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Re-emergence and diversification of a specialized antennal lobe morphology in ithomiine butterflies

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How an organism's sensory system functions is central to how it navigates its environment. The insect olfactory system is a prominent model for investigating how ecological factors impact sensory reception and processing. Notably, work in Lepidoptera led to the discovery of vastly expanded structures, termed macroglomerular complexes (MGCs), within the primary olfactory processing centre. MGCs typically process pheromonal cues, are usually larger in males, and provide classic examples of how variation in the size of neural structures reflects the importance of sensory cues. Though prevalent across moths, MGCs were lost during the origin of butterflies, consistent with evidence that courtship initiation in butterflies is primarily reliant on visual cues, rather than long distance chemical signals. However, an MGC was recently described in a species of ithomiine butterfly, suggesting that this once lost neural adaptation has re-emerged in this tribe. Here, we show that MGC-like morphologies are widely distributed across ithomiines, but vary in both their structure and prevalence of sexual dimorphism. Based on this interspecific variation we suggest that the ithomiine MGC is involved in processing both plant and pheromonal cues, which have similarities in their chemical constitution, and co-evolved with an increased importance of plant derived chemical compounds.

KEY WORDS: Chemical ecology, ithomiini, neural adaptation, olfaction, pheromones, sexual signaling.

An organism'ssensory system is its interface with the rest of the world, the link between its internal and external environments. The manner in which sensory systems vary can reveal how different species are attuned to different cues, the association between cues and behavior, and how behavioral variation maps to the evolution of sensory systems. Lepidopterans have often been used as models to investigate how ecological variability affects the evolution of olfactory systems (Carlsson et al. 2011; Bisch-Knaden et al. 2012; Carlsson et al. 2013; Namiki et al. 2014; Van Dijk et al. 2017), and how the central brain processes olfactory information (Kanzaki et al. 1989; Anton and Hansson 1994; Tabuchi et al. 2013). This includes classic work characterizing pheromones, the olfactory response to these chemical cues, and the manner in which the pheromone processing system evolves (e.g., Butenandt et al. 1959; Klun and Maini 1979; Namiki et al. 2014). Studies on lepidopteran sensory systems have provided crucial insights into how sensory systems function, how separate strands of information are processed and integrated within the brain, and the relationship between sensory systems and ecological variables (Couto et al. 2020).

As in all insects, the primary olfactory processing structure within the lepidopteran brain is the antennal lobe. The antennal lobe is composed of a collection of functional and morphological units, termed glomeruli. Each glomerulus is a synapse dense region composed of the axon terminals of antennal sensory neurons that typically express the same olfactory receptor (Vosshall et al. 2000), local interneurons that refine the olfactory message, and projection neurons that convey information to higher brain

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centers. Odorants elicit activity across a range of olfactory receptors, and associated glomeruli, encoding the odorant identity through the combinatorial activation of glomeruli (Joerges et al. 1997; Galizia et al. 1999; Carlsson et al. 2002; Wang et al. 2003; Hallem and Carlson 2006; Zube et al. 2007). Despite their ecological diversity, within Lepidoptera the antennal lobe is relatively consistent in its structure, being made up of ∼60-80 glomeruli (Rospars 1983; Berg et al. 2002; Kazawa et al. 2009; Heinze and Reppert 2012; Montgomery and Ott 2015; Montgomery et al. 2016; Zhao et al. 2016). However, in moths a prominent morphologically distinct sub-cluster of glomeruli occur at the base of the antennal nerve (Bretschneider 1924; Matsumoto and Hildebrand 1981; Koontz and Schneider 1987). This glomerular cluster is termed a macroglomerular complex (MGC) and is composed of enlarged, "macro" glomeruli (MG), and smaller, associated glomeruli, termed "satellite" glomeruli. These glomeruli often display an extreme degree of sexual dimorphism, being vastly enlarged in males but not in females (Matsumoto and Hildebrand 1981; Koontz and Schneider 1987). Although first identified in moths, MGs/MGCs have subsequently been observed in a diverse range of insects, including in Blattodea, Diptera, Hymenoptera, and Lepidoptera (Chambille et al. 1980; Kelber et al. 2009; Ibba et al. 2010; Kuebler et al. 2010; Couto et al. 2016). MGCs are typically involved in processing pheromonal cues detected by the antennal sensilla, where their corresponding olfactory receptors are highly expressed in a greater number of sensory neurons, providing heightened sensitivity (Warner et al. 2007; Miura et al. 2009). MGs responsive to host plant-related cues have also been reported (Ibba et al. 2010), suggesting they reflect an efficient way of increasing sensitivity to odors with particular biological importance within a species. MGs are therefore classic examples of how neuropil size reflects functional performance, as variation in their volume is generally associated with variation in sensitivity to their corresponding odor (Gronenberg and Hölldobler 1999; el Jundi et al. 2009; Warner et al. 2007; Miura et al. 2009). Furthermore, MGC structure and composition are variable across closely related species, suggesting they may coevolve adaptively with species-specific ecological and behavioral traits (Kondoh et al. 2003; Namiki et al. 2014; Bastin et al. 2018).

While MGCs are ubiquitous in moths (Rospars and Hildebrand 2000; Huetteroth and Schachtner 2005; el Jundi et al. 2009; Løfaldli et al. 2010; Yan et al. 2019), including diurnal species (Stöckl et al. 2016), they are absent in several phylogenetically disparate butterflies (Rospars 1983; Carlsson et al. 2011; Heinze and Reppert 2012; Montgomery et al. 2016) suggesting they were lost at the origin of Papilionoidea. This has been interpreted as reflecting an increased reliance on visual cues, and the decreased importance of long-distance chemical signaling in butterfly mating behaviors (Rospars 1983; Rutowski 1991; Andersson et al. 2007). However, this view is being revisited. Evidence is accumulating that pheromone cues function in interspecific discrimination, sexual attraction, and discrimination, and expedite female acceptance in courtship in a range of butterflies (Andersson et al. 2007; Constanzo and Monteiro 2007; Schulz et al. 2007; Mérot et al. 2015; Chengzhe et al. 2017; Darragh et al. 2017).

