

1 **Larval morphology of the avian parasitic genus *Passeromyia*: playing hide and seek with**  
2 **a parastomal bar**

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20 **Running head:** Larval morphology of *Passeromyia*

21

22 **Key words:** Diptera, confocal laser scanning microscopy, light microscopy, Muscidae, myiasis,  
23 scanning electron microscopy

24

## 25 **Abstract**

26 The enigmatic larvae of the Old World genus *Passeromyia* Rodhain & Villeneuve, 1915  
27 (Diptera: Muscidae) inhabit the nests of birds as saprophages or as haematophagous agents of  
28 myiasis among nestlings. Using light microscopy, confocal laser scanning microscopy and  
29 scanning electron microscopy, we provide the first morphological descriptions of the first,  
30 second and third instar of *P. longicornis* (Macquart, 1851) (Diptera: Muscidae), the first and  
31 third instar of *P. indecora* (Walker, 1858) (Diptera: Muscidae), and we revise the larval  
32 morphology of *P. heterochaeta* (Villeneuve, 1915) (Diptera: Muscidae) and *P. steini* Pont, 1970  
33 (Diptera: Muscidae). We provide a key to the third instar of examined species (excluding *P.*  
34 *steini* and *P. veitchi* Bezzi, 1928 (Diptera: Muscidae)). Examination of the cephaloskeleton  
35 revealed paired rod-like sclerites, named ‘rami’, between the lateral arms of the intermediate  
36 sclerite in the second and third instar larva. We reveal parastomal bars fused apically with the  
37 intermediate sclerite, the absence of which has so far been considered as apomorphic for second  
38 and third instar muscid larvae. Examination of additional material suggests that modified  
39 parastomal bars are not exclusive features of *Passeromyia* but occur widespread in the  
40 Muscidae, and rami may occur widespread in the Cyclorrhapha.

41

## 42 **Introduction**

43 *Passeromyia* Rodhain & Villeneuve, 1915 is a small genus of the fly family Muscidae, limited  
44 to the Old World (Skidmore, 1985). The genus is represented by five or six species, due to the  
45 uncertain position of *P. pruinosa* (Wulp, 1880) (described as *Cyrtonaura pruinosa*) for which  
46 the type specimens are lost (Pont, 1974). Several species have a relatively limited distribution.  
47 *Passeromyia longicornis* (Macquart, 1851) is endemic to Tasmania, *P. indecora* (Walker, 1858)  
48 is known from mainland Australia and Fiji, and *P. veitchi* Bezzi, 1928 occurs only in Fiji (Pont,  
49 1974). *Passeromyia steini* Pont, 1970 and *P. heterochaeta* (Villeneuve, 1915) have wider  
50 distributions; the former from Oriental and Australotropical regions and the latter from  
51 Afrotropical, Palearctic and Oriental regions (Pont, 1974; Grzywacz *et al.*, 2014b). Adult flies  
52 feed on rotting plants, resin and the faeces of mammals and birds (Zumpt, 1965; Pont, 1974).  
53 However, the natural history of the immature stages is what attracts most attention from

