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OPINION

The enduring value of reciprocal illumination in the era of insect phylogenomics: a response to Cai *et al.* (2020)

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Background

Arguably no other group within Coleoptera has received as robust and sustained investigation into their phylogenetic relationships as aquatic beetles (Short, 2018). Among this ecological guild, evolutionary relationships of the families within Dytiscoidea, a clade comprising the charismatic diving beetles (Dytiscidae) and their close relatives, have received particular attention (Ribera *et al.*, 2002; Balke *et al.*, 2005; Balke *et al.*, 2008; Alarie *et al.*, 2011; Hawlitschek *et al.*, 2012; Toussaint *et al.*, 2016). Very recently, four different studies were published investigating the phylogeny of Dytiscoidea, three of which utilized phylogenomic data (Table 1), the most recent by Cai *et al.* (2020).

Cai *et al.* (2020) (hereafter CEA) approached investigating the evolutionary relationships among dytiscoid families by reanalysing the transcriptomic dataset of Vasilikopoulos *et al.* (2019) using different evolutionary models and data trimming regimes. CEA's analyses recovered three different topologies for relationships amongst Dytiscoidea (Fig. 1), two of which (Fig. 1A, B), have been recovered in several previous studies (Table 1). The primary difference among these topologies is the placement of Hygrobiidae, either as sister to (Dytiscidae (Amphizoidae + Aspidytidae)) (Fig. 1A), sister to Amphizoidae + Aspidytidae (Fig. 1B), or as sister to Dytiscidae (Fig. 1C). In CEA, topologies shown in Fig. 1A, C both received maximal (e.g. bootstrap values of 100 and posterior probabilities of 100%) to strong support respectively via their preferred model of evolution. Whereas, CEA's recovery of Hygrobiidae sister to Amphizoidae + Aspidytidae (Fig. 1B) was not as strongly supported, Gustafson *et al.* (2020) recovered this topology primarily with strong to maximal support across all analyses with comprehensive taxon sampling of Dytiscoidea. Rather than treating the three topologies recovered both within their own study and elsewhere as equally viable hypotheses (Table 1), CEA dismissed the relationships shown in Fig. 1A, B as the result of phylogenetic methodological error, promoting Fig. 1C as their preferred tree because it is '… consistent with morphology-based views of dytiscoid relationships.' (Cai *et al.*, 2020: 5).

Here, we address (i) the manner in which CEA approached reconciling conflicting hypotheses about the evolution of Dytiscoidea; and (ii) the misconception that dytiscoid relationships shown in Fig. 1C are the most consistent with morphology-based views in relation to those of Fig. 1A, B.

Choosing among competing topologies: Reciprocal illumination

Phylogenomic datasets present a new challenge in that nodes in recovered trees are often maximally supported (e.g. bootstrap values of 100 or posterior probabilities of 100%). In certain cases, even incorrect species tree topologies can receive strong statistical support due to systematic biases, incomplete lineage sorting, gene tree conflict, biased taxon sampling, model misfit, etc. (Phillips *et al.*, 2004; Philippe *et al.*, 2005; Rodriguez-Ezpeleta *et al.*, 2007; Philippe *et al.*, 2011; Sharma *et al.*, 2014; Prasanna *et al.*, 2020). However, issues with choosing among competing topologies have plagued systematists long before the phylogenomics era. Hennig (1950, 1966) promoted the use of 'wechselseitige Erhellung' or reciprocal illumination, to re-evaluate homology assessments using all available sources (morphological, ecological, biogeographical) in order to understand and resolve evolutionary relationships (Mooi & Gill, 2016). With reciprocal illumination, investigators

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Table 1. Major phylogenetic analyses including Dytiscoidea.

References	Data type	Focal clade	Taxon sampling	Data size	Placement of Hygrobiidae in preferred tree
Cai *et al.* (2020)	Vasilikopoulos *et al.*, (2019)	Dytiscoidea	Incomplete (missing Meruidae)	1 661 023	((Amphizoidae+Aspidytidae)(Hygrobiidae+Dytiscidae)) **C**
Beutel *et al.* (2020)	Morphology	Adephaga	Complete	174	((Amphizoidae+Aspidytidae)(Hygrobiidae+Dytiscidae)) **C**
Gustafson *et al.* (2020)	Phylogenomic (UCE)	Adephaga	Complete	480 223	(Dytiscidae(Hygrobiidae(Amphizoidae+Aspidytidae))) **B**
McKenna *et al.* (2019)	Phylogenomic (transcriptomes)	Coleoptera	Limited (missing Amphizoidae, Hygrobiidae, Meruidae)[a]	1 907 014	N/A
Vasilikopoulos *et al.* (2019)	Phylogenomic (transcriptomes)	Dytiscoidea	Incomplete (missing Meruidae)	4 098 894	(Hygrobiidae(Dytiscidae(Amphizoidae+Aspidytidae))) **A**
Zhang *et al.* (2018)	95 genes	Coleoptera	Limited (missing Amphizoidae, Aspidytidae, Hygrobiidae, Meruidae)	71 406	N/A
Baca *et al.* (2017)	Phylogenomic (UCE)	Hydradephaga	Incomplete (missing Aspidytidae)	83 547	(Hygrobiidae[Amphizoidae+Dytiscidae])
López-López & Vogler (2017)	Mitogenome	Adephaga	Incomplete (missing Amphizoidae)	13 948	(Dytiscidae[Hygrobiidae+Aspidytidae])
Toussaint *et al.* (2016)	11 genes	Dytiscoidea	Complete	5448	(Dytiscidae(Hygrobiidae[Amphizoidae+Aspidytidae[c]])) **B**
McKenna *et al.* (2015)	8 genes	Coleoptera	Complete	8377	(Dytiscidae(Hygrobiidae(Amphizoidae+Aspidytidae))) **B**
Beutel *et al.* (2013)	Morphology	Adephaga	Complete	150	(Aspidytidae(Amphizoidae(Hygrobiidae+Dytiscidae)))
Hawlitschek *et al.* (2012)	5 genes + Morphology	Dytiscoidea	Incomplete (missing Meruidae)	unclear, likely over 4000	(Hygrobiidae(Dytiscidae(Amphizoidae+Aspidytidae))) **A**
Lawrence *et al.* (2011)	Morphology	Coleoptera	Complete	516	(Amphizoidae(Hygrobiidae+Dytiscidae))
Dressler *et al.* (2011)	Morphology	Adephaga	Complete	145	(Aspidytidae(Amphizoidae(Hygrobiidae+Dytiscidae)))
Alarie *et al.* (2011)	Morphology	Dytiscoidea	Complete	28	((Amphizoidae+Aspidytidae)(Hygrobiidae+Dytiscidae))
Maddison *et al.* (2009)	3 genes	Adephaga	Incomplete (missing Aspidytidae, Meruidae)	~3052	Hygrobiidae nested inside Dytiscidae
Balke *et al.* (2008)	6 genes	Dytiscoidea	Complete	~4200	(Hygrobiidae(Dytiscidae(Amphizoidae+Aspidytidae))) **A**
Hunt *et al.* (2007)	3 genes	Coleoptera	Incomplete (missing Amphizoidae, Meruidae)	unclear	(Dytiscidae[Hygrobiidae+Aspidytidae])
Beutel *et al.* (2006)	Morphology	Adephaga	Complete	148	(Aspidytidae(Amphizoidae(Hygrobiidae+Dytiscidae)))
Balke *et al.* (2005)	6 genes + Morphology	Dytiscoidea	Complete	4208	(Hygrobiidae(Dytiscidae(Amphizoidae+Aspidytidae))) **A**
Alarie & Bilton (2005)	Morphology	Adephaga	Complete	23	((Hygrobiidae+Dytiscidae)Noteridae,Amphizoidae,Aspidytidae)
Ribera *et al.* (2002)	3 genes + Morphology	Dytiscoidea	Complete	2943	(Amphizoidae(Aspidytidae(Hygrobiidae+Dytiscidae)))
Ribera *et al.* (2002)	1 gene (18S)	Hydradephaga	Incomplete (missing Aspidytidae, Meruidae)	~2000	Hygrobiidae nested inside Dytiscidae
Shull *et al.* (2001)	1 gene (18S)	Adephaga	Limited (missing Amphizoidae, Aspidytidae, Meruidae)	~2000	Hygrobiidae nested inside Dytiscidae[b]
Beutel & Haas (1996)	Morphology	Adephaga	Incomplete (missing Aspidytidae, Meruidae)	80	(Amphizoidae(Hygrobiidae+Dytiscidae))