One diverse tribe of diurnal butterflies with particular reliance on olfactory cues is the Ithomiini. Ithomiines utilize derivatives of pyrrolizidine alkaloids (PAs) for both chemical defense and intraspecific communication (Pliske 1975a; Pliske 1975b; Pliske et al. 1976; Brown 1984). PAs are sequestered from particular species of plants at the adult stage, with males being significantly more attracted and motivated by these resources than females (Pliske 1975a; Pliske 1975b; Brown 1984). Males provision eggs with PAs through the spermatophore, providing chemical protection to the egg and larvae (Brown 1984). PA-derived pheromones are secreted from "hair pencils," specialized, elongated cells found on the dorsal surface of the androconial gland on the forewing (Schulz et al. 1998). The expression of PA-derived pheromones is believed to represent an honest signal of male quality and facilitates mating receptivity in females (Boppré 1978; Trigo et al. 1994). Male pheromones have been shown to serve a variety of functions across ithomiines, acting purely as an attractant in some species, and as both a short-range female-attractant and long-range male-repellent in other species where males are territorial and defend habitat patches (Pliske 1975a). Consistent with these differences, pheromone blends vary qualitatively and quantitatively across the tribe (Edgar et al. 1976; Brown 1984; Brown 1987; Schulz 1998; Trigo et al. 1994; Trigo et al. 1996; Schulz et al. 2004; Stamm et al. 2019), and male mating strategies range from establishing and defending territories, to gregarious leks, or aggressive "take downs" of females (Pliske 1975a).

The strong sexual dimorphism in adult attraction to PA sources, and the utilization of these chemicals for both chemical defense and pheromones, suggests that olfactory adaptations to detect these compounds have had particular importance in ithomiine evolution. Indeed, the first sexually dimorphic MGC recorded in butterflies was recently described in an ithomiine, *Godyris zavaleta*, where a morphologically distinct cluster of four glomeruli are present in both sexes, with two of these glomeruli being expanded in males (Montgomery and Ott 2015). This observation was surprising given evidence MGCs were lost early in butterfly evolution, with even species of the relatively closely related Danainae subfamily lacking MGCs (Heinze and Reppert, 2012). This strongly suggests the independent, convergent evolution of a once lost neural adaptation in this tribe, a striking example of a reversal in a phylogenetic trend.

However, it remains unclear how prevalent MGCs are across ithomiines, whether this structure functions solely for the localization of PA sources, or whether it is used for long-distance pheromone detection (Montgomery and Ott 2015). Notably, the selection pressures driving dimorphism in these structures may be distinct and more complex than in moths, where male-biased MGCs are typically related to the long-range detection of pheromones released by females (e.g., Matsumoto and Hildebrand 1981). In ithomiines, specialization and male-biased sexual dimorphism in the olfactory system could be related to two distinct selection pressures; (i) detection and acquisition of PA containing plant resources, to which males are more attracted (Pliske 1975a,b; Brown 1984); or (ii) intraspecific interactions dependent on chemical communication, most likely involving male-male detection of chemical traits during territorial interactions, as well as in male-female courtship behaviors. To begin to investigate the distribution, variability, and ecology of ithomiine MGCs, we utilize a comparative approach across 13 diverse species of ithomiines.

Methods

ANIMALS

Specimens were collected in the Estación Científica Yasuní, in the Parque Nacional Yasuní, Orellana Province, Ecuador, during November-December 2011, and September-October 2012, under permit 0033-FAU-MAE-DPO-PNY and exported under permits 001-FAU-MAE-DPO-PNY and 006-EXP-CIEN-FAU-DPO-PNY. Permits were obtained from Parque Nacional Yasuní, Ministerio Del Ambiente, La Dirección Provincial de Orellana. Species representing 12 genera, excluding *Godyris*, were selected on the basis of phylogenetic distribution and available sample size, and represent 8 of the 10 ithomiini subtribes (Table S1; Fig. 1). These species are *Melinaea spp., Mechanitis polymnia, Forbestra olivencia, Methona grandior/curvifascia, Ithomia amarilla, Hypothyris anastasia, Napeogenes larina, Oleria gunila, Hyposcada illinissa, Callithomia lenea, Pseudoscada florula*, and *Hypoleria Sarepta*. To increase the sample size for *Melinaea*, which is in an important phylogenetic position, we include individuals from *M. menophilus, mnasias* and *marsaeus* (see Supporting Information for further discussion). Dissection and fixation of specimens were performed at the Estación Científica Yasuní. Brains were exposed by removing a section of head carapace under HEPES-buffered saline (HBS; 150 mM NaCL; 5mM KCL; 5 mM CaCl2; 25 mM sucrose; 10mM HEPES; ph 7.4), before being fixed with zinc formaldehyde solution (ZnFA; 0.25% [18.4 mM] $ZnCl_2$; 0.788% [135 mM] NaCl; 1.2% [35 mM] sucrose; 1% formaldehyde) for 16–20 h. Extraneous head tissue was then removed, and brains were washed three times in HBS. Samples were transferred to 80% methanol/20% DMSO for at least two hours, then stored in 100% methanol. Samples were kept at room temperature until return to the United Kingdom, then transferred to −20°C.

