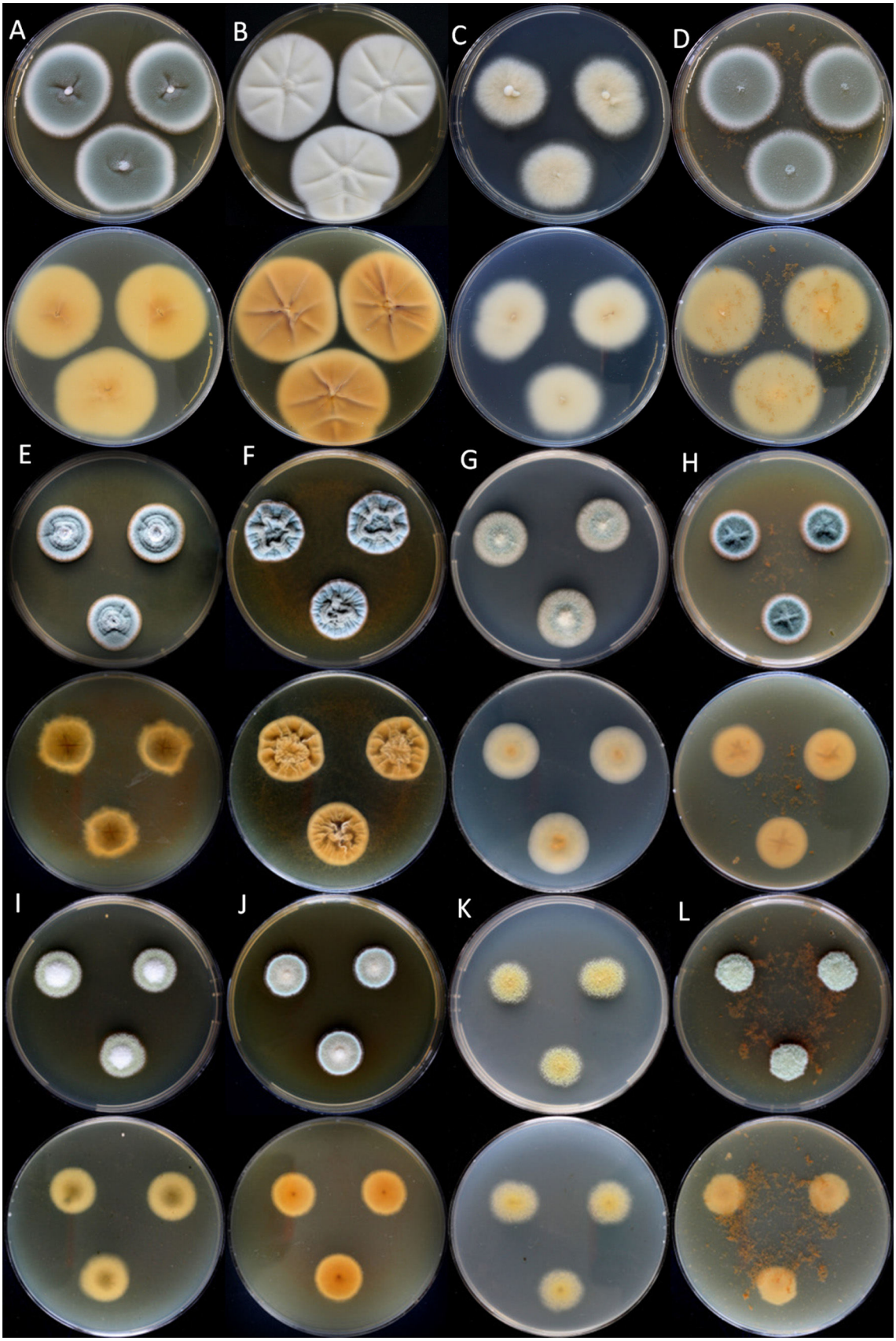
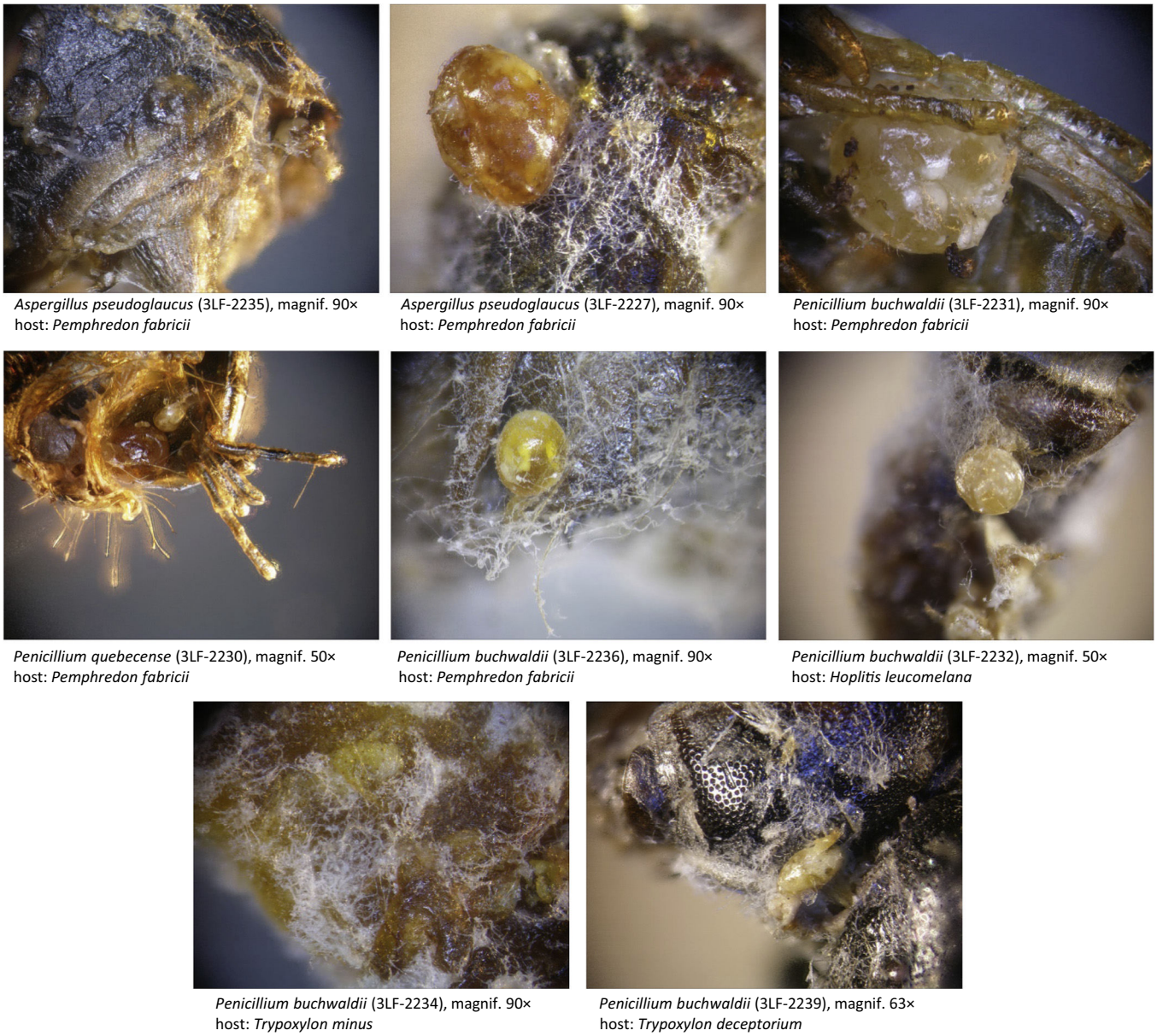


Fig. 3. Colonies and reverse on YESA, CYA, CZA and MEA at 25 °C after 14 d in the dark. *Penicillium quebecense* – strain 3LF-2246 (CCF 5157; A–D); *Penicillium buchwaldii* – strain 3LF-2249 (CCF 5156; E–H); *Aspergillus pseudoglaucus* – strain 3LF-2251 (CCF 5154; I–L), all cultivated on YESA (A, E, I), CYA (B, F, J), CZA (C, G, K) and MEA (D, H, L).



Aspergillus pseudoglaucus (3LF-2235), magnif. 90×
host: *Pemphredon fabricii*

Aspergillus pseudoglaucus (3LF-2227), magnif. 90×
host: *Pemphredon fabricii*

Penicillium buchwaldii (3LF-2231), magnif. 90×
host: *Pemphredon fabricii*

Penicillium quebecense (3LF-2230), magnif. 50×
host: *Pemphredon fabricii*

Penicillium buchwaldii (3LF-2236), magnif. 90×
host: *Pemphredon fabricii*

Penicillium buchwaldii (3LF-2232), magnif. 50×
host: *Hoplitis leucomelana*

Penicillium buchwaldii (3LF-2234), magnif. 90×
host: *Trypoxylon minus*

Penicillium buchwaldii (3LF-2239), magnif. 63×
host: *Trypoxylon deceptorium*

Fig. 4. Representative photographs of fungal infections in the brood of crabronid wasps and megachilid bees found in course of this study.

(Houbraken et al., 2011). So far, this species was known only from its type culture, isolated from the air in a sawmill in Quebec, Canada. Here we report the second isolate of this species, extending its distribution range, identifying additional DNA markers, and reporting the first documented host record of this species.

Another *Penicillium* species identified in course of this study, *P. buchwaldii* of the section *Brevicompacta*, was described only recently as closely related to *P. spathulatum* (Frisvad et al., 2013). Most species in section *Brevicompacta* are commonly found in soil and food (Frisvad and Samson, 2004). Frisvad et al. (2013) isolated and examined 39 strains of *P. buchwaldii*, which originated from indoor air, soil (including a saltern), *Quercus ruber* leaf and several types of food and feedstuffs from multiple European countries, Greenland and Senegal. The diagnostic features include pale beige reverse on CYA agar, bi- and ter-ramulate penicilli, echinulate thick-walled globose conidia and a production of several extrolites of potential clinical interest, namely asperphenamate, citreoisocoumarin, communesin A and B, asperentin and 5'-hydroxyasperentin. So far, this species was known only from sources noted in the publication describing this species. Here we report *P. buchwaldii* as the dominant fungal associate of the exoskeleton of live immature individuals of aculeate hymenoptera, identified in five host species, namely *P. fabricii*, *T. deceptorium*, *T. minus*, *H. leucomelana* and *H. moricei*. We report *P. buchwaldii* for the first time from the Czech Republic and Slovakia, identify additional DNA markers, and identify for the first time the arthropods as potential (and frequent) hosts of this species.

The third species identified in course of this study, *A. pseudoglaucus* (*Eurotium repens*), was identified nearly a century ago by Blochwitz. It belongs to well-known entomopathogenic species of *Aspergillus*, which include also *A. flavus*, *A. parasiticus*, *A. tamarii*, *A. ochraceus*, *A. fumigatus* and *A. versicolor*. All these species are mainly saprophytic but can infect a wide range of insect species, infecting also the brood and adults of honey bees, causing the relatively rare, but well-known disease termed stonebrood, characterized by the formation of hard stone-like mummified cadavers of the brood (Tanada and Kaya, 2012). The section *Aspergillus*, where *A. pseudoglaucus* belongs, comprises of xerophilic fungi,

which grow exceptionally well in highly saline environments, which includes not only the salines, but also foods and feeds preserved with high concentrations of NaCl or sugar (Pitt and Hocking, 1997; Butinar et al., 2005; Hubka et al., 2013), produce numerous mycotoxins (Butinar et al., 2005; Smetanina et al., 2007), and are even able to degrade selected food additives such as the antioxidant 3-tert-butyl-4-hydroxyanisole (BHA) (Doi et al., 1991). Although *A. pseudoglaucus* is relatively uncommon in vertebrates, including humans, it is considered zoonotic. Combined infection by *A. pseudoglaucus* and *Microascus cinereus* was reported to cause maxillary sinusitis in otherwise healthy human male in France subjected to grain dust work exposure (Aznar et al., 1989). Crude mycelial extracts of *A. pseudoglaucus* are toxic to invertebrates as tested on *Artemia salina* larvae. The mycelium contains asperentin, which displays LD_{50} = 86 μg ml^{-1} toward the *A. salina* larvae. Another *A. pseudoglaucus* metabolite, physcion, is considered cytotoxic, and causes 50% growth inhibition of HeLa cells when added at 100 ng ml^{-1} (Podojil et al., 1979). Here we report *A. pseudoglaucus* as an abundant fungal pathogen associated with exoskeletons of the live immature individuals of reed gall-associated aculeate hymenopteran species *P. fabricii*. Interestingly, two of the four genetically confirmed infection events were represented by co-infections with *P. buchwaldii*.

Within the genera *Trypoxylon* and *Hylaeus*, we noticed a trend toward higher infection rates in hymenopteran species, which are not narrowly specialized to use the *Lipara*-induced reed galls as compared to the *Lipara*-induced reed gall specialists *T. deceptorium* and *H. pectoralis*. Such difference might be related to the suboptimal environment provided by reed galls to non-specialists and/or by poor trophic support within monotypic stands of common reed. Foley et al. (2012) have already shown that honey bee larvae fed on a nutritionally poor diet are more susceptible to *A. fumigatus* infections. In contrast, honey bee larvae fed with a diet supplemented with either dandelion or polyfloral pollens increased their resistance toward *A. fumigatus*. Thus, proper nutrition and diverse food resources are definitively among the factors affecting the susceptibility of bee brood toward fungal infections. Shortage of food resources can be caused by various factors. Among them are the presence of agricultural monocultures and simplification in crop rotation, which leads to temporal shortages in food supply linked to agricultural cycles in intensively farmed areas (Decourtye et al., 2010). Also habitat fragmentation and loss of buffer zones of natural and near-natural habitats in intensively farmed areas leads to the loss of natural forage diversity that may be required for optimum nutrition (Kremen et al., 2002). Parasite infections may cause energetic stress such as in case of the microsporidian *N. ceranae* infections (Thompson and Redak, 2008; Mayack and Naug, 2009). And also the absence of elements essential for proper immune responses and/or detoxification of xenobiotics play an important role in nutrition-linked susceptibility of bee brood toward the fungal infections (Johnson et al., 2012). Even if the larvae survive the infection, impairments in development may occur and sub-lethal effects can persist into the adulthood. The stonebrood parasites are ubiquitous, opportunistic species, which have little impact on vertebrates except of immunocompromised individuals (Tell, 2005; Foley et al., 2012). Thus, it is possible that the occurrence of stonebrood and other fungal infections found in course of this study might be associated only with adverse conditions. These may apply either at the level of the health of host individual prior infection, quality of the nest resource and nest construction, or adverse microhabitat conditions.

In conclusion, we have identified the *Lipara*-induced reed galls colonized by aculeate hymenopterans as a microhabitat hosting abundant, although species-poor assemblage of filamentous fungi. We recorded multiple infection events at a large part of sampling sites, thus fungal infections should be considered an important variable driving the abundance of reed gall-associated aculeate hymenoptera. Infections of generalist host species were more frequent than those of reed gall specialists, suggesting that suboptimal conditions decrease the immunocompetence of non-specialized species, which only occasionally nest in reed galls and in surrounding monotypic stands of common reed.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jip.2015.12.007.

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ORIGINAL PAPER

Industrial and post-industrial habitats serve as critical refugia for pioneer species of newly identified arthropod assemblages associated with reed galls

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Abstract Gravel-sand river terraces were nearly eliminated from central European landscape by river channelization. Monotypic stands of common reed (*Phragmites australis*) growing on such terraces are often stressed by drought, which makes them vulnerable to *Lipara* spp. (Diptera: Chloropidae) gallmakers. Although *Lipara* are considered ecosystem engineers, only fragmentary information is available on the biology of their parasitoids and inquilines. We analyzed the assemblages of arthropods (Arachnida, Collembola, Dermaptera, Psocoptera, Thysanoptera, Hemiptera, Raphidioptera,

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Neuroptera, Coleoptera, Diptera, Lepidoptera and Hymenoptera) that emerged from 17,791 *Lipara*-induced galls collected in winter from 30 reed beds in the Czech Republic, 15 of which were situated at (post)industrial sites (gravel-sandpits, tailing ponds, limestone quarries, colliery dumps, and reclaimed lignite open-cast mines) and 15 were in near-natural habitats (medieval fishponds, and river and stream floodplains). The Chao-1 estimator indicated 229.3 ± 18.1 species in reed galls at (post)industrial and 218.1 ± 23.6 species at near-natural sites, with the Sørensen index reaching only 0.58. We identified 18 red-listed species and four new species for the Czech Republic (*Gasteruption phragmiticola*, *Echthrodelphax fairchildii*, *Haplogonatopus oratorius* and *Enclisis* sp.), representing mostly obligate (64 %) or facultative (9 %) reed specialists. We propose that *Lipara* gall-associated assemblages undergo a long-term cyclic ecological succession. During first 10 years after reed bed formation, only *Lipara* spp. and several other species occur. During next decades, the reed beds host species-rich assemblages with numerous pioneer species (*Singa nitidula*, *Polemochartus melas*) that critically depend on presence of prior disturbances. Middle-aged reed beds (near medieval fishponds) are prevalently enriched in common species only (*Oulema duftschmidi*, *Dimorphopterus spinolae*). Habitats with the longest historical continuity (river floodplains) host again species-rich assemblages with several rare species that probably require long-term habitat continuity (*Homalura tarsata*, *Hylaeus moricei*). Landscape dynamics is thus critical for the persistence of a full spectrum of reed gall inquilines, with (post)industrials serving as the only refugia for pioneer species ousted from their key nesting habitats at once cyclically disturbed gravel-sand river terraces.

Keywords Biodiversity conservation · Community structure · Emergence traps · Hydric restoration · Life-history traits · Post-industrial habitats

Introduction

Higher land use intensity substantially alters the associations among the diversities of multiple animal and plant taxa (Manning et al. 2015). Although many previous studies have investigated the effects of land use on the abundances of particular species and the biodiversity of individual taxonomic groups, there are still significant gaps in our understanding of the ecological consequences of land use changes (Allan et al. 2014; Weiner et al. 2014). Understanding these associations is particularly important as the use of inappropriate indicators can lead to poor conservation management decisions and planning, and wrong estimates of wider biodiversity. Particularly where taxa are trophically diverse, forming a mix of secondary consumers, herbivores and omnivores, their diversity is expected to be weakly correlated (Scherber et al. 2010; Weiner et al. 2014; Manning et al. 2015).

Monotypic stands of the common reed *Phragmites australis* serve as important habitats for numerous threatened vertebrates and host diverse communities of invertebrates. Reed beds are frequently protected as nature reserves and form large parts of endangered wetlands. However, the common reed is also considered to be invasive, particularly in North America, and it is also able to swiftly colonize newly formed (post)industrial habitats, such as sandpits, gravel-sandpits, claypits, former open-cast mines and ash deposits (Tscharntke 1992; van der Putten 1997; Čurn et al. 2007; Lelong et al. 2009; Heneberg et al. 2014).

Arthropods utilize common reed as a food source (sap suckers, leaf- and pollen-feeding species) or also as a nesting resource and shelter (stem borers, gall makers, and gall inquilines). Tewksbury et al. (2002) reported 160 species of reed-associated arthropods in Europe, but only 23 species of reed-associated arthropods in North America, where *Phragmites australis* subsp. *americanus* is considered native, but subsp. *australis* is considered an alien taxon. Interestingly, Canavan et al. (2014) reported only six species of arthropods in South Africa, where common reed is considered native, and only a few species were recorded in Australia (Wapshere 1990).

In total, over 100 oligophagous reed stem boring species are known (Tscharntke 1992, 1993, 1999), of which 11 damage reed shoot tops (Narchuk and Kanmiya 1996; Tscharntke 1999; Gudkov et al. 2006). These include nine species of *Lipara* flies (four of which, *L. lucens*, *L. rufitarsis*, *L. pullitarsis* and *L. similis*, occur in the Czech Republic, all inducing cigar-like galls on the top of reed shoots) and two species of *Steneotarsonemus* thread-footed mites (*S. phragmitidis* and *S. gibber*, which induce morphologically different type of galls).

The females of *Lipara* spp. deposit their eggs on the surface of the reed shoot, into which the first instar larvae bore and feed upon the newly emerging leaves. Meanwhile, the gall is formed, and the *Lipara* larvae enter the gall only when its formation is completed. Larvae of *L. lucens* and *L. rufitarsis* gnaw from the top through the growing point and continue their life cycle inside, whereas larvae of *L. pullitarsis* never pass through the growing point and can be found between the enwrapped leaves (De Bruyn 1994). Because of that, *L. lucens* and *L. rufitarsis* attack especially reed shoots of less than 4.5 mm in diameter. Such thin reed stems are usually formed in response to abiotic stress, including the deficiency in water or nutrients or severe contamination by heavy metals. The stressed stems contain less silicate and cause less mortality of gall-inducing first instar chloropid larvae (Tscharntke 1989). Newly formed reed beds are colonized relatively slowly. The $\geq$50 % probability of the presence of the two most abundant gall makers, *L. pullitarsis* and *Giraudiella inclusa*, is reached only in habitats older than 3 and 6 years, respectively, and larger than 25 and 100 m^2, respectively (Athen and Tscharntke 1999).

The reed galls induced by *Lipara* flies host a diverse spectrum of successors. The *Lipara* larvae serve as hosts to parasitoids, some of which are regulated by the physical properties of the galls. The survival of *L. lucens* is higher by 40 % on thicker shoots, which is mainly attributed to the parasitoid *Stenomalina liparae*, which attacks the host larva inside the reed shoot. The ovipositor of *S. liparae* has a mean length of 1.9 $\pm$ 0.2 mm, and when the walls of the shoot are too thick, the parasitoid simply does not reach the larva of *L. lucens*. In contrast, *Polemochartus liparae*, the second most important parasitoid of *L. lucens*, oviposits on the host while it is still attached to the surface of the reed shoot, thus there are no physical barriers to prevent the infestation (De Bruyn 1994). In addition, many inquilines use the *Lipara*-induced galls as a shelter for nesting or overwintering. Some of them also seem to preferentially select galls with narrowly defined physical properties or according to other habitat features, such as the proximity of food sources. In this regard, the previously reported main food source of the wasp *Pemphredon fabricii*, the aphid *Hyalopterus pruni*, shares a similar distribution pattern with its predator—it occurs abundantly at the edge of reed beds, whereas the central parts of large reed beds are subject to infestation that is lower by over one order of magnitude (Tscharntke 1992). Such difference is attributed to the intraseasonal switch of host plants of *H. pruni*—the reed is utilized during the summer, and then, the aphids migrate to their main host, *Prunus* spp. (Dill 1937). Habitat type in general contributes to the variability of reed-associated arthropod assemblages, with only few species considered insensitive to the habitat type (Tscharntke 1989).

Knowledge of the component community of *Lipara* reed gall parasitoids, predators and inquilines is still fragmentary. Few groups of arthropods have been thoroughly studied. These include parasitoids of *Lipara* spp. (e.g., Giraud 1863; Kasparjan 1981; Dely-Draskovits et al. 1994; Nartshuk 2006), dipteran inquilines (Pokorný and Skuhravý 1981; Tscharntke 1999; Grochowska 2008), and aculeate hymenopteran inquilines (Dely-Draskovits et al. 1994; Westrich 2008; Heneberg et al. 2014). Systematically collected data on *Lipara* reed gall inquilines from other groups of arthropods, such as spiders, beetles and hemipterans, are missing, as are large-scale complex studies on *Lipara* gall inquilines, with the exception of the study by Dely-Draskovits et al. (1994).

In this study, we address the arthropod component communities associated with reed galls induced by *Lipara* spp. in their complexity, focusing particularly on the diversity of gall inquilines, which represent a key component of gall assemblages (Sanver and Hawkins 2000). We use the *Lipara* gall communities as a model system to compare the diversity of arthropods belonging to several trophic levels in habitats with strikingly different land use intensity and history. We show that all of the four central European *Lipara* species can be found equally in well-preserved nature reserves and in newly formed (post)industrial habitats. Therefore, we use this opportunity to identify: (1) species that prefer or are limited to the near-natural habitats present in the nature reserves and other well-preserved areas, such as river floodplains and medieval fishponds, (2) species that had the capability to colonize the newly emerging reed beds in the (post)industrial habitats and are equally present in near-natural and (post)industrial habitats, and (3) species that prefer (post)industrial habitats over the near-natural ones due to better availability of bare ground and adjacent xerothermic microenvironments or other yet unknown reasons.

Materials and methods

Study area and sampling sites

The study was carried out at 30 reed bed sites in the Czech Republic (Central Europe, 48°39′–50°59′ N, 12°19′–18°29′ E). Detailed description of sampling sites (Table S1) was provided by Heneberg et al. (2014). Half of selected sampling sites were located to near-natural habitats (15 reed beds, of them 12 near ancient fishponds, and 3 along rivers or streams), representing reed beds spanning 0.2–480 ha and occurring within the altitudinal range 163–452 m a.s.l. It is important to note that despite a continual reed bed presence at the examined sites, the actual extent of most of the reed beds was subject to change in the past, and they were harvested for fuel, animal food, litter, or other purposes, or cultivated in part as meadows or fields. Importantly, the *Lipara* flies occupy prevalently the reed bed ecotones, and thus can easily adjust to gradual changes in the reed bed area. Additional 15 sampling sites were represented by reed beds in (post)industrial habitats. As (post)industrial habitats, we classified any sites formed by mining or quarrying, and water bodies and dumps used for the deposition of ash, slug, waste from metallurgic and chemical industry, waste from uranium processing or spoil from colliery mines. The (post)industrial sites examined in this study thus included gravel-sandpits, tailing ponds, stone quarries, colliery dumps and reclaimed lignite open-cast mines. The reed beds formed there between the years 1922 and 2010, and covered areas 0.2–19 ha within the altitudinal range 157–467 m a.s.l. The sampling sites were chosen to represent the whole spectrum of reed beds present throughout the study area (Fig. 1; Table S2), and to allow an assessment of changes

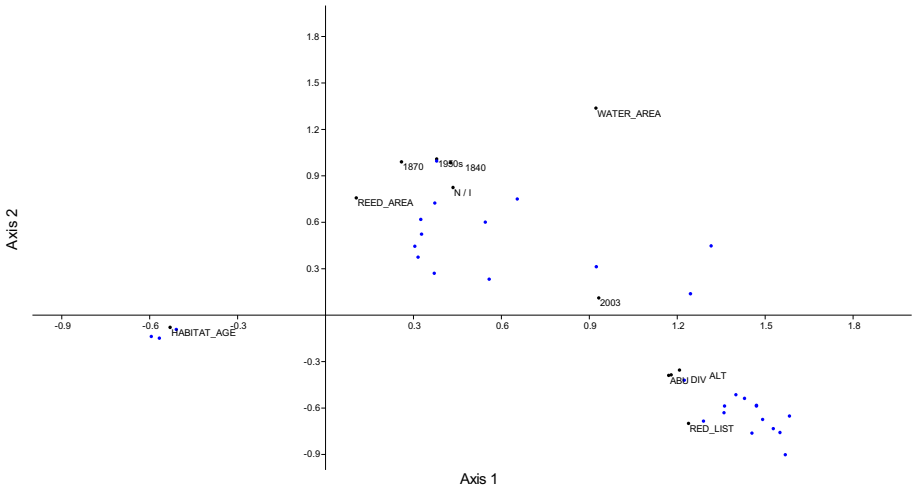

Fig. 1 Correspondence analysis (Benzecri scaling) of the biotic and abiotic variables (*black dots* labeled by *acronyms*) associated with the sampling sites examined in the course of this study (*blue dots*). The resulting factor scores of correspondence analysis are provided in Table S2

associated with the succession of newly emerging reed beds based on a space-for-time substitution paradigm (Pickett 1989).

Sampling

At each sampling site, 300–1000 reed galls were collected between 12 January and 16 March 2013 as described by Heneberg et al. (2014). Briefly, the deformed reed shoots were cut right under the gall, and protruding leaves were also cut out in order to fit collected galls into rearing bags. At each site, the galls induced by *Lipara* spp. were selected randomly, regardless of their position, size or age, reflecting their variation at each sampling site. Arthropods were allowed to rear when exposed to a daylight cycle, at a temperature between 15 and 23 °C for 3–4 months. The rearing bags were sprayed with water several times a week. Plastic bottles with conservation fluid (ethanol or propylene glycol mixed with water and detergent) were installed proximal to the light source; most of the arthropods were captured into the bottles provided. The total number of reed galls sampled reached 17,791, out of which 8820 (49.6 %) were obtained from near-natural habitats, and 8971 (50.4 %) were collected from (post)industrial habitats.

The sampling was performed by Petr Heneberg, Petr Bogusch and Alena Astapenková. Obtained specimens were identified to species by Petr Baňař (Heteroptera), Petr Bogusch (Hymenoptera: Aculeata, selected other taxa), Kamil Holý (Hymenoptera: Parasitica), Petr Janšta (Hymenoptera: Parasitica), Štěpán Kubík (Diptera), Jan Macek (Hymenoptera: Symphyta, Parasitica, Dryinidae), Igor Malenovský (Auchenorrhyncha, Sternorrhyncha), Miroslav Mikát (Coleoptera, Lepidoptera), and Milan Řezáč (Araneae). Albert Damaška, Alois Hamet, Tomáš Kopecký and Jan Pelikán revised selected specimens of Coleoptera; Pavel Tyrner revised selected specimens of Chrysidoidea. The findings of aculeate hymenopterans (except Dryinidae) obtained from this set of reed galls were analyzed previously (Heneberg et al. 2014).

Statistical analyses

All arthropods obtained in course of the rearing experiments were analyzed. To estimate their species richness, Chao-1 estimator, corrected for unseen species, was calculated (Colwell and Coddington 1994). To compare species composition of the analyzed datasets, Sørensen similarity index was calculated. Both indices were calculated in EstimateS 9.1.0. We also calculated basic diversity indices for each of the datasets; these included the total number of species found, the total number of individuals found, dominance (=1 - Simpson index), Brillouin's index (particularly useful for the partially skewed datasets obtained from Moericke traps, which may be selective for species with certain behavioral habits), Margalef's species richness index, equitability, Fisher's alpha and Berger–Parker dominance index. To compare the diversities, we employed Shannon *t* test with bias correction term (Poole 1974). Linear and Spearman correlation coefficients and their significance were calculated when indicated. χ^2 test was used to assess the differences in sex ratios and between the particular habitat types. To analyze the contribution of multiple variables, we applied a correspondence analysis. The resulting factor scores are disclosed in supplementary materials (Tables S2, S3, S4, S5, S6, S7, S8). The correspondence analysis took in account species-specific abundance and the following characteristics of each respective sampling site: altitude [m a.s.l.] (ALT), binary criterion of a presence/absence of near-natural habitat (N_/_I), reed bed area [ha] (REED_AREA), water surface area [ha] (WATER_AREA), habitat age [years] (HABITAT_AGE), relative extent of reed bed in year 2003 [%] (2003) and in 1950s [%] (1950s), relative extent of the water surface area [ha] in 1870s [%] (1870) and in 1840s [%] (1840), number of species reared from reed galls (DIV), number of red-listed species reared from reed galls (RED_LIST), abundance defined as a number of individuals reared per 100 reed galls (ABU). The descriptors of sampling sites were listed in detail in our previous publication (Heneberg et al. 2014). Particularly, the data on the presence of reed beds in the past were retrieved from aerial photographs available from the 1950s onwards, publicly available from http://www.mapy.cz (cited as 28 November 2013) and http://kontaminace.cenia.cz (cited as 28 November 2013). When considering the changes since industrial revolution, the maps created in course of military surveys in nineteenth century were used [Third Military survey initiated by Franz Joseph I. of Austria in 1876–1880, available from http://kontaminace.cenia.cz (cited as 28 November 2013), and Second Military survey initiated by Franz I. of Austria in 1836–1852, available from http://www.mapy.cz (cited as 28 November 2013)]. We used these maps to identify the position and extent of water bodies as they were superimposed over the current maps and orthophotomaps. In the figures, the species names were abbreviated to first three letters from their genus and species names (e.g., *Ischnodemus sabuleti* to Isc_sab). The conservation value of analyzed species was assessed according to the most recent versions of national red lists of spiders (Řezáč et al. 2015) and other arthropods (Farkač et al. 2005). The species included in the Czech Red List were termed as "red-listed" throughout the text, and include all species known as critically endangered (CR), endangered (EN), vulnerable (VU) or least concern [LC—this category refers to those species labeled as "near threatened" (NT) in most other Red Lists but not in that published by Řezáč et al. (2015)]; the other species were termed ecologically sustainable (ES). Together with the red-listed species, we analyzed also newly emerging (NE) species, which were identified in the Czech Republic only recently. The information on habitat specialization were retrieved from Nickel et al. (2002), Kocarek et al. (2005), Macek et al. (2010), Wachmann et al. (2004, 2006, 2007, 2008) and Nentwig et al. (2015). We used the

χ^2 test with Bonferroni correction according to MacDonald and Gardner (2000) to assess the species-specific differences in the species-specific abundance across the study habitats and in sex ratios; in addition, we used uncorrected χ^2 test to test the differences in total abundance between the two habitat types. All the above calculations were performed in PAST 2.14 (Hammer et al. 2001). Data are shown as mean ± SD unless stated otherwise.