54 entomologists, veterinarians and conservation biologists. Larvae of *Passeromyia* are  
55 exclusively found in birds' nests where most species act as obligate parasites of passeriform  
56 nestlings (Skidmore, 1985) and other avian host orders (Pont, 1974; Nelson & Grzywacz,  
57 2017). The association with birds' nests is uncommon among muscid flies (Ferrar, 1987), and  
58 *Passeromyia* and the New World *Philornis* Meinert, 1890 are the only genera containing  
59 members that are obligate parasites of nestlings (Skidmore, 1985; Grzywacz *et al.*, 2015).  
60 Similar to *Philornis*, *Passeromyia* display various larval habits, ranging from coprophagy and  
61 saprophagy through to external and subcutaneous parasitism (Skidmore, 1985). *Passeromyia*  
62 *steini* scavenges on excreta and dead nestlings (Roberts, 1940). *Passeromyia heterochaeta*  
63 pierce the skin of nestlings and feed externally on blood, while *P. indecora* and *P. longicornis*  
64 burrow under the skin of the host to feed on blood or tissue (Skidmore, 1985; Edworthy, 2016).  
65 The pathogenic effects of larval-induced myiasis in birds is well established for *Philornis* with  
66 some species reported to severely impact fitness and population dynamics in New World birds  
67 (Dudaniec *et al.*, 2006; Bulgarella *et al.*, 2015). Less is known about the impacts of parasitism  
68 by *Passeromyia* on their avian hosts, however, growth reductions and increased host mortality  
69 are among the pathogenic effects reported in early accounts (Skidmore, 1985; Poiani, 1993).  
70 More recently, surveys in Tasmania revealed an increase in morbidity and mortality among  
71 nestlings of the endangered forty-spotted pardalote (*Pardalotus quadragintus* Gould, 1838)  
72 associated with parasitism by *P. longicornis* (Edworthy, 2016; Edworthy *et al.*, 2019).  
73 Despite the association of *Passeromyia* larvae with a wide range of bird species and their impact  
74 on host fitness, knowledge of the natural history of the immature stages is fragmentary. For  
75 example, the peculiar first instar larvae of *Passeromyia* are well known for their unique, long  
76 filamentous processes on the anal division, yet details of the cephaloskeleton are scant  
77 (Skidmore, 1985) despite the utility of this feature for diagnostics and interpretation of feeding  
78 habits. Existing literature concerning the larval morphology of *Passeromyia* encompasses two

79 early contributions that were limited in material and scope (Zumt, 1965; Skidmore, 1985). The  
80 well-established relationship between larval cephaloskeletal structure and evolutionary  
81 adaptation to feeding strategy (Roberts, 1970; Ferrar, 1979; Skidmore, 1985) has been utilised  
82 in taxonomic and systematic research (Szpila, 2010; Grzywacz *et al.*, 2021). Morphological  
83 analysis of the larval stages has traditionally involved examination by light microscopy, and  
84 more recently, light microscopy combined with scanning electron microscopy (SEM) (e.g.  
85 Grzywacz, 2013; Velásquez *et al.*, 2013; Grzywacz & Pape, 2014). Due to optical limitations  
86 in resolution, illumination and depth of field, light microscopy is largely inadequate for the  
87 precise recognition of, and interactions between, the minute sclerites in the cephaloskeleton  
88 (Grzywacz *et al.*, 2014a). The application of confocal laser scanning microscopy (CLSM)  
89 overcomes these issues because it allows the visualisation of fine, complex, autofluorescent  
90 larval structures (Grzywacz *et al.*, 2014a). CLSM provides high resolution, high-fidelity  
91 imaging and 3D reconstruction of examined structures (Szpila *et al.*, 2021).  
92 The objectives of this paper are to fill the knowledge gap on the larval morphology of  
93 *Passeromyia* to increase diagnostic capacity, and to demonstrate the advantages of applying  
94 CLSM technology in morphological studies of dipteran larvae. A combination of light  
95 microscopy, SEM and CLSM was applied to newly obtained field-collected specimens, while  
96 light microscopy was applied only to older museum material. All larval stages of *P. longicornis*  
97 and the first and third instars larvae of *P. indecora* were examined for the first time.  
98 Redescriptions of the larval morphology of *P. heterochaeta* and *P. steini* are provided.

99

## 100 **Material and methods**

101 Larvae of *P. longicornis* were collected from the nests of *Pardalotus quadragintus* in 2017–  
102 2018 during fieldwork on Bruny Island (southern Tasmania, Australia). *Passeromyia indecora*  
103 were obtained from a pigeon nestling in October 2015 at Bluewater, Queensland (Australia).

104 For details of the identification and further information on study sites, see Edworthy (2016),  
105 Alves *et al.* (2020) and Nelson & Grzywacz (2017). Previously identified larval specimens of  
106 *P. heterochaeta* and *P. steini* were obtained from collections at the Natural History Museum,  
107 London, UK.