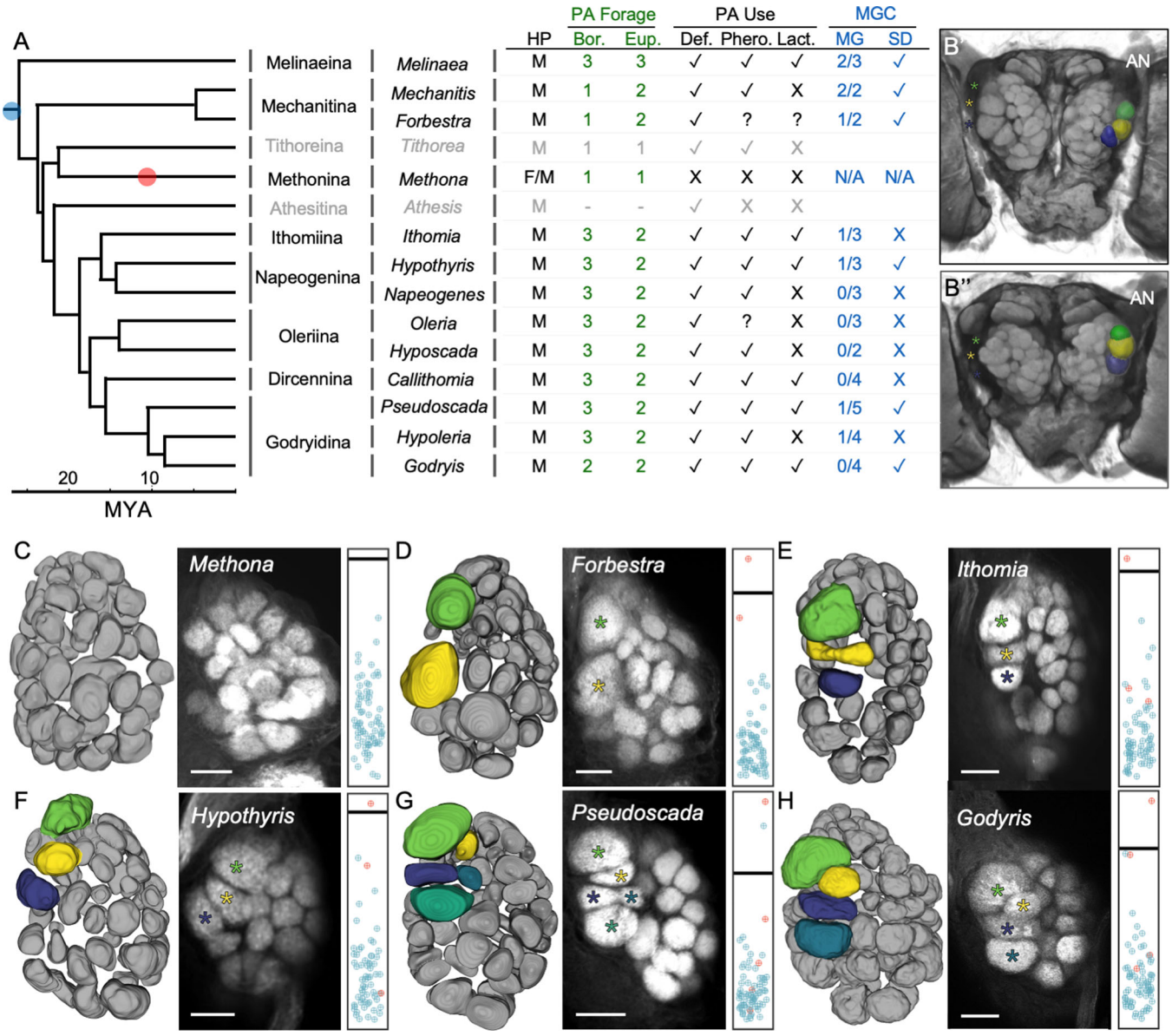
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Samples were rehydrated using serial Tris buffer-methanol solutions (90%, 70%, 50%, 30%, and 0%), each for 10 min. Brains were then incubated for 2 h in NGS-PBS_d (5% Normal Goat Serum, 1% DMSO, 94% 0.1M PBS), before being exposed to anti-SYNORF1 (Antibody 3C11; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, RRID: AB_2315424; Klagges et al., 1996) in solution with PBS_d-NGS, at a ratio of [1:30], for 3.5 days at 4°C. Non-bound antibody was removed by washing with PBS_d (1% DMSO, 99% 0.1M PBS) three times. Goat anti-mouse secondary antibody, Cy2-conjugated (Jackson ImmunoResearch; Cat No. 115−225-146, RRID: AB_2307343, West Grove, PA), was then applied at [1:100] in PBS_d-NGS for 2.5 days at 4°C. Samples were imbued with glycerol through graded exposure in 0.1 M Tris buffer (1%, 2%, 4% each for 2 h, and 8%, 15%, 30%, 60%, 70%, 80%, each for 1 h), and full dehydrated by washing with 100% ethanol (3 × 30 min). Methyl Salicylate was then underlaid, and the brains were allowed to sink. This was repeated twice before transfer to storage vials of methyl salicylate.

CONFOCAL IMAGING AND IMAGE SEGMENTATION

Samples were mounted in methyl salicylate held between two coverslips on either side of a hole bored through an aluminum slide. *Mechanitis* and *Ithomina* were imaged on a Leica SP5 microscope using a 10 × 0.4NA objective. All other species were scanned on an Olympus IX3-SSU using a 10 × 0.4NA objective. As we do not compare raw volumes across species, the use of different microscopes does not affect our subsequent analyses. For each individual, a single stack was taken encompassing the whole of one antennal lobe, chosen at random, with a z-step of 1μm between each optical section and a x-y resolution of 1024 × 1024 pixels. Consistent light detection was ensured by adjusting the laser intensity and gain with depth. To correct for the artefactual shortening of the z-dimension of images due to the air objective lenses, a correction factor of 1.52 was applied to the z-dimension of the image stacks (Heinze and Reppert 2012; Montgomery and Ott 2015). Image segmentation was performed in Amira 5.4.1 (ThermoFisher Scientific; RID: SCR_007353). Volumes of evaluated areas were exported using the *measure statistics* tool. Volumes for surface models were plotted using the *nat* R package (Bates et al. 2020).