Results

Global view on the reed gall universe

We sampled 17,791 reed galls, from which we reared 12,062 arthropod individuals. From the reared arthropods, 6031 individuals emerged from the galls collected at (post)industrial sites (67.2 individuals per 100 galls collected), and an identical amount of 6031 individuals emerged from galls collected at near-natural sites (68.4 individuals per 100 galls collected). Thus, the abundance of arthropods in reed galls at (post)industrial sites was nearly identical to that at the near-natural sites ($\chi^2 = 0.9$, $d_f = 1$, $p > 0.05$).

In total, we recorded 236 species of invertebrates emerging from *Lipara* reed galls, which included 14 species of aculeate hymenopteran inquilines (on which we focused earlier, cf. Heneberg et al. 2014), and 222 species of other invertebrates—Arachnida, Collembola and, particularly, numerous insects of the orders Dermaptera, Psocoptera, Thysanoptera, Hemiptera, Raphidioptera, Neuroptera, Coleoptera, Diptera, Lepidoptera, and Hymenoptera: Symphyta, Parasitica and Aculeata: Dryinidae. Arthropods that are not named explicitly in the above list were absent in the specimens that emerged from the collected reed galls. We attempted to identify all specimens to species, with the exception of few groups (adult Cecidomyidae, and insect larvae in general), which led to the identification of 143 species and another 94 morphospecies of arthropods. The Chao-1 species richness estimator (corrected for unseen species in the samples) indicated a species richness of 229.3 ± 18.1 species in reed galls at (post)industrial sites, and 218.1 ± 23.6 species in reed galls at near-natural sites. Despite the estimated species richness was similar to each other, Shannon diversity *t*-test suggested that the differences in diversity between the (post)industrial and near-natural sites are significant ($p < 0.001$ by bootstrapping; $t = 4.01$, $d_f = 11{,}940$), suggesting that the differences exist at the level of particular orders or lower taxonomical units. Both habitat types hosted diverse assemblages with low dominance, with significantly lower dominance identified at near-natural sites (0.098 and 0.089, respectively; $p = 0.002$ by either bootstrapping or permutation). Supporting the above, the levels of Brillouin (3.14 and 3.02, respectively) and Berger–Parker dominance indices (0.208 and 0.196, respectively) were low at both habitat types. The Margalef's species richness index (20.45 vs. 17.69; $p = 0.04$ and 0.01) and Fisher's alpha (34.66 vs. 29.02; $p = 0.03$ and 0.13) were significantly higher at post-industrial sites, suggesting that despite such habitats are less stabilized, they attract more diverse species spectrum of reed gall inquilines. Importantly, the species composition of the examined component communities overlapped only to a limited extent, with the Sørensen similarity index being equal to just 0.58. The correspondence analysis (Fig. 1) showed that the habitat age is a major environmental factor for *Lipara* gall communities as it was highly correlated with the first ordination axis which explained 63.8 % of variance in the species data. The second ordination axis was largely correlated with the habitat (reed bed) size and explained 18.8 % of variance in the species data.

We identified 18 red-listed species and four species that were new for the Czech Republic (*Gasteruption phragmiticola*, *Echthrodelphax fairchildii*, *Haplogonatopus oratorius* and *Enclisis* sp.), consisting mostly of obligate (64 %) or facultative (9 %) reed specialists. Only a few red-listed species (*Clubiona germanica*, *C. subtilis*, *Gibbaranea omoeda*,[1] *Homalura tarsata* and *Hylaeus moricei*) were confined to sites with a long-term presence of reed, whereas most of the others were found at (post)industrial habitats encompassing relatively small areas, which formed only recently[2] (Fig. 2a; Table S3).

Araneae

We collected 1254 specimens of 32 morphospecies of spiders, 19 of which were identified to species. Nine (47 %) of these species were included on the national Red List (Řezáč et al. 2015), including one species that was considered CR (*Clubiona juvenis*, found at three (post)industrial and two near-natural sites), one EN (*Mendoza canestrinii*), three VU and four LC species. Nine species (47 %) were considered reed bed specialists.

The observed species richness and abundance were nearly identical at the (post)industrial and near-natural sites. A total of 618 individuals of 24 morphospecies emerged from the galls collected at postindustrial sites (6.9 individuals per 100 galls collected), and 636 individuals of 26 morphospecies emerged from the galls collected at near-natural sites (7.2 individuals per 100 galls collected). The Chao-1 estimated species richness was lower at the (post)industrial (23.7 ± 1.1 species) when compared to near-natural sites (30.6 ± 5.3 species). The component communities were similar to each other (Sørensen similarity index 0.71). The differences of the conservation interest consisted of a higher abundance of *Clubiona juvenis* (CR) at (post)industrial sites [19 individuals at 3 (post)industrial sites vs. 3 individuals at 2 near-natural sites] and in the absence of *Mendoza canestrinii* (EN) at (post)industrial sites (0/0 vs. 6/2). The dominant species included *Clubiona phragmitis* (208/9 vs. 79/11), *Singa nitidula*[3] (44/11 vs. 17/6) and *Synageles venator* (51/11 vs. 14/4), which were all more abundant at the (post)industrial sites (Tables 1, S4; Fig. 2b). Of note was the absence of males in *Clubiona subtilis* (Fig. 3).

Heteroptera

We collected 880 specimens of 11 species of true bugs. All specimens were identified to species. No species was included on the national Red List (Farkač et al. 2005); the specimens included the first record of *Dimorphopterus spinolae* in Bohemia.[4] Only four species (36 %), including *D. spinolae*, were considered specialists for Poaceae, including the reed.

[1] *G. omoeda* is considered a species of mountain spruce forests but emerged from reed galls collected in/near an *Alnus glutinosa* forest in the Mesophyticum.

[2] These included, e.g., *Hypsosinga albovittata*, which is a xerothermic species that emerged from reed galls collected at a pine bog and peat meadows with interspersed reed stands in the Mesophyticum.

[3] Some of the dominant spider species were hitherto considered infrequent, with a very limited number of records. For *S. nitidula*, only a single record was known, e.g., for South Bohemia, from where we obtained 21 individuals from five of the seven sampling sites examined in this region.

[4] First record of *Dimorphopterus spinolae* for Bohemia: 1F: Bohdanečský fishpond, Lázně Bohdaneč, PU, 28 January 2013. However, this species is common at numerous sites in Moravia and abroad, where it mainly feeds on *Calamagrostis epigejos* (Wachmann et al. 2007). It causes large-scale damage to reed beds in China, and was even treated with insecticides to suppress its effects (Schaefer and Panizzi 2000).

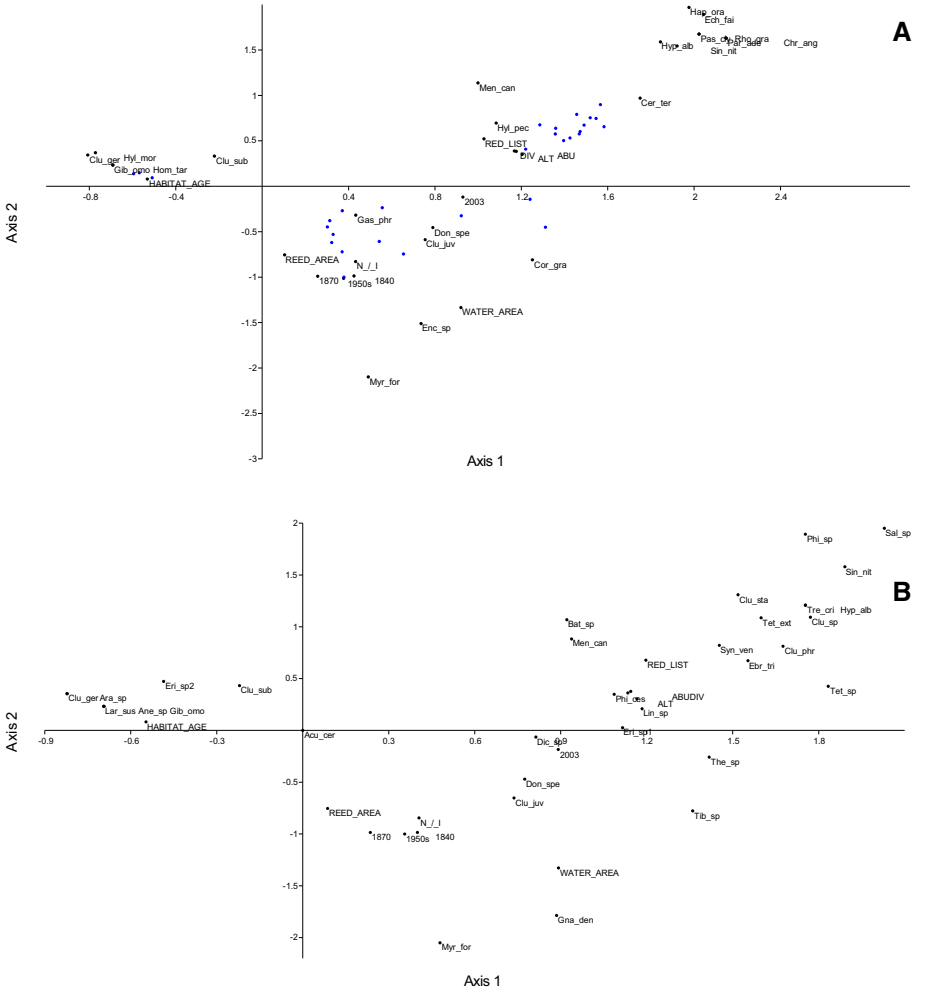

Fig. 2 Correspondence analysis (Benzecri scaling) of the red-listed species (**a**) and Araneae (**b**) superimposed in the Q mode by biotic and abiotic variables (*black dots* labeled by *acronyms*) and the sampling sites examined in the course of this study (*blue dots*). The particular species are indicated by *black dots* labeled by *acronyms*. The resulting factor scores of correspondence analyses are provided in Tables S3 and S4

The observed species richness, but not the abundance, was nearly identical at the (post)industrial and near-natural sites. The species composition differed except for the species with the highest dominance. A total of 52 individuals of 6 species emerged from the galls collected at postindustrial sites (0.6 individuals per 100 galls collected), and 828 individuals of 8 species emerged from the galls collected at near-natural sites (9.4 individuals per 100 galls collected). The Chao-1 estimated species richness differed between the (post)industrial (6.0 $\pm$ 0.2 species) and near-natural sites (13.0 $\pm$ 7.1 species). The component communities differed from each other (Sørensen similarity index 0.46). The only dominant species was *Ischnodemus sabuleti* (34/8 vs. 808/9), which was present at

Table 1 List of spiders (Araneae) reared from the *Lipara*-induced galls collected in January–March 2013 in the Czech Republic

Species	Acronyms	Red List status	Habitat specialization	Number of individuals				
				(Post)industrial sites	Near-natural sites	$p(\chi^2)$	F	M
Aculepeira ceropegia	Acu_cer	ES			1[ab]			
Anelosimus sp.	Ane_sp				1[ab]			
Araniella sp.	Ara_sp			1	2[b]			
Bathyphantes sp.	Bat_sp				1[ab]			
Clubiona germanica	Clu_ger	VU			1		1	0
Clubiona juvenis	Clu_juv	CR	R	19	3	*	13	3
Clubiona phragmitis	Clu_phr	ES	R	208	79[b]	***	41	48
Clubiona stagnatilis	Clu_sta	ES	R	2	12[b]	n.s.	5	9
Clubiona subtilis	Clu_sub	LC	R	15	3	n.s.	18	0
Clubiona sp.	Clu_sp			229	426[b]	***		
Dictyna sp.	Dic_sp			4	12	n.s.		
Donacochara speciosa	Don_spe	LC	R	7	2		3	1
Ebrechtella tricuspidata	Ebr_tri	ES		2	2			
Erigoninae gen. sp. 1	Eri_sp1			2	17[ab]	*		
Erigoninae gen. sp. 2	Eri_sp2				9[ab]			
Gibbaranea omoeda	Gib_omo	VU			1			
Gnathonarium dentatum	Gna_den	ES		1			0	1
Hypsosinga albovittata	Hyp_alb	LC			1[ab]			
Larinioides suspicax	Lar_sus	ES	R	4	2[b]		0	2
Linyphia sp.	Lin_sp			2	1[b]			
Mendoza canestrinii	Men_can	EN	fR		6		2	2
Myrmarachne formicaria	Myr_for	VU	fR	1				
Philodromus sp.	Phi_sp			9	7[b]	n.s.		

Table 1 continued

Species	Acronyms	Red List status	Habitat specialization	Number of individuals				
				(Post)industrial sites	Near-natural sites	$p(\chi^2)$	F	M
Philodromus cespitum	Phi_ces	ES		2			0	2
Salticus sp.	Sal_sp			2				
Singa nitidula	Sin_nit	LC	R	44	17	*	13	9
Synageles venator	Syn_ven	ES	R	51	14	***	17	15
Tetragnatha extenso	Tet_ext	ES	R	2				
Tetragnatha sp.	Tet_sp			6	6	n.s.		
Theridion sp.	The_sp			2	1			
Tibellus sp.	Tib_sp			1				
Trematocephalus cristatus	Tre_cri	ES		2	9[b]	n.s.		

The classification according to the national Red List (Řezáč et al. 2015), obligate (R) and facultative (fR) specialization for reed beds, number of individuals found at post-industrial and near-natural sites, and the ratio of females (F) and males (M) of the adult individuals collected are indicated. The total counts include juvenile individuals (often determined only to the genus level). The number of expected individuals was calculated based on the total number of individuals found and the number of reed galls examined at each habitat type. Species with the total capture rate <10 specimens were excluded from the χ^2 analysis

Significance of observed differences in abundance between (post-)industrial and near-natural sites compared to the expected abundance (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, n.s. = not significant) as revealed by the species-specific χ^2 tests with Bonferroni correction at n = 64

[a] Species found at reed beds alongside rivers, but not at other near-natural habitats

[b] Species more abundant at reed beds alongside rivers compared to reed beds near fish ponds

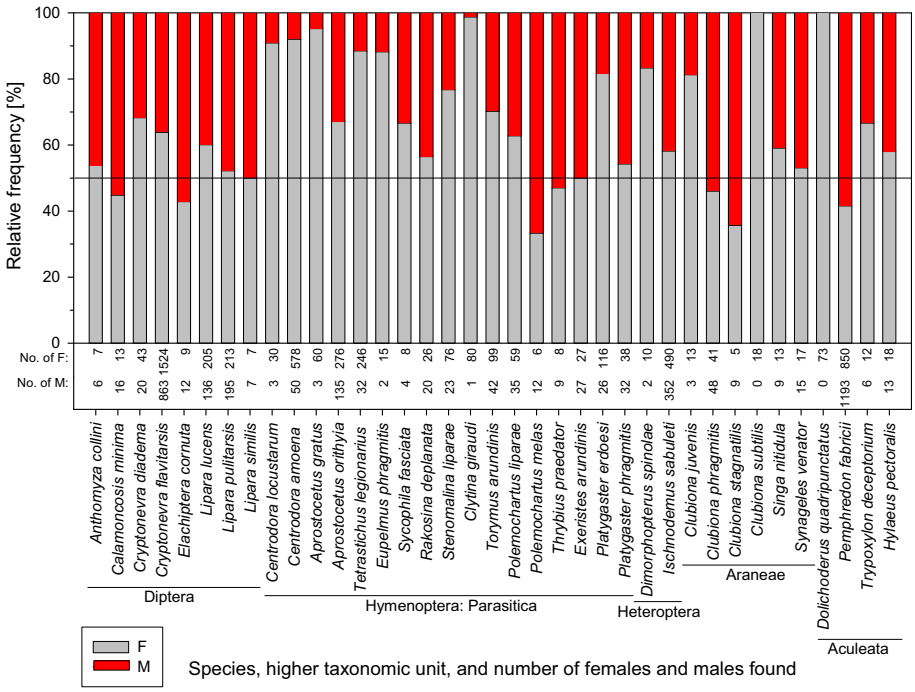

Fig. 3 Sex ratio in species, of which ≥10 individuals emerged from the *Lipara*-induced reed galls collected during the course of this study. Relative and absolute frequencies of males and females are shown

both types of sites, but much more abundant at the near-natural sites. *Dimorphopterus spinolae* (0/0 vs. 12/2) was present only at near-natural sites (Tables 2, S5; Fig. 4a).

Auchenorrhyncha

We collected 113 specimens of 6 morphospecies of planthoppers and leafhoppers, 3 of which were identified to species. One of them (*Paraliburnia adela*) was included on the national Red List as VU species (Farkač et al. 2005) (1/1 vs. 0/0). The observed species richness was identical at the (post)industrial and near-natural sites despite the abundance was higher at (post)industrial sites, and the species composition differed between the two types of sampling sites. In sum 87 individuals of 4 morphospecies emerged from galls collected at postindustrial sites (1.0 individuals per 100 galls collected), and 26 individuals of 4 morphospecies emerged from galls collected at near-natural sites (0.3 individuals per 100 galls collected). Dominant morphospecies included only the nymphs of reed specialist *Chloriona* sp. (82/7 vs. 14/6) present at both types of sites, but more abundant at the near-natural ones (Table 2).

Sternorrhyncha

We collected two specimens of two species of jumping plant lice, identified as *Trioza urticae* and *Cacopsylla saliceti/pulchra* (Table 2). Both emerged from galls collected at

Table 2 List of true bugs (Heteroptera), planthoppers and leafhoppers (Auchenorrhyncha) and jumping plant lice (Sternorrhyncha) reared from the *Lipara*-induced galls collected in January–March 2013 in the Czech Republic

Species	Acronyms	Red List status	Habitat specialization	Number of individuals				
				(Post)industrial sites	Near-natural sites	$p(\chi^2)$	F	M
True bugs (Heteroptera)								
Anthocoris nemoralis	Ant_nem	ES	U		3[ab]		2	1
Dimorphopterus spinolae	Dim_spi	ES	P		12	*	10	2
Gastrodes abietum	Gas_abi	ES	*Picea*	6	1[ab]		7	0
Gastrodes grossipes grossipes	Gas_gro	ES	*Pinus*	3			3	0
Ischnodemus sabuleti	Isc_sab	ES	P	34	808[b]	***	490	352
Leptopterna dolabrata	Lep_dol	ES	P	1			1	0
Lygus pratensis	Lyg_pra	ES	U	6			4	2
Orius (*Orius*) *niger*	Ori_nig	ES	U	2	1[ab]		1	2
Notostira erratica	Not_err	ES	P		1[ab]		1	0
Orthops (*Orthops*) *campestris*	Ort_cam	ES	Apiaceae		1[ab]		1	0
Orthops (*Orthops*) *kalmii*	Ort_kal	ES	Apiaceae		1		1	0
Planthoppers and leafhoppers (Auchenorrhyncha)								
Chloriona sp.	Chl_sp	ES	R	82	14	***		
Idiocerus herrichii	Idi_her	ES	*Salix*	3	2		1	4
Deltocephalinae gen. sp.	Del_sp	ES	P	1				
Paraliburnia cf. *adela*	Par_ade	VU	*Phalaris*	1				
Javesella sp.	Jav_sp	ES	P		1[ab]			

Table 2 continued

Species	Acronyms	Red List status	Habitat specialization	Number of individuals				
				(Post)industrial sites	Near-natural sites	$p(\chi^2)$	F	M
Stenocranus major	Ste_maj	ES	*Phalaris*		9[ab]		4	5
Jumping plant lice (Sternorrhyncha)								
Trioza urticae	Tri_urt	ES	*Urtica*	1			0	1
Cacopsylla saliceti/pulchra	Cac_sal	ES	*Salix*	1			1	0

The classification according to the national Red List (Farkač et al. 2005), habitat specialization: specialization for reed beds (R), Poaceae including reed (P), ubiquitous and polyphagous species (U) and other host plants, number of individuals found at post-industrial and near-natural sites, and the ratio of females (F) and males (M) of the adult individuals collected are indicated. The number of expected individuals was calculated based on the total number of individuals found and the number of reed galls examined at each habitat type. Species with the total capture rate <10 specimens were excluded from the χ^2 analysis

Significance of observed differences in abundance between (post-)industrial and near-natural sites compared to the expected abundance (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, n.s. = not significant) as revealed by the species-specific χ^2 tests with Bonferroni correction at n = 64

[a] Species found at reed beds alongside rivers, but not at other near-natural habitats

[b] Species more abundant at reed beds alongside rivers compared to reed beds near fish ponds

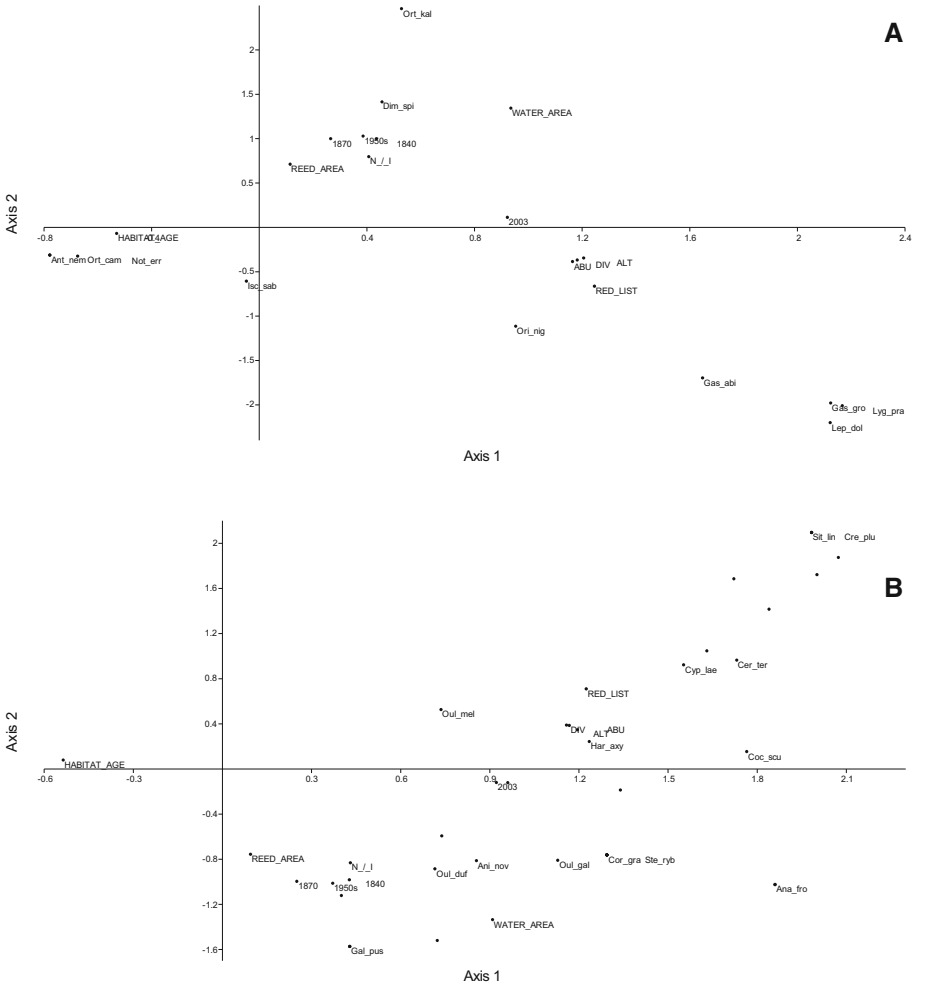

Fig. 4 Correspondence analysis (Benzecri scaling) of Heteroptera (**a**) and Coleoptera (**b**) superimposed in the Q mode by biotic and abiotic variables (*black dots* labeled by *acronyms*) and the sampling sites examined in the course of this study (*blue dots*). The particular species are indicated by *black dots* labeled by *acronyms*. The resulting factor scores of correspondence analyses are provided in Tables S5 and S6

(post)industrial sites, and are recognized as ES species (Farkač et al. 2005) feeding on *Urtica* spp. and *Salix* spp., respectively.

Lepidoptera

We collected four specimens of four morphospecies of moths, three of which were identified to species and all of which were recognized as ES species according to the national Red List (Farkač et al. 2005). Two specimens emerged from the galls collected at (post)industrial sites (*Brachmia inornatella* and *Boudinotiana notha*), and two specimens emerged from the galls collected at near-natural sites (*Ethmia quadrillella* and *Eupithecia*

Table 3 List of beetles (Coleoptera) and moths (Lepidoptera) reared from the *Lipara*-induced galls collected in January–March 2013 in the Czech Republic

Species	Acronyms	Red List status	Habitat specialization	Number of individuals				
				(Post)industrial sites	Near-natural sites	$p(\chi^2)$	F	M
Beetles (Coleoptera)								
Adalia (*Adalia*) *bipunctata*	Ada_bip	ES	U		1			
Anaspis (*Anaspis*) *frontalis*	Ana_fro	ES	N	3			1	2
Anisosticta novemdecimpunctata	Ani_nov	ES	fR	1	3			
Anthonomus (*Furcipus*) *rectirostris*	Ant_rec	ES	N/Rosaceae	1				
Attagenus (*Attagenus*) *pellio*	Att_pel	ES	U	1				
Cardiophorus sp.	Car_sp			1				
Cerapheles terminatus	Cer_ter	VU	fR	4	1[ab]		2	3
Chaetocnema sp.	Cha_sp			1			1	0
Coccidula scutellata	Coc_scu	ES	fR	61	30	n.s.		
Coccinella (*Coccinella*) *quinquepunctata*	Coc_qui	ES	U	1				
Cordicollis gracilis	Cor_gra	VU	fR		2			
Crepidodera plutus	Cre_plu	ES	N/*Salix*	2			0	2
Cyphon laevipennis	Cyp_lae	ES	R	28	13	n.s.		
Dasytes (*Mesodasytes*) *plumbeus*	Das_plu	ES	N/wood		1[ab]		0	1
Demetrias (*Aetophorus*) *imperialis*	Dem_imp	ES	R		1[ab]			
Demetrias (*Demetrias*) *monostigma*	Dem_mon	ES	R	1				
Galerucella (*Neogalerucella*) *pusilla*	Gal_pus	ES	N/*Lythrum*		2			
Harmonia axyridis	Har_axy	ES	U	1	1[ab]			
Isochnus sequensi	Iso_seq	ES	N/Salicaceae		1			
Lathridiidae: Corticariinae gen. sp.					1			

Table 3 continued

Species	Acronyms	Red List status	Habitat specialization	Number of individuals				
				(Post)industrial sites	Near-natural sites	$p(\chi^2)$	F	M
Nedyus quadrimaculatus	Ned_qua	ES	N/*Urtica*		1			
Odacantha (*Odacantha*) *melanura*	Oda_mel	ES	fR		1			
Orchestes (*Alyctus*) *testaceus*	Orc_tes	ES	N/*Alnus*	1				
Oulema duftschmidi	Oul_duf	ES	P	1	4	n.s.	N/D	4
Oulema duftschmidi / *melanopus*		ES	P	27	70	*	97	1
Oulema gallaeciana	Oul_gal	ES	P	1	2[b]		N/D	3
Oulema melanopus	Oul_mel	ES	P	20	27[ab]		N/D	47
Paradromius (*Manodromius*) *linearis*	Par_lin	ES	fR	1				
Paradromius (*Paradromius*) *longiceps*	Par_lon	ES	R		1			
Phalacridae gen. sp.	Pha_sp			1	2			
Phyllotreta vittula	Phy_vit	ES	P		1		1	0
Pria dulcamarae	Pri_dul	ES	N/*Solanum*		1		1	0
Rhinusa sp.	Rhi_sp				1			
Sitona lineatus	Sit_lin	ES	N/Viciaceae	1			0	1
Stephostethus rybinskii	Ste_ryb	ES	fR		1			
Coleoptera: larvae				73	158[b]	***		
Moths (Lepidoptera)								
Ethmia quadrillella	Eth_qua	ES	N/Boraginaceae	0	1		1	0
Brachmia inornatella	Bra_ino	ES	R	1	0		0	1
Boudinotiana notha	Bou_not	ES	N/Salicaceae	1	0		1	0
Eupithecia sp.	Eup_sp	ES		0	1[ab]		1	0

Table 3 continued

Species	Acronyms	Red List status	Habitat specialization	Number of individuals				
				(Post)industrial sites	Near-natural sites	$p(\chi^2)$	F	M
Lepidoptera-larvae				14	8[b]	n.s.		

The classification according to the national Red List (Farkač et al. 2005), habitat specialization: obligate (R) and facultative (fR) specialization for reed beds, Poaceae including reed beds (P), ubiquitous species (U), and species, which occur on other plant species only (N), number of individuals found at post-industrial and near-natural sites, and the ratio of females (F) and males (M) of the adult individuals collected are indicated. The number of expected individuals was calculated based on the total number of individuals found and the number of reed galls examined at each habitat type. Species with the total capture rate <10 specimens were excluded from the χ^2 analysis

Significance of observed differences in abundance between (post-)industrial and near-natural sites compared to the expected abundance (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, n.s. = not significant) as revealed by the species-specific χ^2 tests with Bonferroni correction at $n = 64$

[a] Species found at reed beds alongside rivers, but not at other near-natural habitats

[b] Species more abundant at reed beds alongside rivers compared to reed beds near fish ponds

sp.) (Table 3). *Ethmia quadrillella* represents an uncommon species of mesophilous sites and wetlands, developing on roots of Boraginaceae. *Brachmia inornatella* represents a species rare in Central Europe, developing in stems of *Phragmites australis*, and is considered the only reed bed specialist among the Lepidoptera, which emerged from the reed galls during the course of this study.

Coleoptera

We collected 328 specimens of 34 morphospecies of beetles, of which 29 were identified to species. Only two species were included on the national Red List (Farkač et al. 2005); they were classified as VU (*Cerapheles terminatus*, 4/2 vs. 1/1, and *Cordicollis gracilis*, 0/0 vs. 2/1). In total 15 species (36 %) were considered specialists for Poaceae, including the reed beds, and four species (14 %) were considered ubiquitous saprophages and aphidophages. For 10 species (34 %), there was no prior evidence on their overwintering and/or development in reed galls (*Dasytes plumbeus*, *Pria dulcamarae*, *Anaspis frontalis*, *Galerucella pusilla*, *Crepidodera plutus*, *Anthonomus rectirostris*, *Isochnus sequensi*, *Orchestes testaceus*, *Nedyus quadrimaculatus* and *Sitona lineatus*).

The observed species richness and abundance were nearly identical at the (post)industrial and near-natural sites. The species composition was similar, except that the species found at low frequency were randomly distributed between the (post)industrial and near-natural sites. Nevertheless, all of the dominant species were found at both types of sampling sites. A total of 159 individuals of 20 morphospecies emerged from the galls collected at postindustrial sites (1.8 individuals per 100 galls collected), and 169 individuals of 23 morphospecies emerged from the galls collected at near-natural sites (1.9 individuals per 100 galls collected). Most of the species were captured in low numbers, which caused that the Chao-1 estimated species richness was high in both analyzed habitats but was associated with a high degree of uncertainty, reaching 66.2 ± 34.4 species at the (post)industrial sites and 38.5 ± 11.6 species at near-natural sites. The component communities differed from each other (Sørensen similarity index 0.43). The dominant species included *Oulema melanopus* (males: 20/8 vs. 27/7),[5] *Coccidula scutellata* (61/6 vs. 30/6) and *Cyphon laevipennis* (28/4 vs. 13/4). All of these species were present at both types of sites, but the latter two species were more abundant at the post-industrial sites (Tables 3, S6; Fig. 4b).

Hymenoptera: Symphyta

We collected 15 specimens of seven morphospecies of sawflies, six of which were identified to species; all recognized as ES species according to the national Red List (Farkač et al. 2005). Four morphospecies emerged from galls collected at (post)industrial sites (*Pontania brevicornis*, *Ametastegia glabrata*, *Cladius brullei* and *Pontania* sp.), and four emerged from galls collected at near-natural sites (*Euura gemmacinerae*, *Amauronematus viduatus*, *Ametastegia glabrata*, *Brachythops flavens*) (Table 4). All the species represented ubiquitous species, for which common reed did not serve as a host plant, the reed galls were used only to pupate. The only dominant species was *Ametastegia glabrata* (males: 2/1 vs. 5/3).

[5] Males of *O. melanopus* were approximately 10× more abundant than males of *O. duftschmidi*. Females of these two species were 1.9× more abundant than males at both types of sampling sites (27/10 vs. 70/6 of females) but were indistinguishable from each other.