108 Examination by light microscopy involved destructive (*P. indecora* and *P. longicornis*) and  
109 non-destructive (*P. heterochaeta* and *P. steini*) protocols. The destructive protocol involved  
110 slide-mounting the anterior body region of specimens in Hoyer's medium using concave slides.  
111 The non-destructive examination of museum collection material was preceded by the  
112 dehydration of larval specimens by serially increasing the ethanol (EtOH) concentration (80%,  
113 90%, 99.5%) followed by immersion in methyl salicylate (Niederegger *et al.*, 2011). Whole  
114 specimens were examined under a stereomicroscope without slide-mounting, after which the  
115 methyl salicylate was washed off and the larvae were rehydrated by decreasing the EtOH  
116 concentration (99.5%, 90%, 80%). Slide-mounted specimens were examined and photographed  
117 with a Nikon 8400 digital camera mounted on a Nikon Eclipse E200 microscope (Nikon Corp.,  
118 Tokyo, Japan).

119 SEM preparation involved dehydration of specimens at 80.0%, 90.0% and 99.5% EtOH,  
120 followed by critical point drying in carbon dioxide (CO<sub>2</sub>) with an Autosamdri®-815, Series A  
121 critical point dryer (Tousimis Research Corp., Rockville, MD, U.S.A.). Larvae were  
122 subsequently mounted on aluminium stubs using double-sided adhesive tape and sputter coated  
123 with gold for 210 s (30 nm of coating) using a JEOL JFC 2300HR high-resolution fine coater  
124 (JEOL Ltd., Tokyo, Japan). SEM images were taken with a JEOL scanning microscope (JSM-  
125 6335F; JEOL Ltd., Tokyo, Japan).

126 According to Grzywacz *et al.* (2014a), material stored in Hoyer's medium is not adequate for  
127 examination by CLSM due to strong absorption of the emitted light by soft tissues and limited  
128 autofluorescence of the cephaloskeleton. Therefore, larval specimens prepared with Hoyer's

129 medium and newly obtained ethanol-preserved material were transferred to 10% potassium  
130 hydroxide (KOH). Material was prepared according to the protocol by Szpila *et al.* (2021) with  
131 the following modifications: 1) adjustment of the tissue maceration interval for specimens of  
132 various sizes (16–18 h for first instar larvae to 24–50 h for second and third instars larvae) to  
133 avoid over-maceration and consequent CLSM image quality reduction; 2) the placement of  
134 larvae in a drop of viscous glycerine. Following tissue maceration in KOH, larval specimens  
135 were transferred to 80% EtOH to dehydrate for 15 minutes. Each sample was placed in a drop  
136 of glycerine on a cavity slide and covered with a coverslip. Sequential scanning of samples at  
137 various excitation wavelengths (488 nm, 561 nm, 633 nm) was conducted using a Leica TCS  
138 SP8 Confocal Laser Scanning Microscope (Leica Microsystems, Wetzlar, Germany). However,  
139 the use of the 488 nm laser was limited to the third instar of *P. indecora* and second instar of *P.*  
140 *longicornis* to prevent imaging of soft tissue remains that obscured fine sclerites of the  
141 cephaloskeleton. Specimens were examined under a 40x oil immersion objective with a  
142 numerical aperture (N.A.) of 1.3. After acquisition, all individual images were assembled to  
143 generate maximum intensity projections (MIP) using LAS AF V3.3 software and 3D  
144 visualization using LAS X 3D Viewer program. To obtain the appropriate image quality and  
145 sufficient data to generate a 3D model, the number of z-steps was individually adjusted to 234,  
146 328–391 and 433 z-frames for sequential larval stages of *P. longicornis* and 304 and 305–555  
147 z-frames for the first and third larval instars of *P. indecora*, respectively. Specimens were  
148 deposited in the collection of the Department of Ecology and Biogeography, Nicolaus  
149 Copernicus University, Toruń, Poland. Larval terminology follows Courtney *et al.* (2000) with  
150 several modifications to general morphology proposed by Szpila & Pape (2005b). Family-  
151 specific structures follow Skidmore's (1985) terminology with a few modifications proposed  
152 by Grzywacz (2013).

153

154 **Results**

155 *Material examined*

156

157 *Passeromyia heterochaeta* (Villeneuve, 1915)

158 Four second instar larvae, label: “C.27: accession”; one third instar larva, label: “C.27:  
159 accession”, two first instar larvae and one second instar larva, label: “*Passeromyia* sp. ex  
160 Mauritius Kestrel // C.27:9”, three third instar larvae, label: “1927 Victoria // C.27:9”; NHM  
161 London.