Putative MGCs were identified on the basis of internal fibrous structure and location at the base of the antennal nerve, as described in Montgomery and Ott (2015). To formally examine



			PA Forage		PA Use			MGC	
		HP	Bor.	Eup.	Def.	Phero.	Lact.	MG	SD
Melinaeina	*Melinaea*	M	3	3	✓	✓	✓	2/3	✓
Mechanitina	*Mechanitis*	M	1	2	✓	✓	X	2/2	✓
	Forbestra	M	1	2	✓	?	?	1/2	✓
Tithoreina	*Tithorea*	M	1	1	✓	✓	X		
Methonina	*Methona*	F/M	1	1	X	X	X	N/A	N/A
Athesitina	*Athesis*	M	-	-	✓	X	X		
Ithomiina	*Ithomia*	M	3	2	✓	✓	✓	1/3	X
Napeogenina	*Hypothyris*	M	3	2	✓	✓	✓	1/3	✓
	Napeogenes	M	3	2	✓	✓	X	0/3	X
Oleriina	*Oleria*	M	3	2	✓	?	X	0/3	X
	Hyposcada	M	3	2	✓	✓	X	0/2	X
Dircennina	*Callithomia*	M	3	2	✓	✓	✓	0/4	X
Godryidina	*Pseudoscada*	M	3	2	✓	✓	✓	1/5	✓
	Hypoleria	M	3	2	✓	✓	X	1/4	X
	Godryis	M	2	2	✓	✓	✓	0/4	✓

Figure 1. (A) Ithomiine phylogeny illustrating variation in PA usage and summarizing results on MGC morphology. The phylogeny is based upon Chazot et al. (2019) and displays ithomiini subtribes and representative genera (Brower et al. 2014) with associated PA foraging data, PA usage, and putative MGC data. Subtribes where MGC was not evaluated are shown in grey. HP denotes presence or absence of hair pencils, and the sex where these are present is indicated by F/M for female and male. PA foraging is divided between attraction to withered Boraginaceae plants (Bor.), and the flowers and vegetation of Eupatorieae plants (Eup) based on Brown (1984). Attraction to these categories is subdivided into 1 – weak attraction by both sexes, 2 – strong male and moderate female attraction, and 3 – strong male and no female attraction. PA use is categorized into three primary categories; use of PAs for defense (Def) based on cuticular PAs reported by Trigo et al. (1996), PAs as pheromones (Phero), and whether these PA pheromones have been reported to contain lactones in at least one species of this genus (Lact) based on Schulz et al. 2004 and Stamm et al. 2019. '?' indicates missing data. MGC information is given for number of MGs and total MGC glomeruli (MG) and whether there is evidence of sexual dimorphism in any MGC glomeruli (SD). On the phylogeny, the blue circle indicates the presumed origin of the MGC, and the red circle indicates a loss in *Methona*. (B) Volume rendering of synapsin (3C11) immunofluorescence depicting brain anatomy, highlighting the position and morphology of MGC highlighted in color, in *Oleria gunilla* (B') and (B'') *Napeogenes larina*. (C-H) Surface model of full glomeruli segmentations, an example of anti-synapsin immunofluorescence in an antennal lobe confocal section, and the distribution of glomerular volumes in illustrative genera. In glomerular distribution plots, the discrimination threshold above which a glomerulus is considered an MG is indicated in black, and glomeruli within the putative MGCs are highlighted in red. The genera shown are *Methona*, *Forbestra*, *Ithomia*, *Hypothyris*, *Pseudoscada*, and *Godyris*, (C-H) respectively. For full results see Figs. S2-14.

the presence of MGs, each individual glomerulus was segmented in two focal male ALs for each species. In *Methona*, four males were analyzed in this way to confirm the apparent lack of both MGs and MGCs (see Results). Males were chosen for this initial assessment as the MGs clustered around the base of the antennal nerve are larger in *Godyris* males, and no enlarged glomeruli outside this cluster are observed in females (Montgomery and Ott 2015), as is also commonly observed across moths (Rospars and Hildebrand, 1992; Huetteroth and Schachtner 2005; el Jundi et al. 2009; Løfaldli et al. 2010; Yan et al. 2019). Three tests were used to evaluate whether MGs were present in focal individuals, based on methodologies used in previous publications (Kelber et al. 2009; Kuebler et al. 2010; Montgomery and Ott 2015). As the method used by Kelber et al. (2009) was found to be the most conservative (see Supporting Information) we focus on these results in the main text. Under this method, a glomerulus is considered to be an MG if its volume is greater than the 90th percentile of glomeruli volumes plus k times the difference between the 10th and 90th percentiles (Kuebler et al. 2010). We discriminated between MGs and normal glomeruli using a threshold k value of 1.5, which defines a moderate outlier (Sachs 1988). MGs were considered to be present if they passed the discrimination threshold in both fully segmented males. We note that our definition of an MG is dependent upon the volumetric distribution of the other glomeruli, meaning that expansion of non-MGC glomeruli may obscure the presence of glomeruli expanded to a similar degree as MGs in other genera. We therefore provide the glomeruli size distributions for all species (Figs. S2-14).

For subsequent evaluations of sexual dimorphism, we scanned multiple males and females for each species in the same way as described above. In total 178 individuals were measured for this dataset with an average of 15 individuals/species. Samples size for each species are provided in Table S1, and range from 2 to 12 males and 4 to 11 females. In all individuals of both sexes, we then segmented: (i) all glomeruli comprising the putative MGC, including both MGs and closely associated "satellite" glomeruli. Satellite glomeruli were included where they form a distinct unit of glomeruli with the MGs, which are together raised with respect to the anterior surface of the remaining glomeruli, and have the fibrous internal structure characteristic of MGCs (Montgomery and Ott 2015); (ii) the combined volume of all glomeruli; and (iii) total antennal lobe volume including glomeruli and the internal antennal lobe hub. Data for *Godyris zavalata* were taken from Montgomery and Ott (2015) but images were checked for consistency with newly obtained data.