Table 4 List of Hymenoptera: Parasitica, Symphyta and Aculeata (Dryinidae) reared from the Lipara-induced galls collected in January–March 2013 in the Czech Republic

Species	Acronyms	Red List status	Habitat specialization	Number of individuals				
				(Post)industrial sites	Near-natural sites	$p(\chi^2)$	F	M
Parasitica: Ceraphronoidea								
Ceraphronidae gen. sp. 1	Cer_sp				1[ab]		2	1
Ceraphronidae gen. sp. 2				2				
Dendrocerus serricornis	Den_ser	ES	U	1				
Parasitica: Chalcidoidea								
Aphelinus gen. sp. 1				1			0	1
Aphytis gen. sp. 1				1			1	0
Centrodora cf. *acridiphagus*	Cen_acr	ES	N/D	17	42[b]	n.s.	59	0
Centrodora amoena	Cen_amo	ES	fR	325	296[b]	n.s.	578	50
Centrodora locustarum	Cen_loc	ES	fR	1	32[ab]	***	30	3
Centrodora sp. 1		ES		3			2	1
Centrodora sp. 2		ES		3			2	1
Centrodora sp. 3		ES			7[ab]		7	0
Boucekiella sp. 1	Bou_sp			23		***	12	11
Encyrtidae gen. 1 sp. 1				1		***	1	0
Encyrtidae gen. 2 sp. 1				3			2	1
Encyrtidae gen. 3 sp. 1					141		92	49
Encyrtidae gen. 4 sp. 1				1			0	1
Encyrtidae gen. 5 sp. 1					4		2	2
Encyrtidae gen. 6 sp. 1				1			0	1
Boucekiella depressa	Bou_dep	ES	R		2		2	0
Aprostocetus gratus	Apr_gra	ES	R	18	14	n.s.	60	3
Aprostocetus orithyia	Apr_ori	ES	R	220	190[b]	n.s.	276	135
Aprostocetus sp. 1				7	2		7	2
Eulophidae gen. 1 sp. 1				3	15[b]	n.s.	6	12

Table 4 continued

Species	Acronyms	Red List status	Habitat specialization	Number of individuals				
				(Post)industrial sites	Near-natural sites	$p(\chi^2)$	F	M
Eulophinae gen. 2 sp. 1				1	2		1	2
Eulophinae gen. 3 sp. 1				1			1	0
Melittobia sp. 1				33	12[ab]	n.s.	45	0
Melittobia sp. 2				3	8[b]	n.s.	11	0
Pediobius sp. 1				4	47[b]	***	46	7
Pediobius sp. 2				1	1[ab]		2	0
Pediobius sp. 3				3			3	0
Pronotalia sp. 1				27	32	n.s.	54	5
Tetrastichinae gen. 1 sp. 1				2	4		1	5
Tetrastichinae gen. 1 sp. 2				3	9	n.s.	3	9
Tetrastichinae gen. 2 sp. 1				29		***	26	3
Tetrastichinae gen. 3 sp. 1				9	8	n.s.	16	1
Tetrastichinae gen. 4 sp. 1					5[b]		4	1
Tetrastichinae gen. 5 sp. 1				3			3	0
Tetrastichinae gen. 6 sp. 1				1	6		7	0
Tetrastichinae gen. 7 sp. 1				6	3[b]		9	0
Tetrastichinae gen. 8 sp. 1				1			1	0
Tetrastichus legionarius	Tet_leg	ES	R	172	106	**	246	32
Tetrastichus sp.				2			2	0
Eupelmus (sg. *Macroneura*) sp.					1[ab]			
Eupelmus phragmitis	Eup_phr	ES	R	16	1	*	15	2
Eurytoma sp. 1				2	6[b]		4	4
Eurytoma sp. 2				1	5		5	1
Eurytoma sp. 3				1			1	0

Table 4 continued

Species	Acronyms	Red List status	Habitat specialization	Number of individuals				
				(Post)industrial sites	Near-natural sites	$p(\chi^2)$	F	M
Tetramesa phragmitis	Tet_phr	ES	R	4	3[b]		6	1
Sycophila fasciata	Syc_fas	ES	R	9	3	n.s.	8	4
Anagrus sp.				26	2[b]	***	17	10
Asaphes suspensus	Asa_sus	ES	U	3			0	3
Callitula elongata	Cal_elo	ES	R	4	2[ab]		6	0
Gyrinophagus sp. 1				24	11[b]	n.s.	35	0
Gyrinophagus sp. 2				7			6	1
Pachyneuron sp.				2			1	1
Rakosina deplanata	Rak_dep	ES	R	31	15	n.s.	26	20
Stenomalina liparae	Ste_lip	ES	R	65	32[b]	n.s.	76	23
Clytina giraudi	Cly_gir	ES	R	47	34[b]	n.s.	80	1
Torymus arundinis	Tor_aru	ES	R	62	79[b]	n.s.	99	42
Chalcidoidea gen. sp.				4				
Platygastroidea / Chalcidoidea gen. sp.				1				
Parasitica: Cynipoidea								
Alloxysta fulviceps	All_ful	ES	fR/*Praon*	2				
Rhoptromeris sp.				1	2			
Parasitica: Evanioidea								
Gasteruption phragmiticola	Gas_phr	NE	R	1	2[ab]		3	0
Parasitica: Ichneumonoidea								
Braconidae gen. sp.				2	1		1	2
Polemochartus liparae	Pol_lip	ES	R	58	36[b]	n.s.	59	35
Polemochartus melas	Pol_mel	ES	R	20	2	n.s.	6	12

Table 4 continued

Species	Acronyms	Red List status	Habitat specialization	Number of individuals				
				(Post)industrial sites	Near-natural sites	$p(\chi^2)$	F	M
Diaeretus sp.				1	3^{ab}			
Praon sp.				2				
Bracon sp.					3^{ab}			
Ichneumonidae gen. sp.				23		***		
Cryptinae gen. sp.				2	1		2	1
Gambrus ornatus	Gam_orn	ES	P		3^{b}		2	1
Hoplocryptus centricolor	Hop_cen	ES	R	1			1	0
Hoplocryptus sp.				1			0	1
Thrybius praedator	Thr_pra	ES	R	9	8^{b}	n.s.	8	9
Ctenopelmatinae gen. sp.					1^{ab}		1	0
Endromopoda detrita	End_det	ES	P	4	3^{b}		7	0
Ephialtes sp.				2			1	1
Exeristes arundinis	Exe_aru	ES	R	45	8	***	27	27
Pimplinae gen. sp.				1			1	0
Polysphincta rufipes	Pol_ruf	ES	U	2			2	1
Zatypota percontatoria	Zat_per	ES	U		2		1	1
Enclisis sp.	Enc_sp	NE			1		0	1
Parasitica: Platygastroidea								
Inostemma sp.				4	3^{b}		4	3
Platygaster erdoesi	Pla_erd	ES	U/*Cassida*	33	109^{b}	***	116	26
Platygaster phragmitis	Pla_phr	ES	R	38	31^{b}	n.s.	38	32
Scelio sp. 1					1		1	0
Scelionidae gen. 1 sp. 1				2	5		6	1
Scelionidae gen. 1 sp. 2				2			0	2

Table 4 continued

Species	Acronyms	Red List status	Habitat specialization	Number of individuals				
				(Post)industrial sites	Near-natural sites	$p(\chi^2)$	F	M
Scelionidae gen. 1 sp. 3				11	1	n.s.	10	2
Scelionidae gen. 2 sp. 1				2			2	0
Scelionidae gen. 3 sp. 1					4		3	1
Parasitica: Proctotrupoidea								
Trichopria nigra	Tri_nig	ES	U		1			
Symphyta								
Amauronematus viduatus	Ama_vid	ES	U		1		1	0
Ametastegia glabrata	Ame_gla	ES	U	2	5[b]		4	3
Brachythops flavens	Bra_fla	ES	U		3		2	1
Cladius brullei	Cla_bru	ES	U	1			1	0
Euura gemmacinerae	Euu_gem	ES	U		1[b]			
Pontania brevicornis	Pon_bre	ES	U	1				
Pontania sp.			U	1			1	0
Aculeata: Chrysidoidea, Dryinidae								
Echthrodelphax fairchildii	Ech_fai	NE	P	5				
Gonatopus clavipes	Gon_cla	ES	P	1				
Gonatopus distinctus	Gon_dis	ES	P	1				
Haplogonatopus oratorius	Hap_ora	NE	P	1				

The classification according to the national Red List (Farkač et al. 2005), habitat specialization: obligate (R) and facultative (fR) specialization for reed beds, Poaceae including reed beds (P), and ubiquitous species (U) or hosted by such specialists, number of individuals found at post-industrial and near-natural sites, and the ratio of females (F) and males (M) of the adult individuals collected are indicated. The number of expected individuals was calculated based on the total number of individuals found and the number of reed galls examined at each habitat type. Species with the total capture rate <10 specimens were excluded from the χ^2 analysis

Significance of observed differences in abundance between (post-)industrial and near-natural sites compared to the expected abundance (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, n.s. = not significant) as revealed by the species-specific χ^2 tests with Bonferroni correction at $n = 64$

[a] Species found at reed beds alongside rivers, but not at other near-natural habitats

[b] Species more abundant at reed beds alongside rivers compared to reed beds near fish ponds

Hymenoptera: Parasitica and Aculeata (Dryinidae)

We collected 2938 specimens of 99 morphospecies of parasitic hymenopterans. Of them, 36 morphospecies were identified to species. The particular superfamilies identified included Ceraphronoidea (3 morphospecies), Chalcidoidea (59 morphospecies), Cynipoidea (2 morphospecies), Evanioidea (a single species), Ichneumonoidea (20 morphospecies), Platygastroidea (9 morphospecies), Proctotrupoidea (a single species) and Chrysidoidea (4 species). The national Red List of parasitic hymenopterans is very short in extent because of uncertainties due to limited or aged information available. No species found were included in the national Red List (Farkač et al. 2005), but at least four were considered rare in the study area (*Eupelmus phragmitis*, *Tetramesa phragmitis*, *Rakosina deplanata* and *Callitula elongata*), and another four species were new for the Czech Republic.[6] Altogether 28 species (67 %) were considered specialists for Poaceae, being frequently strictly confined to the reed beds and reed galls induced by the *Lipara* flies. The remaining 33 % of species were considered ubiquitous. There were no species considered specialists for other habitats.

The observed (but not estimated) species richness and abundance were similar at the (post)industrial and near-natural sites. The species composition differed, except for the species with the highest dominance. A total of 1524 individuals of 84 morphospecies emerged from the galls collected at postindustrial sites (17.0 individuals per 100 galls collected), and 1414 individuals of 61 morphospecies emerged from the galls collected at near-natural sites (16.0 individuals per 100 galls collected). The Chao-1 estimated species richness differed between the (post)industrial (99.9 ± 9.3 species) and near-natural sites (66.5 ± 4.5 species). The component communities were relatively similar to each other (Sørensen similarity index 0.60). The dominant species were *Centrodora amoena*[7] (325/14 vs. 296/12), *Aprostocetus orithyia*[8] (220/8 vs. 190/8), *Tetrastichus legionarius* (172/3 vs. 106/9), *Platygaster erdoesi* (33/5 vs. 109/8), *Torymus arundinis* (62/7 vs. 79/8) and an unidentified species of Encyrtidae gen. sp. found at near-natural sites in the Pannonian part of Moravia only (0/0 vs. 141/2). Several unidentified morphospecies, and *Gambrus ornatus* (0/0 vs. 3/3), *Centrodora locustarum* (1/1 vs. 32/2) and *Platygaster erdoesi* (33/5 vs. 109/8) were present prevalently at near-natural sites. Several unidentified morphospecies,

[6] The new species for the Czech Republic included *Gasteruption phragmiticola* (1 ex.: fishpond Baroch, Hrobice, PU, 28 January 2013, 1 ex.: Knovízský stream, Olovnice, ME, 16 February 2013, 1 ex.: disused ash/slag deposit of the lignite power station Triangl, Olešník, CB, 16 March 2013), *Enclisis* sp. (1 ex.: fishpond Proudnice, Žíželice-Hradištko, KO, 25 January 2013), *Haplogonatopus oratorius* (1 ex.: sandpit Dobříň, LT, 2 February 2013) and *Echthrodelphax fairchildii* (1 ex.: sandpit Dobříň, LT, 2 February 2013, 2 ex.: spoil heap Mariánské Radčice, MO, 3 February 2013, 1 ex.: spoil heap Pokrok, Duchcov, TE, 3 February 2013, 1 ex.: gravel-sandpit Vojkovice, ME, 17 February 2013).

[7] Nartshuk (2006) questioned the association of *Centrodora amoena* (Aphelinidae) with their *Lipara* hosts proposed by Fulmek (1968), with Orthoptera serving as the only confirmed hosts. In our material from *Lipara*-induced galls, *Centrodora amoena* was a dominant species (628 individuals emerged), with Orthoptera completely absent in the examined dataset. Therefore, it is likely that the initial observation by Fulmek was correct.

[8] *Aprostocetus orithyia* and *A. gratus* were reported as specialized parasites of *Giraudiella inclusa* by Tscharntke et al. (1991), who also questioned the previous record of *A. orithyia* association with *Lipara lucens* (Graham 1987) and questioned all of the other host records of *A. gratus* (which was never associated with *Lipara* flies or any other dipterans reported in our study). In particular, *A. orithyia* was a dominant species in our dataset (410 individuals emerged). Although our materials contained hundreds of potential cecidomyid hosts, it is important to note that all of this material originated from the microhabitat (galls) that was induced exclusively by *Lipara* flies. Therefore, Graham was probably correct when reporting it from *Lipara* galls, but it remains to be tested whether the *Lipara* spp. themselves can host these two species.

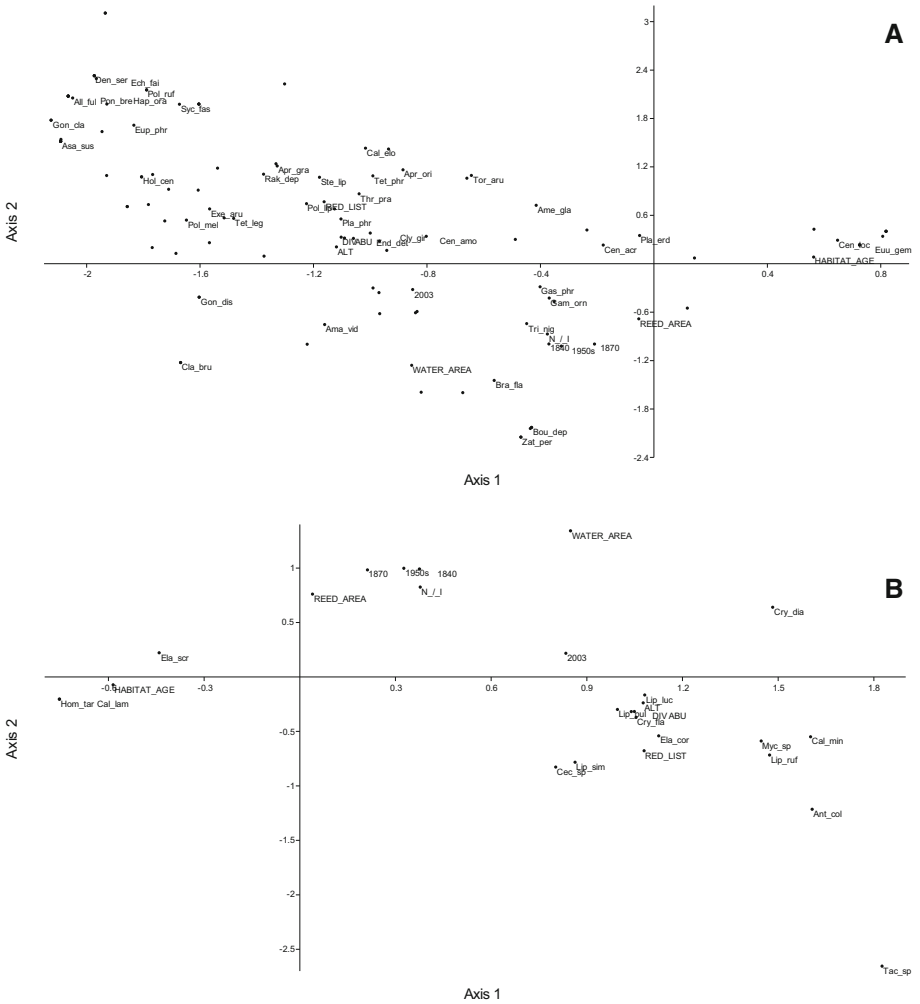

Fig. 5 Correspondence analysis (Benzecri scaling) of Hymenoptera: Parasitica and Aculeata (Dryinidae) (**a**) and Diptera (**b**) superimposed in the Q mode by biotic and abiotic variables (*black dots* labeled by *acronyms*) and the sampling sites examined in the course of this study (*blue dots*). The particular species are indicated by *black dots* labeled by *acronyms*. The resulting factor scores of correspondence analyses are provided in Tables S7 and S8

and *Eupelmus phragmitis* (16/2 vs. 1/1), *Polemochartus melas* (16/5 vs. 2/2) and *Exeristes arundinis* (45/8 vs. 8/3) were present prevalently at (post)industrial sites (Tables 4, S7; Fig. 5a). Besides *Lipara* spp. and other dipterans, the putative host spectrum of parasitic hymenopterans found included lepidopterans (*Gambrus ornatus*), other hymenopterans (*Holocryptus centricolor*, *Thrybius praedator*, *Endromopoda detrita*), spiders (*Polysphincta rufipes*, *Zatypota percontatoria*), and leafhoppers and planthoppers (*Gonatopus clavipes*, *G. distinctus*). Numerous species were skewed towards females; among them were *Clytina giraudi* (99 %), *Aprostocetus gratus* (95 %), *Centrodora amoena* (92 %), *Centrodora locustarum* (91 %), *Tetrastichus legionarius* (88 %) and *Eupelmus phragmitis* (88 %) (Fig. 3).

Diptera

We collected 4021 specimens of 15 morphospecies of Diptera. Only the species of Chloropidae (11 species) and Anthomyzidae (1 species) were identified to species. Cecidomyiidae (729 ex.), Mycetophilidae (3 ex.) and Tachinidae (5 ex.) were not identified to species. The national Red List of Chloropidae is very short in extent because of uncertainties due to limited or aged information available. Only a single species, *Homalura tarsata*, was included on the national Red List (Farkač et al. 2005). The dipterans found were represented by reed gall-inducing species of the genus *Lipara* (four species), obligate reed gall specialists (three species: *Calamoncosis minima*, *Cryptonevra diadema* and *Cryptonevra flavitarsis*), two facultative reed gall inquilines (*Anthomyza collini* and *Calamoncosis laminiformis*), two facultative reed herbivores (*Elachiptera cornuta* and *Elachiptera scrobiculata*), and a species associated with reed beds, but without sufficient data to classify its feeding and nesting strategy (*Homalura tarsata*). There were no ubiquitous species, and no species were considered specialists for other habitats.

The observed species richness, abundance and composition were similar at the (post)industrial and near-natural sites. A total of 2055 individuals of 12 morphospecies emerged from the galls collected at postindustrial sites (22.9 individuals per 100 galls collected), and 1966 individuals of 14 morphospecies emerged from the galls collected at near-natural sites (22.3 individuals per 100 galls collected). The Chao-1 estimated species richness was lower at the (post)industrial (11.0 $\pm$ 0.4 species) compared to near-natural sites (17.0 $\pm$ 4.2 species). The component communities were similar to each other (Sørensen similarity index 0.88). *Cryptonevra flavitarsis* was recognized as a highly dominant species and was the only invertebrate species found at all sampling sites in course of this study (1203/15 vs. 1180/15). Several species were more prevalent at the (post)industrial sites, including *Anthomyza collini* (11/6 vs. 2/1), *Calamoncosis minima* (24/6 vs. 5/1) and *Cryptonevra diadema* (57/8 vs. 6/1).

We non-selectively collected both the 0.5 year-old galls and the older galls when present at the sampling site; therefore we were able to evaluate the species composition of gall-inducing *Lipara* flies. The most common species were *L. pullitarsis* (181/13 vs. 227/15) and *L. lucens* (185/15 vs. 156/14), whereas less common species were *L. similis* (9/4 vs. 5/3, present in Bohemia only) and *L. rufitarsis* (4/2 vs. 1/1, present in both the Moravian and Bohemian thermophyticum) (Tables 5, S8; Fig. 5b). Consistent with the ecological characteristics of *Lipara* flies, *L. rufitarsis* was associated with the most recently emerged reed beds, whereas *L. similis* was associated with well-established habitats, which were usually more eutrophicated and thus allowed the growth of higher and thicker reed stands. We found skewed sex ratios in multiple dipteran species; the populations of *Cryptonevra diadema* and *C. flavitarsis* were both skewed towards the females (68 and 64 %, respectively). Similarly, the reared *Lipara lucens* were slightly skewed towards females (60 %), but the sex ratio of *L. pullitarsis* and *L. similis* was equal (Fig. 3).

Other taxa and larvae

The emergence traps contained also the following taxa, which originated from the collected reed galls: Pseudoscorpiones: *Chelifer cancroides* (0/0 vs. 1/1), Neuroptera: *Semidalis aleurodiformis* (4/2 vs. 0/0), Raphidioptera: *Raphidia notata* (0/0 vs. 1/1),

Table 5 List of flies and midges (Diptera) reared from the Lipara-induced galls collected in January–March 2013 in the Czech Republic

Species	Acronyms	Red List status	Habitat specialization	Number of individuals				
				(Post)industrial sites	Near-natural sites	$p(\chi^2)$	F	M
Anthomyza collini	Ant_col	ES	R	11	2	n.s.	7	6
Calamoncosis laminiformis	Cal_lam	ES	P		1[ab]		0	1
Calamoncosis minima	Cal_min	ES	R	24	5	*	13	16
Cecidomyiidae gen. sp.	Cec_sp			360	369[b]	n.s.	484	245
Cryptonevra diadema	Cry_dia	ES	R	57	6[ab]	***	43	20
Cryptonevra flavitarsis	Cry_fla	ES	R	1203	1180	n.s.	1524	863
Elachiptera cornuta	Ela_cor	ES	P	14	7[b]	n.s.	9	12
Elachiptera scrobiculata	Ela_scr	ES	P		5[b]		2	3
Homalura tarsata	Hom_tar	EN	R		1[ab]		1	0
Lipara lucens	Lip_luc	ES	R	185	156	n.s.	205	136
Lipara pullitarsis	Lip_pul	ES	R	181	227[b]	n.s.	213	195
Lipara rufitarsis	Lip_ruf	ES	R	4	1		5	5
Lipara similis	Lip_sim	ES	R	9	5[b]	n.s.	7	7
Mycetophilidae gen. sp.				2	1		3	0
Tachinidae gen. sp.				5			5	0

The classification according to the national Red List (Farkač et al. 2005), habitat specialization: obligate (R) and facultative (fR) specialization for reed beds, Poaceae including reed beds (P), ubiquitous species (U), and species, which occur on other plant species only (N), number of individuals found at post-industrial and near-natural sites, and the ratio of females (F) and males (M) of the adult individuals collected are indicated. The number of expected individuals was calculated based on the total number of individuals found and the number of reed galls examined at each habitat type. Species with the total capture rate <10 specimens were excluded from the χ^2 analysis

Significance of observed differences in abundance between (post-)industrial and near-natural sites compared to the expected abundance (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, n.s. = not significant) as revealed by the species-specific χ^2 tests with Bonferroni correction at $n = 64$

[a] Species found at reed beds alongside rivers, but not at other near-natural habitats

[b] Species more abundant at reed beds alongside rivers compared to reed beds near fish ponds

Dermaptera: *Forficula auricularia* (0/0 vs. 2/2), Collembola (35/8 vs. 18/5), Thysanoptera (3/2 vs. 1/1), and Psocoptera (0/0 vs. 1/1). Additionally, the emergence traps contained larvae of Coleoptera (73/10 vs. 158/12), Lepidoptera (14/5 vs. 8/3), Diptera (2/2 vs. 9/2) and Hymenoptera (1/1 vs. 0/0).

Discussion

The species composition of arthropod assemblages associated with reed galls strongly differed between the near-natural and (post)industrial sites, and both habitats hosted very diverse assemblages of reed gall inquilines. In agreement with Athen and Tscharntke (1999), we revealed the habitat age and size as key drivers of the species composition of site-specific assemblages. However, Athen and Tscharntke used a different time scale, focusing on sewage purification plants aged 2–11 only years and spanning just 10–2500 m^2 in size. They found that the diversity of the insect assemblages attacking these newly formed small reed beds increases with the age and size of these habitats. Extending the scale of their variables, we analyzed the (post)industrial habitats of 6–91 years of age, near-natural sites (fishponds) aged up to 599 years and several reed beds in floodplains along the meandering rivers expected to be present onsite since the last glacial period. We also scaled up the variability in the area of examined habitats up to 480 ha for near-natural habitats and up to 19 ha for (post)industrial habitats. We found that the model provided by Athen and Tscharntke (1999) is valid only in early successional stages of limited area. The initial increase in the abundance and, particularly, the species-richness of reed gallers and their parasitoids lasts for only a few years. Later, the species diversity associated with the reed galls becomes stabilized, and, instead, the assemblages seem to undergo a process of succession instead of enrichment (Table 6; Fig. 6). This situation clearly resembles that well known from forests subject to an initial disturbance followed by a long-term ecological succession (cf. Attiwill 1994; Hubbell et al. 1999). Higher microhabitat heterogeneity associated with increased vegetation diversity surrounding, and sometimes interspersing, the newly forming reed bed (Haddad et al. 2001; Hawkins and Porter 2003), and the presence of rare stress-tolerant plants preferred by certain threatened herbivores (Nickel and Hildebrandt 2003; Dennis et al. 2004) may significantly contribute to the diversity of newly formed, disturbed and only patchily colonized sites. However, the availability of the key resource, the common reed, from the very beginning of the establishment of such sites probably causes the absence of any transient increase in the species richness of reed gall-associated arthropods. Thus, the situation does not resemble the previously reported species succession gradients of aculeate hymenopterans, vascular plants and some other taxa at early successional stages of dry post-quarrying and post-mining sites (cf. Heneberg et al. 2013; Prach et al. 2013).

Our data confirmed previous conclusions of other large biodiversity assessments conducted in other habitats, such as grasslands (Allan et al. 2014; Manning et al. 2015), heathlands (Cameron and Leather 2012), mosaic temperate landscapes (Duelli and Obrist 1998; Oertli et al. 2005), or tropical forests (Lawton et al. 1998), showing that the higher land use intensity substantially alters the study environment, affecting differentially the trophically diverse taxa. Importantly, we found that the higher land use is associated with a formation of replacement niches for pioneer species that are only rarely found in the latter stages of reed bed succession. These species occurred previously at active river terraces, which were freshly formed from sand or gravel-sand, but such habitats were nearly completely destroyed in the study region as well as throughout large parts of the industrialized world. Thus, higher land use (in terms of mining, quarrying and associated activities) led to a formation of specific habitats instead of habitat deterioration when focusing on the reed beds. Despite the newly formed reed beds served as important strongholds for pioneer species of invertebrates ousted from the surrounding cultural landscape, they did not host the whole species spectrum associated with *Lipara*-induced

Table 6 Characteristic features of developmental stages of *Lipara*-induced reed gall-associated assemblages

Reed bed age (habitat)	Species richness	Red-listed species	Common species
<10 years	Low	None	Only >50 % occupancy of reed beds by *Lipara pullitarsis* at ≥25 m^2 and ≥3 years of reed bed age. Increase in parasitism of *L. pullitarsis* by *Stenomalina liparae* from 5 to 35 % during first 10 years following the reedbed formation (see Athen and Tscharntke 1999 for details)
10–100 years (post-industrial areas, gravels and river terraces)	High	*Echthrodelphax fairchildii*, *Passaloecus clypealis*, *Rhopalum gracile*	**Araneae:** *Clubiona phragmitis*, *Clubiona stagnatilis*, *Singa nitidula*, *Tetragnatha extensa*, **Heteroptera:** *Gastrodes abietum*, *Gastrodes grossipes*, *Lygus pratensis*, **Coleoptera:** *Cerapheles terminatus*, *Cyphon laevipennis*, *Coccidula scutellata*, **Hymenoptera:** *Alloxysta fulvices*, *Eupelmus phragmitis*, *Polemochartus melas*, *Polysphincta rufipes*, *Sycophila fasciata*, **Diptera:** *Anthomyza collini*, *Calamoncosis minima*, *Lipara rufitarsis*, *Cryptonevra diadema*
100–700 years (ancient fishponds)	High	None	**Araneae:** *Myrmarachne formicaria*, **Heteroptera:** *Dimorphopterus spinolae*, **Coleoptera:** *Anisosticta novemdecimpunctata*, *Oulema duftschmidi*, **Hymenoptera:** *Ametastegia glabrata*, *Brachythops flavens*, *Gambrus ornatus*, **Diptera:** none
>1000 years (meandering river floodplains)	High	*Homalura tarsata*, *Hylaeus moricei*	**Araneae:** *Clubiona germanica*, *Clubiona subtilis*, *Gibbaranea omoeda*, **Heteroptera:** *Ischnodemus sabuleti*, **Coleoptera:** none, **Hymenoptera:** *Centrodora locustarum*, *Platygaster erdoesi*, **Diptera:** *Elachiptera scrobiculata*
Species insensitive to habitat age	N/A	*Donachocara speciosa*, *Hylaeus pectoralis*	**Araneae:** none, **Heteroptera:** none, **Coleoptera:** none, **Hymenoptera:** *Aprostocetus orithyia*, *Centrodora amoena*, *Pemphredon fabricii*, *Torymus arundinis*, **Diptera:** *Cryptonevra flavitarsis*, *Lipara lucens*, *Lipara pullitarsis*

galls. Shared environmental drivers are expected to play a role in positive associations among species within the analyzed assemblages (cf. Wolters et al. 2006; Qian and Ricklefs 2008).

We identified numerous species-specific associations of reed gall-associated arthropods that differed from previously published data. In contrast to the findings by Tscharntke (1999), only some of the phytophagous insects found by us in reed galls were

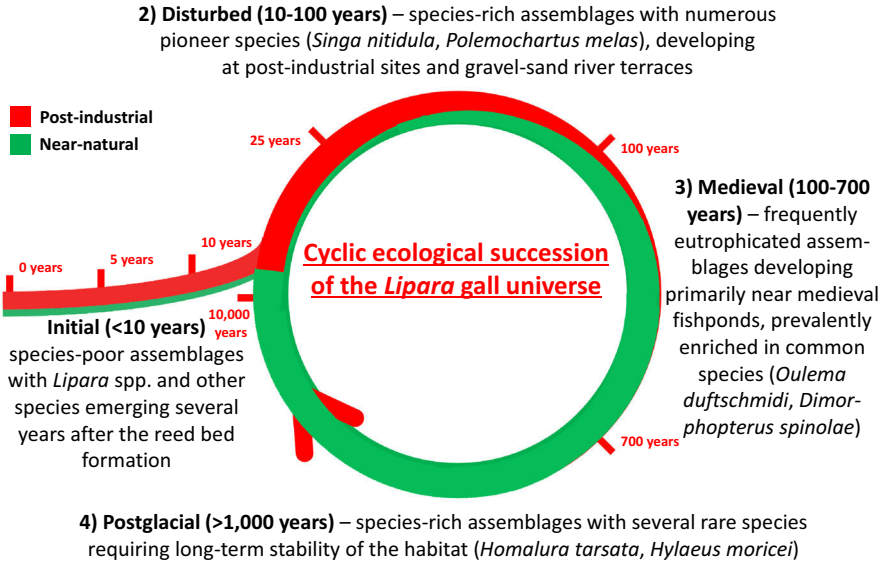

Fig. 6 Cyclic ecological succession of *Lipara*-induced reed gall-associated assemblages. The initial stages were analyzed in detail by Athen and Tscharntke (1999); the assemblages formed at the latter three stages of reed bed evolution were examined in course of this study. There is only negligible share of early successional reed beds developing spontaneously in intensively cultivated central European landscape, which highlights the importance of (post-)industrial sites as refugia

monophagous on reed; some were facultative common reed herbivores (e.g., *Elachiptera cornuta* and *E. scrobiculata*), and many others used the galls only as a shelter for overwintering but were trophically associated with other plant species (Table 2).

The reed galls served as an important winter niche for spiders collected by reed bed passerines, which typically specialize for *Clubiona juvenis*, *C. phragmitis* and *Singa* spp. (Schmidt et al. 2005). Five *Clubiona* spp., including the CR *C. juvenis*, were particularly abundant in the collected material. When comparing our data to previous studies on reed beds (Fig. 7), we found that the species composition of spider assemblage associated with reed galls collected in winter (this study) differs from those found by employing a broad range of sampling techniques in Czech and French reed beds in spring (Holec 2000; Schmidt et al. 2005), and that it strongly differs even from that obtained by collecting spiders on dead reed stalks in Belgium in winter (Decleer 1988). Only 3 species were found in all 4 of these studies, whereas over 20 spider species were found exclusively in the reed galls during the course of this study (Fig. 7). Schmidt et al. (2005) reported that the dominant reed gall-associated species *C. juvenis* is negatively affected by reed cutting, which may be consistent with frequent overwintering of this species in reed galls, which are typically destroyed by such reed management.