Data size given is in base pairs for molecular data and characters for morphological data. Bold letters indicate corresponding topology shown in Fig. 1.

[a] Contrary to statements by CEA, the phylogenomic analysis of McKenna *et al.* (2019) did not include Hygrobiidae and Amphizoidae (see figure 1 and supplemental materials of McKenna *et al.*, 2019). These taxa were part of the diversification rate analysis only, with the molecular data used for these taxa coming from McKenna *et al.* (2015) (see figure 2 and supplemental materials of McKenna *et al.*, 2019).

[b] Contrary to what CEA state, the results of Shull *et al.* (2001) did not suggest Hygrobiidae was sister to Dytiscidae, Hygrobiidae was recovered as being nested inside Dytiscidae suggesting hygrobiids are dytiscids.

[c] Aspidytidae recovered as a paraphyletic grade into Amphizoidae.

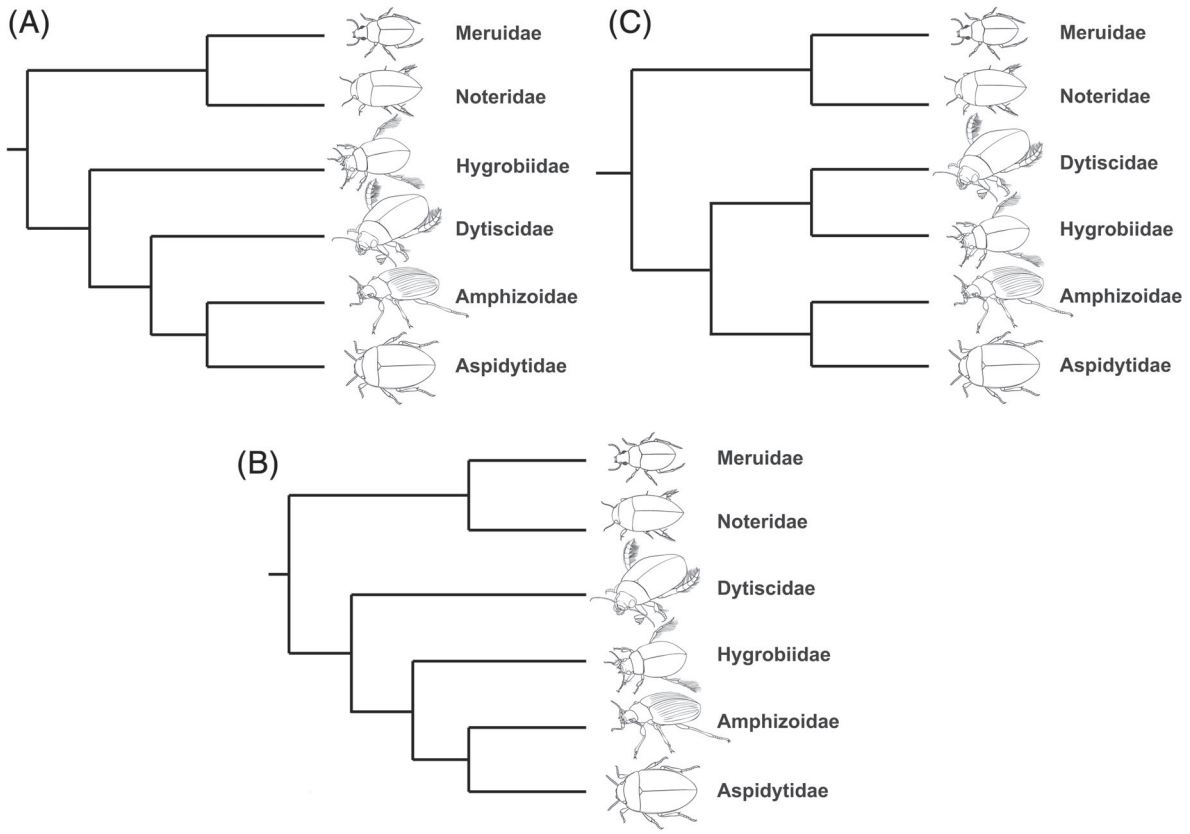

Fig. 1. Current prevailing phylogenetic hypotheses with regards to relationships of the Dytiscoidea.

use the results of current analyses as evidence to correct errors in prior conclusions and to inform about potentially spurious phylogenetic relationships or dubious homology assessments. Thus, reciprocal illumination serves as a philosophical test of the explanatory power of a hypothesis (in this case a tree topology) in relation to broader evolutionary theory, making it testable and thus preferable to empirical observations (e.g. re-analysis of datasets under different assumptions), as the explanatory power of such empirical tests are limited by the narrower circumstances under which the results were extracted (Grant, 2002). CEA relied almost exclusively on this latter option, reanalysing datasets under different evolutionary models and trimming settings, thus limiting the broader explanatory power of their results to a single model under a particular trimming regime. Furthermore, the trimming regimes implemented by CEA are recommended for the analysis of closely related species (Vasilikopoulos *et al.*, 2020), not higher-level taxa like families, whose ancestors likely diverged hundreds of millions of years ago (Hawlitschek *et al.*, 2012). Thus, CEA's results were obtained under biologically unrealistic settings (Vasilikopoulos *et al.*, 2020), further limiting their explanatory power to an unrealistic dimension. The kinds of evidence utilized by reciprocal illumination, on the other hand, like complex traits and features, can be used to defensibly choose among competing hypotheses of homology and tree topologies (Grant & Kluge, 2004; Mooi, 2016). This last aspect of reciprocal illumination is increasingly relevant in the phylogenomic era when competing trees are often maximally supported, with differing topologies offering conflicting hypotheses about the evolution of a particular group.

CEA provided several examples of morphological features to support their preferred topology (Fig. 1C) with a sister-group relationship between Dytiscidae and Hygrobiidae [emphasis added]:

> 'The sister-group relationship between Hygrobiidae and Dytiscidae was proposed by Burmeister (1976) based on *morphology of the ovipositor* and by Ruhnau (1986) based on *larval morphology*. Both adult Dytiscidae and Hygrobiidae also share the *presence of prothoracic glands*, among other characters (Forsyth, 1970; Beutel, 1986, 1988).' (Cai *et al.*, 2020: 5).

We revisited the literature CEA cited among others, in order to re-examine the morphology in light of the three different topologies for Dytiscoidea (Fig. 1A, B, C) for the purposes of reciprocal illumination.