STATISTICAL ANALYSES

In each species, linear models were used to test whether sexual dimorphism is present in the MGC glomeruli using the total volume of non-MGC glomeruli, calculated by subtracting the volume of MGC glomeruli from the total glomerular volume, as an additional factor to control for overall size. We also examined sexual dimorphism in total glomerular volume minus MGC volumes, correcting for the antennal lobe hub volume, a region of low synaptic density that is composed of tracts interlinking glomeruli and higher brain areas, to account for size variation. Normality of the residual errors was assessed using a Shapiro-Wilks test, and equal variance a Breusch-Pagan test. Data were seen to be normal in all cases. Where variance of errors was not equal, we reassessed the data with robust error linear models. These corroborated the results of the linear models in all cases. Multiple testing was accounted for using a sequential Bonferroni correction (Benhamini and Hochberg 1995). As the sample size is limited in some species, we also report the Hedge's g effect size (Hedges and Olkin 1985) for all statistical tests.

Results

ITHOMIINES VARY IN THE PRESENCE AND FORM OF THE MACRO-GLOMERULAR COMPLEX

Ithomiine antennal lobes are similar in structure to other Lepidoptera, with glomeruli (averaging between 64 and 74 glomeruli; Table S2) positioned around a central fibrous region, the "AL hub," which contains projections between glomeruli and downstream targets (Huetteroth and Schachtner 2005; el Jundi et al. 2009; Heinze and Reppert 2012; Montgomery and Ott 2015). Of the 13 species included in our analysis, MGs that passed our statistical threshold were observed in seven: *Melinaea*, *Mechanitis*, *Forbestra*, *Ithomia*, *Hypothryris*, *Pseudoscada*, and *Hypoleria* (Fig. 1). These structures were all components of a multiglomerular complex (MGC) located at the dorsal base of the antennal nerve, with the exception of *P. florula* where we observe one MG within the MGC, and a second on the dorsomedial antennal lobe surface. Two MGs within the putative MGC cluster are observed in *Mechanitis* and *Melinaea* (Table S2, Figs. S3-S4). In species lacking MGs, with the exception of *Methona*, we can however still identify a glomerular complex at the dorsal base of the antennal nerve that is distinct from other glomeruli in the remaining four species (Fig. 1; Figs. S2-14). Despite the variable presence of a formally determined MG within this structure, we refer to it as an MGC throughout as we hypothesize it has a homologous function across species, with differential expansion of individual glomeruli.

These MGCs occur in a corresponding position to that observed in *Godyris*, but the degree to which this structure is raised with respect to the antennal lobe surface is variable. Oleriina species exhibit a more homogenous glomerular surface than those of other subtribes, whereas the MGC is particularly

Table 1. Statistical tests for MGC glomeruli sexual dimorphism, evaluating the influence of sex with the total non-MGC glomeruli volume as a second factor. Significant results are highlighted in bold.

			Linear Model (Structure ~ Sex + All Glomeruli)				
Genera	Structure	Estimate	Std. Error	*t* Value*	*p*-Value	Bonferroni *p*-value	Hedge's *g**
Melinaea	Glom1	49,267	25,269	1.950	0.099		1.702
	Glom2	12,290	10,126	1.214	0.270		1.059
	Glom3	51,579	17,799	2.898	0.027	0.081	2.529
Mechanitis	Glom1	30,930	14,994	2.063	0.069		1.346
	Glom2	**43,309**	**12,909**	**3.355**	**0.008**	**0.017**	2.190
Forbestra	Glom1	28,997	11,792	2.459	0.028	0.056	1.369
	Glom2	48,043	**16,962**	**2.832**	**0.013**	**0.027**	1.577
Ithomia	Glom1	5,042	11,732	0.430	0.673		0.201
	Glom2	4,730	3,098	1.527	0.145		0.713
	Glom3	11,941	6,044	1.976	0.065		0.922
Hypothyris	**Glom1**	46,094	**15,897**	**2.900**	**0.011**	**0.033**	1.513
	Glom2	−2,678	8,762	−0.306	0.764		−0.159
	Glom3	7,646	10,458	0.731	0.476		0.381
Napeogenes	Glom1	−6,120	12,716	−0.481	0.638		−0.268
	Glom2	28,951	21,363	1.355	0.197		0.754
	Glom3	1,878	12,216	0.154	0.880		0.086
Oleria	Glom1	22,264	17,922	1.242	0.254		0.775
	Glom2	15,645	13,485	1.160	0.284		0.724
	Glom3	18,143	15,229	1.191	0.272		0.744
Hyposcada	Glom1	29,946	28,744	1.042	0.338		0.650
	Glom2	−533	14,175	−0.038	0.971		−0.023
Calithomia	Glom1	3,470	7,307	0.475	0.648		0.308
	Glom2	311	11,999	0.026	0.980		0.017
	Glom3	6,356	5,675	1.12	0.295		0.727
	Glom4	2,691	6,805	0.395	0.703		0.257
Peudoscada	**Glom1**	31,996	**5,733**	**5.582**	**<0.001**	**<0.001**	2.815
	Glom2	2,488	2,473	1.006	0.330		0.507
	Glom3	5,870	**1,542**	**3.807**	**0.002**	**0.010**	1.920
	Glom4	2,737	2,083	1.314	0.209		0.663
	Glom5	22,187	**4,648**	**4.774**	**<0.001**	**0.001**	2.408
Hypoleria	Glom1	4,508	6,862	0.657	0.523		0.356
	Glom2	12,863	12,080	1.065	0.306		0.577
	Glom3	−908	6,499	−0.140	0.891		−0.076
	Glom4	8,911	5,547	1.607	0.132		0.870
Godryis	**Glom1**	**40,294**	**12,560**	**3.198**	**0.007**	**0.028**	1.677
	Glom2	−4,044	6,453	−0.627	0.542		−0.329
	Glom3	9,095	10,606	0.858	0.407		0.450
	Glom4	**49,099**	**8,686**	**5.653**	**<0.001**	**<0.001**	2.965