We recorded four species of hymenopteran parasitoids for the first time in the Czech Republic. *Echthrodelphax fairchildii* (Dryinidae) is a semi-solitary ectoparasitoid of Delphacidae planthoppers, originally described from Hawaii, and known from Bangladesh, India, Indonesia, Philippines, Malaysia, Thailand, Vietnam, Japan (Olmi 1984), China (He and Xu 2002) and Romania (Nagy 1967, questioned by Olmi 1984). We found this species at four sampling sites (all in (post)industrial habitats), thus it should be considered a

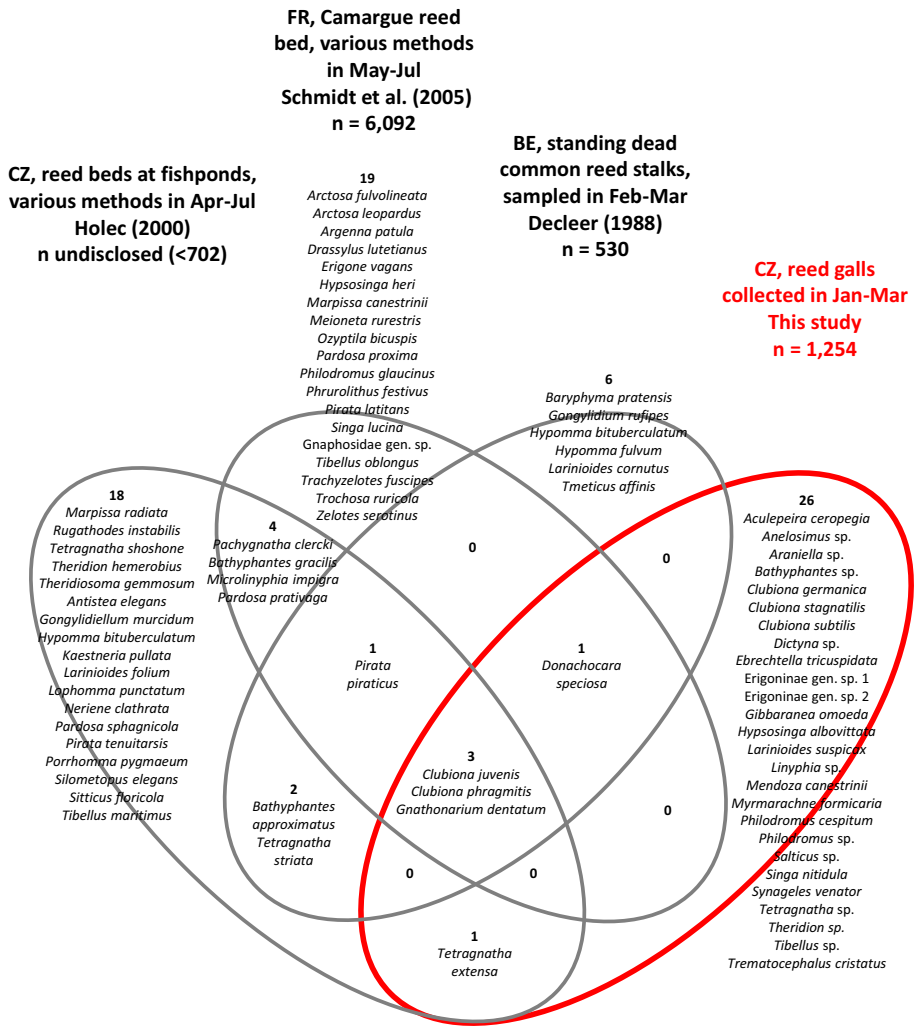

Fig. 7 Species composition of local microhabitat-specific reed bed-associated component communities of spiders strongly differed from each other as shown by the Venn diagram. Four datasets are compared: (*1*) spiders of Czech reed beds at fishponds, collected by various methods in April–July (Holec 2000; n < 702), (*2*) spiders of French reed beds in Camargue, collected by various methods in May–July (Schmidt et al. 2005; n = 6092), (*3*) spiders collected from common reed stalks in Belgium in February–March (Decleer 1988; n = 530) and (*4*) spiders identified in course of this study in the Czech Republic, which emerged from reed galls collected in January–March (n = 1254)

stable member of the Czech entomofauna. *Haplogonatopus oratorius* (Dryinidae) is also a parasitoid of Delphacidae planthoppers (Guglielmino and Olmi 1997, 2006; He and Xu 2002) with a Palearctic and Oriental distribution. In Europe, it has been only rarely found, reported from Karelia, Romania, Italy, Austria and England (Olmi 1984, 1999). *Gasteruption phragmiticola* (Evanioidea: Gasteruptiidae) was described a decade ago as a new species from Germany (Saure 2006) and confirmed in Germany by Westrich (2008), who reported it from the galls induced by *Lipara lucens* as a parasitoid of *Hylaeus* spp.

(Hymenoptera: Aculeata: Colletidae). We also found an unidentified member of the genus *Enclisis* (Ichneumonidae) for the first time in the Czech Republic. *Enclisis* is a small Palearctic genus with only six European and one Chinese species. The information on their biology is limited; all members of this genus are considered idiobiont ectoparasitoids of various Coleoptera and Hymenoptera nesting in wood (Bordera and Hernández-Rodríguez 2003).

Due to the quantitative and non-discriminatory nature of the rearing method used, the collected material provides a unique opportunity to evaluate unbiased species-specific sex ratios. We found skewed ratios across multiple taxonomic groups, including the *Lipara* flies and parasitic hymenopterans (Fig. 3). To our knowledge, the sex ratios in *Lipara* spp. and *Cryptonevra* spp. were not previously studied. In parasitic hymenopterans, the sex ratio is subject to complicated regulation, many species are arrhenotokous, and sometimes females only mate as soon as they emerge with the males emerging in the same host nest. Therefore, more females are produced under these conditions, generating only sufficient numbers of males to fertilize the females (Godfray 1994). The host size, ratio of different hosts, rate of oviposition and even temperature extremes can influence the sex ratio in Parasitica (Fisher et al. 1999). Regarding spiders, some, such as *Dysdera hungarica*, may develop parthenogenetic clones (Řezáč et al. 2007). It remains to be investigated, whether the absence of males in the examined reed bed populations of *Clubiona subtilis* was due to the yet undiscovered parthenogenesis or whether their males simply overwinter in different microhabitats.

Conclusions and conservation implications

Reed beds are often subject to cutting, herbicide treatment and complete eradication. In some parts of the world, they are considered alien. With widespread eutrophication they often invade formerly nitrogen- and nutrient-poor habitats even in their native distribution range, including the Czech Republic. Reed harvesting allows the silting of reed beds and enhances plant species diversity in the undergrowth (Decleer 1990; Cowie et al. 1992; Hawke and José 1996; Schmidt et al. 2005), but limits the nesting resources of early breeding passerines (Baldi and Moskat 1995; Poulin and Lefebvre 2002) and removes overwintering stages of arthropods (Pühringer 1975; Ditlhogo et al. 1992; Schmidt et al. 2005). The early ecological succession of arthropods in reed beds encompassing very small areas was studied by Athen and Tscharntke (1999), who showed that the keystone species, *Lipara pullitarsis*, colonizes nearly all of the available reed beds just within a few years after their formation, followed shortly by the dominant parasitoids, such as *Stenomalina liparae*. In this report, we addressed, for the first time, long-term changes of arthropod assemblages associated with *Lipara*-induced reed galls. We identified a cyclic and long-term nature of the succession (Fig. 6). The reed beds formed in recent decades host a diverse assemblage of pioneer species that are only rarely found in later stages of the reed bed succession. We assume that such newly formed reed beds may occur at active river terraces, which are freshly formed from sand or gravel-sand in regions where the rivers are not subject to extensive regulations such as in Central Europe. In an intensively cultivated central European cultural landscape with channelized rivers, such newly forming habitats are available nearly exclusively in post-industrial areas, which thus play a key role in the survival of the pioneer species of arthropods associated with reed galls. The later stages of the ecological succession of the studied assemblages are associated with a different

spectrum of species, which are typically found in the Czech Republic in the vicinity of ancient fishponds that are nowadays largely protected as nature reserves and form also key hotspots of diversity of reed passerines. However, the reed gall assemblages in these centuries old habitats still differ from the reed beds that occur in floodplains along rivers and streams and probably have the longest historical continuity, going back perhaps to the last glacial period. When subject to a severe disturbance, or when a new reed bed is formed, the pioneer species absent near both the fishpond- and river-associated reed beds emerge again, completing the cyclic succession nature of the *Lipara*-induced reed gall arthropod assemblages. Efficient evidence-based conservation of such assemblages thus should focus on the whole spectrum of reed beds, including recently formed ones (particularly those stressed by drought or other factors), as well as on the large reed beds that have been present for a long period of time. Of particular importance are the ecotones and the availability of diverse food sources in the vicinity of reed beds (bogs, dry grasslands, shrubs and trees) because numerous species of the *Lipara*-induced reed gall arthropod assemblages utilize the galls only as a shelter or a nesting resource but do not depend on the common reed as a food source.

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Compliance with ethical standards

Conflict of Interest The authors declare that they have no conflict of interest.

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3.5 ASTAPENKOVÁ A., HENEBERG P. & BOGUSCH P. 2017: Larvae and Nests of Aculeata Hymenoptera (Hymenoptera: Aculeata) Nesting in Reed Galls Induced by *Lipara* spp. (Diptera: Chloropidae) with a Review of Species Recorded. Part II. *PLoS ONE* **12**: e0169592.

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RESEARCH ARTICLE

Larvae and Nests of Aculeate Hymenoptera (Hymenoptera: Aculeata) Nesting in Reed Galls Induced by *Lipara* spp. (Diptera: Chloropidae) with a Review of Species Recorded. Part II.

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Abstract

The ability of aculeate Hymenoptera to utilize wetlands is poorly understood, and descriptions of their nests and developmental stages are largely absent. Here we present results based on our survey of hymenopterans using galls induced by *Lipara* spp. flies on common reed *Phragmites australis* in the years 2015–2016. We studied 20,704 galls, of which 9,446 were longitudinally cut and the brood from them reared in the laboratory, while the remaining 11,258 galls reared in rearing bags also in laboratory conditions. We recorded eight species that were previously not known to nest in reed galls: cuckoo wasps *Chrysis rutilans* and *Trichrysis pumilionis*, solitary wasps *Stenodynerus chevrieranus* and *Stenodynerus clypeopictus*, and bees *Pseudoanthidium tenellum*, *Stelis punctulatissima*, *Hylaeus communis* and *Hylaeus confusus*. Forty five species of Hymenoptera: Aculeata are known to be associated with reed galls, of which 36 make their nests there, and the other are six parasitoids of the family Chrysididae and three cuckoo bees of the genus *Stelis*. Of these species, *Pemphredon fabricii* and in southern Europe also *Heriades rubicola* are very common in reed galls, followed by *Hylaeus pectoralis* and two species of the genus *Trypoxylon*. We also found new host-parasite associations: *Chrysis angustula* in nests of *Pemphredon fabricii*, *Chrysis rutilans* in nests of *Stenodynerus clypeopictus*, *Trichrysis pumilionis* in nests of *Trypoxylon deceptorium*, and *Stelis breviuscula* in nests of *Heriades rubicola*. We provide new descriptions of the nests of seven species nesting in reed galls and morphology of mature larvae of eight species nesting in reed galls and two parasitoids and one nest cleptoparasite. The larvae are usually very similar to those of related species but possess characteristics that make them easy to distinguish from related species. Our results show that common reeds are not only expansive and harmful, but very important for many insect species associated with habitats dominated by this plant species.

Introduction

Hymenoptera, together with Diptera, Coleoptera and Lepidoptera, represent the four most diverse insect groups, not only according to their species richness but also with regard to variability of life strategies [1–3]. Hymenopterans nesting in various kinds of cavities are related to their various nesting and foraging strategies. Cavity nesters use not only holes in wood, reed stalks or plant stems for their nesting, but they can also adopt quite unexpected places, such as empty snail shells, cavities in old walls or in the reed roofs of buildings. Also, various types of galls host numerous very rare species, represented frequently by narrow habitat specialists [3–6]. Several digger wasps of the family Crabronidae nest in galls induced by the gall wasps of the family Cynipidae [7–9], and a whole group of Aculeata species use the cigar-shaped galls induced by frit flies of the genus *Lipara* (Diptera: Chloropidae) [5–6, 10–12].

The gall-nesting Aculeata are species of various families, which form a specific guild. This ecological group is very heterogeneous, containing reed gall specialists, wetland species (that only occasionally use reed galls for nesting), and ubiquitous species that nest in various types of cavities as well as in reed galls [6]. Although several hymenopterans, which nest in reed galls, are bioindicative, they have been rarely studied. Previous data has often been vague, such as "some species use cigar reed galls for their nesting" [2–4]. Despite being published more than a century ago, many of these works have been used by authors in recent monographic studies on Hymenoptera e.g., [3–4, 13–14]. There have been a variety of species-specific reports, Wolf [10] recorded *Pemphredon fabricii* (that time known as *Pemphredon lethifer*) together with several *Lipara* spp. and their parasitoids in reed galls collected at a single sampling site in Germany. Dely-Draskovits et al. [11] found this species as well as unidentified *Hylaeus* sp. together with many parasitoid species in reed galls collected in Hungary. In southern Germany, six species were recorded by [12]: *Pemphredon fabricii* (as *P. lethifer*), *Hylaeus pectoralis*, *Trypoxylon deceptorium* (as *Trypoxylon attenuatum*), *Trichrysis cyanea*, *Hoplitis leucomelana* (as *Osmia leucomelana*), and *Stenodynerus xanthomelas*. In monograph on *Lipara* of Fennoscandia, 26 species of aculeate Hymenoptera and 3 parasitic cuckoo wasps in their nests were reported [15]. Our previous results showed that 13 species nest in reed galls in Czech reed beds located in river floodplains, fishponds and post-industrial sites, and, summarizing all available information, we stated that 29 species are known to nest in reed galls, and were shown to be parasitized by two nest cleptoparasites and four parasitoids of the family Chrysididae [5, 6]. Thus, the community of hymenopterans nesting in reed galls is rich and highly variable.

Recent studies conducted in Europe showed that four species of *Lipara* and their hymenopteran inquilines are distributed across multiple countries [15]. Among their inquilines, *Pemphredon fabricii* is eudominant, usually comprising more than 90% of all aculeate nests and reared aculeate individuals [5–6], followed by *Hylaeus pectoralis* and *Trypoxylon deceptorium*. Most of the other species that have been recorded as nesting in reed galls, are relatively rare and probably use them only occasionally. There are also differences among habitat types, different species occur in large reed beds in fishponds or lake reservations, small sparse reeds on wet meadows, or short-stemmed reed beds in tailing ponds of power stations [see 5].

In this article, we provide a complete list of species recorded in reed galls including sources and countries of occurrence. We focus on aculeate species for which nests and larvae have never been previously described. We also describe, for the first time, the structure of nests and the morphology of mature larvae of eight species that nest in reed galls and three parasitic species. This contribution should thus be considered as a follow-up to our paper published 2015 in this journal [6]. By analyzing an extensive set of reed galls collected across multiple European countries, we were able to collect significant amounts of data even for rare species, which

are of special interest for conservation reasons, and thus provide the first available data set on the differences in their larval morphology and nesting preferences.

Materials and Methods

Study sites and sampling

We collected 20,704 galls induced by *Lipara* spp. on common reeds from 47 sampling sites located across central Europe (Fig 1), the sites included: northern Poland (15 sites), Hungary (14 sites), Czech Republic (8 sites), northern Italy (5 sites), Slovenia (3 sites) and Slovakia (2 sites). All localities along with coordinates are listed in S1 Table. Study of plants and animals was possible at all localities without any restriction, except the following: Czolpino, Gardna Wielka, Kluki and Rowy, permission issued by Slowinski National Park headquarters, Smoldzino, Poland, signed by Dr. Ireneusz Izydorek; Apajpuszta, Baks, Izsák, Munkastelep, Orgovány, and Sándorfalva, permission issued by Kiskunság National Park, signed by Ferenc Pál Szabó; Dubno Nature Reserve and Zbytka Nature Reserve, permission issued by Královéhradecký kraj, signed by Jan Novák; Slanisko u Nesytu National Nature Reserve and Slanisko Novosedly Nature Reserve by Pálava Protected Landscape Area, signed by Pavel Dedek; and Břehyně-Pecopala National Nature Reserve, Jestřebské slatiny Nature Reserve, Novozámecký rybník National Nature Reserve and Swamp National Nature Monument, permission issued by Kokořínsko Protected Landscape Area, signed by Věra Lucie Válová. The field studies did not involve any protected animals and no CITES species.

Only galls older than 1 year (greyish or darker in appearance, usually without leaves and often with the apex broken) were collected because our focus was on cavity nesting Hymenoptera (bees and wasps) and not on the *Lipara* spp. (inducing the galls) or their parasitoids. We collected reed galls from 15 Jan to 8 Mar 2015 and additional material from 17 to 22 Jan 2016. In late winter and early spring, mature larvae are present in their nests, and their rearing is easier than if they are collected before hibernation in the autumn [5]. Typically, at least 500 reed galls were collected from each sampling site, of which 200 were longitudinally cut and their contents analyzed, while the remainder were allowed to develop. In the 2016 sampling, only around 200 galls were collected, all of which were cut and none of which were measured. Additionally, galls $<$ 1-year-old (with *Lipara* spp. or their parasitoids present) were removed from analyses, thus the total number of galls analyzed from each site was slightly less than the number collected. At localities with limited availability of galls, we collected only 200–300 galls, which were all either longitudinally cut or reared. To measure dimensions of the cavity within random galls, a subset of less than one year old galls, containing *Lipara* spp. flies, were collected and subsequently measured. In total, 9,446 reed galls were cut and their contents were studied; the other 11,258 were reared in rearing bags in the same way used by [5–6].

Data acquisition

In the longitudinally cut reed galls, we studied the material of the walls separating the brood cells (henceforth termed bars) and the closing plugs at the top of each nest (henceforth termed corks), the structure and number of brood cells, and also the morphology and coloration of larvae and pupae. In the descriptions, "first cell" means the bottom, i.e., first-built cell of the nest. The "last cell" means the uppermost cell, i.e., the one nearest to the nest entrance. When the larvae were in cocoons, we removed part of the larvae from the cocoons but left the others inside. For each species, we first tried to rear the adults. For nests containing more than three larvae, we conserved part of the brood for morphological studies. To rear the larvale, the living larvae were taken from the nests, placed in Eppendorf 1.5 ml micro-tubes, which were plugged with cotton wool, left at the room temperature with ambient moisture, and reared similarly as

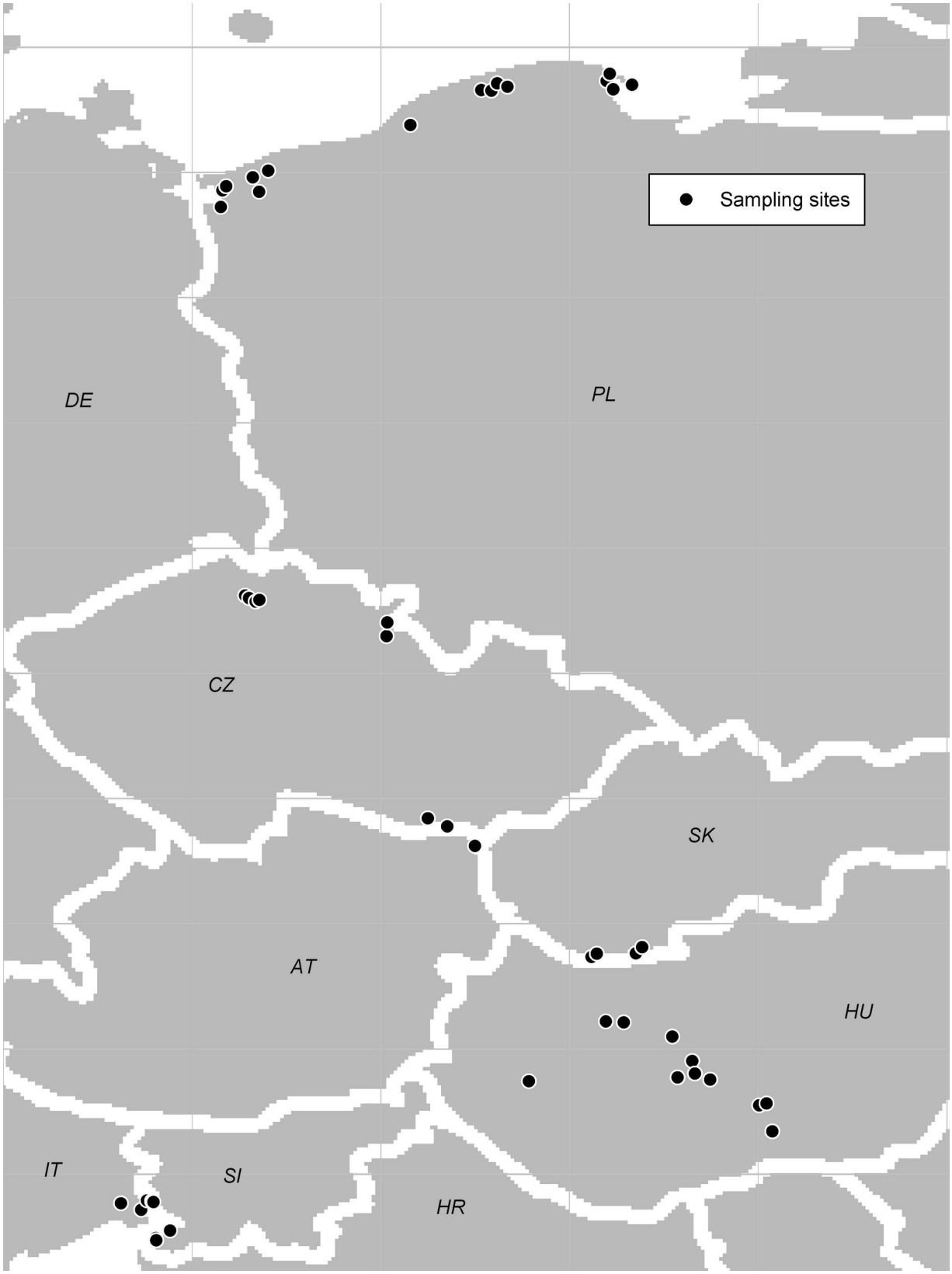

Fig 1. Map of central Europe with the localities studied.

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described by [6]. The adults usually hatched within three to four weeks after pupation, after which they were fixed, similarly to unreared larvae, i.e., in 96% ethanol. Only members of the family Megachilidae were left to develop in their cocoons, usually for two to three months. We measured the maximum diameter of each reed gall and the diameter of the reed stem just below the reed galls. For a random set of galls with *P. fabricii*, and for all well-preserved galls occupied by other aculeate species (except of mixed or parasitized nests), we measured also the length of the reed gall, the length of the nest (from the base of the nest to the cork) and the width of the cavity within the gall.

The reed galls allowed to develop were placed into rearing sacs as described by [5], and allowed to hatch for twelve weeks. The reared individuals were fixed in 20% ethylene glycol solution supplemented with a mixture of ionic and anionic detergents and later transferred to 96% ethanol.

The obtained material was identified by the first author and verified by the last author. Representative specimens (including the nests of each species) are available in the collections of Petr Bogusch (University of Hradec Králové, Czech Republic). We adopted nomenclature used in [14] and [16].

We documented the representative part of the nests using a digital camera (photographs of whole nests) and a macro-photographing apparatus consisting of a macro-camera Canon attached to a stereo microscope (brood cells, whole larvae). Documentation included photographs of nests shared by multiple species of aculeate Hymenoptera and of the parasitized nests. We took photos of living larvae as well as the larvae fixed in Pampel solution (30 volumes of distilled water, 15 volumes of 96% ethanol, 6 of formaldehyde and 4 volumes of glacial acetic acid) as described by [17]. To describe the morphology of larval specimens, we transferred some larvae (but never more than a sample of larvae present in a single nest–part of the larvae were allowed to rear to find out the species) into Pampel solution. After we took the photographs of the intact larvae, we focused on their sclerotized parts. For this purpose, we placed the larvae into 10% solution of hot (60˚C) potassium hydroxide, for 12 hours, to dilute all parts of the body except the integument. Then we colored the integument in 5% Chlorazol Black E (Sigma Aldrich) for 2 seconds and moved the specimens into 96% ethanol for conservation. To observe the identification features, we placed the integument into glycerol and separately observed the head, mouthparts, spiracles and other important parts of the integument under a light microscope. We used the same specimens for the study of small structures such as setae, sensillae or mouthparts. We drew figures of (1) the head with a focus on the clypeus, labrum, maxillae, and labium; (2) the mandibles from the anterior view; and (3) the spiracles of larvae of each species.

Data analysis

The data are shown as means ± SD unless stated otherwise. We analyzed the dataset sampled in 2015 in detail. This included 17,032 *Lipara*-induced reed galls from 34 sampling sites, which were either cut (6,449 galls) or allowed to develop (10,583 galls). Only the species of which we collected five or more individuals were included. We analyzed the occupancy rate of three different size-categories of reed stems and four size-categories of reed galls. To analyze the overall datasets, we employed one-way ANOVA. To analyze the species-specific differences between the observed and expected occupancy rates in the particular size-categories of reed galls and reed stems, we used χ^2 tests. As "expected frequencies," we used two groups of comparators, one formed by galls occupied by *Pemphredon fabricii* and the other one formed by random galls collected by our group in 2014. These comparative data sets for gall width and stem width (*Pemphredon fabricii* and random galls data sets) were retrieved from [6].

To analyze the correlations between gall width, stem width, and number of larvae, we calculated linear correlation coefficients r, and Spearman´s D for each species of which we found more than five individuals. To perform this analysis, we merged our data with the data set obtained by [6], which did not perform such analyses but accumulated significant amounts of data. In addition to species-specific correlations, we also calculated the same correlations for random galls (i.e., all those collected in 2014 and 2015). The calculations included the adult specimens of aculeate Hymenoptera as well as their parasitoids obtained in course of this study by hatching the imagines from longitudinally cut galls, and those reared directly from galls. We also calculated Pearson´s correlation coefficients for the inner and outer dimensions of the galls, the numbers of larvae and the ratios of outer to inner dimensions of galls. All calculations were performed using PAST v 2.14; graphs were prepared using SigmaPlot v 8.0.

Results

Hymenoptera: Aculeata nesting in cigar galls

Our data, combined with literary sources, suggest that the *Lipara*-induced reed galls host nests of 36 species of Hymenoptera: Aculeata (Table 1). The same nesting resource is also associated with nine species of hymenopteran parasites (six parasitic species of the family Chrysididae and three nest cleptoparasites of the genus *Stelis*).

In this study, we recorded these five species nesting in *Lipara*-induced reed galls for the first time: solitary wasps *Stenodynerus chevrieranus* and *Stenodynerus clypeopictus*, the mason bee *Pseudoanthidium tenellum*, and bees *Hylaeus communis* and *Hylaeus confusus*. Of these species, *S. clypeopictus* seems to be especially dependent on nesting in reed galls because this very rare species has been recorded quite frequently in reed galls. We also recorded two new parasitoids: *Chrysis rutilans* in nests of *S. clypeopictus* from several localities (Hungary: Bödi-Szék, Orgovány and Sándorfalva and Slovakia: Virt), which is also the first ever record of this host association. We have also recorded *Trichrysis pumilionis* (syn. *Chrysidea pumila*) in nests of *Trypoxylon deceptorium* (Hungary: Sándorfalva, Szabadszállás), and we confirmed previous reports of the nest of cleptoparasite *Stelis punctulatissima* in the nest of *Hoplitis leucomelana* (Hungary: Bödi-Szék), *Chrysis angustula* in the nests of *Pemphredon fabricii* (Czech Republic: Novozámecký rybník), and *Trichrysis cyanea* dominating in the nests of *Trypoxylon deceptorium* and *Trypoxylon minus* (Czech Republic, multiple localities, Slovakia: Virt). We found the parasitoid species, *Trichrysis cyanea* infrequently in the nests of *Pemphredon fabricii* (Czech Republic: Břehyně and Hungary: Orgovány), which also appears to be a novel discovery. We also report for the first time that *Stelis breviuscula* is a nest cleptoparasite of *Heriades rubicola* (Slovakia: Virt) and that *S. breviuscula* was very abundant in the nests of *H. rubicola* at this (i.e., Slovak) sampling site. We also confirmed the well-known host association of *Stelis ornatula* with *Hoplitis leucomelana*.

The Aculeata associated with *Lipara*-induced reed galls can be divided into three main groups:

1. Species preferring reed galls. These include only *Pemphredon fabricii*, *Hylaeus pectoralis* and probably *Stenodynerus clypeopictus*.
2. Species nesting in reed stalks or other types of cavities and frequently nesting in reed galls. This group is represented by *Trypoxylon deceptorium*, *Hylaeus moricei*, *Heriades rubicola*, and *Passaloecus clypealis*.
3. Species nesting in various types of cavities and accidentally or very rarely nesting also in reed galls. This group includes all other species found so far in reed galls, even though some

Table 1. Review of all Hymenoptera: Aculeata recorded as nesting in or parasitizing reed galls. Country codes: CZ–Czech Republic, DE–Germany, HU–Hungary, IT–Italy, PL–Poland, SI–Slovenia, SK–Slovakia,??? –unknown. Sources under the numbers used in References chapter.

Family/Species	Country	Literary source
Chrysididae		
Chrysis angustula Schenck, 1856 *	CZ	5, 6, this study
Chrysis rutilans Olivier, 1790 *	HU, SK	this study
Holopyga fastuosa generosa Förster, 1853 *	CZ	6
Pseudomalus auratus (Linnaeus, 1761) *	CZ	5, 15
Trichrysis cyanea (Linnaeus, 1761) *	CZ, DE, HU	5, 6, 12, this study
Trichrysis pumilionis Linsenmaier, 1987 *	HU	this study
Formicidae		
Dolichoderus quadripunctatus (Linnaeus, 1771)	CZ	5
Vespidae		
Stenodynerus chevrieranus (Saussure, 1855)	HU, IT	this study
Stenodynerus clypeopictus (Kostylev, 1940)	HU, SK	this study
Stenodynerus xanthomelas (Herrich-Schaeffer, 1839)	DE	12
Symmorphus bifasciatus (Linnaeus, 1761)	CZ	5, 6, 15, this study
Symmorphus fuscipes (Herrich-Schaeffer, 1839)	???	15
Crabronidae		
Ectemnius confinis (Walker, 1871)	CZ	5, 6
Nitela spinolae Latreille, 1809	CZ	5, 6, this study
Passaloecus clypealis Faester, 1947	CZ, PL	5, 6, 15, this study
Passaloecus corniger Shuckard, 1837	???	15
Passaloecus gracilis (Curtis, 1834)	???	15
Passaloecus singularis Dahlbom, 1844	???	15
Pemphredon fabricii (Müller, 1911)	CZ, DE, HU, IT, PL, SI, SK	5, 6, 11, 12, this study
Pemphredon inornata Say, 1824	???	15
Pemphredon lethifer (Shuckard, 1837)	???	12, 15
Pemphredon rugifer (Dahlbom, 1844)	???	15
Pemphredon wesmaeli (Morawitz, 1864)	???	15
Rhopalum clavipes (Linnaeus, 1758)	???	15
Rhopalum gracile Wesmael, 1852	CZ, IT	5, 6
Trypoxylon attenuatum Smith, 1851	???	12, 15
Trypoxylon deceptorium Antropov, 1991	CZ, DE, HU, IT, PL, SK	5, 6, 12, this study
Trypoxylon figulus (Linnaeus, 1758)	???	15
Trypoxylon minus Beaumont, 1945	CZ, PL, SK	5, 6, this study
Megachilidae		
Chelostoma campanularum (Kirby, 1802)	CZ	6
Heriades rubicola Pérez, 1890	CZ, HU, IT, SI, SK	6, this study
Hoplitis leucomelana (Kirby, 1802)	CZ, DE, HU, PL	5, 6, 12, 15, this study
Megachile centuncularis (Linnaeus, 1758)	???	15
Megachile versicolor Smith, 1844	???	15
Pseudoanthidium lituratum (Panzer, 1801)	CZ	6
Pseudoanthidium tenellum (Mocsáry, 1881)	HU	this study
Stelis breviuscula (Nylander, 1848) *	CZ, SK	6, this study
Stelis ornatula (Klug, 1807) *	CZ, PL	6, this study
Stelis punctulatissima (Kirby, 1802) *	CZ, HU	this study

(*Continued*)

Table 1. (*Continued*)

Family/Species	Country	Literary source
Colletidae		
Hylaeus communis Nylander, 1852	CZ	this study
Hylaeus confusus Nylander, 1852	HU	this study
Hylaeus gracilicornis (Morawitz, 1867)	???	15
Hylaeus incongruus Förster, 1871	CZ	6
Hylaeus moricei (Friese, 1898)	CZ	5, 6, 15, this study
Hylaeus pectoralis Förster, 1871	CZ, HU, PL, SK	5, 6, 12, 15, 27, this study

* marked are parasitic species.

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of these species are quite common in reed galls, this is because they form large populations, of which only a very small percentage use reed galls for nesting. This group is represented by, e.g., *Symmorphus bifasciatus*, *Trypoxylon minus* and *Hoplitis leucomelana*.

The first two groups are sensitive to changes in habitat surrounding reed beds and to the frequency of disturbances affecting the reed beds themselves, therefore they can be used as bioindicators of well-preserved reed beds within intensively cultivated landscapes.