Prothoracic and pygidial defence glands

Forsyth, in a series of papers (Forsyth, 1968; Forsyth, 1970; Forsyth, 1972), documented the anatomical structure of the

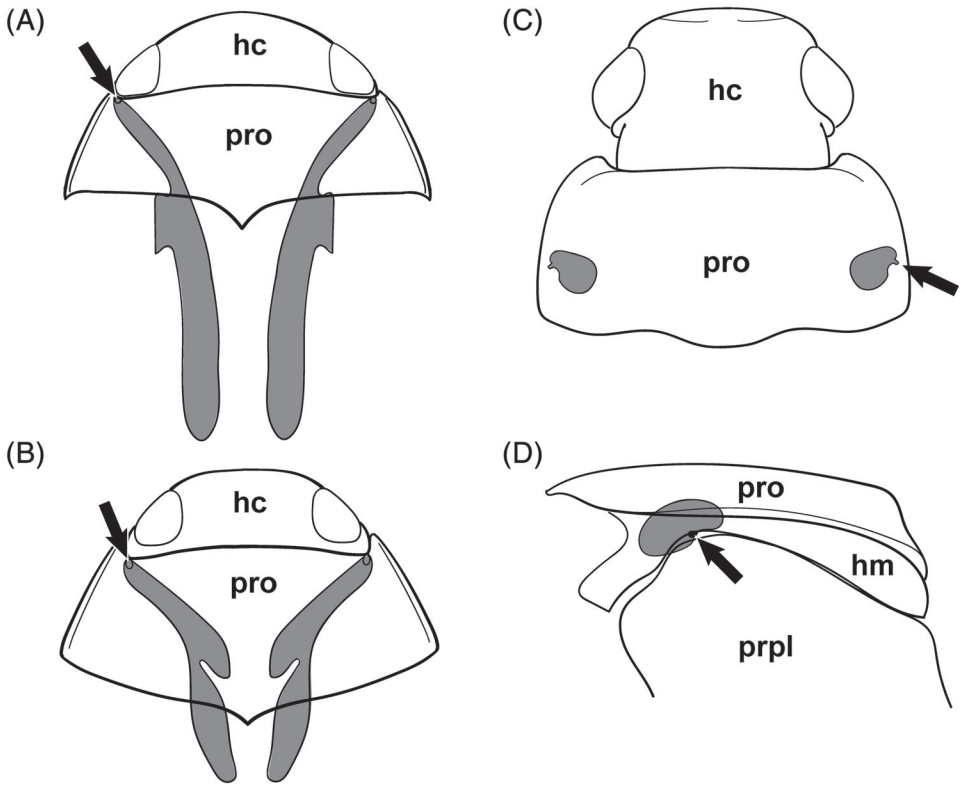

Fig. 2. Prothoracic defence gland shape and position. The gland is shaded grey with arrows indicating location of the gland's opening. The head capsule has been added to the illustrations to show anterior vs. posterior directions. (A) Dorsal view of *Laccophilus minutus* L. and (B) the same of *Hyphydrus ovatus* L. after Dettner (2014). The former was selected as a member of the clade sister to the larger diving beetle subfamilies for which the prothoracic gland has been illustrated, and the later as representative of Hydroporinae which together with Hydrodytinae was recovered as sister to the aforementioned clade (see Gustafson *et al.*, 2020). (C) Dorsal view and (D) right lateral view of *Hygrobia hermanii* (Fabricus) after Forsyth (1970). hc, head capsule; pro, prothorax; hm, hypomeron; prpl, propleuron. Not to scale.

defensive glands, both gross and cellular, across all currently recognized adephagan families, with the exception of the more recently discovered Aspidytidae (Ribera *et al.*, 2002) and Meruidae (Spangler & Steiner, 2005). Adephagans have two general types of paired defence glands: those located towards the apex of the abdomen, the pygidial defence glands, and those situated within the prothorax, the prothoracic defence glands. Pygidial defence glands occur in all adephagan beetles (Forsyth, 1968, 1970, 1972). Prothoracic glands are only known to occur in the families Hygrobiidae and Dytiscidae (Forsyth, 1968, 1970, 1972; Beutel *et al.*, 2006; Dettner, 2019). In general, prothoracic exocrine glands are present in various beetle families (e.g. Chrysomelidae, Erotylidae, Histeridae, Pyrochroidae, Staphylinidae) (Dettner, 1987), however, complex prothoracic glands like those found in Dytiscidae and Hygrobiidae (Forsyth, 1968, 1970), are rare and known to have evolved outside these two families only in Tenebrionidae (e.g. *Tribolium* Macleay, *Diaperis* Geoffroy, *Zophobas* Dejean) (Roth, 1943; Sokoloff, 1975; Tschinkel, 1975). However, within Tenebrionidae prothoracic glands have potentially evolved multiple times (Tschinkel, 1975).

The prothoracic glands of all Dytiscidae (Fig. 2A, B) are similar in being elongate, sac-like structures that are situated dorsally, with their openings positioned anterolaterally (most Dytiscidae), or more anteromedially (*Cybister* Curtis*, Hydaticus* Leach*, Dytiscus* L.) into the cervical membrane, with gland reservoirs that are not covered by muscles (Forsyth, 1968; Dettner, 2014). Depletion of the reservoir content is achieved through turgor pressure generated by contraction of the tergo-sternal muscles (Forsyth, 1968; Dettner, 2014). In Hygrobiidae, the prothoracic glands (Fig. 2C) are short, reniform structures situated dorsally, with their openings placed posterolaterally on the propleuron (Fig. 2D), with gland reservoirs that are covered by muscles dorsally (Forsyth, 1970; Dettner, 2019). Depletion of the reservoir's content is achieved through contraction of the covering muscles (Forsyth, 1970). Thus, the prothoracic glands in Hygrobiidae and Dytiscidae do not share the same position in the prothorax; they are structurally different in form and they do not secrete in the same manner. Furthermore, upon molestation Dytiscidae will deplete their prothoracic glands (Dettner, 2014, 2019). Hygrobiidae, on the contrary, are not known to deplete their prothoracic glands upon molestation, instead exhibiting a stridulating behaviour giving them their common name of 'squeak beetles' (Aiken, 1985; Dettner, 2019). This suggests the prothoracic glands may be used in different ways in these two families. Although it is

possible that homologous structures can shift position and change in both structure and function over evolutionary time, no known intermediate forms exist between the prothoracic glands of Dytiscidae (Fig. 2A, B) and Hygrobiidae (Fig. 2C, D) among any of the extant species (reviewed by Dettner, 2019), and these structures have not been described in any fossil taxa, to provide evidence in support of this possibility. Given the above and that no known intermediate forms exist between these two prothoracic glands, they do not meet any of Remane's (1952) criteria for objectively identifying primary homology (de Pinna, 1991). Therefore, it is unsurprising that Forsyth (1970) concluded:

> '… Hygrobiidae and Dytiscidae are unique within Caraboidea in that both have thoracic defense glands. These have probably been evolved independently in the two groups.' (Forsyth, 1970: 68).