***Negative values are in the direction of females, positive values in the direction of males**

pronounced in species of Dircennina and Godyridina (Figs. S11-14). The composition of the MGC is also highly variable across the tribe. In the basal Mechanitina, the MGC contains two glomeruli. In representatives of Melinaeina, Ithomiina, Napeogenina, and Olerinia we see a single additional satellite glomerulus. Despite being within *Olerinia*, *Hyposcada* does not share this satellite glomeruli, and the two observed MGC glomeruli are of reduced size. The MGC observed in Dircennina and Godyridina have acquired further satellite glomeruli, with their MGCs containing four glomeruli, or five in the case of *Pseudoscada*. The most morphologically complex MGC is observed in *Pseudoscada*, with one MG and four satellite glomeruli.

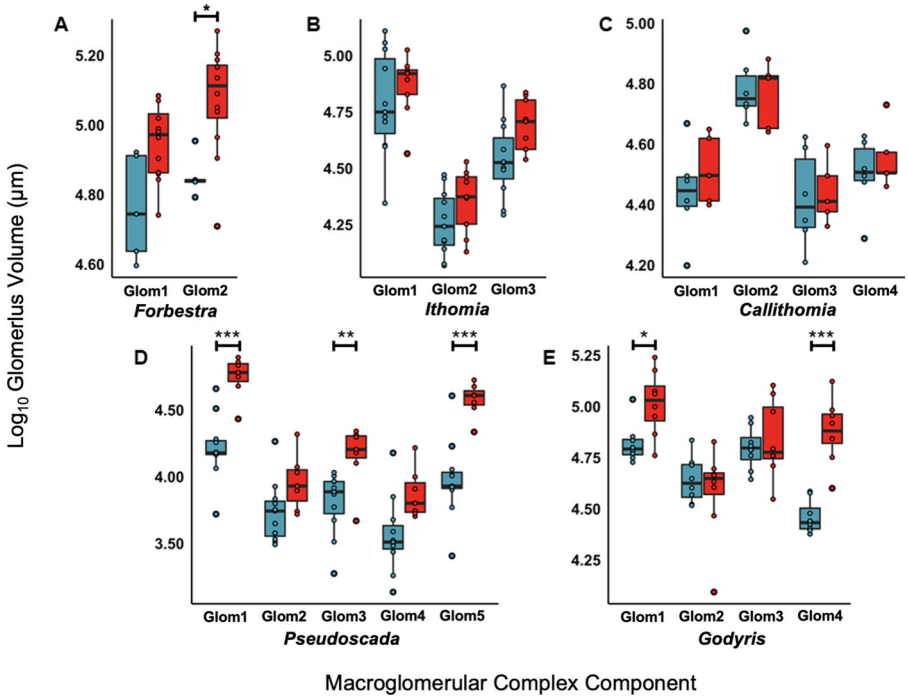


Figure 2. Varying levels of sexual dimorphism observed in MGC glomeruli (numbered dorsally to ventrally as 'Glom') volumes across selected ithomiine genera showing (A-E) *Forbestra* (♀5, ♂12), *Ithomia* (♀11, ♂9), *Callithomia* (♀5, ♂6), *Pseudoscada* (♀11, ♂7), *Godyris* (♀8, ♂8), respectively. Females are shown in blue, males are shown in red. Significance denoted by: *<0.05, **<0.01, ***<0.001. For full results see Figs. S2-14

Table 2. Results of Statistical tests antennal lobe (AL) sexual dimorphism, evaluating the influence of sex with the AL-hub volume as a second factor. Significant results highlighted in bold.

	Linear Model (AL ~ Sex + AL Hub)				
Genera	Estimate	Std. Error	*t* Value*	*p*-Value	Hedge's *g**
Melinaea	−22,946	844,708	−0.027	0.979	−0.024
Mechanitis	238,652	487,445	0.490	0.636	0.320
Forbestra	361,659	278,020	1.301	0.214	0.724
Ithomia	73,192	246,454	0.297	0.770	0.139
Hypothyris	−104,491	473,039	−0.221	0.828	−0.115
Napeogenes	157,416	316,589	0.497	0.627	0.277
Oleria	−63,231	229,058	−0.276	0.790	−0.172
Hyposcada	**-1,377,952**	**315,420**	**-4.369**	**0.005**	**-2.727**
Calithomia	−450,012	275,856	−1.631	0.141	−1.059
Peudoscada	67,279	149,040	0.451	0.658	0.228
Hypoleria	−481,089	376,968	−1.276	0.224	−0.691
Godryis	57,206	279,056	0.205	0.841	0.108

*Negative values are in the direction of females, positive values in the direction of males

VARIATION IN SEXUAL DIMORPHISM IN ANTENNAL LOBE STRUCTURES

Within the MGC, the clusters identified in males are also observed in females. However, the size of these glomeruli is often reduced in females, with correspondingly variable levels of sexual dimorphism across ithomiines. The size of at least one glomerulus is sexually dimorphic in *Mechanitis*, *Forbestra*, *Hypothyris*, *Pseudoscada*, and *Godyris* (Table 1a). In all cases.

These glomeruli are larger in males, with no examples of female expanded glomeruli within the identified clusters. Male-biased sexual dimorphism is limited to a single glomerulus in all genera with the exception of *Pseudoscada*, which has three sexually dimorphic glomeruli, again, all larger in males (Table 1a; Figure 2). After multiple test correction, we do not observe dimorphism in any MGC glomeruli of *Melinaea, Ithomia, Napeogenes, Hyposcada, Oleria, Calithomia, and Hypoleria* (Table 1a). However, we note that in many non-significant tests we observe effect sizes that are comparable with those observed in sexually dimorphic genera (Table 1a) (Cohen 1988). This may suggest that with increased sample size some of these genera would also display significant levels of sexual dimorphism. However, in all cases with "large" effect sizes (Hedge's $g > 0.8$; Cohen 1988) the glomeruli are enlarged in males. In contrast, the total volume of non-MGC glomeruli were seen to be sexually monomorphic, with the exception of *Hyposcada* (Table 1b, Table 2).