Structure of nests of selected species

In our previous study, we described the structure of nests of the most common species nesting in reed galls [6]. Here, we provide descriptions of nests of seven less common species that also can be found nesting in reed galls, with notes on their differences from nests of similar and related species. The occupancy of nests, by a particular species, changed with the differences in stem width (one-way ANOVA: sum of sqrs = 234.9, $d_f = 13$, F = 11.8, $p << 0.001$), gall width (sum of sqrs = 1031.1, $d_f = 13$, F = 10.0, $p << 0.001$); also the differences in the number of larvae per nest was species-specific (sum of sqrs = 476.2, $d_f = 12$, F = 12.2, $p << 0.001$). We thus analyzed the preferences of particular aculeate hymenopteran species for specific reed gall width, length, and also width of the inner space of the gall and a length of the nest, all correlated with each other, with the number of larvae within the nests and with the gall:nest width and length ratios (Tables 2 and 3, Fig 2A). Besides the data collected in 2015 and 2016 (Table 3), we re-analyzed the data collected in 2014 [6].

The analysis of gall and nest measurements (Table 2) shows that most of the species use only the inner space of the gall but *Pemphredon fabricii* and in several cases also *Heriades rubicola* and *Hylaeus pectoralis* extend brood cells also to the space between the reed leaves outside of the cavity of the gall. Additionally, *P. fabricii* and *H. rubicola* have usually longer nests with more brood cells than the others, whereas *Hylaeus pectoralis* also has usually longer nests but with less brood cells because it makes very often empty spaces (false brood cells) inside the gall. *Stenodynerus* spp. and *Trypoxylon* spp. do not use the whole cavity of the gall and they also frequently settle only the top parts of galls, in which the basal parts were settled by another species, usually *P. fabricii*. In this regard, it is important to note that we did not find any nest of *S. chevrieranus* occupying the whole gall–in both two nests examined, this species settled in the empty space of the gall pre-occupied in part by *P. fabricii*). The ratio of gall versus nest lengths did not differ significantly among most of the species, with the exception of *Trypoxylon minus*, which usually made short nests in long galls. Also the ratio of gall versus nest widths did not show any species-specific pattern except of *P. fabricii* and *S. clypeopictus*, which preferred

Table 2. Species-specific preferences for the cavity dimensions. The cavity dimensions were measured as nest length (length of the nest from the base to the plug) and nest width (cavity width), and they were compared with the gall length (Gall:nest length ratio) and width (Gall:nest width ratio). The measures are expressed as means ± SD (range) for N≥3; for lower N, individual measurements are indicated. Note that particularly the *P. fabricii* nests extend often out of the cavity and their upper parts may be surrounded by dry leaves only, thus the nests could sometimes be longer than the galls in which they are located. For species with N≥10, the Pearson's correlation coefficients were calculated in order to correlate the nest length and width with the gall length and width, with the number of mature larvae contained within the nests and with the gall:nest length and width ratios.

	Nest length	Nest width	Pearson's correlation coefficient						Gall: nest length ratio	Gall: nest width ratio	Pearson's correlation coefficient				N
			Nest length	Nest width	Nest length	Gall length	Nest width	Gall width			Nest length	Gall length	Nest width	Gall width	
Species			Gall length	Gall width	N of larvae	N of larvae	N of larvae	N of larvae			Length ratio	Length ratio	Width ratio	Width ratio	
Heriades rubicola	51.6 ± 13.1 (25–90)	3.9 ± 0.6 (2.5–5)	0.752	0.364	0.657	0.542	0.228	0.063	1.29 ± 0.24 (0.90–1.94)	1.87 ± 0.35 (1.33–3.00)	-0.591	0.061	-0.698	0.398	40
Hoplitis leucomelana	34.6 ± 10.4 (20–59)	3.3 ± 0.4 (3–4)	0.783	0.328	0.644	0.533	-0.092	0.304	1.56 ± 0.33 (1.05–2.59)	1.79 ± 0.29 (1.33–2.33)	-0.532	0.077	-0.413	0.721	31
Hylaeus confusus	49, 54	3, 3							1.12, 1.24	1.67, 2.00					2
Hylaeus moricei	37.7 ± 6.3 (29–44)	3.3 ± 0.5 (3–4)							1.14 ± 0.11 (1.00–1.27)	2.03 ± 0.45 (1.67–2.67)					3
Hylaeus pectoralis	44.6 ± 10.8 (23–73)	3.5 ± 0.6 (3–5)	0.726	0.331	0.586	0.464	-0.038	0.322	1.28 ± 0.21 (0.81–1.80)	1.83 ± 0.32 (1.25–2.67)	-0.610	0.073	-0.610	0.537	50
Passaloecus clypealis	41.0 ± 2.3 (39–45)	3.8 ± 0.4 (3–4)							1.45 ± 0.19 (1.23–1.73)	1.73 ± 0.04 (1.67–1.75)					4
Pemphredon fabricii	50.5 ± 14.4 (12–78)	3.3 ± 0.8 (2–5)	0.271	0.704	0.839	0.175	-0.070	0.054	1.16 ± 0.64 (0.57–5.00)	2.89 ± 0.66 (1.67–6.00)	-0.697	0.210	-0.184	0.555	53
Stenodynerus clypeopictus	35.7 ± 13.1 (17–53)	3.6 ± 0.5 (3–4)	0.175	0.684	0.336	-0.356	-0.237	-0.382	1.80 ± 0.94 (1.05–3.76)	1.88 ± 0.27 (1.50–2.43)	-0.839	0.364	0.078	0.777	10
Symmorphus bifasciatus	27, 30	3.5, 3.6							1.59, 2.00	1.43, 1.81					2
Trypoxylon deceptorium	30.8 ± 8.3 (18–57)	3.2 ± 0.5 (2.5–4.5)	0.432	0.412	0.727	0.210	0.060	-0.092	1.75 ± 0.49 (1.14–3.11)	1.84 ± 0.27 (1.33–2.40)	-0.681	0.302	-0.627	0.433	29
Trypoxylon minus	35.3 ± 12.0 (10–70)	3.3 ± 0.6 (2–5)	0.738	0.735	0.394	0.327	0.201	0.223	1.84 ± 0.76 (1.14–5.00)	2.00 ± 0.25 (1.50–2.50)	-0.737	-0.252	-0.540	0.159	29
Random galls with *Lipara* spp.	N/D	3.0 ± 0.71 (2–4.5)		0.438					N/D	2.69 ± 0.69 (1.50–4.50)			-0.545	0.482	50

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Table 3. Occupancy rate of four reed gall size-categories. The χ^2 test was employed to compare the observed frequencies (data collected in 2015 during the course of this study) with two types of expected frequencies, namely with the frequencies of galls (i) occupied by *Pemphredon fabricii* and (ii) random galls collected by our group in 2014 [6]. Only species with n ≥ 5 are shown.

Species	Reed gall width				*p* (χ^2 test)	
	< 5 mm	5–9.5 mm	10–14.5 mm	≥ 15 mm	Obs. vs random	Obs. vs *P. fabricii*
Chrysis angustula	0	0	1	0		
Heriades rubicola	0	14	5	0	3.0 E-01	**4.3 E-02**
Hylaeus confusus	0	2	0	0		
Hylaeus incongruus	0	0	1	0		
Hylaeus moricei	0	5	0	0	2.3 E-01	**5.1 E-02**
Stelis punctulatissima	0	1	0	0		
Stenodynerus clypeopictus	0	9	2	0	3.0 E-01	**5.3 E-02**
Trypoxylon minus	0	26	4	0	**4.0 E-03**	**3.5 E-05**
Comparators:						
Pemphredon fabricii	0	469	512	48	**5.4 E-21**	N/A
Random galls	267	3416	2381	287	N/A	**7.0 E-63**

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equally wide cavities in thin as well as in wide galls. All other species settled in galls, which displayed positive correlation of the cavity width with the gall:nest width ratio. Thus, only *P. fabricii* and *S. clypeopictus* settled narrow galls as long as the cavity proportions were useful for them, which was not recorded in the other species.

The analysis of width of galls and stems indicated that some species are associated with galls of specific width. Particularly, *Symmorphus bifasciatus* and *Thyridanthrax fenestratus* were associated characteristically with galls of significantly smaller diameter compared to both random galls or galls occupied by *Pemphredon fabricii*. We also analyzed the preferences of particular aculeate hymenopteran species for specific reed stem width (Table 4, Fig 2B). Besides the data collected in 2015 (Table 4), we re-analyzed the data collected in 2014 [6], which indicated that some aculeate species differ in their stem width preferences. In particular, *Hylaeus pectoralis* was associated with the widest available stems, and thus differed significantly from *Pemphredon fabricii*. *Trypoxylon deceptorium* was more prevalent in thin stems, and thus differed significantly in this parameter from randomly collected reed galls; this was also true for *Trypoxylon minus* and *Pemphredon fabricii*. In multiple species, the number of larvae per nests correlated with the reed gall width but not with the reed stem width (Table 5), which is consistent with the preferences of most species for galls induced by *Lipara lucens*, which forms thick galls even on very thin stems. Such correlations suggest that females of these species are limited by the dimensions of available cavities, i.e., galls. Photos of nests of all species are in Figs 3 and 4.

Stenodynerus clypeopictus. The nests of *Stenodynerus clypeopictus* usually consisted of 1–2 brood cells (range 1–4; median 1; mean 1.8 ± 1.07 cell per nest; n = 10). The brood cells were quite long (length 8.1 ± 0.91 mm; median 8 mm; width 3.3 ± 0.42 mm; median 3.25 mm; n = 19) and were separated by bars made of soil, sometimes mixed with plant debris (approximately 3 mm thick). In some cases, the soil bar was surrounded on both sides by a layer of plant debris mixed with larval feces. The nests were made at the base of the gall cavity. However, in galls with a very narrow base, the brood cells were placed in the wider part of the gall, and the base of the gall was not filled with anything. The brood cells were placed in the gall one after the other. When there were smaller numbers of cells, the upper part of the gall was filled

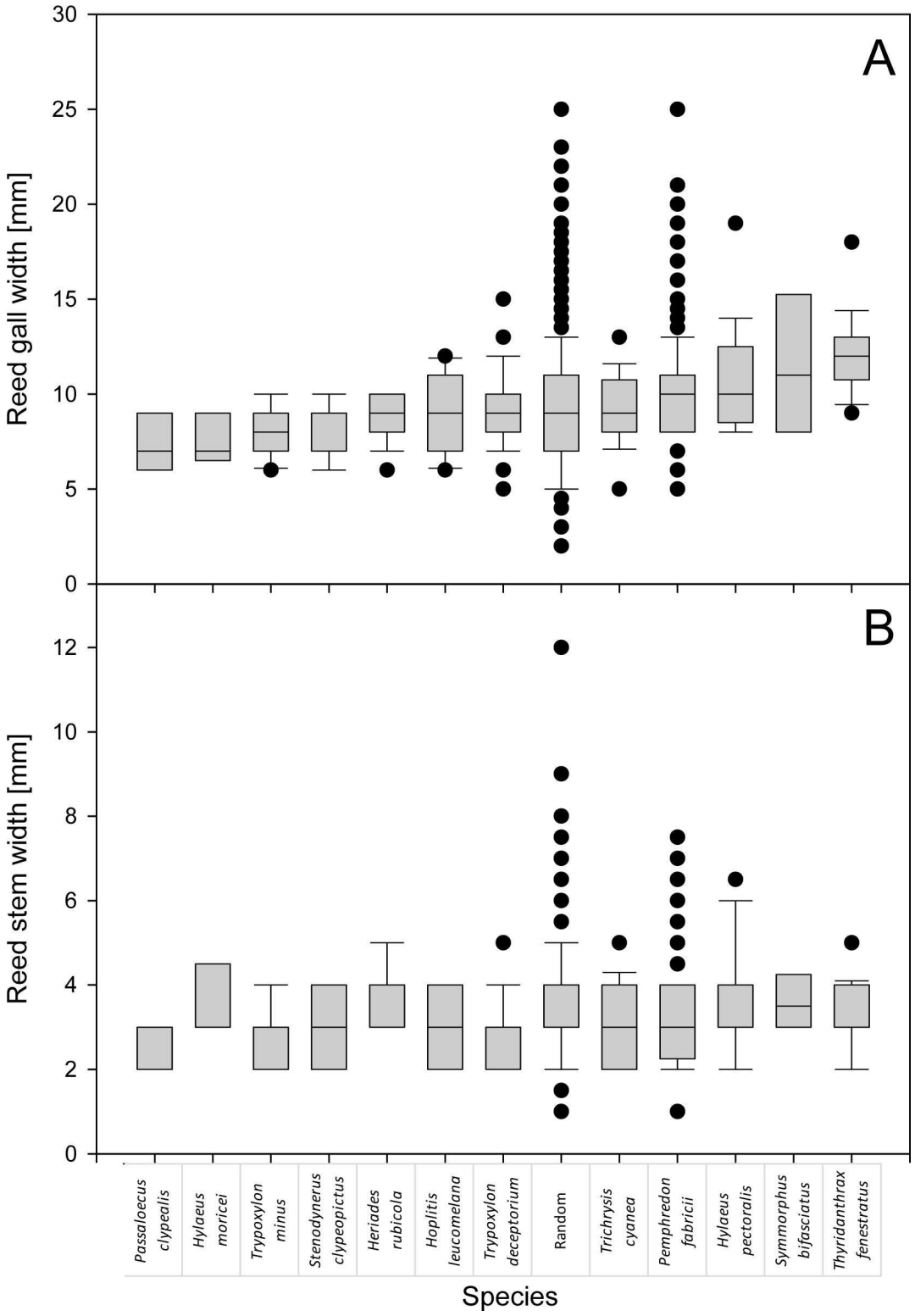

Fig 2. Species-specific preferences for a particular gall (A) and stem (B) width. The lines within the boxes show medians, the boxes denote the 25th and 75th percentiles, the whiskers indicate the 10th and 90th percentiles, black points denote outlying points below the 10th and above the 90th percentiles.

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Table 4. Occupancy rate of three reed stem size-categories. The χ^2 test was employed to compare the observed frequencies (data collected in 2015 and 2016 during the course of this study) with two types of expected frequencies, namely with the frequencies of galls i) occupied by *Pemphredon fabricii* and ii) random galls collected by our group in 2014 [6]. Only species with n $\geq$ 5 are shown.

Species	Reed stem width			p (χ^2 test)	
	> 4 mm	4–5.5 mm	$\geq$ 6 mm	Obs. vs random	Obs. vs *P. fabricii*
Chrysis angustula	1	1	0		
Heriades rubicola	16	13	0	1.6 E-01	**2.3 E-02**
Hylaeus confusus	2	0	0		
Hylaeus incongruus	1	0	0		
Hylaeus moricei	4	2	0	8.1 E-01	8.2 E-01
Stelis punctulatissima	1	1	0		
Stenodynerus clypeopictus	11	3	0	3.7 E-01	8.8 E-01
Trypoxylon minus	30	5	0	**1.2 E-02**	3.2 E-01
Comparators:					
Pemphredon fabricii	953	298	18	**3.9 E-25**	N/A
Random galls	4899	2515	506	N/A	**0.0 E+00**

doi:10.1371/journal.pone.0169592.t004

Table 5. Correlation analysis of the number of larvae per nest with gall dimensions suggests that gall size is the limiting factor for females of aculeate hymenopterans. To analyze the correlations of gall width, stem width, and number of larvae, we calculated linear correlation coefficients r, and Spearman´s D for each species with more than five individuals. To perform this analysis, we merged our data with the data set obtained by [6]. Besides the species-specific correlations, we also calculated the correlation for random galls (all those collected in 2014 and 2015).

	Linear correlation r						Spearman´s D					
	Gall width		Gall width		No. of larvae		Gall width		Gall width		No. of larvae	
Species	vs. No. of larvae		vs. stem width		vs. stem width		vs. No. of larvae		vs. stem width		vs. stem width	
Heriades rubicola	−0.05		0.59	**	0.01		1109		399	*	971	
Hoplitis leucomelana	0.72	*	0.57		0.28		40	*	69		109	
Hylaeus pectoralis	0.38		0.61	***	0.07		2043		1268	**	2775	
Pemphredon fabricii	0.45	***	0.56	***	0.32	***	1.0E +08	***	7.2E +07	***	1.2E +08	***
Stenodynerus clypeopictus	−0.36		0.64	*	0.00		245		68	*	188	
Symmorphus bifasciatus	0.92	**	0.74		0.50		6		8		8	
Trichrysis cyanea	0.52	*	0.36		0.14		296	*	362		488	
Trypoxylon deceptorium	0.59	***	0.49	**	0.09		4305	***	4450	**	8050	
Trypoxylon minus	−0.02		0.45		0.02		37		17		33	
Random galls	N/A		0.44	***	N/A		N/A		2.5E +10	***	N/A	

Asterisks show the significance of the results–

* significant

** highly significant

*** very highly significant.

doi:10.1371/journal.pone.0169592.t005

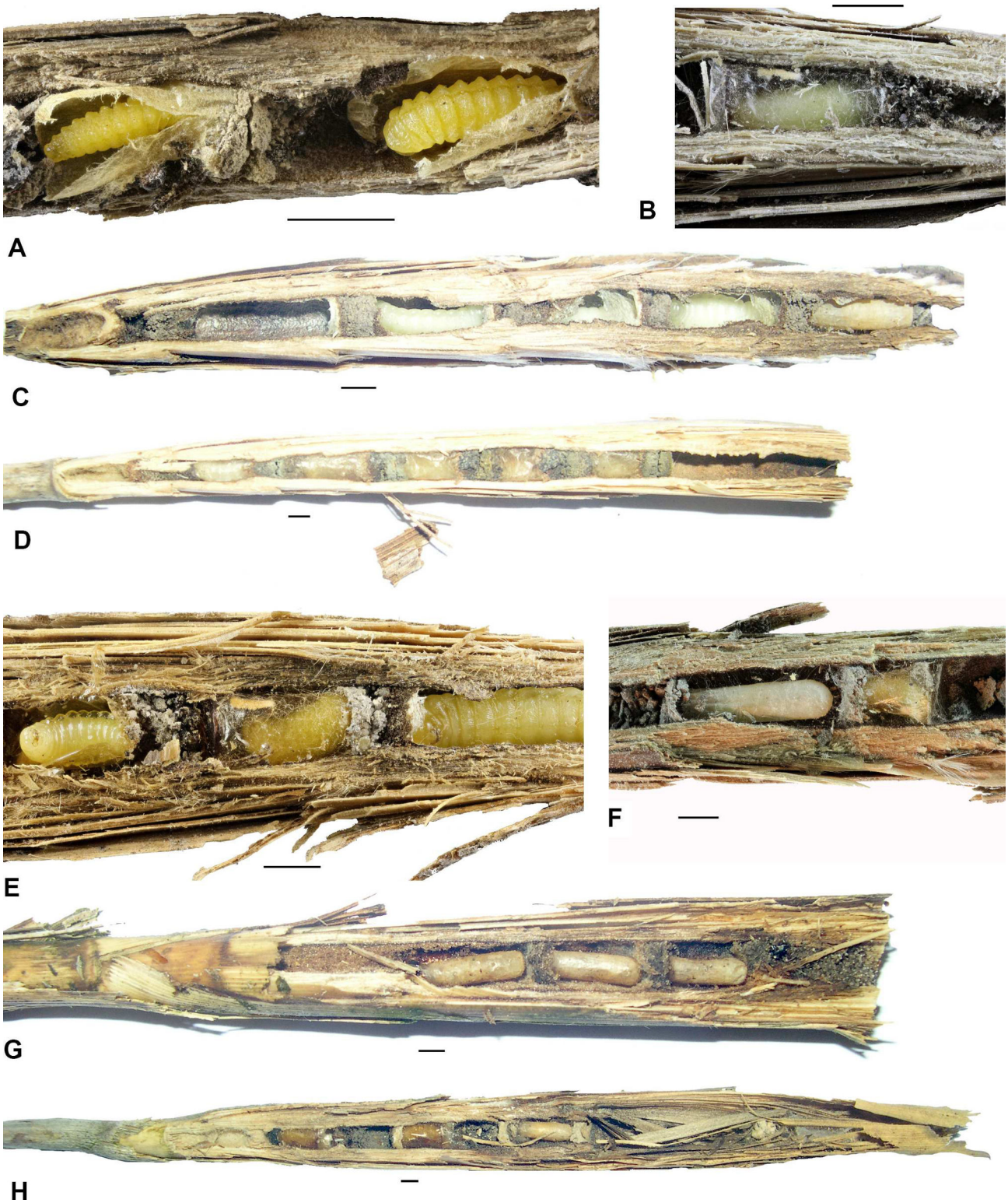

Fig 3. Photos of nests and parts of the nests of aculeate Hymenoptera in reed galls. A–*Symmorphus bifasciatus*, part of a nest with two larvae in cocoons, B–*Stenodynerus chevrieranus*, brood cell, C–*Stenodynerus clypeopictus*, nest with three white colored larvae and *Trypoxylon* sp. in the last brood cell, D–*S. clypeopictus*, nest with five brood cells, E–*S. clypeopictus*, details of a nest with one larva of this species and two larvae of *Chrysis rutilans*, F–*Trypoxylon deceptorium*, details of a nest with one larva in a cocoon and one short cocoon with larva of *Trichrysis pumilionis*, G–*T. minus*, nest with three brood cells, H–*T. minus*, nest with one cocoon of this species and two cocoons of *Trichrysis cyanea*. Measurements show 2 mm.

doi:10.1371/journal.pone.0169592.g003

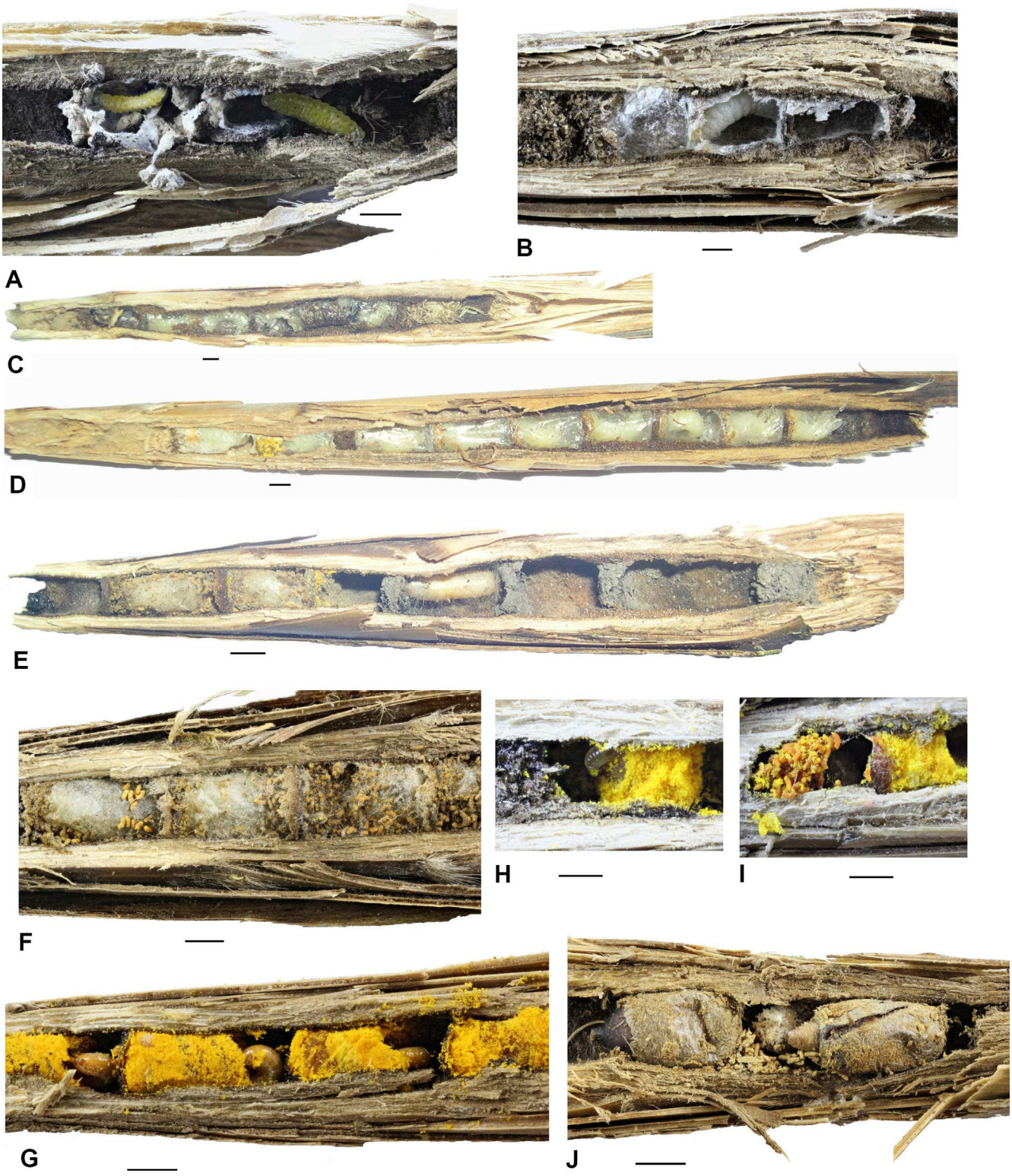

Fig 4. Photos of nests and parts of the nests of aculeate Hymenoptera in reed galls. A–*Passaloecus clypealis*, nest with two brood cells with yellow larvae, B–*Hylaeus moricei*, details of a nest with three brood cells with one larva, C–*H. moricei*, nest with six brood cells, D–*Heriades rubicola*, nest with nine brood cells, E–*H. rubicola*, nest with two brood cells and the rest full of brood cells and one cocoon of *Trypoxylon* sp., F–*H. rubicola*, details of a nest with three brood cells and larval feces on their surface, G–*H. rubicola*, details of brood cells with young larvae on yellow pollen, H–*H. rubicola*, brood cell with young larva, I–*H. rubicola*, brood cell with premature larva with the rest of the pollen and feces, J–*Stelis breviuscula*, brood cells of characteristic shape in a nest of *H. rubicola*. Scale bars show 2 mm.

doi:10.1371/journal.pone.0169592.g004

with a substrate consisting of soil and small sand or silt grains (smaller than 0.5 mm). The surface of the brood cells was covered by a shiny silk-like layer made by secretions from the female's Dufour's glands, the layer on the inner surface of the brood cell was creamy-white, bright and opaque, and made from the silk of mature larvae (Fig 3B–3E).

We recorded cuckoo wasps, *Chrysis rutilans*, in the nests of *S. clypeopictus* at three localities in Hungary (Bödi-Szék, Orgovány, Sándorfalva) and one locality in the south of Slovakia (Virt). This species was discovered to be a parasite of *S. clypeopictus* and seems to typically be a parasite of small-sized species of solitary wasps. *Chrysis rutilans* had shiny and slightly conical cocoons, which were glued to the bars on the bottom side but not glued on the upper side. The cocoons were transparent and significantly shorter than brood cells of *S. clypeopictus*. Both ends of the cocoons were reddish brown (Fig 3E).

Stenodynerus chevrieranus. We found only one nest of *Stenodynerus chevrieranus*. It consisted of 2 brood cells and was located near Fonyód, close to Lake Balaton in Hungary. The nest was at the end of a gall occupied by *Pemphredon fabricii*. Brood cells were covered by a shiny creamy-white layer, very similar to that of *S. clypeopictus*. The bars between the brood cells were made of soil and were only around 1 mm thick (shorter than at *S. clypeopictus*). The end of the nest was filled with soil and grit.

Symmorphus bifasciatus. Two nests of this species, from the 2016 season, were comprised of two and three brood cells. They were very similar to those previously described by [6]. The bars between the brood cells were made of soil and small grit. The quite thick cork (3–4 mm) was made of soil and there were no empty cells in the gall. Light-brownish cocoons were made of silk, and slightly extended toward the head of the pupa. The cocoons did not fill the whole brood cell, but rather only about two thirds of it. We found remnants of larvae and adults of chrysomelid beetles (*Galleruca* sp.) in the brood cells (Fig 3A).

Passaloecus clypealis. *Passaloecus clypealis* nests were very similar in their general appearance with those of *Pemphredon fabricii* but had much smaller brood cells (length 5.5 mm ± 0.55 mm; median 5.2 mm; width 2.8 mm ± 0.54 mm; median 2.8 mm; n = 7). The nests were comprised of 4–6 brood cells (median 4; mean 4.5 ± 0.87 cell per nest; n = 4 nests). Between the brood cells, there was usually a filling of large (0.5–0.8 mm) grit glued by soil and other unidentified materials. The walls of the brood cells were covered with a white silk-like layer that created white cocoons made by the larva after it defecated. The base of the nest was filled with grit; the cork was made of grit, silk, and aphid parts. Brood cells were separated from each other by ≈1 mm thick bars of soil. The empty parts between the brood cells were filled with unidentified materials, probably a mixture of larval feces and soil particles. Larvae pupated in the brood cells covered with silk but without making cocoons (Fig 4A).

***Trypoxylon deceptorium* and *T. minus*.** Nests of both species were described by [6]. We also improved our knowledge of *T. minus* nests based on additional material from Poland and the Czech Republic. We usually recorded 1–3 brood cell per nest (range 1–4; median 2; mean 2.2 ± 0.99 cells per nest; n = 28). The nest structure was similar to that described by [6]. We also recorded the cuckoo wasp *Trichrysis cyanea* as a parasitoid in nests of *T. minus* (see Figs 2F–2H and 4E).

We confirmed the cuckoo wasp *Trichrysis pumilionis* as a parasite in the nests of *T. deceptorium* in Hungary (Sándorfalva and Szabadszállás). The cocoons of this cuckoo wasp had the same structure as cocoons of *T. cyanea*, which parasitizes nests of *Trypoxylon* spp. across central Europe. However, only one larva of *T. pumilionis* was in each of the nests, so we did not have sufficient data to describe the morphology of the larva of this species.

Heriades rubicola. The nests of *Heriades rubicola* usually consisted of 3–4 brood cells, less frequently up to 7 (range 1–7; median 4; mean 3.7 ± 1.6 cell per nest; n = 23). Brood cells

(length 6.1 mm ± 0.88 mm; median 6 mm; width 3.6 mm ± 0.48 mm; median 3.5 mm; n = 61) were placed in the cavity one after another and separated from each other by very thin bars (less than 1 mm) consisting of the same material as that filling the gall. The nests comprised the whole gall with no empty cells as protection against parasitoids. The top of the gall was filled with a substance made of resin and chewed plant tissues (light brown pieces of reed or grass leaves). The cork was just behind the last cell and was made of silt grains and resin (hard and sticky mass). In some cases, the narrow base of the gall was filled with silt grains. Mature larvae were placed in brownish, silk and cellophane-like cocoons (similar to cocoons of *Hoplitis leucomelana*). The cocoons were hard (not easy to open) and made of silk. Imagines hatched about 2–3 months after pupation, which is a very long time in comparison to other species. The males hatched first, about 1–2 weeks before females. Nests of this species consisted of the horn-shaped feces of the larvae, which remained on the surface of the cocoons and were often found to be getting moldy (Fig 4D–4F). Nests with young larvae collected in summer were full of brood cells with yellow-orange pollen of Asteraceae. The brood cells were filled to one-half to two-thirds of their volume with pollen. Larvae fed directly on the pollen, higher instars had a brownish coloration (Fig 4G–4I).

At Virt in southern Slovakia, nests of *H. rubicola* were frequently parasitized by *Stelis breviuscula* (seven of 45 nests of *H. rubicola* were parasitized by 15 individuals of *S. breviuscula*), whose brood was typically placed in brownish oval cocoons with a cusp on the bottom and on the top (Fig 4J).

***Hylaeus confusus*.** The nests of *Hylaeus confusus* were very similar to *H. pectoralis;* we did not find any significant differences. Two nests found in our survey were comprised of two and three brood cells.

***Hylaeus moricei*.** The nests of *Hylaeus moricei* were very similar to the nests of *H. pectoralis* but smaller and usually with more brood cells (two nests of our dataset comprised three and six brood cells), whose length was 5.0 mm ± 1.24 mm; median 4.3 mm; and width 3.2 mm ± 0.39 mm; median 3.2 mm; n = 9. The nests usually did not occupy the whole gall but no empty cells were placed between the brood cells with larvae. The bases of the galls were filled with chewed plant particles (> 1 mm). Above the filling was a space of very similar size to that of brood cells, then chewed plant particles again, and then brood cells that were only separated by thin cellophane-like layers. The walls of the brood cells were also covered by a cellophane-like material, which was made by the female and consisted of secretions from her Dufour's gland. At the end of the gall from Novozámecký rybník, there was a cork made of plant particles and mud and elongated pieces of leaves (possibly reed leaves) on top. Mature larvae were very close to each other, but without cocoons (Fig 4B and 4C).

Description of mature larvae

We analyzed mature larvae of eight species of aculeate Hymenoptera nesting in reed galls induced by *Lipara* spp., two of their parasitoid species, and one nest cleptoparasite found in their nests. Below, we provide descriptions of mature larvae, including photos of whole larvae from lateral and ventral views (Figs 5 and 6) and drawings of the main identification characteristics–head capsules, mandibles, and spiracles (Figs 7 and 8).

***Chrysis angustula* (Fig 7A–7C).** Mature larvae of this species have been described previously by [18]. However, the species was divided into two species later by [19] so we cannot state larva of which of them was described previously.