Subsequent researchers have since agreed that homology of these glands between the two taxa seems questionable (Dettner, 1985, 1987, 2014, 2019) and have even considered them nonhomologous (Miller, 2001) and the likely result of convergent evolution (Kavanaugh, 1986; Lawrence *et al.*, 2011). Others have contested that the glands are homologous, such as Burmeister (1976) who thought it unlikely such a 'differentiated' organ could result from convergent evolution, and most notably, Beutel (1986) who similarly thought convergent evolution of this gland was unlikely and cited the glands' similar sieve plates as evidence of common ancestry. Although it is true that both prothoracic glands in Hygrobiidae and Dytiscidae do have the secretory cell duct openings clustered together into groupings called sieve plates, the arrangement of these sieve plates is different. In *Hygrobia* Latreille they are positioned primarily along the lateral margin of the gland reservoir where they are unobstructed by the muscles dorsally covering the gland (Forsyth, 1970). In Dytiscidae the sieve plates are distributed randomly over the basal half of the reservoir only, additionally in between each sieve plate are many inwardly directed spine-like invaginations that are not found in the prothoracic glands of *Hygrobia* (Forsyth, 1968, 1970). Thus, the homology of the prothoracic sieve plates also seems questionable given the positional and structural differences (i.e. having spine-like invaginations separating them in Dytiscidae). Subsequent cladistic analyses coding the prothoracic defence gland as a simple binary present-or-absent character have recovered it either as the only unambiguous synapomorphy uniting Hygrobiidae + Dytiscidae (Beutel & Haas, 1996), or in combination with the larval trochanteral annulus, position of the larval cerebrum (Beutel *et al.*, 2006; Beutel *et al.*, 2020) and most recently with the elongate larval antennomere 1 (Beutel *et al.*, 2020). However, Baehr's (1979) detailed cladistic study of the prothoracic musculature of Adephaga and phylogenetic analyses utilizing molecular data from multiple genes other than the primary use of mitochondrial genes (Table 1), have failed to provide support for the synapomorphy of the prothoracic gland (de Pinna, 1991), instead corroborating the likely convergent evolutionary origins of these structures.

Although most attention has been paid to the prothoracic glands in terms of the phylogeny of Adephaga, upon reviewing the morphology of the pygidial glands, they are more phylogenetically informative and their homology has never been disputed. In other beetle groups that use chemical defence, such as the Tenebrionidae and Pyrochroidae, abdominal defence glands have also been found to provide a wealth of phylogenetically informative characters (Tschinkel, 1975; Dettner, 1984). The paired pygidial glands of all adephagans are located towards the apex of the abdomen on either side of the hind gut above the reproductive tract, with their opening situated behind the eighth tergite (Forsyth, 1968, 1970, 1972). The glands themselves consist of a large sac-like reservoir and associated secretory lobe attached via a collecting canal (Fig. 3), with an efferent duct and valve near the gland opening (Forsyth, 1968, 1970, 1972). The secretory lobe offers multiple characters that are reviewed here. For this discussion, we utilize the relationships recovered in a recent phylogenomic analysis of Adephaga with comprehensive taxon sampling at the family level (Gustafson *et al.*, 2020). In most Adephaga, the secretory lobe consists of a single elongate tube-like structure (Fig. 3A). This type of secretory lobe occurs in Gyrinidae based on examination of *Gyrinus* Geoffroy (Forsyth, 1968; Dettner, 1985) and *Enhydrus* Laporte (Barth, 1960) in Gyrininae, but most importantly also in *Spanglerogyrus* Folkerts (Burmeister, 1990b: shown in Fig. 4) the sister to all living Gyrinidae (Miller & Bergsten, 2012; Gustafson *et al.*, 2017). As Gyrinidae is the sister group to all other adephagans it is reasonable to assume this type of secretory lobe as the plesiomorphic state. A single elongate secretory lobe (Fig. 3A) is also known in *Cicindela* L. (Forsyth, 1970), with other Cicindelinae secretory lobes having yet to be studied. In Gustafson *et al.* (2020), Cicindelinae was recovered as sister to both Carabidae and Trachypachidae. In all non-cicindeline Carabidae studied by Forsyth (1970, 1972) (71 species representing 32 tribes), and Rhysodidae (i.e. *Rhysodes arcuatus* Chevrolat), the secretory cells are aggregated at the end of long collecting canals into lobular structures called acini (Fig. 3B). Interestingly, Trachypachidae also have this same type of secretory lobe that led Forsyth (1972) to comment:

> '[The pygidial glands of Trachypachidae] show greater similarity to [Carabidae] than do those of Cicindelidae, (Forsyth, 1970) which Crowson prefers to include as a tribe of Carabidae.' (Forsyth, 1972: 267).

Therefore, a pygidial gland with a secretory lobe composed of acini is likely a potential synapomorphy uniting Trachypachidae and Carabidae, possibly to the exclusion of Cicindelinae. This is consistent with the phylogenetic relationships recovered by Gustafson *et al.* (2020) and could potentially provide further morphological support for Cicindelinae as a family distinct from Carabidae (as hinted at by Forsyth in the quote above), pending study of the secretory lobe of Platychilini, which was recently recovered as sister to all other cicindelines (Gough *et al.*, 2020). In Haliplidae, the sister group to Dytiscoidea, most genera have the plesiomorphic simple-elongate-tube-type of secretory lobe (Fig. 3A), for example *Haliplus* Latreille (Forsyth, 1968; Dettner & Böhner, 200) and *Brychius* Thomson (Dettner & Böhner, 2009). *Peltodytes* Régimbart on the other hand appears

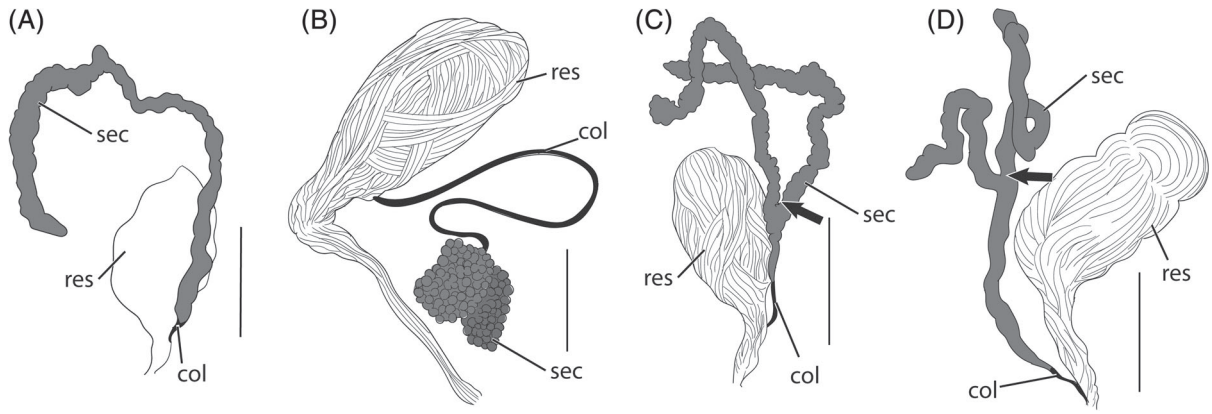

Fig. 3. Pygidial defence glands of Adephaga. (A) Simple elongate tube type secretory lobe as exhibited by *Hyphydrus ovatus* L. after Forsyth (1968). (B) Acini type secretory lobe as exhibited by *Carabus problematicus* Herbst, after Forsyth (1972). (C) Bifurcate type secretory lobe as exhibited by *Hygrobia hermanni* (Fabricus), after Forsyth (1970). (D) Bifurcate type secretory lobe as exhibited by *Amphizoa insolens* LeConte, after Forsyth (1970). Arrows indicate origin of the bifurcation. Res, reservoir; col, collecting canal – shaded black; sec, secretory lobe – shaded grey. Scale bar of A = 0.5 mm, B–D = 1 mm.

to have the acini-type secretory lobe (Fig. 3B), supporting its placement within Haliplidae as sister to the reamining genera (Dettner & Böhner, 2009; Gustafson *et al.*, 2020).