162

163 *Passeromyia indecora* (Walker, 1858)

164 Two first and four third instar larvae; Queensland, Australia; L. Nelson leg.

165

166 *Passeromyia longicornis* (Macquart, 1851)

167 Five first, 26 second and 30 third instar larvae; Bruny Island, southern Tasmania (Australia); F.  
168 Alves de Amorim and D. Stojanovic leg.

169

170 *Passeromyia steini* Pont, 1970

171 One second instar larva, label: “C.27:9”; NHM London.

172

173 Given the numerous morphological similarities between members of the genus, descriptions of  
174 the four *Passeromyia* species are combined to avoid repetition, and species-specific traits are  
175 highlighted within the combined description.

176

177 *First instar cephaloskeleton*

178 The cephaloskeleton of the first instar consists of paired mouthhooks (*mh*), a labrum (*lb*), an  
179 unpaired intermediate sclerite (*is*), paired parastomal bars (*pb*) and a basal sclerite (*bs*) with  
180 paired vertical plates (*vp*) and an incomplete (Fig. 3A) dorsal bridge (*db*) (Figs. 1A, B, E; 2A,  
181 B; 3A, B; 4A–C). The *mh* are symmetrical, approximately triangular in shape (Figs. 2A, B; 3A;  
182 4A), dorsally concave with an upwardly curved basal part. Each *mh* is apically equipped with  
183 teeth: three in *P. longicornis* (Figs. 1A; 3A) and two in *P. indecora* (Figs. 1E; 4A). Suprabuccal  
184 teeth (*sub*) are fused with the *mh* (Figs. 1B; 3A, B), protrude forward and are the least  
185 sclerotized component of the cephaloskeleton. At the base of the *mh*, two rod-shaped dental  
186 sclerites (*ds*) are present ventrally (Figs. 1A, E; 3A; 4A, C). The labrum (Figs. 1B; 3B; 4A) is  
187 in the form of a minute process extending upwards from the apical part of an epistomal sclerite  
188 (*es*). The paired *pb* are long and slender (Figs. 1A, E; 2A, B), fused apically with a fine, flattened  
189 *es* (Figs. 1B; 3B; 4B). The *es* is equipped with four, ovoid-shaped appendages, identical in size  
190 and shape (Figs. 3B; 4B). An intermediate sclerite is equipped with a broad crossbeam (*crs*)  
191 (Fig. 3B), *is* arms taper slightly towards the *bs* in lateral view. The massive *bs* (Fig. 2A, B)  
192 consists of paired structures on left and right sides, a broad *vp* with a slender dorsal cornu (*dc*)  
193 and much narrower ventral cornu (*vc*). The upper edge of the *bs*, posteriorly to the *db*, is slightly  
194 arched (convex) in *P. indecora* (Figs. 1E; 2A), while concave in *P. longicornis* (Figs. 1A; 2B).  
195 The *vc* is slightly shorter than *dc*, strongly extended apically and with a clearly visible dorsal  
196 extension (*de*) in *P. longicornis* (Figs. 1A; 2B).

197

### 198 *Second instar cephaloskeleton*

199 In the cephaloskeleton of the second instar (Figs 1C, I; 2D, G), *mh* are symmetrical and well-  
200 separated (Fig. 3E). In lateral view, each *mh* contains a deep incision running from the antero-  
201 ventral edge parallel to the long axis. The incision divides the *mh* into a robust dorsal and a  
202 slender ventral part (Figs. 1C, I; 3E). The dorsal part of *mh* is strongly widened apically with