Material: Czech Republic, Bohemia bor., Zahrádky, Novozámecký rybník National Natural Reserve, terrestrial reed bed surrounding fishpond, 08.iii.2015, 1 larva, P. Bogusch et A. Astapenková lgt., P. Bogusch det. (coll. P. Bogusch).

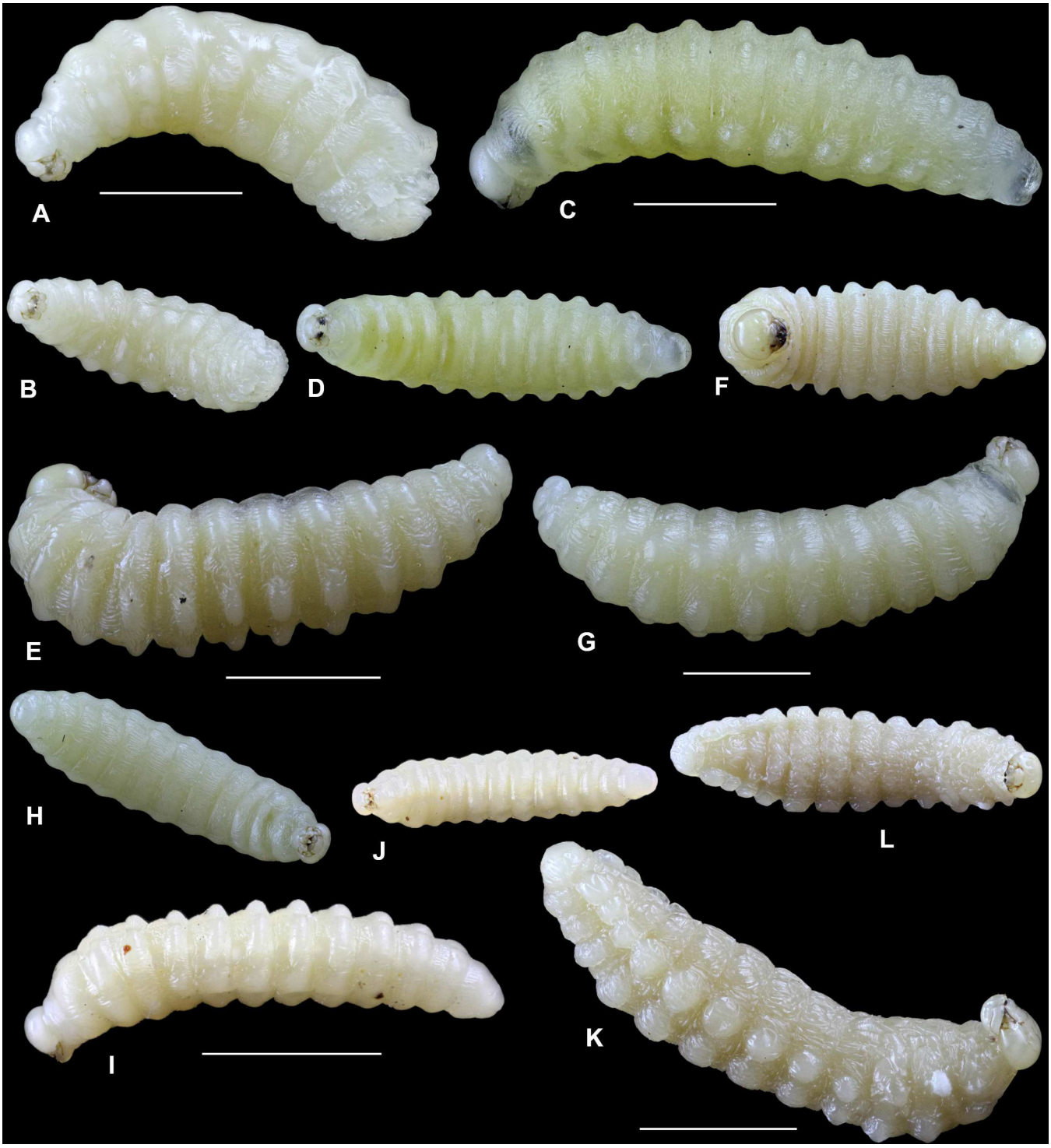

Fig 5. Larvae of aculeate Hymenoptera in reed galls. A–B–*Chrysis rutilans*, lateral and ventral view; C–D–*Symmorphus bifasciatus*; E–F–*Stenodynerus chevrieranus*; G–H–*S. clypeopictus*; I–J–*Passaloecus clypealis*; K–L–*Trypoxylon minus*. Measurements show 2 mm.

doi:10.1371/journal.pone.0169592.g005

Body: Short and robust, white colored, length 5.92 mm; width 2.22 mm (n = 1). Fusiform in shape with well-developed dorsal posterior lobes reaching pleurae of segments. Pleural lobes

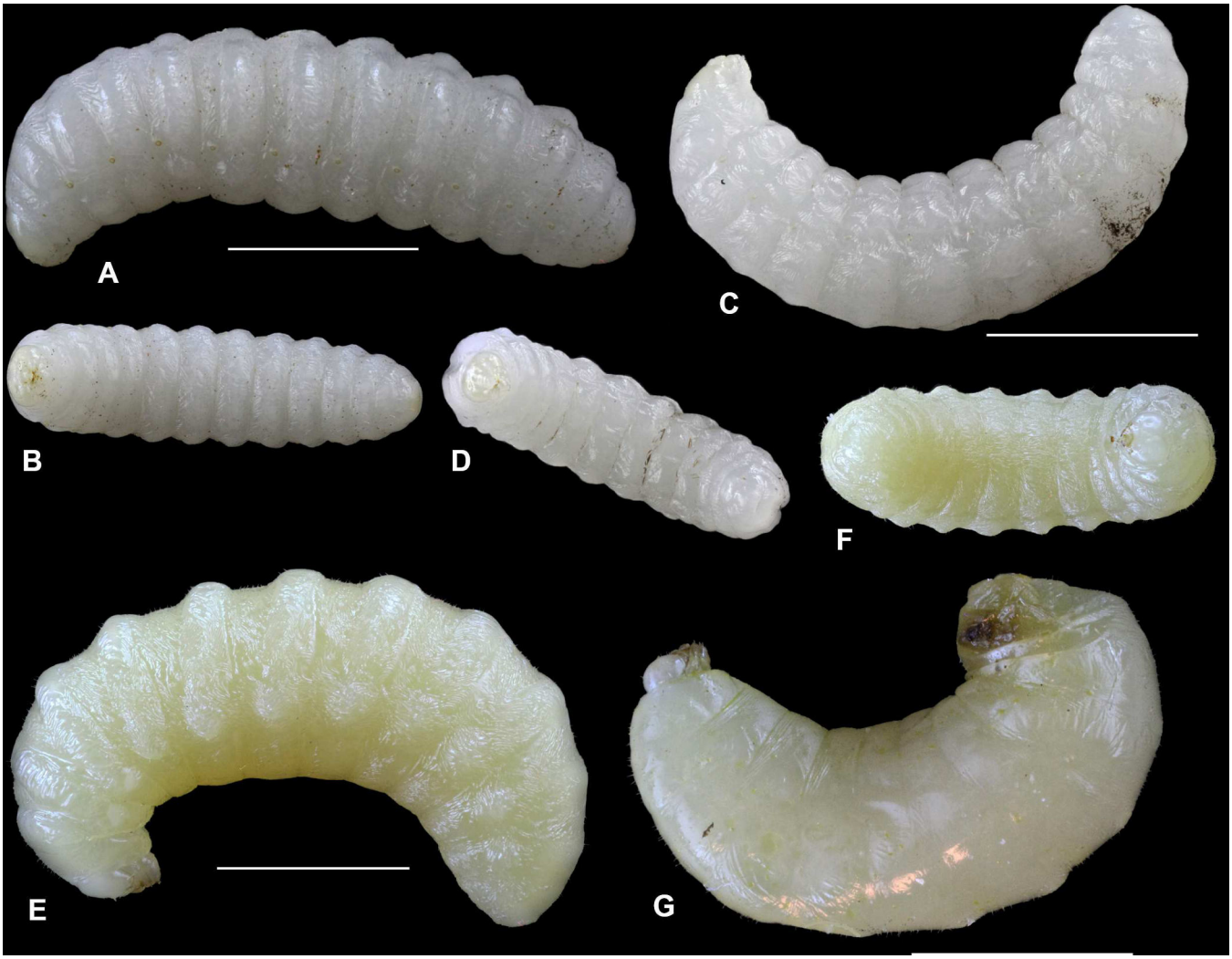

Fig 6. Larvae of aculeate Hymenoptera in reed galls. A–B–*Hylaeus confusus*, lateral and ventral view; C–D–*Hylaeus moricei*; E–F–*Heriades rubicola*; G–*Stelis breviuscula*. Measurements show 2 mm.

doi:10.1371/journal.pone.0169592.g006

well developed and forming a line. Not dorsoventrally flattened. Last abdominal segmen-trounded, very short, and narrower than other segments. Anus terminal is a transverse slit. Integument almost smooth, bearing only a few short setae. Spiracles well sclerotized, brownish; atrium has a very wide margin (more than half the width of the pore), short, with only one septum.

Head and mouthparts: Head well visible, more than half the width of the abdominal segments. Rounded, with typical frontoclypeal suture in the middle, width 0.91 mm, height 1.15 mm, width:height ratio < 1. Pale and unpigmented except for brownish marking on the following structures: antennal orbits, frontoclypeal suture, anterior and posterior tentorial arms, labrum, teeth and joints of mandibles, some parts of the maxillae, and labium. Antennal orbits clearly visible, with four very small sensory cones in the membrane. Head with punctures bearing setae, mostly on vertex, above antennal orbits and above joints of mandibles. Clypeus and labrum not visibly separated, clypeus less sclerotized, on apical part of clypeus 12 setae (six on each side). Labrum longer than clypeus, sclerotized especially on the apical part, with slight

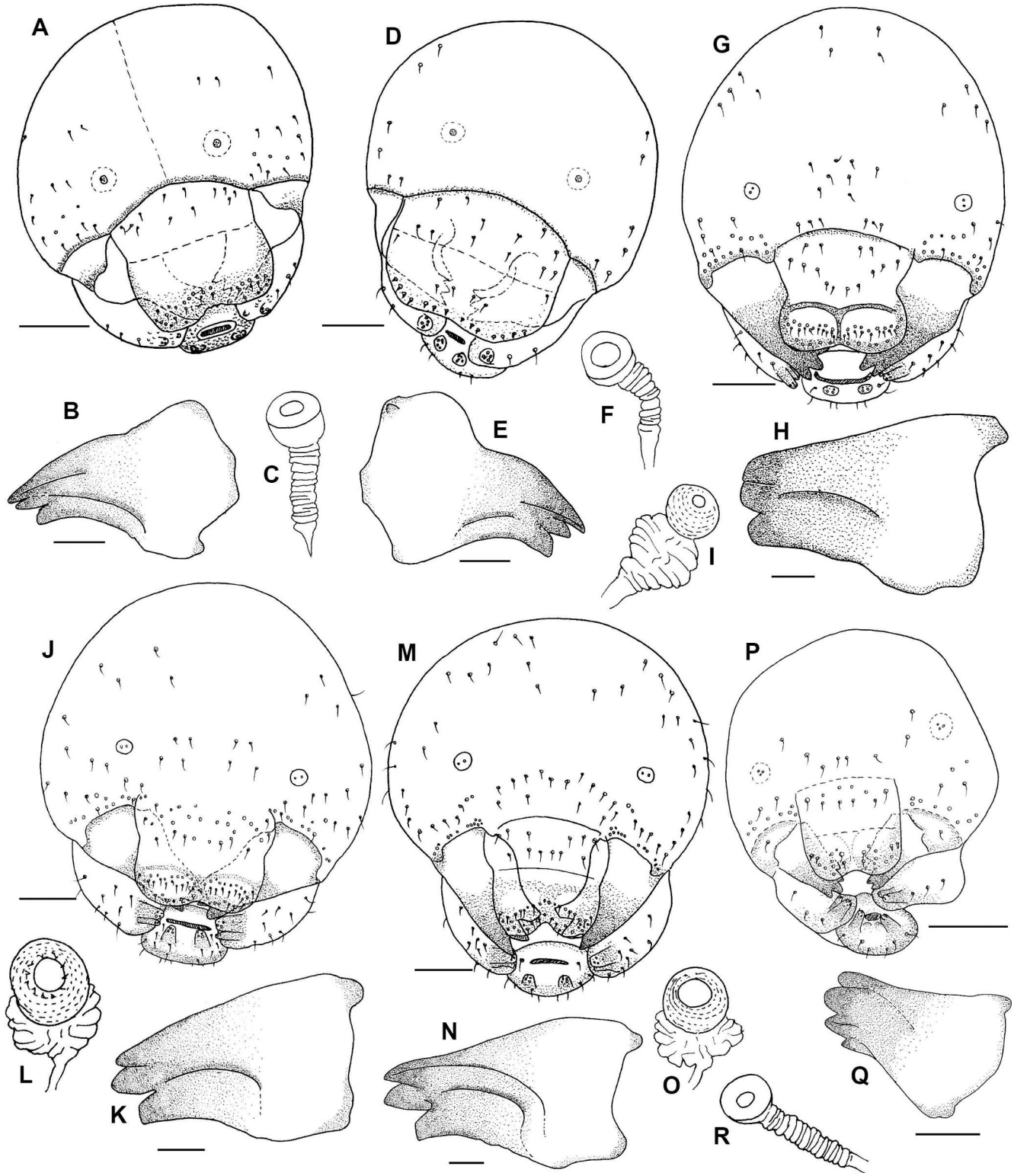

Fig 7. Morphology of larvae of aculeate Hymenoptera in reed galls. A–C–*Chrysis angustula*; D–F–*Chrysis rutilans*; G–I–*Symmorphus bifasciatus*; J–L–*Stenodynerus clypeopictus*; M–O–*Stenodynerus chevrieranus*; P–R–*Passaloecus clypealis*, all species head capsule frontal view, mandible lateral view, spiracle. Measurements show 0,2 mm in drawings of heads and 0,1 mm in drawings of mandibles.

doi:10.1371/journal.pone.0169592.g007

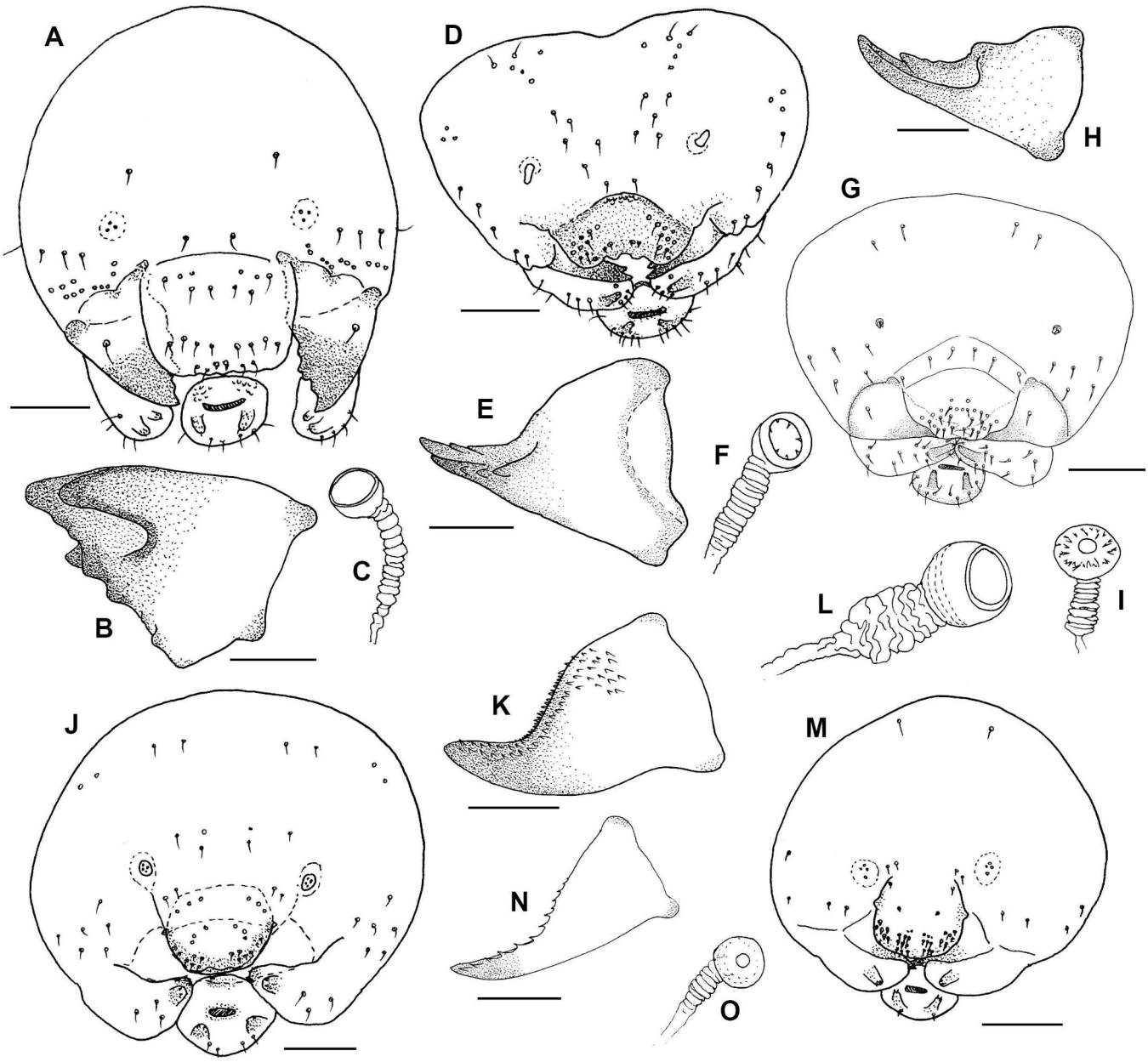

Fig 8. Morphology of larvae of aculeate Hymenoptera in reed galls. A–C–*Trypoxylon minus*; D–F–*Heriades rubicola*; G–I–*Stelis breviuscula*; J–L–*Hylaeus confusus*; M–O–*Hylaeus moricei*, all species head capsule frontal view, mandible lateral view, spiracle. Measurements show 0,2 mm in drawings of heads and 0,1 mm in drawings of mandibles.

doi:10.1371/journal.pone.0169592.g008

emargination in the middle, with 16 sensillae on each side, five of them bearing setae. Sensillae located near apical lobes of labrum but mostly on the sides. Mandible (length 0.41 mm) with at least three teeth; apical tooth is longer and sharper than the other teeth. Maxillae with four conspicuous setae on each side. Galea short but easily visible, sclerotized, with five sensillae, one of them elongated. Maxillar palpus short and smaller than the galea, with two short sensillae at the distal end. Labium rugous has a wide salivary slit. Labial palpus sclerotized and short, with four conical sensillae and one elongated sensilla.

***Chrysis rutilans* (Figs 5A, 5B and 7D–7F).** Mature larvae of this species have not been previously described.

Material: Hungary centr., Kiskunság National Park, Orgovány env., terrestrial reed bed, 24.ii.2015, 2 larvae; Dunatetétlen env., Bödi-Szék, salt marsh and terrestrial reed bed, 24.ii.2015, 2 larvae; Hungary mer., Kiskunság National Park, Sándorfalva env., terrestrial reed bed, 25.ii.2015, 1 larva, all P. Bogusch et P. Heneberg lgt., all P. Bogusch det. (coll. P. Bogusch).

Body: Short and robust, widest in the hind part, white colored, length 5.72 ± 0.59 mm; width 2.12 ± 0.04 mm (n = 5). Fusiform in shape with well-developed dorsal posterior lobes reaching pleurae of segments. Pleural lobes well developed and forming a line. Not dorsoventrally flattened. Last abdominal segment very short, rounded, and narrower than other segments. Anus terminal is a transverse slit. Integument almost smooth, with only a few short setae. Spiracles sclerotized, brownish, with a wide margin (margin wider than half the width of the atrium). Atrium with one septum, shorter than wide.

Head and mouthparts: Head large but slightly narrower than thorax, longer than wide: width 1.00 mm, height 1.17 mm, width:height ratio < 1. Frontoclypeal suture in the middle. Most of the head pale and unpigmented except for brownish markings on the following structures: frontoclypeal suture, clypeus (only very slightly), labrum, teeth and joints of the mandibles, apex of the maxillae and labium. Antennal orbits small, unsclerotized, with three sensory cones in the membrane. Six setae on each side of the head, most of them above the joints of the mandibles. Clypeus two and a half times longer than it is wide, with two setae on the apex and four setae in the middle on each side. Labrum shaped like a rounded rectangle, slightly sclerotized, with only slight serrations on the apical margin, without setae and sensillae. Five prominent setae on each side of labrum and a row of small sensillae posterior to them. Mandible (length 0.38 mm) has three teeth, with the ones on the outer side being a bit longer and sharper. Maxillae blunt, poorly sclerotized at the ends, with three conspicuous setae on each side. Galea short but easily visible, with four short sensillae and one elongated sensilla. Maxillar palpus poorly visible with one large and one small sensilla. Labium contained a wide salivary slit, with rough structures at the end and three setae on each side. Labial palpi have four conical sensillae and one elongated sensilla. Hypopharynx easily visible, not serrated.

***Stenodynerus chevrieranus* (Figs 5E, 5F and 7M–7O).** Mature larvae of this species have not been previously described.

Material: Hungary occ., Fonyód env., reed bed near Lake Balaton, 25.ii.2015, 2 larvae, P. Bogusch et P. Heneberg lgt., P. Bogusch det. (coll. P. Bogusch).

Body: Length 7.7–7.8 mm; width 2.0–2.1 mm (n = 2). Yellow or yellowish in color. Posterior parts of segments have distinct lobes, pleural lobes also well developed, pleural lobes less distinct on the prothorax and mesothorax and most distinct on the central abdominal segments. Last abdominal segment larger and knob-shaped, with a transverse slit anus. Integument forms a fine cuticle with smooth parts, every segment bears tens of small sensillae. Spiracles well developed, funnel shaped, with a very narrow margin around the atrium, in six unbroken lines.

Head and mouthparts: Head rounded with a suture in the middle of the vertex, suture failed to reach the clypeus. Head longer than wide, width 1.09–1.1 mm, height 1.22–1.25 mm, width:height ratio < 1. Head pale and unpigmented except for brownish markings on the following structures: antennal orbits, apical part of clypeus, labrum, mandibles, maxillae, galeae, and labium. Antennal orbits large and rounded with three sensillae. Head has punctures bearing setae, mostly on the frons and above mandibular joints. Three groups of small sensillae (3 + 3 + 7) are present, but only on the mandibular joints. Clypeus wider than long, apical part sclerotized and rugous with six sensillae bearing setae on each side. Labrum sclerotized, its margin has two lobes on each side, a depression in the middle with numerous sensillae. Labrum has

more than 15 sensillae bearing setae on each side. Epipharynx with small teeth. Mandible (length 0.49 mm) with three large teeth of similar size, inner tooth shorter, blunt, and quadratic. Maxillae slightly sclerotized with nine interspersed conspicuous setae. Galea elongated, well sclerotized, brownish, and with three conical sensillae and one elongated sensilla in the apical part. Maxillar palpus wider than galea, narrowing sharply toward the apex, with two small sensillae at the apex. Labium sclerotized and curved, wide salivary slit, and five setae on each side. Labial palpus short, cylindrical, with three conical sensillae and one elongated sensilla.

***Stenodynerus clypeopictus* (Figs 5G, 5H, 7J and 7K).** Mature larvae of this species have not been previously described.

Material: Hungary centr., Kiskunság National Park, Orgovány env., terrestrial reed bed, 24.ii.2015, 7 larvae; Dunatetétlen env., Bödi-Szék, salt marsh and terrestrial reed bed, 24.ii.2015, 7 larvae; Hungary mer., Kiskunság National Park, Sándorfalva env., terrestrial reed bed, 25.ii.2015, 1 larva, all P. Bogusch et P. Heneberg lgt., all P. Bogusch det. (coll. P. Bogusch).

Body: Length 9.57 ± 1.27 mm; width 2.22 ± 0.19 mm (n = 15). Color yellow or yellowish but in some cases also white or whitish. Elongated, slim, dorsoventrally flattened body. Posterior parts of the segments on the dorsal part form distinct lobes, pleural lobes also well developed, pleural lobes less developed on the prothorax and mesothorax and largest on the central abdominal segments. The last abdominal segment rounded with a transverse slit anus. Integument smooth with small inconspicuous setae.

Head and mouthparts: Head smallish, only slightly wider than half the width of the thoracic segments, width 1.22 mm, height 1.38 mm, width:height ratio < 1. Head has a suture on the vertex, which failed to reach the clypeal margin. Head pale and unpigmented except for brownish markings on the following structures: antennal orbits, clypeus and labrum (only very slightly), teeth and joints of mandibles, maxillae, galeae, maxillar palpi, and labium. Antennal orbits flat with three sensory cones in the membrane. Head has many punctures bearing setae, mostly on the vertex, around antennal orbits, and near joints of the mandibles. Clypeus short, length:width ratio 1:4, the front part straight, not sclerotized, bearing five setae and nine sensillae on each side. Labrum with two lobes slightly raised, depression in the middle, 12 setae and at least 18 sensillae on each side. Epipharynx has numerous tooth-like processes. Mandible (length 0.50 mm) with three conspicuous rounded teeth. Maxilla rounded, slightly sclerotized, with nine setae on each side. Galea conspicuous and sclerotized, with three conical sensillae and one elongated sensilla. Maxillar palpus narrowed towards the end, longer than galea, with two sensillae at the end. Labium with wide salivary slit (width almost equal to the whole labium), with ten setae on each side. Labial palpus bearing threelarge and one smaller sensillae.

Remarks: Spiracles very similar to those of *S. chevrieranus*.

***Symmorphus bifasciatus* (Figs 5C, 5D and 7G–7I).** Mature larvae of this species have not been previously described.

Material: Czech Republic, Bohemia centr., Mělník env., terrestrial reed bed surrounding complex of abandoned sandpits, 22.ix.2015, 3 larvae, P. Bogusch et A. Astapenková lgt.; Bohemia or., Rzy, terrestrial reed bed surrounding fishpond, 31.xii.2015, 2 larvae, P. Bogusch lgt., all P. Bogusch det. (coll. P. Bogusch).

Body: Length 7.9–8.1 mm; width 2 mm (n = 2). Yellowish in color. Posterior parts of the dorsal segments form distinct lobes, pleural lobes also well developed, pleural lobes less developed on the prothorax and mesothorax and largest on the central abdominal segments. All lobes much less distinct than in both *Stenodynerus* species. The last abdominal segment knob-like with a transverse slit anus. Integument rough, with many very small spinules (well visible under 400× magnification). Spiracles easily visible, brownish, with a round atrium. Atrial margin very thin, with seven lines.

Head and mouthparts: Head easily visible, narrower than thorax, width 1.10 mm, height 1.32 mm, width:height ratio < 1. Head with suture on the vertex towards the frons, but not reaching the clypeal margin. Head pale and unpigmented except for brownish markings on the following structures: antennal orbits, clypeus, labrum, teeth and joints of mandibles, maxillae, and labium. Large antennal orbits, with two sensillae. Head with many pits bearing setae, mostly on the vertex, above the clypeus and joints of the mandibles. Clypeus long, sclerotized, but only at the base and at the end; the basal clypeal suture well developed. Eight setae on each side of the clypeus. Labrum shorter than clypeus, with a depression in the middle full of setae and sensillae. Apical margin of labrum and central part is sclerotized. Labrum with two main lines of setae and sensillae, several sensillae also located near the apical margin (altogether ten setae and 15 sensillae). Epipharynx with tooth-like processes. Mandible (length 0.49 mm) straight, with three teeth; the inner tooth shorter. Maxilla blunt, with eight setae on a side, slightly sclerotized. Galea elongated, easily visible, sclerotized with three conical sensillae and one elongated sensilla at the end. Maxillar palpus is elongated, sharply narrowing toward the end, with two sensory cones in the membrane. Labium rugous and slightly sclerotized, with a wide salivary slit, and with six conspicuous setae on each side. Labial palpus short, with three conical sensillae and one elongated sensilla.

***Passaloecus clypealis* (Figs 5I, 5J and 7P–7R).** Mature larvae of this species were described by [20] but only very briefly, with no comments on the head and mouthparts.

Material: Czech Republic, Bohemia occ., Soseň env., Plaviště Nature Monument, reed bed at fishpond, 10 larvae; Bohemia mer., Třeboňsko Protected Landscape Area, Smržov env., loose reeds at the dam on the Smržovský Dolní rybník, 4 larvae, all from nests in reed galls installed artificially and exposed between 25.v.-30.vi.2015, P. Heneberg lgt.; Bohemia or., Železné hory Protected Landscape Area, Strádovka Nature Reserve, terrestrial reed bed from a fishpond, from nests in reed galls installed artificially and exposed between 20.v.-15.viii.2015, 4 larvae, P. Bogusch lgt., all P. Bogusch det. (coll. P. Bogusch).

Body: Length 5.32 ± 0.40 mm; width 1.00 ± 0.14 mm (n = 28). Body color pale yellowish to yellow-ochre in darker specimens. Head quite large, almost as wide as the body. The posterior parts of the segments form distinct lobes on the dorsal part, pleural lobes well developed, less developed on the prothorax and mesothorax and largest on the central abdominal segments. Bbody slightly flattened dorsoventrally. The last abdominal segment knob-like. Integument rugose with sparse but large setae. Spiracles clearly visible, small, funnel-shaped, with very a small atrium, the atrial margin thicker than the atrial pore.

Head and mouthparts: Head rounded, without any suture, width 0.76 mm, height 0.78 mm, width:height ratio > 1. Head pale and unpigmented except for brownish markings on the following structures: at the end of the clypeus, labrum, mandibles, maxillae, and labium. Small antennal orbits, with three sensory cones in the membrane. Head has many pits bearing setae, mostly above the mandibular joints and on the frons. A group of eight sensillae located just above the mandibular joints. Clypeus quadratic and sclerotized at the end and two times wider than it is long; each side has two conspicuous setae and three sensillae. Labrum sclerotized with very slight emargination; each side has five large setae and seven sensillae. Mandible (length 0.29 mm) very sclerotized, with four large teeth. Maxilla sclerotized, with three big setae on each side. Galea elongated with three elongated sensory cones and one conical sensilla at the end. Maxillar palpus elongated with three elongated sensory cones at the end. Labium sclerotized, with a narrow salivary slit; each side has three large setae. Labial palpus elongated, with three elongated sensillae apically.

***Trypoxylon minus* (Figs 5K, 5L and 8A–8C).** Mature larvae of this species have not been described previously.

Material: Hungary bor., Pákozd env., Velencei-tó lake, terrestrial reed bed, 23.ii.2015, 3 larvae; Hungary mer., Kiskunság National Park, Munkastelep, terrestrial reed bed, 25.ii.2015, 2 larvae, all P. Bogusch et P. Heneberg lgt.; Poland bor., Lunowo, terrestrial reed bed, 30.i.2015, 3 larvae; Troszyn env., terrestrial reed bed, 30.i.2015, 3 larvae; Slowinski National Park, Rowy, terrestrial reed bed near the sea, 01.ii.2015, 4 larvae; Slowinski National Park, Gardna Wielka, terrestrial reed bed at Gardno lake, 01.ii.2015, 2 larvae; Jastarnia, terrestrial reed bed at Hel peninsula, 02.ii.2015, 2 larvae, all P. Bogusch et P. Heneberg lgt.; Czech Republic; Bohemia bor., Zahrádky, Novozámecký rybník National Natural Reserve, terrestrial reed bed surrounding a fishpond, 08.iii.2015, 17 larvae; Jestřebí env., Jestřebské slatiny National Natural Monument, terrestrial reed beds surrounding streams, 08.iii.2015, 4 larvae; Doksy env., Břehyně-Pecopala National Natural Reserve, terrestrial reed bed surrounding a fishpond, 08.iii.2015, 22 larvae; Doksy, Swamp National Natural Monument, peat bog and terrestrial reed bed, 08.iii.2015, 1 larva, all P. Bogusch et A. Astapenková lgt., all P. Bogusch det. (coll. P. Bogusch).

Body: Length 7.17 ± 0.28 mm; width 1.59 ± 0.28 mm (n = 11). Whitish, white or pale ochre in color. Body elongated, slim, and dorsoventrally flattened. Posterior parts of segments have distinct lobes; pleural lobes well developed, lobes are less developed on prothorax and most distinct and widest on last few abdominal segments. Last abdominal segment small and narrower than the head. Integument forms a fine skin with many large setae and small spinulae between them. Spiracles pale brown, atrium very faintly marked with five lines and the subatrium unarmed.