Discussion

Within Lepidoptera, significant variation has been observed in the volume of olfactory processing areas. In moths, males have vastly expanded glomeruli in the antennal lobe (MGs/MGCs) that are typically sexually dimorphic and responsive to pheromones (Bretschneider 1924; Matsumoto and Hildebrand 1981; Koontz and Schneider 1987). Here, we present evidence for the widespread distribution of MGCs across the Ithomiini, the only tribe of butterflies currently known to possess this kind of olfactory specialization (Montgomery and Ott 2015). We provide evidence that, having secondarily re-evolved this antennal lobe specialization, ithomiine MGCs have diversified, and are variable across species in both composition and the degree of sexual dimorphism.

Two hypotheses have been proposed to explain why ithomiines have secondarily acquired a trait lost at the base of butterflies. First, ithomiine MGCs could reflect a heightened sensitivity of males to PA resources, increasing their foraging efficiency (Montgomery and Ott 2015). Ithomiine expressed PAs are plant-derived chemicals utilized for several key fitness traits; chemical defense, pheromone synthesis, and nuptial gifts (Pliske 1975a; Pliske 1975b; Pliske et al. 1976; Brown 1984). Scarcity of PA resources, or competition among males, may have increased the selective pressure for sensitivity to PAs and led to the expansion of glomeruli processing these odor cues. This hypothesis would predict a consistent pattern of MG presence and sexual dimorphism across all ithomiines that are attracted to PA-containing plants. Our results suggest an MGC, or at least a positionally homologous glomerular cluster, is indeed identifiable in all members of the tribe, with the exception of *Methona*. The absence of an MGC in *Methona* may be consistent with a relationship between PA attraction and the presence of an MGC, as this genus displays only "weak" attraction to PA-containing plants in both sexes (Brown 1984). However, *Methona* also does not strongly rely on PAs for chemical defense, nuptial gifts, or pheromone precursors (Brown 1987; McClure et al. 2019), making it difficult to disentangle roles of PA attraction and mode of PA use in determining in the presence of an MGC. In addition, although present, *Hyposcada* has an MGC of reduced size and complexity (Figure 1, S10) but maintains a high level of attraction to PA sources (Brown 1984). Hence, our results are not wholly consistent with the prediction of a simple, direct link between PA attraction and MGC morphology. Instead, we find that both the presence of a statistically significant MG and the presence and degree of sexual dimorphism vary across ithomiine genera that are all strongly attracted to PAs. This variation argues against a singular role for the MGC in PA foraging.

The second hypothesis for the origin of the ithomiine MGC suggests a role in intraspecific communication using chemical cues (Montgomery and Ott 2015). Across ithomiines, male pheromones are used in the courtship of females, but some lineages additionally use these pheromones in male territorial defense (Pliske 1975a; Pliske 1975b). The structural diversification of the ithomiine MGC may, therefore, relate to *how*, rather than merely *if*, PAs are used in sexual communication. In many of these species, males are repulsed by lactones, which are present in the pheromone blend of phylogenetically disparate ithomiines (Schulz et al. 2004) and used in male territorial interactions (Pliske 1975a; Pliske 1975b). Genera with more structurally complex MGCs (>2 glomeruli) either include species whose pheromone blends contain lactones (*Melinaea*, *Ithomia, Hypothyris, Calithomia*, *Pseudoscada*, and *Godyris* (Schulz et al. 2004), or species that lack pheromonal lactones but instead have other novel pheromone blends, utilizing compounds that are not observed in other ithomiines (*Napeogenes* and *Oleria*; Schulz et al. 2004; Stamm et al. 2019). In contrast, in *Hyposcada*, in which lactones are absent and the expression of PA-derived pheromones is reduced (Schulz et al. 2004; Stamm et al. 2019), we observe an apparent secondary reduction in both the volume and number of MGC glomeruli. While there is variation beyond these crude groupings, and we lack chemical data for some genera such as *Forbestra*, these features suggest a potential role of an expanded number of MGC glomeruli and the processing of additional pheromone components.

Consistent with this role, the position of the ithomiine MGC is also comparable to the putative pheromone processing cluster of glomeruli in *Danaus plexippus*, which shares a similar fibrous structure (Heinze and Reppert, 2012; Montgomery and Ott 2015) but which is not volumetrically enlarged. The important role that PAs play in the ecology of Danaid species may suggest that this

glomerulus was ancestrally sensitive to PA-derived chemicals in Danaids and ithomiines, but was elaborated in ithomiines (Brown 1987; Shulz et al. 1988). A reasonable question is then, why we have not observed the anatomical specializations that we see in ithomiines in Danaids? We suggest that this may be due to the limited nature of the data on the Danaid olfactory system, with only *D. plexippus* being formally described (Norlander and Edwards 1968; Heinze and Reppert, 2012). Like other Danainae, *D. plexippus* has PA-derived pheromonal compounds and appears to use these compounds to break off male-male courtship pursuits (Pliske 1975c). However, unlike its congeneric species *D. gilippus* (Myers and Brower 1969), removal of abdominal hair pencils has minimal effect on male mating success in *D. plexippus* (Pliske 1975c). Indeed, *D. plexippus'* aggressive male mating strategy, which can involve "aerial take downs" of females (Pliske 1975c) is also similar to behaviours observed in *Methona* (Pliske 1975a), which we find also lack MGCs. It is therefore possible some Danaids may yet have a greater degree of derived antennal lobe specialization depending on their mating behavior. Indeed, the pheromone blends of several other Danaids have similar and overlapping PA-derived compounds to those that we observe in ithomiines (Nishida et al. 1996; Honda et al. 2006).