203 two well-developed teeth directed ventrally. The rod-like ventral arm of the *mh* is shorter than  
204 dorsal part and is joined apically with the dorsal part of the *mh*. The pair of dental sclerites (*ds*)  
205 (Fig. 3E) are tightly appressed to the ventral side of the *mh*. Paired accessory stomal sclerites  
206 (*acc*) similar in size and shape to the *ds*, are positioned ventrally to the middle part of the paired  
207 *mh* (Fig. 1C, I). The *is* is H-shaped in dorsal view (Fig. 3D), laterally angular and elongated  
208 with a broad crossbeam (*crs*). Arms of the *is* are anterodorsally equipped with a parastomal bar  
209 (*pb*) as upwardly directed rods (Figs. 1C, I; 3C), pointed in *P. longicornis* (Fig. 2D) and blunt-  
210 ended in *P. steini* (Fig. 2G) (see discussion below). The connection between parastomal bar and  
211 basal sclerite has atrophied. Additionally, the dorsal margins of the *is* carry fine extensions  
212 directed posteriorly (Fig. 3C). A pair of slender labial sclerites (*ls*) and an epistomal sclerite lie  
213 freely (Figs. 2D, G; 3C) between the anterior part of the *is*. Well-sclerotized rod-like rami (*r*,  
214 see discussion below) (Figs. 1C, I; 2D, G; 3C, D) lie between the arms of the *is*. Rami may  
215 extend posteriorly towards *bs*. Posterior extensions of *r* may be well developed or in reduced  
216 form (Fig. 3D), lie freely between the *vp* and are irregular, straight or wavy in shape. The *vp* is  
217 broader than the *vc* and *dc* (Fig. 2D, G). The *dc* is ellipsoidal, mostly of uniform width, straight,  
218 and twice as long as the *vc*. Dorsal extension (*de*) of the *vc* is strongly sclerotized (Fig. 2D, G).  
219 In *P. steini*, the *vc* is more strongly sclerotised posteriorly than in *P. longicornis*. The *vp* and  
220 the *vc* are not connected transversely (Fig. 3D). The texture of the sclerites is more irregular  
221 than the smooth texture of the first instar cephaloskeleton (Fig. 3C, E).

222

### 223 *Third instar cephaloskeleton*

224 In the third instar (Figs. 1D, F–H; 2E, F, H, I; 3F; 4D–F), the *mh* are symmetrical, distal parts  
225 of each *mh* curved downwards (Fig. 4D). In *P. heterochaeta*, the distal part of each *mh* is slender  
226 and sharply pointed (Fig. 2H, I) while slightly shorter and blunt in *P. indecora* (Fig. 2E). Ventral  
227 to the robust basal part of the *mh* are additional paired sclerites (Fig. 2E, F, H, I). The

228 anteriormost additional sclerites are paired oral bars (*ob*) (Fig. 1D, F, G, H), partially  
229 sclerotized, consisting of a single fine tooth connected to the *mh* by a movable tubular joint  
230 (Fig. 4D). Below the massive basal part of each *mh* is a triangular *ds* and the *acc*. The *es* and  
231 the pair of *ls* (Fig. 4D) lie freely between or below the anterior region of the *is*, respectively.  
232 The *is* is H-shaped with a broad *crs* directed posteroventrally in lateral view. Each arm of the  
233 *is* carries anterodorsally a parastomal bar (*pb*) in the form of a strong rod-like extension (Figs.  
234 2E, F, H, I; 3F; 4E), more or less raised upwards. The parastomal bar tapers apically with a  
235 highly irregular texture (Fig. 3F). The connection between *pb* and basal sclerite has atrophied.  
236 The rami (Figs. 2E, F, H, I; 3F; 4E, F), located in a similar position to the second instar, are  
237 slender, straight and either solid or fractured ending anteriorly with a small hook in *P.*  
238 *longicornis* (Fig. 3F) or without a hook in *P. indecora* (Fig. 4E). The long basal sclerite consists  
239 of broad, paired *vp* with associated pairs of slender *dc* and *vc*. The *vc* are connected  
240 anterodorsally by a narrow, arcuate *db* (Fig. 4F); the ventral bridge (*vb*) is incomplete (Fig. 4F).  
241 The *dc* is slightly longer than the *vc*. In *P. longicornis*, the *vc* appears shorter due to its poorly  
242 sclerotized lobe-shaped posterior end, which extends to the same extent as the *dc*. In all species,  
243 the *vc* has a prominent hump-shaped *de* (Figs. 1H; 2H, I). The hypopharynx bears indistinct  
244 longitudinal ridges.