Head and mouthparts: Head width 0.89 mm, height 0.99 mm, width:height ratio < 1. Head pale and unpigmented except for brownish markings on the following structures: antennal orbits, mandibles and joints, galeae slightly pigmented, maxillar and labial palpi. Antennal orbits contain three sensory cones in the membrane. Head has many pits bearing setae, mostly on the vertex, above the clypeus, and above the mandibles; one seta also on the mandible. Clypeus only very slightly sclerotized, wide, with six conspicuous setae in one line, and six sensillae close to the base of the clypeus. Labrum slightly sclerotized, narrower than the clypeus, square-shaped, end rugous; there are nine conspicuous setae on each side. Apical margin of labrum has slight emargination in the middle. Epipharynx has many tooth-like processes laterally. Mandible (length 0.34 mm) has five small teeth and three tooth-like processes laterally. Maxilla slightly sclerotized with six conspicuous setae on each side. Galea elongated with three conical sensillae and one elongated sensilla at the end. Maxillar palpus elongated, with two elongated sensillae. Labium has a wide salivary slit, its width more than half the width of the labium, with three large setae on each side. Labial palpus elongated, with two small sensillae and one elongated sensilla at the end. Hypopharynx clearly visible and conspicuously serrated.

Heriades rubicola **(Figs 6E, 6F and 8D–8F).** Mature larvae of this small species have not been previously described.

Material: Hungary bor. occ., Pákozd env., Velencei-tó lake env., terrestrial reed bed, 23.ii.2015, 3 larvae; Hungary centr., Kiskunság National Park, Izsák, Kolón-tó lake, reed bed at meadow, 24.ii.2015, 5 larvae; Kiskunság National Park, Orgovány env., terrestrial reed bed, 24.ii.2015, 20 larvae; Dunatetétlen env., Bödi-Szék, salt marsh and terrestrial reed bed, 24.ii.2015, 4 larvae; Hungary mer., Kiskunság National Park, Munkastelep env., terrestrial reed bed at saline lake, 25.ii.2015, 48 larvae; Slovenia mer., Portorož env., terrestrial reed bed near sea, 26.ii.2015, 4 larvae, P. Bogusch et P. Heneberg lgt., all P. Bogusch det. (coll. P. Bogusch).

Body: Length 5.65 ± 0.44 mm; width 1.45 ± 0.11 mm (n = 25). Color white or whitish, in some cases very slightly yellow or ochre colored. Head very small, about half of the width of the abdominal segments. Slightly dorsoventrally flattened. Posterior parts of segments form transverse welts on the dorsal part, but very poorly formed and poorly separated from other parts of the segment. Last abdominal segments rounded, narrower than other segments, and

bear a transverse anus. Conspicuous, large setae on the body; more so on abdominal segments. Integument with small dense setae. Spiracles normal, atrium armed with quite a thick margin, with at least seven lines, lines not disrupted.

Head and mouthparts: Head easily visible but very small (usually smaller than half the width of the thoracic and abdominal segments) and heart-shaped, width 0.99 mm, height 0.81 mm, width:height ratio > 1. Pale and unpigmented except for brownish markings on the following structures: anterior and posterior tentorial arms, labrum, mandibles and their condyli, maxillar and labial palpi. Antennal orbits elongated along the entire segment, with two short setae at the end. Head with many pits bearing stout setae, mostly on sides and on frons above the clypeus. Slight depression in the middle of head, reaching anterior clypeal margin. Clypeus poorly visible, not well separated from other head parts, with six setae. Labrum trapezoidal and slightly sclerotized, its margin has a lobe on each side, another small lobe on each side towards the middle, as well as a small depression in the middle. Rugous structure in the middle at the base of labrum, and five setae and eight sensillae on each side of the labrum. Mandible (length 0.30 mm) sharply narrowed with three teeth, two apical teeth are blunt and the one on the outer side somewhat longer (about one width of tooth apex) than the second, the third tooth small and lateral. Mandibles have a sclerotized apex with teeth, joints and basal edge. Maxillae blunt, with many prominent setae on each side: three at the base of the galea, four around the galea, and three at the end of the maxilla. Galea elongated with two elongated sensillae at the end and one small conical sensilla. Maxillar palpus short and rudimentary. Labium with rugous structure on apical part, wide transverse salivary slit. Seven conspicuous setae on each side of labium. Labial palpus with two elongated sensillae and one small conical sensilla at the end.

***Stelis breviuscula* (Figs 6G and 8G–8I).** Mature larvae of this species were only briefly described by [21].

Material: Slovakia mer., Virt and Marcelová env., terrestrial reed bed at Patinský kanál, 20. i.2016, 3 larvae, P. Bogusch lgt., all P. Bogusch det. (coll. P. Bogusch).

Body: Length 5.11 ± 0.17 mm; width 1.42 ± 0.12 mm (n = 3). Color white or whitish, some parts of body (anus and head) light brown. Head very small, about half the width of the abdominal segments. Slightly dorsoventrally flattened. Posterior parts of segments form transverse welts on the dorsal side, but very poorly formed and poorly separated from other segment parts. Last abdominal segment rounded and somewhat narrower than other segments, with a transverse anus. Large conspicuous setae on body, more so on abdominal segments. Spiracles with wide margin, serrated, atrium thick-walled, with at least five septa.

Head and mouthparts: Head heart-shaped, clearly visible although very small, width 0.7 mm, height 0.61 mm, width:height ratio > 1. Pale and unpigmented except for brownish markings on the following structures: antennal orbits, labrum, mandibles with joints, maxillae with palpus and galea, labium with palpus. Very small, elongated antennal orbits with two elongated setae. Head has many pits bearing setae, mostly on sides above mandibular joints and on the vertex. Clypeus trapezoidal, slightly sclerotized, with four setae on each side of the apical part. Labrum slightly sclerotized, with lobes on each side, and a small depression in the middle. Rugous structure in the middle at the base of the labrum, seven thick setae and six sensillae on each side. Mandible (length 0.29 mm) has two teeth, one tooth shorter than the other. One prominent seta near the base of the mandible. Maxilla has tubular and elongated galea, with three sensillae at the end. Maxillar palpi elongated, narrower than the galea, with two sensillae at the end. Maxilla with at least ten big setae on each side, three of them located near the apex in front of galea. Labium small, with five setae on each side, and a wide salivary slit. Labial palpi elongated, with three sensillae at the end.

***Hylaeus confusus* (Figs 6A, 6B and 8J–8L).** Mature larvae of this species were previously described by [22] but the description lacked some important characteristics, e.g., the chaetotaxy.

Material: Hungary bor., Pákozd env., Velencei-tó lake env., terrestrial reed bed, 23.ii.2015, 3 larvae; Hungary occ., Fonyód env., terrestrial reed bed near Lake Balaton, 25.ii.2015, 2 larvae, all P. Bogusch et P. Heneberg lgt., all P. Bogusch det. (coll. P. Bogusch).

Body: Length 7.75 ± 0.05 mm; width 1.95 ± 0.05 mm (n = 5). Color white or whitish. Head medium large, only slightly narrower than the rest of the body. Only slightly dorsoventrally flattened. Posterior parts of segments have transverse welts on dorsal side, but they are very poorly formed and poorly separated from other segment parts. Last abdominal segment very short. Integument smooth with very few setae and sensillae. Spiracles yellowish, large, round, atrium wide, with only a thin margin, with more than seven lines.

Head and mouthparts: Head easily visible, width 1.18 mm, height 1.08 mm, width:height ratio $>$ 1. Pale and unpigmented except for brownish markings on the following structures: antennal orbits, lateral clypeal teeth, apex of labrum, mandibles and their condyli, apical part of maxillae, galeae, labial palpi. Antennal orbits without arms and have three sensory cones in the membrane. Head has several setae, most of them on the sides above mandibular joints. Clypeus ribbed with one sclerotized tooth on each side, also three sensillae on each side. Labrum rugous, has apical margin with two very slight lobes on each side, and no emargination in the middle. Labrum with three setae on each side, with at least 16 short, stiff setae below them towards the apical part of labrum. Mandible (length 0.32 mm) with one tooth. Mandibles without joints, have sclerotized apex with tooth. Maxilla has three setae on each side, maxillar palpus large, elongated, with three sensory cones at the end. Labium with narrow salivary slit and two setae on each side. Labial palpus has two sensillae at the end. Hypopharynx rough and easily visible.

***Hylaeus moricei* (Figs 6C, 6D and 8M–8O).** Mature larva of this species have not been previously described, even though [22] described the nests and larvae of most of the central European species in this genus.

Material: Czech Republic, Bohemia bor. or., Zlíč env., Dubno Natural Reserve, terrestrial reed bed surroundings, 15.i.2015, 6 larvae; Bohemia bor., Zahrádky, Novozámecký rybník National Natural Reserve, terrestrial reed bed surrounding fishpond, 08.iii.2015, 3 larvae, all P. Bogusch et A. Astapenková lgt., P. Bogusch det. (coll. P. Bogusch).

Body: Length 5.46 ± 0.41 mm; width 1.26 ± 0.20 mm (n = 3). Color white or whitish. Head medium large, slightly narrower than the rest of the body. Only slightly dorsoventrally flattened. Posterior parts of segments form transverse welts on dorsal side, but very poorly formed and poorly separated from other parts of the segment. Last abdominal segment rounded. Integument smooth with only a few short setae and sensillae. Spiracles normal, atrium funnel-shaped, with a very narrow margin, with eight lines.

Head and mouthparts: Head easily visible, rounded, medium large, width 0.87 mm, height 0.79 mm, width:height ratio $>$ 1. Pale and unpigmented except for brownish markings on the mandibles, mostly at the apex and condyli. Antennal orbits large, larger than two thirds of the length of the whole mandible, with three sensory cones in the membrane. Head almost without setae. Clypeus poorly visible, rectangular, quite narrow, with four small setae. Labrum very narrow and only slightly sclerotized, rounded without a depression in the middle. Apical part of labrum has large conspicuous setae (at least ten setae) and some sensillae. Mandible (length 0.32 mm) with one tooth that neither sharp nor blunt, and with eight small teeth on the inner side. Mandibles sclerotized only at the apex, joints sclerotized only very slightly and not darkened. Maxillae rounded at the ends with no setae, with six sensillae at each end. Galea well developed but not sclerotized, with two elongated sensillae and one small conical sensilla at the

end. Maxillar palpus poorly visible and rudimentary. Labium has narrow salivary slit and wide blunt palpi, with four small setae on each side of the labium. Labial palpus has two elongated sensillae and one small conical sensilla at the end.

Discussion

The total number of species recorded nesting in abandoned reed galls was surprisingly high and shows that these specific shelters are being used frequently by several, and occasionally by some species of aculeate Hymenoptera. These reed galls host not only nesting hymenopterans, but also insects and invertebrates of other groups, e.g., spiders, beetles, etc. Bogusch et al. [23] examined these inhabitants and showed that most of them use reed galls as a hiding or overwintering place, and some of these species are very rare across Europe. Reed galls host species bound to reed beds with long historical continuity as well as pioneer species. Among the Aculeata recorded in reed galls, several records were, in our opinion, very questionable. First was *Stenodynerus xanthomelas*, which was recorded by [12]. This species occurs in dry steppe habitats and is, in appearance, very similar to *S. clypeopictus*, which was first recorded as nesting in reed galls in this study. We believe that the author misidentified the species. Additionally, the records of *Pemphredon* spp. except for *P. fabricii* by [15] are also questionable. During four years of comprehensive studies of reed galls, we have checked more than 5,000 individuals of *Pemphredon* from reed galls and all were represented by only a single species–*P. fabricii*. Though the identification of species within the genus *Pemphredon* is not trivial [24–25], we assume that records of other species from this genus nesting in reed galls are based on incorrect identifications made by non-specialists. A similar situation probably exists with species of the genus *Passaloecus* other than *P. clypealis*. Even *P. clypealis* is quite a rare species, inhabiting reed galls only sporadically. Also doubtful are the records of two species of leafcutter bees (*Megachile centuncularis* and *M. versicolor*) published by [15]. Both of these species are very common and they use various cavities for their nesting. We have recorded nests of *M. versicolor* between reed stalks at an artificially made nest site (P. Bogusch, pers. obs.), but we did not find any nests of these species in reed galls examined in this study, nor in those examined previously. Finally, some species have been published under their synonyms. This situation is common especially for the genera *Pemphredon* and *Trypoxylon*, whose taxonomy has only very recently been elucidated. Records of *Trypoxylon figulus* and *T. attenuatum* almost certainly also represent *T. minus* and *T. deceptorium* (described by [26] and [27], respectively) and those of *Pemphredon lethifer* are represented by *P. fabricii* (resurrected from the synonymy by [25]).

Aculeate Hymenoptera are unique in using reed galls not only as a shelter, but also as a nesting place. Even though the galls do not have clearly visible holes in them, several species are able to access the inside through the top of the gall and make their nest inside. It is interesting that most such species use small-size prey or pollen, and nectar for their brood, and species using bigger prey usually do not use reed galls for nesting. This proposal is supported by [5], who showed that several very common reed bed species (frequently being captured in color pan traps) have never been recorded in reed galls within the reed bed sites where they occur. *Anoplius caviventris*, which catches big spiders of the genus *Clubiona*, and *Gymnomerus laevipes*, which hunts chrysomelid larvae, are probably unable to get inside the gall with these larger prey; as a result they more frequently use reed stalks, with larger openings, for nesting.

Several species nesting in reed galls are very abundant when appropriate nesting resources are available. In particular, *Pemphredon fabricii*, and also *Heriades rubicola* in southern parts of central Europe, can be found in one out of every two or every three checked galls. It remains to be determined, whether these species are specialized for nesting in reed galls or prefer reed galls over other cavities like reed stalks or plant stems. Some species, such as *Hylaeus pectoralis*

or *Stenodynerus clypeopictus*, are much less common in reed galls. However, these species are also very rare in general, and form only limited populations in their habitats. These rare species are specialized to very specific habitats, such as reed beds connected with wet meadows with an abundance of flowering plants (*H. pectoralis*), and saline reed bed marshes (*S. clypeopictus*). They probably prefer reed galls for their nesting over other kinds of cavities, and *H. pectoralis* is usually classified in the literature as a specialist for nesting in reed galls [5–6, 12, 28] but with no evaluation or comparison to other cavity types.

The majority of the species being found in reed galls are cavity nesters with broad ecological preferences. These usually common species can be found nearly everywhere and in some cases are able to use reed galls for their nesting. Typical members of this group include *Trypoxylon minus*, *Symmorphus bifasciatus*, and *Hoplitis leucomelana*. These species are all very abundant in various habitats across Europe [3–4, 13]. The cuckoo wasp *Trichrysis cyanea* [5–6] and cuckoo bees of the genus *Stelis* (here recorded *S. ornatula* and *S. punctulatissima*) are their nest parasites. *Trichrysis cyanea* has a very broad ability to parasitize nests of many species. This species usually invades nests of wasps collecting spiders as prey for their larvae [3, 29]; however, we have occasionally found it in the nests of *Pemphredon fabricii*, provisioning its nests with aphids.

Numerous analyzed species displayed preferences for reed galls with specific parameters such as the width of the reed galls and reed stems. The widest galls were preferred by eudominant *P. fabricii*, but also by *Hylaeus pectoralis*, *Symmorphus bifasciatus*, and *Thyridanthrax fenestratus*. In contrast, narrower galls were more frequently occupied by *Passaloecus clypealis*, *Hylaeus moricei*, *Trypoxylon minus*, and *Stenodynerus clypeopictus* (which is not very surprising since they, especially the first two mentioned species, are very small). Importantly, the narrowest galls were not occupied by any hymenopteran species or were occupied by a negligible share of the eudominant species *P. fabricii*. This applied particularly to galls less than 5 mm in diameter (Fig 2A). However, species preferring large galls as well as those found in intermediate-sized galls were limited by the width of the galls as suggested by the correlation of gall width and the number of larvae present within the galls. Due to a difference in species-specific numbers of nests analyzed, this was especially prominent for the eudominant *P. fabricii*. However, the same relationship was also significant for less frequently examined species, such as *Trypoxylon deceptorium*, *Symmorphus bifasciatus*, and *Hoplitis leucomelana*, and even for the most common parasitoid, *Trichrysis cyanea* (Table 4). The analysis of reed stem width suggested that some species were strictly limited to thin stems. These included *Passaloecus clypealis*, *Trypoxylon minus*, and *Trypoxylon deceptorium*. Thus, these species probably represent those limited only to galls induced by *L. lucens* and *L. pulitarsis* [6, 30]. In contrast, species such as *Hylaeus moricei*, *Heriades rubicola*, *Hylaeus pectoralis*, *Symmorphus bifasciatus*, and *Thyridanthrax fenestratus* were found to be associated with larger stems, which typically occur in reed beds less stressed by drought or other factors. There were no species that preferred stems with widths $\geq$ 5 mm over other stem widths, despite galls on such stems being available; only *P. fabricii* and *Hylaeus pectoralis*, and even then only rarely, were found in galls on such stems (Fig 2B).

The length of the gall is also very important and different species settle the inner space of the gall in many ways. *Pemphredon fabricii* and *Heriades rubicola* make longest nests with higher number of brood cells than the other species, whilst both species very often extend their nest from the gall cavity into the soft innerspace among the leaves (the same was recorded several times also at *Hylaeus pectoralis*). In the contrary, *Trypoxylon minus* usually makes short nests with 1–2 brood cells even in very long and big galls. *Hylaeus pectoralis* often uses false brood cells as a defence against parasitoids. *Stenodynerus* spp. and *Trypoxylon* spp. prefer

broader cavities and make short nests and thus very often occupy galls used by *Pemphredon fabricii* with some innerspace left.

Most of the species nesting in reed galls have nests very similar to one another. They use mud or sand to create bars between brood cells and also to make the closing cork for the nest. This is typical for Crabronidae and Vespidae, but atypical for bees, which use plant matter for the same purpose. Related *Hylaeus* species had nests which, in general appearance, were very similar to that recorded for the genus *Hylaeus*: only nests of small species such as *H. moricei* differ in size from those of larger species such as *H. pectoralis* and *H. confusus* (which are very similar and for which species identification is not trivial). The same situation applies for both species of the genus *Trypoxylon*, *T. deceptorium*, and *T. minus*, for which no species-specific differences have been found. Nest structures seem to be autapomorphic for the particular examined families since very similar nests can be found among the species of the same family or subfamily. Both species of *Stenodynerus* have nests that are also very similar to *Symmorphus bifasciatus* (all species are classified within the family Vespidae). Also, *Pemphredon fabricii* and *Passaloecus clypealis* of the subfamily Pemphredoninae (family Crabronidae) have nests very similar to each other, differing (at first sight) only in the size of brood cells and larvae. Nest structures of species described in this study correspond with previous descriptions of nests of the same species (*Trypoxylon minus* by [6]; *Passaloecus clypealis* by [7]; *Hylaeus confusus* by [22, 29]; and *Symmorphus bifasciatus* by [6]) or with descriptions of nests of phylogenetically closely related species (*Symmorphus bifasciatus* to the nest of *Symmorphus mutinensis* described by [31], *Trypoxylon minus* to the nest of *T. deceptorium* described by [6, 32], and *Heriades rubicola* to the nest of *H. truncorum* described by [21]). Nests of both species of *Stenodynerus* and *Hylaeus moricei* were described for the first time in this study.

The mature larvae show many more differences than the nests, especially with regard to the structure of mouthparts. However, comparison with previous descriptions was very problematic. Most of the larvae described in our study have not been described previously; this was true for *Chrysis angustula*, *C. rutilans*, *Stenodynerus chevrieranus*, *S. clypeopictus*, *Symmorphus bifasciatus*, *Trypoxylon minus*, *Heriades rubicola*, and *Hylaeus moricei*. Larvae of these species possess typical characteristics that are seen in the larvae of the groups [7–8, 21, 22, 31, 33–34], but are easily distinguishable from related species. For example, the larva of *Heriades rubicola* have inner mandibular teeth moved slightly laterally and possess two rows of small lateral teeth on the mandibles, which differs from those of related species of this genus by the presence of these small lateral teeth (see [21] for comparison). Larvae of previously described species correspond with the descriptions but the comparison is very problematic due to the fact that the authors of previous descriptions did not study some important characteristics, i.e., chaetotaxy (case of *Hylaeus confusus* described previously by [22] or *Stelis breviuscula* by [21]).

Mature larvae of cuckoo wasps (Chrysididae) are short and thick in general appearance, with only weak chaetotaxy on the body surface. The last few abdominal segments are the widest of the whole body. They have round heads with a wide labrum and mandibles with three sharp teeth and a typical concavity on the outer part of the mandible. *Chrysis angustula* has this concavity, but it is much less pronounced in comparison with *C. rutilans*; it also has markedly more sclerotized mouthparts than *C. rutilans*. The size of the whole body, as well as the size of various parts of the body, are very similar in both species. Also, the larvae of Vespidae, as we have described them, have three teeth on their mandibles. *Symmorphus bifasciatus* is typified by sclerotization of the margin of the labrum; its mandibles have very blunt teeth. Both species of *Stenodynerus* have a slightly bilobed labrum. They can be distinguished from each other by the shape of the labral lobes–*S. chevrieranus* has much narrower labrum with well-developed lobes. Its mandibular teeth are much sharper than *S. clypeopictus*. Spiracles of all three species of Vespidae have short and wide subatriums and a cylindrical atrium with a

very narrow opening. A mature larva of *Passaloecus clypealis* is, in general appearance, very similar to larvae of *Pemphredon* and to larvae of other *Passaloecus* species described by [7]. This same author also provided a description of the larva of *P. clypealis*, but the description deals only with the general appearance of the body and does not comment on the mouthparts and head capsule, which are the most important characteristics. The mandible has four blunt teeth and the large maxillae and labium and clypeus are similar to those of larvae of *Pemphredon* and are typical features of larvae of *P. clypealis*. Larvae of other species of *Passaloecus*, as described by [7], also have four mandibular teeth but only one is apical. Only larvae of *Passaloecus corniger* and *Passaloecus eremita* have three apical teeth and one lateral, but *P. eremita* has very sharp, pincer-like teeth. Orientation of the teeth of larva of *P. corniger* is slightly different than that of the larva of *P. clypealis*. Larvae of *Trypoxylon* are typified by the lobes on body segments and also their mandibles have many small teeth [6, 32]. Bogusch et al. [6] found six teeth on the mandible of the larva of *T. deceptorium*, and five main teeth and three smaller, marginal teeth have been found on the mandible of larva of *T. minus*. The last segment of the body, which has the anus, has a very different shape in the two species [32]. *Heriades rubicola* has larvae characteristic of the family Megachilidae, with many setae and sensillae on the body surface, a small heart-shaped head, and mandibles with two teeth. Typical for this genus are mandibles that narrow sharply toward the apex [21], which are also present in the previously undescribed larva of *H. rubicola*. In contrast to other species of the genus, this species has an inner mandibular tooth placed more laterally and two rows of small tooth-like processes. Of interest is a similar difference in *Stelis breviuscula*, the only species of the genus that parasitizes the nests of *Heriades*. Species of the genus *Hylaeus* typically have nearly unsclerotized head and mandibles with only one tooth. Larvae of *H. confusus* typically have very blunt and robust mandibles with many small tooth-like processes on the inner side and our description corresponds with that given by [22]. The small larvae of *H. moricei* have sharp mandibles with only one row of very large tooth-like processes. It also differs with regard to the structure of the spiracle; the atrium is round and the opening is very narrow.

Despite the fact that reed beds are often subject to cutting, herbicide treatment, and complete eradication, the keystone species associated with such sites, frit flies *Lipara* spp., particularly *L. lucens*, use these sites as an important nesting resource, which allows for the survival of numerous rare species that are of interest from a conservation perspective. In this study we enlarged the list of aculeate hymenopterans associated with *Lipara*-induced reed galls to 36 nesting species, which are parasitized by another six species of aculeate parasitoids and three species of cuckoo bees. Species such as *Stenodynerus clypeopictus*, *Passaloecus clypealis*, *Rhopalum gracile*, *Hylaeus moricei*, and *Hylaeus pectoralis* can be used in nature conservation as diagnostic species relative to the quality of reed bed reservations. Prior to this study, knowledge of their nesting biology and larval morphology was almost completely absent. In this contribution, we attempted to fill in our knowledge gaps, thus providing an important tool for efficient conservation of the wetland habitats, facilitating future research, and allowing, for the first time, identification of the larvae of aculeate hymenopterans nesting in reed galls across multiple European countries.

Supporting Information

S1 Table. List of the localities with coordinates.
(XLSX)

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Formal analysis: PH.

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Investigation: AA PB PH.

Methodology: PH PB AA.

Project administration: PB PH.

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Visualization: PB.

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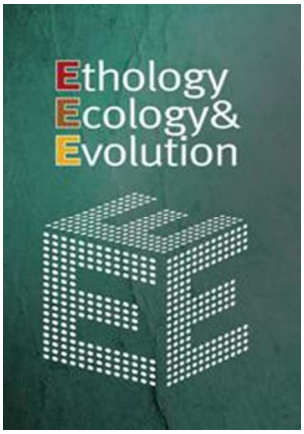
3.6 BOGUSCH P., HAVELKA J., ASTAPENKOVÁ A. & HENEBERG P. 2017: New type of progressive provisioning as a characteristic parental behavior of the crabronid wasp Pemphredon fabricii (Hymenoptera: Crabronidae). *Ethology*, *Ecology & Evolution*, accepted.



New type of progressive provisioning as a characteristic parental behavior of the crabronid wasp Pemphredon fabricii (Hymenoptera Crabronidae)

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Keywords:	nesting biology, Lipara, reed bed, aphids, phenology, predators and parasites

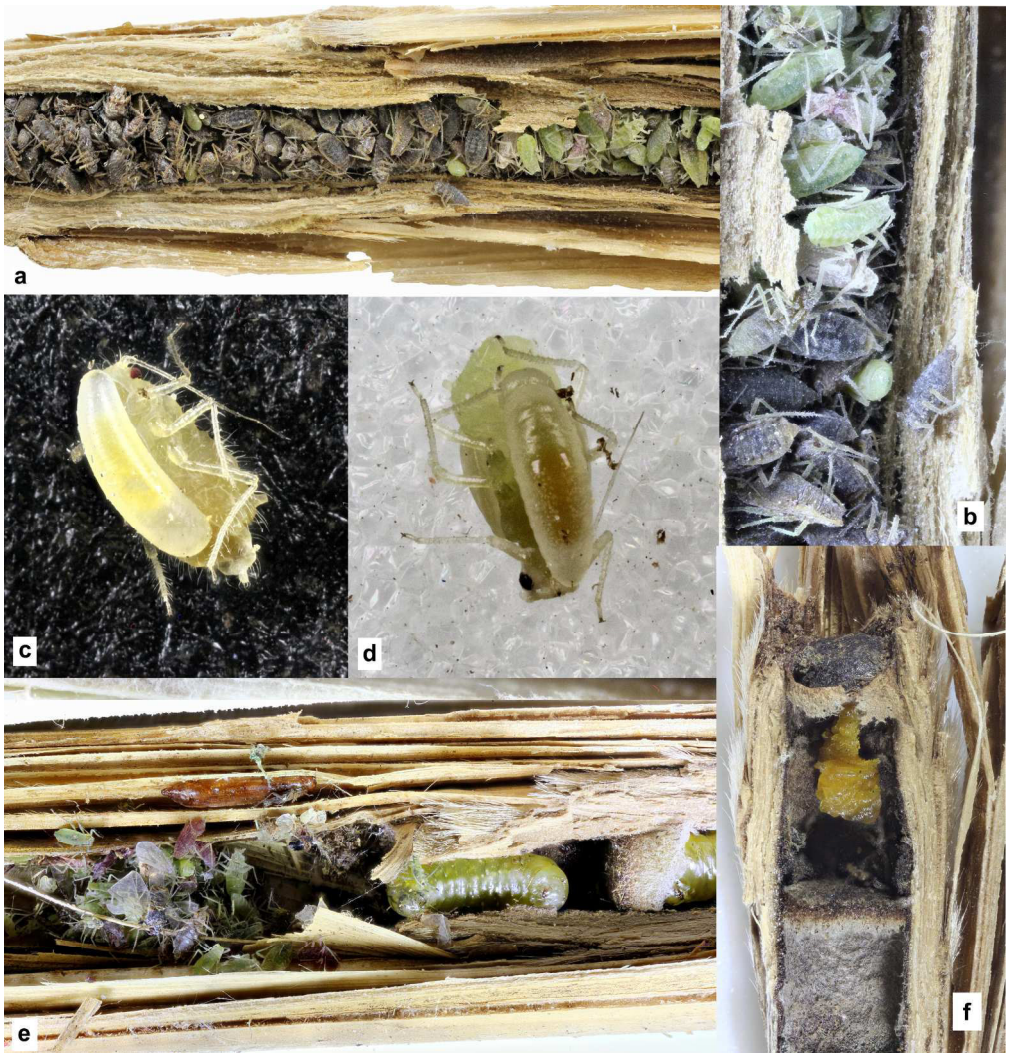

Fig. 2. – Nesting of Pemphredon fabricii. a) reed gall cavity filled with aphids, b) detail on the aphids in the cavity, c) egg on aphid, d) young larva of first instar on aphid, e) P. fabricii nest with mature larvae of which the smallest feed on aphids at the top of the nest, f) brood cells of P. fabricii destroyed by malachiid larvae with remnants of eaten larva of P. fabricii.

209x219mm (300 x 300 DPI)

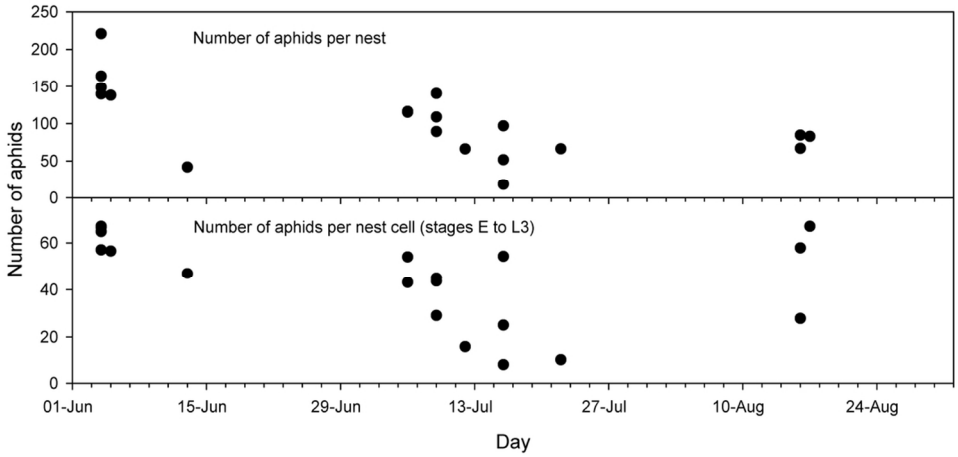

Fig. 3. – Number of aphids per nest and per brood cell (n = 27,623 aphids from 2,472 immature P. fabricii).

104x50mm (300 x 300 DPI)

New type of progressive provisioning as a characteristic parental behavior of the crabronid wasp *Pemphredon fabricii* (Hymenoptera Crabronidae)

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Running head: New type of progressive provisioning

Bees and stinging wasps (Hymenoptera Aculeata) are well known for the great variety of their nesting resources, which include cavities such as empty reed galls. The majority of the species are mass provisioners, and they do not take any care of their brood after provisioning of the nest. *Pemphredon fabricii* (Crabronidae) nests in abandoned reed galls of *Lipara* (Diptera Chloropidae) frit flies. However, *P. fabricii* uses the different here described type of late progressive provisioning. Nesting females do not make separate chambers for larvae, but instead fill the interior space of the gall with paralyzed aphids and lay single egg at body surface of one to eight aphids out of the total amount of aphids provisioned. Larvae are polyphagous, and are provisioned with at least 21 aphid species. *Hyalopterus pruni* is the most common prey, since it feeds on common reed in summer. Before pupation, the larvae sort in the cavity from the biggest (turning to females) at the base to the smallest (turning to males) at the apex. In about 20% of nests, the nesting female brings fresh aphids to feed the smallest larvae at the apex of the nest, while the bigger larvae at the bottom reach maturity much earlier. Similar care on larvae at the end of their development was never reported in any other insect species. Nests of *P. fabricii* are commonly attacked by two predator beetle and 14 parasitoid species. All these parasites are generalists, and *P. fabricii* serves as their satellite host.

KEY WORDS: nesting biology, *Lipara*, reed bed, aphids, phenology, predators and parasites.

INTRODUCTION

Nesting insects, similarly to other nesting animals such as birds, use multiple strategies allowing to raise their fitness. The most of nesting insect species use mass provisioning of their nests, which means that the nesting female brings all the food, lays egg and then does not take any care for the brood (Goulet & Huber 1993; O'Neill 2001). In the contrary, several groups are progressive provisioners, which means that the adult female provides fresh food to its larvae until their development is complete (Evans 1966; Asís et al. 2011; Melo et al. 2011). This type of behavior is well known in many social insect groups, such as Blattodea Isoptera, Hymenoptera Formicidae, Vespidae (Vespinae and Polistinae) and Apidae (Apinae) and was several times reported also among their solitary relatives. Within the order Hymenoptera, progressive provisioning of eusocial bees such the honeybees, bumblebees, or bees of the tribe Allodapini, has been well studied (Field 2005; Michener 2007).