Within Dytiscoidea, Noteridae + Meruidae are consistently supported as being the sister lineage to the remaining families (Fig. 1, Table 1). The pygidial glands of Meruidae remain undescribed, but within Noteridae, *Noterus* Clairville, is known to the have the plesiomorphic simple-elongate-tube-type of secretory lobe (Fig. 3A) (Forsyth, 1968; Dettner, 1985). In nearly all Dytiscidae studied, they similarly possess the plesiomorphic simple-elongate-tube-type of secretory lobe (Fig. 3A). Within the subfamily Hydroporinae which was recovered in a clade with Hydrodytinae as being reciprocally monophyletic to the other subfamilies, *Nebrioporus* Régimbart (Dettner, 2014), *Stictotarsus* Zimmermann (Forsyth, 1968) and *Hyphydrus* Illiger (Forsyth, 1968; Dettner, 1985) exhibit this type of secretory lobe. In Laccophilinae (e.g. *Laccophilus* Leach (Fig. 3A) (Forsyth, 1968; Dettner, 1985) and Copelatinae (e.g. *Copelatus* Erichson [Dettner, 1985]) which are representative of the clades that are sequential sister lineages to the Agabinae, Colymbetinae, Dytiscinae and Cybistrinae, the same type of secretory lobe is found. In Agabinae [e.g. *Ilybius* Erichson (Forsyth, 1968)] and Cybistrinae (e.g. *Cybister*) an unmodified secretory lobe similar to the other dytiscid subfamilies is also encountered. Colymbetinae (e.g. *Colymbetes* Clairville) and Dytiscinae (e.g. *Dytiscus*) (Dettner, 1985; Dettner, 2014; Dettner, 2019) are among the most derived subfamilies (Gustafson *et al.*, 2020) and show a slight modification to the secretory lobe, not seen elsewhere in Dytiscidae. The secretory lobe still consists primarily of a single elongate tube-like structure, however, it is branched apically (Dettner, 1985; Dettner, 2014). These branched secretory lobes could potentially be associated with increased pygidial gland secretion in *Colymbetes* (Dettner, 2019) and *Dytiscus* (2014).

The secretory lobes of Hygrobiidae [e.g. *Hygrobia hermanni* (Fabricius) (see Forsyth, 1970)] and Amphizoidae (e.g. *Amphizoa insolens* LeConte (see Forsyth, 1970), *A. lecontei* Matthews (see Dettner, 2019), *A. davidi* Lucas (see Li *et al.*, 2015) differ from the two aforementioned secretory lobe types (single elongate lobe: Fig. 3A, or acini: Fig. 3B) at both the gross anatomical level and in microstructure. In these two families the secretory lobe is strongly bifurcate (Fig. 3C, D) with the ducts of the secretory cells clustered together into sieve plates as they enter the axial canals (Forsyth, 1970). The secretory cell ducts of the pygidial glands of all other adephagans studied, with the exception of *Brachinus* Weber (Forsyth, 1972) in Carabidae, are not clustered together into sieve plates (Forsyth, 1968, 1970, 1972). In *Amphizoa lecontei* there is also an additional branch off one of the 'arms' distal to the bifurcation (Dettner, 2019), as well as in *A. davidi* (Li *et al.*, 2015), supporting apical branching as a potential secondary modification for increased pygidial grand secretion. Therefore, bifurcate secretory lobes (Fig. 3C, D) of the pygidial defence glands appear to be a synapomorphy of Hygrobiidae + Amphizoidae. Given the monophyly of Aspidytidae with relation to Amphizoidae is in question (Toussaint *et al.*, 2016; Vasilikopoulos *et al.*, 2019), the secretory lobes of Aspidytidae are likely similarly bifurcate. Beutel (1986) previously proposed this character as a synapomorphy of *Hygrobia* and *Amphizoa* LeConte as well, but suggested it also included Dytiscidae [translated from German]:

> 'This situation [of bifurcate pygidial glands] can possibly be regarded as a derived basic plan characteristic of the Amphizoidae + Hygrobiidae + Dytiscidae, whereby the unbranched pygidial gland of most Dytiscidae is interpreted as secondary.' (Beutel, 1986: 47).

However, given that an unbranched secretory lobe is found in Noteridae, the sister group to all other Dytiscoidea except Meruidae (whose secretory lobe form is undescribed), Haliplidae, the sister group to Dytiscoidea, Cicindelinae (recently recovered as sister to Carabidae + Trachypachidae (Gustafson *et al.*, 2020) and Gyrinidae (including *Spanglerogyrus*) the sister group to all other adephagans, it seems most appropriate to regard this state as plesiomorphic, rather than secondarily derived. Furthermore, even if we were to assume the bifurcate

structure of the secretory lobe was lost in Dytiscidae, one would not necessary expect an associated change in the microstructure; so if the single elongate tube of Dytiscidae were in fact secondarily derived, we would expect some evidence of the ancestral sieve plates to be present, which is not the case.

The chemistry of pygidial gland secretions has also been demonstrated to serve as excellent phylogenetic markers in chemically defended beetles, for example the low molecular alkaloids in *Stenus* Latreille staphylinid beetles (Betz *et al.*, 2018). In adephagan beetles, pygidial gland chemistry has frequently been used in the past to infer phylogenetic relationships or explore evolutionary trends (Dettner, 1985, 1987, 1990; Dettner & Böhner, 2009). Dettner (1990) quantified the average number of steps in the biosynthetic pathways necessary for synthesizing the main constituents of pygidial gland secretions in dytiscoid beetles. This was done as a way to identify which components should be regarded as derived characters, as indicated through comparatively more biogenetic steps necessary for their synthesis, allowing evolutionary relationships among dytiscoids to be inferred through presence of shared derived features. In general, Dettner (1990) identified Amphizoida, Dytiscidae and Hygrobiidae, as having shared derived biosynthetic pathways. The pygidial gland secretions of Hygrobiidae have traces of benzoic acid and 4-hydroxybenzaldehyde, that in Dytiscidae make up some of the main gland constituents (Dettner, 1985, 1987, 2019). Hygrobiidae also share biosynthetic precursors in common with Amphizoidae, such as the amino acid methionine, which in the former is used to produce *a*-hydroxy acids and lactides, and dimethyl disulphide in the latter (Dettner, 1990). However, Amphizoidae and Dytiscidae, particularly Hydroporinae, were found to share the most derived pygidial gland chemical components (Dettner, 1990). Notably, both Dytiscidae and Amphizoidae are able to produce the compound marginalin, which was found to be the most derived feature in terms of number of biogenetic steps necessary for synthesis (Dettner, 1990). Marginalin gives the pygidial gland secretions a yellow colour (Dettner, 2014, 2019). However, marginalin is only known from several members of Agabinae (e.g. *Agabus labiatus* (Brahm)*, A. undulatus* (Schrank) and *A. serricornis* (Paykull) (Dettner, 1985, 2014, 2019) and Dytiscinae (e.g. *Dytiscus marginalis* L. (Dettner, 2014, 2019), which are both relatively derived members of Dytiscidae (Gustafson *et al.*, 2020) and even within these genera, some congeners are not known to produce marginalin (Dettner, 2014, 2019). The pygidial gland secretions of Aspidytidae remain unknown, as do those of Meruidae. In general, the biosynthetic pathways and number of biogenetic steps necessary to produce the chemical constituents of the pygidial gland secretions seem to most strongly support the evolutionary relationships in Fig. 1A rather than those promoted by CEA (Fig. 1C).