While our data are consistent with a role for the MGC in sexual communication, sexual dimorphism is variable across the tribe, showing no clear phylogenetic pattern. In ithomiines, sexual dimorphism could also conceivably arise from sex-specific selection on either foraging behavior or intraspecific interactions, or both. First, as discussed above, males of most ithomiines are strongly attracted to PA resources, with sensitivity to these compounds being essential for foraging success. However, once mature, they also become a source of PA-derived volatile compounds themselves (Brown 1984; Trigo et al. 1996). Male-biased sexual dimorphism may therefore be a mechanism for increased sensitivity to PAs and/or to overcome chronic self-exposure to PAs, as chronic exposure to odorants can result in the expansion of responsive glomeruli through increased innervation by inhibitory antennal lobe local neurons (Devaud et al. 2001; Sachse et al. 2007; Anton et al. 2015), a form of inhibitory physiological adaptation. This hypothesis would predict varying degrees of sexual dimorphism across individuals and species, depending on both the amount of pheromone utilized by males, and the similarity of compounds in the male-pheromone blend, PAs used for chemical defense, and attractive plant PA-derived volatiles. We also note that our samples are wild-caught individuals and some of our volumetric estimates show high levels of intraspecific variation, which could be consistent with a plastic response to differential exposure to PAs/PA-derivatives. Second, variable levels of sexual dimorphism could reflect differences in the distances over which males and females use chemical communication. PA-derived pheromones have been hypothesized to represent honest signals of male quality (Boppré 1978; Trigo et al. 1994), which may be salient for both reproductively receptive females, and males during male-male competition. Males of species with heightened territoriality may be under increased selection to detect males entering their territory, leading to increased olfactory sensitivity. Since territorial behavior has been linked to pheromonal lactones (Pliske 1975a) this would predict a greater degree of dimorphism in glomeruli processing these cues. In our data, all but one of the species with detectable dimorphism produce pheromonal lactones, however there are also exceptions, such as *Mechanitis*, which is sexually dimorphic but lacks lactones, and *Callithomia*, which produces lactones but is not sexually dimorphic.

Hence, while neither hypothesis solely explains the emergence and full diversity of ithomiine MGCs, the combination of selection associated with finding and utilizing PAs for pheromonal compounds, and variation in the territorial and display behaviors of male ithomiines, likely explain both the origin and diversification of this structure. Indeed, the coincidence of these two mechanisms may, in itself, be the driving force behind the emergence of MGC. Ithomiines need to discriminate accurately between differing PA chemicals with distinct behavioral implications. The importance of this task increases as the respective behavioral responses diverge, as in the case of PA-lactone aversive males being attracted to plant PA-volatiles. One means of increasing the difference in neural response between glomeruli to chemicals with overlapping receptor activity profiles is through lateral inhibition (Wilson and Laurent 2005; Olsen and Wilson 2008). Expansion of a proto-MGC glomerular cluster may be the result of an increase in local neuron innervation in order to sharpen the neural response from PA responsive glomeruli. Further, this would also suggest a pathway towards the evolution of a compartmentalized MGC, away from the rest of the AL glomeruli, whereby the amount of lateral connections between PA responsive glomeruli increases and connectedness to other AL glomeruli reduces. We see suggestive evidence towards this with species with the most morphologically distinct MGCs typically possessing lactones in their pheromone blend, which have an increased similarity to attractive plant PA volatiles relative to other ithomiine pheromones (Fig. S15).

However, currently the mechanism behind the neural innovation we describe is unclear, but will be informative to explore. Unlike moths, in which pheromone signals are processed by a separate subset of glomeruli from other odors (Kanzaki and Shibuya 1983; Christensen and Hildebrand 1987; Hansson et al. 1991), the limited data available for butterflies suggest they have no specialized pathway for pheromone cues, instead these stimuli produce a neural response in glomeruli that are also responsive to plant odors (Larsdotter-Mellström et al. 2016). The evolution of an MGC in the context in which the pheromone response path-

way is integrated into general odor processing raises several questions: (i) did the MGC originate from existing glomeruli that were sensitive to plant emitted PAs, becoming specialized to detect PA-derived pheromones? (ii) were MGC glomeruli, and their associated olfactory receptors, produced by co-option of existing receptors or duplication of PA-sensitive receptors? (iii) do pheromonal responses remain integrated with general odors during olfactory processing or have parallel pathways evolved in ithomiines? Answering these questions would provide a novel case study in the evolution of sensory and neural traits.

In conclusion, we have identified a highly dynamic cluster of glomeruli in the ithomiine tribe that includes significantly expanded MGs. To date, ithomiines are the only tribe of butterflies reported to have acquired this antennal lobe specialization, strongly implying it has evolved secondarily. We show that ithomiine MGCs are variable in the presence, composition, and degree of sexual dimorphism. Our data support the hypothesis that foraging for plant-derived chemical defense may be the ancestral source of selection pressure favoring the evolution of MGCs in this tribe, with their subsequent elaboration associated with the diversification of ithomiine pheromonal cues. This line of communication could be particularly important in ithomiines as they form multi-species mimicry rings, with ecological convergence within co-mimetic species (Elias et al. 2008), potentially rendering long range visual mating cues less reliable.

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AUTHOR CONTRIBUTIONS

S.H.M. conceived the project and performed the field collections. S.H.M. and B.J.M. designed the current analysis. B.J.M. and A.A. collected the data with support from A.C. and S.H.M. B.J.M. analyzed the data and drafted the manuscript. B.J.M., A.C., and S.H.M. provided intellectual content and revised the manuscript. All authors approved the final version.

DATA ARCHIVING

All data is provided in the supplementary files and has been archived on DataDryad, and is available at: https://doi.org/10.5061/dryad.8sf7m0cp5.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary material
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