Several groups of solitary bees and wasps also use progressive provisioning, including the North-American digger wasps of the family Crabronidae: *Bembecinus quinquespinus* and *Bembix americana* (Evans 1966; O'Neill 1985), European *Bembix merceti*, *Bembix rostrata* and *Bembix troglodytes* (Larsson & Tengö 1989; Coelho et al. 2008; Asís et al. 2011), and also *Bembecinus tridens* (Polidori et al. 2007). Turillazzi et al. (2014) described communal behavior and indirect proofs of progressive provisioning by small digger wasps of the genus *Spilomena*, and confirmed previous similar observations of progressive provisioning in the Australian and American relatives of this genus (West-Eberhard 1977; Matthews & Naumann 1989; Matthews 1991). Augul et al. (2013) reported progressive provisioning in members of the related family Sphecidae, namely in the subgenus *Eremochares* of the genus *Ammophila*. Among the bees, progressive provisioning is known only within the genus *Ceratina*, which displays such behavior in multiple regions (Michener & Eickwort 1966; Sakagami & Laroca 1971; Sakagami & Maeta 1977; Rehan & Richards 2010; Mikát et al. 2016). In some species, the female provides its brood with fresh food only during the first part of their development. This situation is called "truncated progressive provisioning" and has been

observed in the eumenid wasp *Abispa ephippium* by Matthews and Matthews (2009), and in several species of digger wasps of the genus *Bembix* by Ballesteros et al. (2012).

Wasps in the superfamily Apoidea comprise of four families: Ampulicidae, Sphecidae, Heterogynaidae and Crabronidae. In contrast to the rest of the superfamily (Apiformes), this rather paraphyletic group, called the "Spheciformes", uses paralyzed insects or spiders as a food for their brood and usually provision their nests with one to over a hundred of prey individuals (Goulet & Huber 1993; Blösch 2000, 2012; Macek et al. 2010). It is well known that most of the speciform species do not display any type of social behavior. Spheciformes are typical mass provisioners – they finish provisioning their nest, then close the nest and the paralyzed prey inside serves as food for the larvae, which develop quickly over a period of 2 to 5 weeks, depending on the species and weather conditions (Danks 1971; O'Neill 2001). Thus, in temperate zones, the Spheciformes spend most of their lifetime as mature larvae or prepupae, which are waiting to overwinter, and then pupate the following spring. Two generations per year have been observed in some spheciform species (Blösch 2000).

Pemphredon fabricii is a species with poorly understood biology. It was, for many years, considered to be a synonym for *Pemphredon lethifer*. Smissen (2003) resurrected it from the synonymy and other authors found out that it is very common and numerous in reed beds and uses old abandoned galls on the common reed for making its nests (Wolf 1988; Nartshuk & Andersson 2013; Heneberg et al. 2014). These galls are made by frit flies of the genus *Lipara*, and the old abandoned galls, with very thick sclerenchymatic walls, are also very suitable nest cavities s for various other species of aculeate Hymenoptera (Bogusch et al. 2015; Astapenková et al. 2017), and for overwintering and development of many other species of invertebrates (Nartshuk 2006; Bogusch et al. 2016). This species is the commonest aculeate inhabitant of *Lipara*-induced galls, being found in some localities in every other, or every third, suitable gall (Heneberg et al. 2014; Bogusch et al. 2015).

Nesting biology of *Pemphredon fabricii* has been addressed by several authors, but usually only briefly. Most of the authors only collected galls in winter and reared invertebrates from them

(Wolf 1988; Dely-Draskovits et al. 1994; Westrich 2008; Heneberg et al. 2014); as a result, few have studied aspects of *P. fabricii* nesting. Wolf (1988) found that *P. fabricii* occurs in galls of *Lipara lucens* and *Lipara rufitarsis*. This author also stated that nests of *P. fabricii* usually consist of 6–10 brood cells, which conflicts with observations by Bogusch et al. (2015) and Astapenková et al. (2017) who found that nests of this species usually consisted of only four brood cells. Wolf (1988) also published that nesting females of this species collected various species of aphids [also published previously by Lomholdt (1976) and Schmidt (1984)] and preferred aphids that fed on *Saponaria officinalis*. Danks (1971) confirmed that *P. fabricii* has two generations annually and is proterandric, i.e., males emerge earlier than females. The lifespan of adult males was 4 weeks, while females lived 2 weeks longer (Danks 1971). Oehlke (1970) and Blösch (2000) provided an overview of the parasitic species in nests of *P. fabricii*; they documented three cuckoo wasps (*Omalus aeneus*, *Pseudomalus auratus,* and *Trichrysis cyanea*), four ichneumonid species (*Hoplocryptus binotatulus*, *Hoplocryptus signatorius*, *Perithous divinator,* and *Perithous mediator*), and two chalcidids of the family Torymidae (*Diomorus calcaratus* and *Eupelmus neozonus*). Bogusch et al. (2015) reported that the golden wasps *Chrysis angustula* and *Trichrysis cyanea,* and the anthracid fly *Thyridanthrax fenestratus* (Diptera Bombyliidae), also developed in nests of *P. fabricii*.

Here we address the newly revealed and surprising aspects of *P. fabricii* biology, which we found during our studies of hymenopterans nesting in reed galls. By examining the nests in reed galls during the whole year and whole following nesting season, we aimed to find out (i) how does the nesting female arrange the interspace of the gall to make there brood cells, (ii) which species of aphids does the female use as a food for the larvae, (iii) how many generations per year does *P. fabricii* have, (iv) which developmental stage does overwinter and (v) which parasitoids and predators are commonly found in nests of this species. Here, we address all major aspects of the nesting biology of *P. fabricii* with surprising new type of progressive provisioning discovered, and briefly comment also other parts of its biology, e.g., phenology, prey composition, predators, and parasites.

MATERIAL AND METHODS

For the studies on the nesting biology and phenology of *Pemphredon fabricii*, we collected 60 old cigar galls induced by *Lipara* spp. at 27 locations distributed across the Czech Republic and one in Slovakia. The sampling was done in 2015 at four different times: the first half of June, the first half of July, the second half of August, and the second half of September, so there were 5 weeks between each two consequent sampling times. Before the start of the sampling, several old galls were opened to find out, whether the adults of *P. fabricii* were "on wings" or still in pupae. Collected galls were immediately (i.e., within 48 hr) longitudinally cut and the inner contents were studied. We recorded the number of brood of *P. fabricii*-eggs, first instar larvae (VL1), young larvae (L2-3), big larvae (L4-5), mature larvae after defecation or praepupae (ML), pupae (P), and adults [usually represented by nesting females or freshly shed adults before rearing (AD)]. All members of the brood were kept at an ambient laboratory temperature and regularly checked, particularly to determine whether the large and mature larvae had pupated this year or not, and how long the pupal stage lasted. We also studied the number of aphid prey in the gall and identified the species. We also collected and cut at least 200 reed galls per sampling site at six additional locations in the Czech Republic during the spring months to analyze the phenology of *P. fabricii*. The sampling sites were chosen based on our previous knowledge of *P. fabricii* prevalence; we studied the contents of the reed galls by cutting them and recording which stages of the species were found in each nest.

We also performed similar studies in 2016. In this second round of experiments, we shortened the time between gall samplings to just 6–8 days. The shorter intervals allowed us to study, in detail, the phenology of the species during the nesting season. Every week, we collected 50–100 galls from 4–6 sampling sites, and we cut these galls longitudinally within 24 hr and studied their contents in the same manner described above.

All the material of the brood, adults, and prey (aphids) was transferred into 75% alcohol and stored in the University of Hradec Králové insect collection. The larvae of beetles that damaged nests of *P. fabricii* were identified by the first author according to Klausnitzer (1996). It was not possible to record data blind because our study involved animals with focal distribution in the field. We

performed the calculations using EstimateS 9.1.0 and PAST v. 2.14. Data are shown as mean ± SD, unless stated otherwise.

RESULTS

Nesting biology and progressive provisioning

Pemphredon fabricii hatched in May, while males were out of the nests about a week before the females. The females started to make their nests in second half of May and made and provisioned new nests until the first half of August. This species had two generations per year – second generation reared in first days of July and started to make nests in first half of the same month (see Fig. 1). More detailed phenology is described in Supplemental Online Material S1 Phenology.

Pemphredon fabricii females built nests in old abandoned galls. One-year-old galls were occupied just after the *Lipara* adults hatched, but older galls were used for nesting, as well. To occupy the gall, the female *P. fabricii* usually took out the empty puparium of the *Lipara* (although, we have found galls with nests of *P. fabricii* containing old *Lipara* puparia), cleaned the inside space of the gall, and then started to fill the gall with paralyzed aphids. They did not form any brood cells or chambers (Fig. 2a-b). The female laid one egg per aphid, we found in total one to eight eggs per nest. The egg was elongated with a length of about 1.5 mm, which was similar to the length of the aphid (Fig. 2c). Larva hatched after 2–4 days and the 1st instar fed on the aphid on which the egg was laid (Fig. 2d). After it shed to the 2nd instar, it moved from the first aphid and started feeding on other aphids nearby. Larvae of the 2nd and 3rd instars were very active and ate all the aphids in their vicinity. The larva usually ate the internal contents of the aphid, leaving the antennae, legs, and parts of the integument.

We found that the development of *P. fabricii* consisted of five larval instars. The larvae were dark grey-greenish with a darker gut visible through a semitransparent cuticle. After 7–20 days the larva stopped feeding and all larvae in one gall sorted one after the other in a row inside the gall. Every larva defecated, shed into the prepupa and changed color, usually to yellow or orange. Then it started to build a cocoon from its own silk mixed with feces, and sometimes with other materials from

inside of the gall. The cocoon was placed on the inner side of the gall and was dark brownish or dark grey. There were no septa of mud or leaf matter between the larval chambers as in other species of digger wasps that nest in reed galls.

In most cases, the female did not fill the entire interior space of the gall with aphids or the larvae after feeding did not comprise the whole interior space of the gall. Thus, the female could use the remainder of the gall for the next generation of larvae. In all cases, we observed that, before pupation, the larvae sorted in a bottom to top direction from the largest ones near the bottom of the gall (larvae at the bottom: length 8.9 ± 2.1 mm, n = 37 larvae) to the smallest ones at the top of the gall (larvae at the top: length 5.8 ± 1.9 mm, n = 37 larvae). The smallest larvae were one-third shorter than the longest larvae at the bottom. In some cases, the female put freshly hunted aphids into the top part of the nest and the smallest larvae fed on them once the large larvae at the base had finished feeding and turned into prepupae. We named this newly identified form of progressive provisioning "late progressive provisioning" because of its unique character, which consists of the fact that the nesting female returned into the nest and took care on her brood at the end of its development. We observed the late progressive provisioning during both years; in 42 nests in 2015 and in 28 nests in 2016 (21% and 18% of all nests with aphids). When the females displayed this special type of progressive provisioning, they provisioned several aphids (in 95.3% of cases they provisioned less than 50 aphids; the maximum was 102 aphids) in the top part of the gall but did not lay any eggs on them. Thus, these newly brought aphids served exclusively as a food source for the smallest larvae in the nest (Fig. 3e). Thus, late progressive provisioning of the smallest larvae at the end of their development is likely an important part of the nesting behavior of this species.

Larvae from both generations had usually very high hatching rate, 87.2% of defecated larvae turned adult (n = 687) so the using of progressive provisioning is probably a very good investment into the brood.

Prey

Females of *P. fabricii* hunted various species of aphids. We have recorded 21 species of aphids in nests of *P. fabricii* (Table 1). Some aphid species were used more often than others. In this

study, the most common species was *Hyalopterus pruni*. The first generation of this aphid feeds on the common reed (*Phragmites australis*). Also, polyphagous species *Aphis fabae* and *Aphis ruborum*, which feed on *Rubus* spp., were also very commonly used for feeding larvae of *P. fabricii*. *Rubus* spp. were present in, or near, all reed beds positive for this aphid species. Thus, *P. fabricii* is polyphagous and feeds its larvae with any species of aphids abundantly available in the vicinity of its nest.

Altogether, we studied 196 nests with 2,472 individuals of brood of *P. fabricii*. From these nests, we retrieved 27,623 aphids provisioned as a prey to the larvae found in the nests. The nesting female usually provisioned more than one hundred aphids (139.72 ± 87.27 aphids per nest, n = 179 nests with aphids; the highest number of aphids found was 475 per nest) into the gall and, quite often, the entire interior space of the gall was full of aphids (Fig. 2a-b). Despite the number of aphids per nest decreased over the course of the nesting season (Fig. 3a), this trend was fully attributed to the decrease in the number of eggs or young larvae per nest in samples examined later in the nesting season. Thus, when plotted as a number of aphids per egg or immature larva (up to L3), the number remained stable regardless of the time of nesting (Fig. 3b).

Predators and parasites

Nests of *Pemphredon fabricii* were attacked by the following predators and parasites:

Larvae of malachiid beetles *Anthocomus coccineus* and *Malachius aeneus* (Coleoptera Malachiidae) were common inside the galls and were found in the vast majority of examined sampling sites (25 of 28 localities in 2015, and 29 of 40 localities in 2016). The beetle larvae damaged the whole nest of *P. fabricii* and were eating both larvae and pupae (Fig. 2f). We found them primarily in nests with mature larvae during the nesting season, and much less so in winter months.

Three species of parasitoids of the genus *Gasteruption* (Hymenoptera Gasteruptiidae), three species of cuckoo wasps (Hymenoptera: Chrysididae) and one anthracid fly (Diptera Bombyliidae) were recorded in nests of *Pemphredon fabricii* (Table 2). These included first records of *Omalus aeneus*, *Pseudomalus auratus*, *Gasteruption assectator*, *Gasteruption nigrescens* and *Gasteruption phragmiticola* parasitizing the nests of *P. fabricii*.

DISCUSSION

Late progressive provisioning is the most important aspect of the nesting behavior of *P. fabricii* that we have discovered. No authors have published information on this type of progressive provisioning by any species of bees or stinging wasps. Contrary to typical progressive provisioning (Asís et al. 2011; Melo et al. 2011), *P. fabricii* females feed only one or a few larvae (not all of their brood) in a respective nest. They feed the larvae with fresh aphids only at the end of their larval development, which contrasts with truncated progressive provisioning as reported by Matthews and Matthews (2009) or Ballesteros et al. (2012). Previously reported forms of truncated progressive provisioning consisted of the female feeding the larvae at the beginning of their development only.

Late progressive provisioning occurred at a frequency high enough (around 20%) to state that this behavior is common for *P. fabricii* and the nesting female probably increases her fitness by feeding her weakest larvae. If we imagine that the female feeds only one or two larvae with only up to 50 aphids, it is not a very big investment to her brood in contrast to the provisioning of the whole nest. Species related to *P. fabricii* create brood cells with number of prey and one egg and closing plug at the end of the nest, which is typical for mass provisioners (Blösch 2000), except several species of the genus *Spilomena*, which use communal nesting and probably also classic type of progressive provisioning (Turillazzi et al. 2014). Similar type of nest provisioning with quantity of prey brought and creating no nesting chambers was reported in *Rhopalum clavipes* and *Rhopalum coarctatum*, by Danks (1971) and Blösch (2000), and in two related species of the genus *Pemphredon*: *P. inornata* and *P. lethifer* (Janvier 1960; Danks 1971; Blösch 2000). Females of these species fill the whole cavity with many specimens of small prey, barklice (Psocoptera) in the case of *Rhopalum*, and aphids in the case of both *Pemphredon* species. Most of the other genera of Crabronidae nesting in cavities have linear nests with brood cells separated by thin septa, which are made by the female, and their nests are closed with thick closing plugs made of mud, resin or plant material (Janvier 1961; Danks 1971; Lomholdt 1976; Blösch 2000). Also species nesting in circular cynipid galls make separated brood cells in the gall (Janvier 1961; Bloomers 2008).

Although Wolf (1988) reported that *P. fabricii* prefers aphids that feed on *Saponaria officinalis*, we did not record any aphid species connected with the mentioned plant in *P. fabricii* nests. Females collecting aphids also do not move only through the reed, as reported by Blösch (2000). Instead, the females also venture into the neighboring vicinity – trees, meadows and ruderals. Nevertheless, they do not collect aphids at sites distant from nests because they need to collect large numbers of paralyzed aphids (the highest number of aphids found in one nest was 475). Most of the galls with more than 100 aphids inside contained more than five eggs. However, at the end of autumn when mature larvae are prepared to overwinter, most of the galls contained four or less larvae (Bogusch et al. 2015). Part of this mortality could be attributed to losses due to predation, parasitation, or, less often, fungal infections. It remains to be elucidated, whether direct competition with other larvae within the nest also plays its role. When keeping larvae in laboratory, more than 87% of larvae, which reached maturity, turned well into the adults (P. Bogusch unpublished data). Brood predators of *P. fabricii* were very common and the broods of this species are likely their preferred prey. Larvae of two beetles in the family Malachiidae were very common in nests of *P. fabricii* during the summer nesting season, but much rarer during other times of the year. They were found damaging the cocoons and eating the larvae, leaving only their integuments inside the nests. We found larvae of various instars of these species, with *Anthocomus coccineus* being 8 times more abundant than the another species. Somewhat less common were the parasitic species. *Thyridanthrax fenestratus* was numerous in nests of *P. fabricii* at some sampling sites (Bogusch et al. 2015; P. Bogusch unpublished data). This species is a generalist attacking nests of various Crabronidae and Sphecidae, previously better known from species nesting in the ground (Oldroyd 1969). Interestingly, this parasitoid attacked the nests of *P. fabricii* only in the Pannonian lowland despite it is common in other parts of Europe as well (Bogusch et al. 2015; P. Bogusch unpublished data). All other parasitic species found are also generalists and most of them attack nests of *P. fabricii* only accidentally. Other causes of death include relatively sparse fungal infections (Heneberg et al. 2016) and often observed damage of whole galls by birds (Westrich 2008).

In conclusion, we provided the evidence suggesting the establishment of a new type of progressive provisioning, and addressed other surprising aspects of the nest biology of *P. fabricii*, which contradicted previously published data on this eudominant but poorly understood reed gall inquiline.

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Fig. 1. — Proportion of galls with selected instars and stages of *Pemphredon fabricii* during the nesting season, i.e., from 28 May to 2 August 2016. (a) Eggs through 3rd instar larvae (n = 399 nests), (b) 4th instar larvae through mature larvae (n = 606 nests), (c) pupae and imagines (n = 126 nests). Each data point is based on the examination of 252–435 *Lipara*-induced reed galls collected at four or five independent sampling sites. To minimize the effects on local populations of study insects, each sampling site was sampled only once or twice during the nesting season. The total number of reed galls examined was 2,842.

Fig. 2. — Nesting of *Pemphredon fabricii*. (a) reed gall cavity filled with aphids, (b) detail on the aphids in the cavity, (c) egg on aphid, (d) young larva of first instar on aphid, (e) *P. fabricii* nest with mature larvae of which the smallest feed on aphids at the top of the nest, (f) brood cells of *P. fabricii* destroyed by malachiid larvae with remnants of eaten larva of *P. fabricii*.

Fig. 3. — Number of aphids per nest and per brood cell (n = 27,623 aphids from 2,472 immature *P. fabricii*).

Supplemental Online Material S1 Phenology

Altogether, we recorded 7,249 specimens from different developmental stages of *Pemphredon fabricii* at 86 independent locations on 34 different days. We found that the study population of *P. fabricii* (7,249 individuals studied at 86 independent locations) had two generations per year. The first generation started nesting at the end of May (first observed nest with aphids and eggs was on 19 May). The nesting season lasted until the beginning of August while only a few females were making fresh nests. Only several individuals were observed in late August and at the beginning of September (with the last observation on 3 September) – both males and females frequently used galls for spending the night. We also noticed dead females in galls with nests collected in winter – these adults probably spent nights there (which is a common behavior of *P. fabricii*) and then died at the end of nesting season.

Larval development of the first generation lasted around 2 weeks depending on the temperature. In larvae kept under laboratory conditions, the total length of the development lasted until they pupated, which took 7–16 days, and the adults of the 2nd generation hatched after 6–13 days. They started nesting several days after they hatched and we recorded a slight nesting overlap of the two generations; several females from the 1st generation still produced fresh nests, while young females of the second generation started to make their own nests, too. Females of *P. fabricii* usually produced 5–10 nests per season, which usually took less than 4 weeks. Larvae of the 2nd generation matured in their cocoons and overwintered. They pupated the following year in April and May, when the pupal stage lasted 10–21 days; in summer, it lasted 6–13 days, with some variation due to ambient temperatures.

The phenology of *P. fabricii* is shown in Fig. S1. Each graph shows the share of one of the six stages of *P. fabricii* development. We did not perform detailed sampling between the 20 August and the 19 March because the proportions of all developmental stages in these months were the same as at the end of this time period. In Fig. S1, each point shows the relative abundance (% of the respective developmental stage) of all brood collected on the same day. Eggs and fresh larvae were the most abundant in June (more than 60% of all contents of settled galls), while their abundance decreased

through July and August, and they were completely absent from nests that were analyzed from September to the following May. A similar situation was seen with young larvae (2nd and 3rd instars) and older premature larvae (4th and 5th instars), which were the most abundant in July (21%). Mature larvae were present during the whole year except during April and May, when all brood turned to pupae and the adults were hatching. Few mature larvae were found in June (usually only at the end of the month when larvae of the 1st generation started to turn into pupae), whereas in July they comprised 40%, and in August 98.5%, of all nest contents. Pupae were the most abundant in April (99%), May (70% – some hatched), and during the nesting season (June and July, around 10%) when the 2nd generation hatched. Freshly hatched adults were the most abundant in the nests in May (1st generation) and July (2nd generation).

A week-to-week study of the phenology of *P. fabricii* during the nesting season indicated that there were two egg laying peaks – the first peak was seen at the beginning of June (7 June, 46% of all brood in nests) and in the second half of July (21 July, 35%), which confirmed the existence of two generations of *P. fabricii* per season. We found a similar two-peak distribution when checking the presence of young larvae. Older larvae could be found during the whole nesting season, as well as at the end of July and at the beginning of August (those were the last nests with active larvae just before reaching maturity). Mature larvae were the most abundant at the end of the nesting season and were totally absent at the beginning of the nesting season (29 May). The first rise in the number of mature larvae was in mid-June (12 June, 37%, larvae of 1st generation pupating), then their abundance fell (all brood were present as pupae, or eggs, or young larvae in fresh nests); their share rose again during the nesting season of the 2nd generation in July and the beginning of August. Pupae and adults could be found in nests from 12 June to 5 July.

Fig. S1. — Proportion of selected instars and stages of *Pemphredon fabricii* during the year (n = 7,249 individuals, of that, 561 eggs or 1st instar larvae, 340 2nd or 3rd instar larvae, 420 4th or 5th instar larvae, 4,229 mature larvae, 1,381 pupae, and 318 imagines). The data was obtained from 86 independent samplings. The individual data points represent means based on the collections performed on a particular day at several independent sampling sites (n = 34 sampling days). Data

before 19 March and after 20 August are pooled together since there were only mature larvae present in the nests collected between 20 August and 19 March of the following year. Lines indicate moving averages.

Table 1.

Aphid species recorded in *Pemphredon fabricii* nests. Numbers show the abundance of aphids

(+ = 1 nest, 1 = 2–5 nests, 2 = 5–20 nests, 3 = more than 20 nests).

Aphid species	Color	Host plant	Dominance
Anoecia vagans (Koch 1856)	brown	*Cornus* spp., *Elymus repens*	+
Aphis craccivora Koch 1854	black, dark brown	polyphagous	2
Aphis fabae Scopoli 1763	brown, black	polyphagous	3
Aphis farinosa Gmelin 1790	dark green	*Salix* spp.	1
Aphis ruborum (Borner 1932)	dark green	*Rubus* spp.	3
Aphis sedi Kaltenbach 1843	dark green	*Sedum* spp.	+
Cavariella pastinacae (Linnaeus 1758)	green	*Heracleum* spp., *Salix* spp.	+
Chaitophorus capreae (Mosley 1841)	green	*Salix caprea, Salix alba*	+
Chaitophorus leucomelas Koch 1854	green	*Populus nigra, Populus tremula*	2
Chaitophorus populialbae (Boyer de Fonscolombe 1841)	green	*Populus tremula*	+
Chaitophorus salijaponicus niger Mordvilko 1929	black	*Salix caprea, Salix alba*	+
Chaitophorus truncatus (Hausmann 1802)	light green	*Salix alba, Salix fragilis*	2
Dysaphis plantaginea (Passerini 1860)	dark grey-reddish	*Malus* spp., *Plantago* spp.	1
Glyphina betulae (Linnaeus 1758)	dark green	*Betula pendula*	1
Hyalopterus pruni (Geoffroy 1762)	grey-purple	*Prunus* spp., *Phragmites australis*	3
Macrosiphoniella sp.	brown	N/D	+
Metopolophium dirhodum (Walker 1849)	green	*Phalaris arundinacea, Phragmites australis*	+
Myzus cerasi (Fabricius 1775)	green with black markings	*Prunus* spp., *Galium* spp.	+
Ovatus crataegarius (Walker 1850)	green	*Crataegus* spp., *Mentha* spp.	+
Pterocomma populeum Kaltenbach 1843	brown	*Populus nigra, Salix alba*	+
Sipha maydis Passerini 1860	black	*Elymus repens, Calamagrostis* spp.	+

Table 2.

Predators and parasites known from nests of *Pemphredon fabricii*.

Order Family	Species	Note, first report of host-parasite interaction
Coleoptera Malachiidae	*Anthocomus coccineus*	predator of larvae and pupae, new host record
	Malachius aeneus	predator of larvae and pupae, new host record
Diptera Bombyliidae	*Thyridanthrax fenestratus*	parasitoid (Bogusch et al. 2015)
Hymenoptera Ichneumonidae	*Hoplocryptus binotatulus*	parasitoid (Oehlke 1970)
	Hoplocryptus signatorius	parasitoid (Oehlke 1970)
	Perithous divinator	parasitoid (Oehlke 1970)
	Perithous mediator	parasitoid (Oehlke 1970)
Hymenoptera Gasteruptiidae	*Gasteruption assectator*	parasitoid, new host record
	Gasteruption nigrescens	parasitoid, new host record
	Gasteruption phragmiticola	parasitoid, new host record
Hymenoptera Torymidae	*Diomorus calcaratus*	parasitoid (Oehlke 1970)
	Eupelmus neozonus	parasitoid (Oehlke 1970)
Hymenoptera Chrysididae	*Chrysis angustula*	parasitoid (Bogusch et al. 2015)
	Omalus aeneus	parasitoid, new host record
	Pseudomalus auratus	parasitoid (Oehlke 1970)
	Trichrysis cyanea	parasitoid (Oehlke 1970)

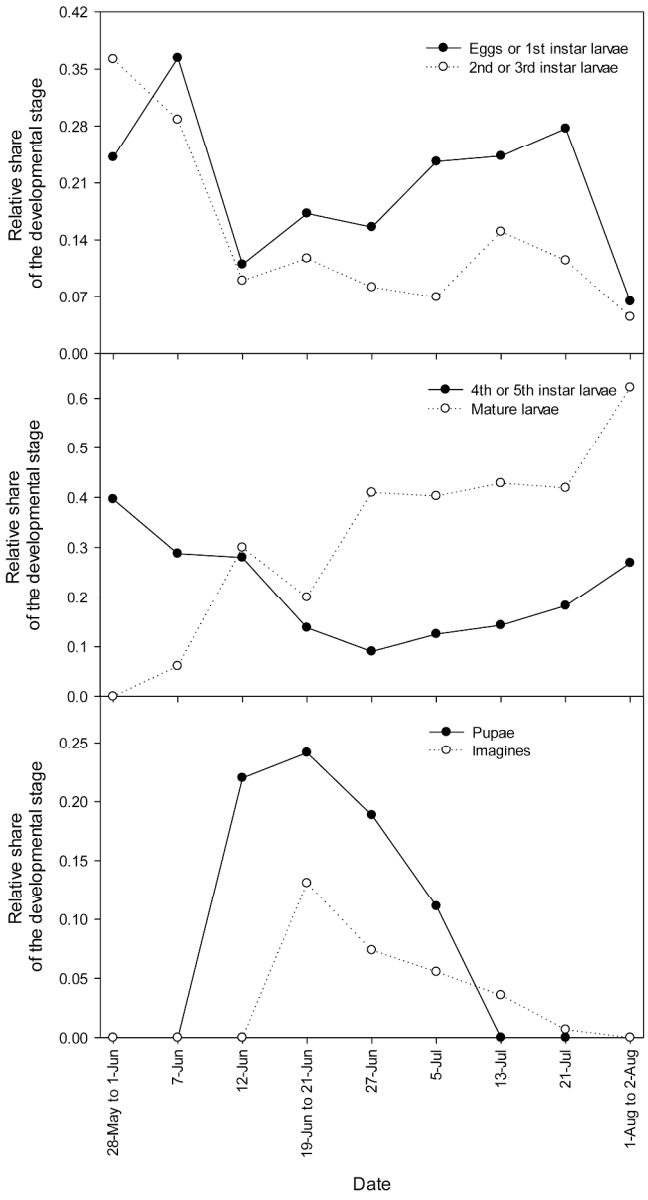
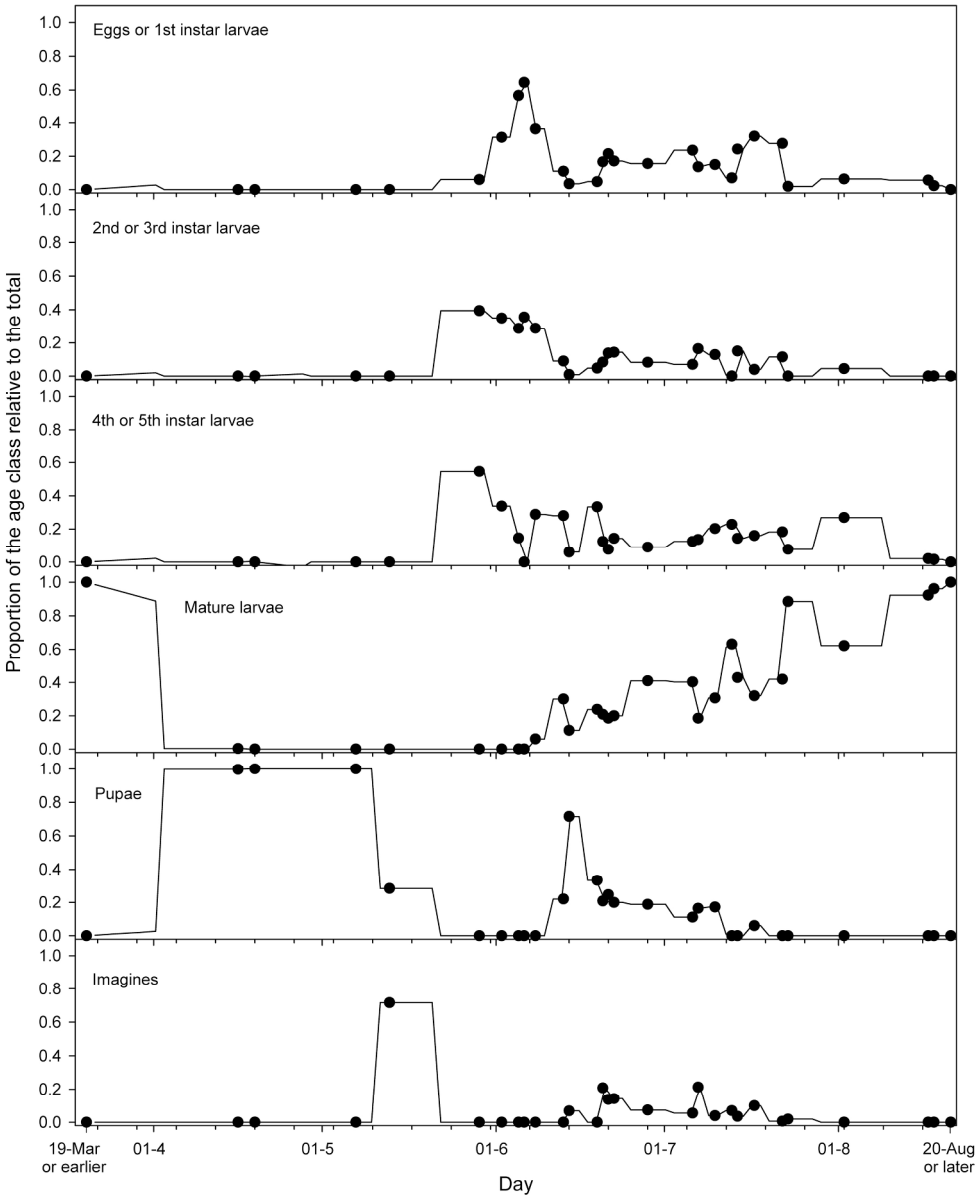


Fig. 1. — Proportion of galls with selected instars and stages of Pemphredon fabricii during the nesting season, i.e., from 28 May to 2 August 2016. (a) Eggs through 3rd instar larvae (n = 399 nests), (b) 4th instar larvae through mature larvae (n = 606 nests), (c) pupae and imagines (n = 126 nests). Each data point is based on the examination of 252–435 Lipara-induced reed galls collected at four or five independent sampling sites. To minimize the effects on local populations of study insects, each sampling site was sampled only once or twice during the nesting season. The total number of reed galls examined was 2,842.

262x484mm (300 x 300 DPI)



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