Larval morphology

For larval morphology supporting a sister group relationship of Hygrobiidae + Dytiscidae, CEA cite Ruhnau (1986) who proposed at least four different features uniting these two taxa. Two of these (characters 36: tarsal claw with spinulae and character 37: presence of a vertical line behind eyes) are either homoplasious across a broad sampling of taxa [spinulae occur on the tarsal claws of numerous other adephagan larvae (Nilsson, 1988) or of questionable homology and have not been treated as viable synapomorphies since that publication. Indeed, Ruhnau (1986) questioned the homology of the vertical line behind the eyes as indicated by introducing character 37 with a '?'. The two characters that have persisted as valid synapomorphies from Ruhnau's (1986) work on larvae are the presence of a trochanteral annulus and an elongate larval antennomere 1.

The trochanteral annulus is a line occurring on the trochanter of the larva in all *Hygrobia* species (Alarie *et al.*, 2004; Michat *et al.*, 2014a) and all Dytiscidae, but which is potentially absent in all other adephagan larvae (Meinert, 1901; Bertrand, 1972; Bousquet & Goulet, 1984; Nilsson, 1988; Alarie *et al.*, 2011; Michat *et al.*, 2017). The exact function of the annulus, whether a sulcus allowing increased flexibility, or an internal ridge providing support, is also currently unclear and its presence may affect the position of muscles originating from within the trochanter (Verhoeff, 1903; Ruhnau, 1986). A similar feature (i.e. trochanteral annulus) is found in larvae of the trichopteran genus *Limnephilus* Leach and has been suggested as providing elasticity to the trochanter (Tindall, 1963). The larva of the hydrophiloid *Spercheus* Kugelann also appears to have a membranous division of the trochanter (Fikáček, 2019: shown in fig. 19.3K) possibly increasing elasticity as well. Alarie *et al.* (2011) proposed the trochanteral annulus of Hygrobiidae and Dytiscidae functions similarly, increasing flexibility for the purpose of improving swimming capability in combination with secondary setae of the legs. Michat *et al.* (2017) concluded the absence of secondary natatory setae on the legs as the likely plesiomorphic state for dytiscid larvae, with numerous independent acquisitions occurring subsequently in several dytiscid groups. Therefore, the presence of similar natatory setae in larval Hygrobiidae is also very possibly a result of convergent evolution, rather than shared ancestry with Dytiscidae (Michat *et al.*, 2017). If indeed the trochanteral annulus is directly related to swimming ability as suggested by Alarie *et al.* (2011), then this feature, like the natatory setae, could also be homoplasious in Dytiscidae and Hygrobiidae, rather than synapomorphic. Evidence appears to be mounting for the convergent evolution of a trochanteral annulus outside that of *Limnephilus* Trichoptera (Verhoeff, 1903) and *Spercheus* hydrophiloids (Fikáček, 2019). Ruhnau (1986: 252) stated 'as a certain convergence *Haliplus* spp. show somewhat like a transverse line of weakness in the posterior wall of their trochanters.' And just recently, Michat *et al.* (2020) provided a detailed description of the larvae of two species of *Haliplus*, where they also recognized the trochanter was divided by an incipient annulus. Closer investigation into the trochanter of larval *Haliplus* species is warranted to help confirm if this lineage also shows independent acquisition of a trochanteral annulus.

Ruhnau (1986) suggested the larval antennomere 1 of Hygrobiidae and Dytiscidae was clearly elongate, being at least twice as long as broad, and thus a synapomorphy uniting the two

families. Beutel *et al.* (2020) recently utilized this character in their morphological dataset:

> '[character] 124. **Shape of antennomere 1: (0) not elongated; (1) distinctly longer than wide. The larval antennomere 1 is strongly elongated in Hygrobiidae and Dytiscidae (Ruhnau, 1986) but not in other groups including the other dytiscoid families … ' (Beutel *et al.*, 2020: Supplementum 1: 13).

Although it is true that most dytiscids have an antennomere 1 that is distinctly longer than wide, this character state varies considerably within Dytiscidae (Alarie *et al.*, 2011). For example, an antennomere 1 that is not distinctly longer than wide occurs in larvae of Copelatinae [i.e. *Copelatus* (Michat & Torres, 2009)], Agabinae [i.e. *Hydrotrupes* Sharp (Alarie *et al.*, 1998; Alarie *et al.*, 2019)], Laccophilinae [i.e. *Laccomimus* Toledo & Michat (Toledo & Michat, 2015)] and Hydroporinae [i.e. *Huxelhydrus* Sharp and *Laccornellus* Roughley & Wolfe (Alarie & Michat, 2007; Michat *et al.*, 2018)], amongst others. Furthermore, even though some *Hygrobia* have an elongate antennomere 1, like *H. nigra* (Clark) (Michat *et al.*, 2014a), others like *H. wattsi* Hendrich do not have antennomere 1 particularly longer than wide (Figs. 3, 4 Alarie *et al.*, 2004). Thus, variability in this feature is present within both Hygrobiidae and Dytiscidae. This character had not previously been employed in morphological analyses (Beutel & Haas, 1996; Beutel *et al.*, 2006; Dressler *et al.*, 2011; Beutel *et al.*, 2013), potentially due to such variability.

The position of the larval cerebrum in the anterior part of the head was proposed as a putative synapomorphy for Hygrobiidae + Dytiscidae by Alarie *et al.* (2004) and recovered as a synapomorphy in subsequent cladistic analyses (Beutel *et al.*, 2006; Dressler *et al.*, 2011; Beutel *et al.*, 2020). However, this character suffers from both problematic homology and character coding. With regards to homology, the observed position of the cerebrum could be a result of modification of other features without common origins. For example, larvae of Hygrobiidae have numerous morphological adaptions that appear associated with their highly specialized diet on oligochaete worms and chironomid larvae (Balfour-Browne, 1922; Cuppen, 2000; Alarie *et al.*, 2004; Michat *et al.*, 2014a). Among these is a voluminous pharynx (Alarie *et al.*, 2004 fig. 24, ph) likely used to suck in vermiform prey (Bertrand, 1972; Alarie *et al.*, 2004). Thus, accommodation of the enlarged pharynx could have resulted in an anterior shifting of the cerebrum of *Hygrobia*, not only correlating these two characters, but causing the anterior position of the cerebrum in *Hygrobia* and dytiscids to be a result of homoplasy. Although it was suggested all larval dytiscids have a cerebrum situated anteriorly (Beutel *et al.*, 2020 supplemental figures), examination of figs 41 and 47 in De Marzo (1979), which illustrate the head of members of Hydroporinae, shows the cerebrum does not appear situated anteriorly, but instead posteriorly. This could be a symplesiomorphy shared with other adephagans. Alternatively, it is entirely possible that the cerebrum of *Hyphydrus* (De Marzo, 1979: fig. 47) and other hydroporines, is not in a different position relative to that of other dytiscid larvae, but appears posteriad due to elongation of the anterior portion of the cephalic capsule into the nasale, another structure adapted for specialized feeding habits (Matta, 1983; Friis *et al.*, 2003; Hayashi & Ohba, 2018). Furthermore, this could also be a result of shortening the posterior region of the cephalic capsule due to a developmental trade-off for lengthening of the nasale, given an increase in certain structures is known to result in compensatory decreases in other anatomical features (Nijhout & Wheeler, 1996; Moczek & Nijhout, 2004), including those located near the exaggerated trait (Emlen, 2001). These aspects render homology assessment of the position of the larval cerebrum both within Dytiscidae, and among dytiscids and hygrobiids problematic. From a character coding aspect, Alarie *et al.* (2004) stated the larval cerebrum of *Amphizoa* is also shifted anteriorly (even if very slightly). Comparing fig. 5 depicting the larva of *Amphizoa lecontei* from Beutel (1991) to figs. 7 and 33 in De Marzo (1979) showing *Hydaticus transversalis* (Pontoppidan) and *Liopterus haemorroidalis* (Fabricius), respectively, the position of the cerebrum appears similar. Additionally, the position of the cerebrum of larval *Aspidytes niobe* Ribera, Beutel, Balke & Vogler shown in Fig. 2 of Balke *et al.* (2005) is similar to that of the aforementioned larvae as well. Thus, if this character is to continue to be used as a binary character: position of cerebrum: (0) posterior part of head; (1) anterior part of head, as in Beutel *et al.* (2020), *Amphizoa* and *Aspidytes* Ribera, Beutel, Balke & Vogler should also be coded as 1 along with Dytiscidae and Hygrobiidae. However, given the issues with assessing the homology of this character and thus establishing consensus on any type of homology statement, it may be appropriate to exclude this character in future morphological datasets.

Morphology of the ovipositor and female reproductive tract

CEA cited Burmeister (1976) for morphological features of the ovipositor uniting Hygrobiidae + Dytiscidae. Indeed, Burmeister's phylogeny depicts this relationship, however, a closer look at fig. 52 reveals character 26 as being the shared derived feature uniting these taxa. Indeed, Burmeister (1976) states (translated from German):

> 'Since the skeleton of *Hygrobia* and Dytiscidae has no derived features in the area of the ovipositor which do not also characterize *Amphizoa*, the possession of this [prothoracic defense] gland is considered a synapomorphy for *Hygrobia* and Dytiscidae and in the following scheme (fig. 52) this feature (26!) is listed.' (Burmeister, 1976: 251).

Later, Burmeister (1990a) again provided numerous synapomorphies associated with the female reproductive tract, but all of these only convincingly united Hygrobiidae, Amphizoidae and Dytiscidae. Even though Burmeister (1990a) did propose two characters uniting Hygrobiidae and Dytiscidae, these involved features questionably observed in preserved specimens and difficult to homologize such as the '*capability* for extreme protraction of coxosterna and tergum IX and genital appendages [emphasis added]' (Burmeister, 1990a: 253). and 'gonocoxosterna ventrally close together in *resting position*

[emphasis added]' (Burmeister, 1990a: 253). Thus, Burmeister (1990a) again relied largely upon the prothoracic glands and the morphology of the immature stages proposed by Ruhnau (1986) for uniting Hygrobiidae and Dytiscidae as discussed above. Miller (2001) conducted a thorough re-examination of the female reproductive tract of these three taxa and similarly found convincing synapomorphies uniting all three families but none supporting Hygrobiidae + Dytiscidae alone.

Morphological view of relationships among Hygrobiidae, Dytiscidae and Amphizoidae

Based on our review none of the morphological features cited by CEA unambiguously support a sister relationship between Hygrobiidae and Dytiscidae. On the contrary, the most complex and unique morphological feature found in these two groups,

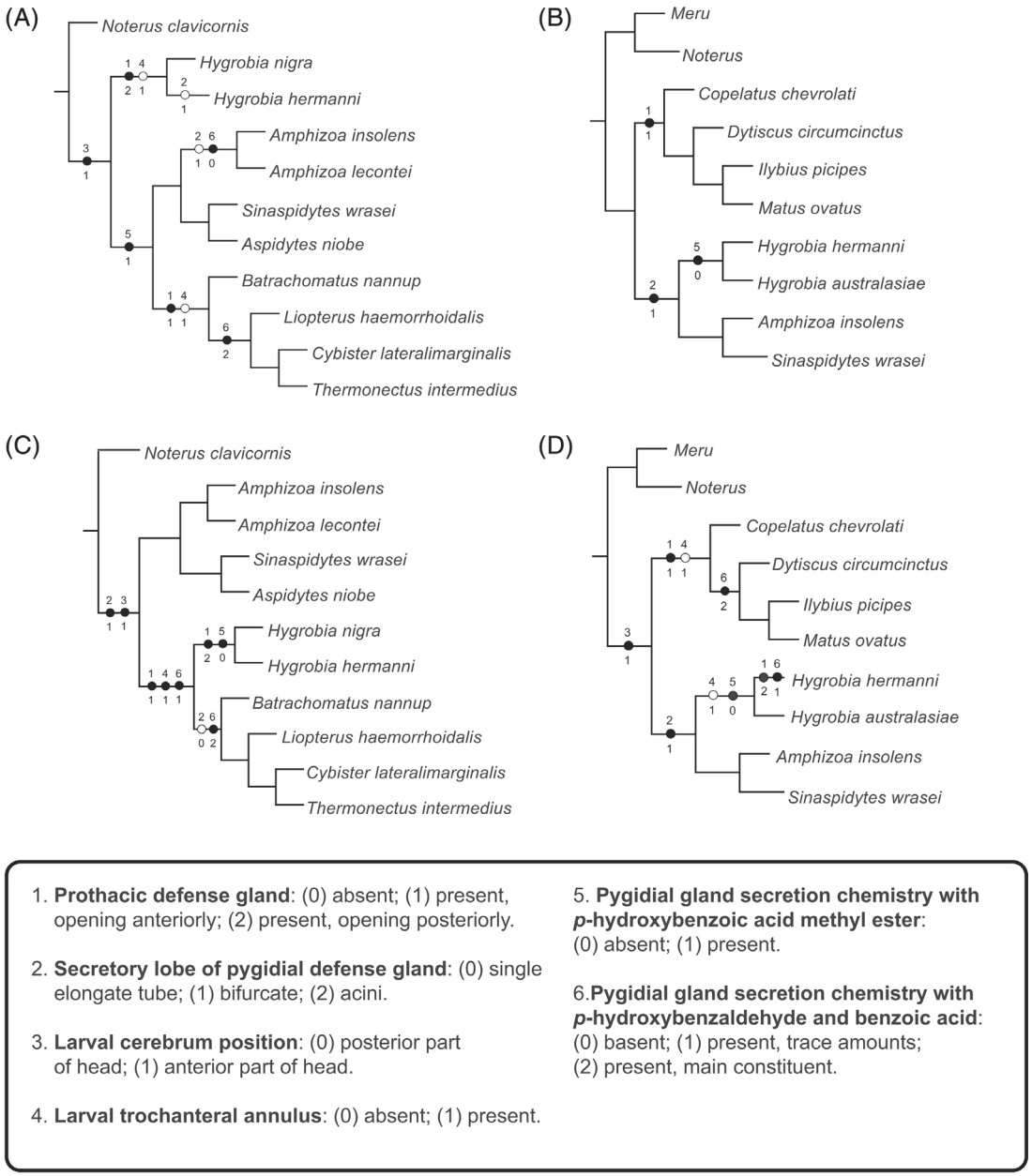

Fig. 4. Results of character mapping onto the preferred phylogenomic trees of (A) Vasilikopoulos *et al.*, (2019) DELTRAN character reconstruction; (B) Gustafson *et al.*, (2020) unambiguous synapomorphies; (C) Cai *et al.*, (2020) ACCTRAN character reconstruction; and (D) Gustafson *et al.*, (2020) DELTRAN character reconstruction. Numbers above circles at nodes indicate character number and correspond to the list of characters provided at the bottom of the figure, with number below indicating character state. Black circles show synapomorphic characters, white circles indicate homoplasious features.

the prothoracic glands, shows strong evidence for the convergent evolutionary origins of these structures. Additionally, the pygidial glands that are undoubtedly homologous but have been largely overlooked, present a potential synapomorphy uniting Hygrobiidae, Amphizoidae, and likely Aspidytidae as well. In Mesquite (Maddison & Maddison, 2019) we coded the characters discussed above based on the literature cited and the discussion presented here (with the exception of the larval antennomere 1 form character). The specifics of our character coding can be found in Fig. 4 as well as in the File S1, including specific assumptions made for coding certain taxa. Using Winclada we mapped these characters onto the dytiscoid clade from the preferred trees of Vasilikopoulos *et al.* (2019) (Figs. 4A, S1), Gustafson *et al.* (2020) (with the dytiscid taxa pruned comparably to the other studies, Figs. 4B, D, S2) and CEA (Figs. 4C, S3). Characters were mapped under three different optimizations: (i) unambiguous (only characters with nonconflicting, unambiguous character state transformations mapped), (ii) accelerated transformation (acctran); and (iii) delayed transformation (deltran) (Farris, 1970; Swofford & Maddison, 1987) onto each topology (Figs. S1–S3). The CEA topology had one 'unambiguous' synapomorphies uniting Hygrobiidae + Dytiscidae: the larval trochanteral annulus (Fig. S3). Under acctran in the CEA topology (Figs. 1C, S3) Dytiscidae is reconstructed as having secondarily lost the bifurcate secretory lobe, an unlikely scenario requiring loss of both macro- and micro anatomical structures as discussed above. deltran in the CEA topology (Fig. S3) suggests the bifurcate secretory lobe is convergent between Hygrobiidae and Amphizoidae, a similarly unlikely scenario. The topology of Vasilikopoulos *et al.* (2019) under deltran, as suspected, is particularly in line with evidence of evolutionary relationships as inferred through chemistry of the pygidial gland secretions (Fig. 1A). Here, the presence of *p*-hydroxybenzoic acid methyl ester is recovered as a synapomorphy uniting Amphizoidae (including Aspidytidae whose secretions remain unknown) and Dytiscidae. Although not supporting secondary homology of the prothoracic gland (Figs. 4A, S1) as an unambiguously synapomorphy under deltran reconstruction, this topology also optimizes as the unlikely secondary loss of bifurcate secretory lobes in Dytiscidae under acctran (Fig. S1). The topology by Gustafson *et al.* (2020) recovers the bifurcate secretory lobe of the pygidial gland as an unambiguous synapomorphy uniting Hygrobiidae, Amphizoidae and Aspidytidae (Fig. 4B). This topology under deltran optimization is particularly compelling (Fig. 4D), with the prothoracic glands an independently evolved synapomorphy of Dytiscidae and likely Hygrobiidae as well (pending description of prothoracic glands outside of *H. hermanni*), with the trochanteral annulus homoplasious in the two families. Optimizing with acctran on this topology (Fig. S2) also supports prothoracic glands as synapomorphies in these two families respectively, but instead reconstructs the trochanteral annulus as a synapomorphy for all dytiscoids except Noteridae and Meruidae, with loss occurring in Amphizoidae + Aspidytidae, which is not implausible considering larvae of these two families primarily crawl over objects submerged in water, rather than swim (Edwards, 1950; Alarie & Bilton, 2005; Michat *et al.*, 2014b). Ultimately, character mapping helps to drive home the conclusion that the results of CEA are not consistent with morphology-based views of dytiscoid relationships and certainly not more so than the other two alternative topologies (Fig. 1A, B). Instead, it seems that CEA's results are most consistent with that of Beutel *et al.* (2020) only, although this topology has never before been recovered by either molecular- or morphological analysis (Table 1).

Concluding remarks

In the age of phylogenomics, competing tree topologies often receive strong to maximum support in spite of conflicting relationships, as exhibited by the three competing hypotheses regarding the phylogeny of Dytiscoidea (Fig. 1) (Vasilikopoulos *et al.*, 2019; Cai *et al.*, 2020; Gustafson *et al.*, 2020). Here, we utilized reciprocal illumination to explore the broader explanatory power of these three trees in the light of complex biological processes and structures, from the biogenetic steps required to synthesize complex chemicals found in the pygidial gland secretions of adephagan beetles, to the morphological structures of the glands themselves. This exercise revealed the two topologies dismissed by CEA (Fig. 1A, B) as being spurious and the result of error in phylogenetic inference, are supported by shared chemical constituents in the pygidial gland secretions requiring complex biosynthetic pathways for synthesis (Fig. 4A) and unambiguous morphological synapomorphies evident in the secretory lobe of the pygidial gland (Fig. 4C) respectively. CEA argued in favour of their tree topology (Fig. 1C) because it is the most '… consistent with morphology-based views of dytiscoid relationships.' It is clear through our use of reciprocal illumination, that this tree topology is neither the most consistent with morphology, nor with pygidial gland chemistry. It is also clear, that morphology-based views of the relationship between Hygrobiidae and Dytiscidae have, in the past, greatly hinged upon interpretation of the homology of prothoracic glands. We have shown that these complex structures do not meet any of Remane's (1952) criteria for objectively identifying primary homology. Additionally, all phylogenetic studies utilizing large quantities of molecular data, with the exception of the recent study by CEA whose topology was recovered utilizing biologically unrealistic trimming regimes (Vasilikopoulos *et al.*, 2020), have not provided evidence for the secondary homology of these structures (Table 1) (de Pinna, 1991). Therefore, contrary to the statement by CEA:

> 'Based on this tree of Dytiscoidea, it will now be possible to address and test a series of hypotheses regarding the evolution of many critical morphological innovations in Dytiscoidea' (Cai *et al.*, 2020: 6),

a phylogeny where the prothoracic glands are recovered as homologous, like Fig. 1C, inhibits our ability to address and test a series of hypotheses regarding the convergent evolution of these morphological innovations in Dytiscoidea. It is our hope that in the future, alternative phylogenetic hypotheses

are given careful consideration, especially through the use of reciprocal illumination. Additionally, we hope more attention will be paid to the pygidial defence glands for understanding the morphological evolution of Adephaga as these are both phylogenetically informative and their homology is not in question.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Appendix S1. Information regarding assumptions used in character coding, morphological character matrices implemented for ancestral state reconstructions with the Vasilikopoulos et al., 2019 taxa and the Gustafson et al., 2020 taxa, and results from all ancestral state reconstructions using Winclada for the three different topologies are available in this file. topologies are available in this file.

Figure S1. Character mapping onto the preferred phylogeny of Vasilikopoulos *et al.*, 2019.

Figure S2. Character mapping onto the preferred phylogeny of Gustafson *et al.*, 2020.

Figure S3. Character mapping onto the preferred phylogeny of Cai *et al.*, 2020.

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Data availability statement

The morphological character matrices and results from all ancestral state reconstructions are provided in the supporting information file S1.

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