245

#### 246 *External morphology of all instars*

247 Anterior spiracle in the first instar presents as a simple opening (Fig. 5A), while in the second  
248 and third instars it is fan-shaped and composed of four to six lobes (Fig. 6B). The body surface  
249 is extensively spiculate. The anterior surface of the thoracic and abdominal segments bears  
250 anterior spinose bands (*asb*) (Figs. 2A, B, D–I; 5A; 6A, B). These bands are well defined and  
251 complete on *t1–a5* (Fig. 6D), yet may be indistinct on *a5*. On *a6–7*, *asb* are indistinguishable  
252 (Fig. 5F, G). Spines of the *asb* are arranged in rows on *t1–a1* (Figs. 5A; 6B), in rows and

253 polygonal clusters on *a2–3* (Fig. 6C, D) and polygonal clusters on *a4–5*. The spines are dark or  
254 light-brown, with colouration intensity decreasing posteriorly (Fig. 2A, B, D, E). The mid and  
255 posterior regions of each segment are covered with minute spines (Fig. 5F, G), often wart-like,  
256 light coloured or colourless, blunt-ended or pointed and arranged in polygonal clusters (Figs.  
257 5G, H; 6D). The width of the *asb* is irregular, extended posterodorsally and posterolaterally  
258 with irregular groups of spines. Spines forming the *asb* are larger and darker than those covering  
259 the rest of the body. Spines are relatively long, single or double pointed, arranged individually  
260 or in short rows (Figs. 5B, C; 6E).

261 The first thoracic segment is equipped with a broad and complete band of spines, followed by  
262 a transverse cleft approximately reaching the middle of the segment (Figs. 2A, B, D–I; 5A; 6A,  
263 B). This spinose band is further equipped ventrally with an additional patch of spines beyond  
264 the main broad band and cleft (Figs. 2A, B, D, E, G, I; 6A, B).

265 In the first instar, spines are distinct, light-coloured on the first thoracic segment and colourless  
266 on the remaining segments (Fig. 2A, B).

267 In the second instar, the *asb* are composed of distinct, dark coloured spines, arranged  
268 individually or in very short rows (Fig. 2D, G). In the second instar of *P. steini*, the *asb* on *t1–*  
269 *a5* are distinct with brownish-dark spines and indistinct on *a6–7*. In *P. longicornis*, *t1–a3* have  
270 dark spines, while *a4–5* are colourless. On *a6–7*, the *asb* are indistinct, yet on *a7*, strong,  
271 individually-arranged, dark-pointed spines are present posteriorly.

272 The *asb* spines of the third instar of *P. longicornis* are dark on the thoracic segments and *a1–2*,  
273 light brown on *a3* and colourless on *a4–a5*. In *P. indecora*, spines of the *asb* are dark on thoracic  
274 segments and *a1–3*, and light brown on *a4–a5*. In the second and third instars of *P.*  
275 *heterochaeta*, the whole body surface is covered with dark spines or wart-like protuberances.  
276 Both spines and wart-like prominences are distinctly coloured and well visible on the body  
277 surface as well as on *a6–7*.

278 In the first instar the middle part of segments *a1–7* features a transverse crevice (*cr*) present  
279 ventrally (Fig. 5F). Elliptical lateral creeping welts (*lcw*) are covered by minute spines. A  
280 bubble membrane was not found. Anterior part of each abdominal segment contains a ventral  
281 creeping welt (*vcw*) (Figs. 5F, 6C). In the first instar *vcw* are fold-like and anteriorly covered  
282 with spines (Fig. 5F). In the third instar *vcw* are devoid of spines and bulge-shaped (Fig. 6C).  
283 The first welt, positioned on *a1*, is less distinct compared to those on the remaining segments.  
284 Spines surrounding *vcw* are always coloured, brown to light brown. In the first instar, the  
285 posteroventral surface of *a1–7* has two pairs of bulge-like prominences (Fig. 5F, G), while the  
286 second and third instars have three pairs (Fig. 6C, D).  
287 The surface of the anal division (*ad*) is covered with brown pointed spines or blunted wart-like  
288 spines (Figs. 2C; 5G, H). The anal division ventrally has an anal plate (*ap*) and subanal papillae  
289 (*sa*) distinctly protuberant in a tube-like form (Figs. 5G; 6G). The *ap* is relatively small and  
290 triangular (Fig. 6G). A postanal papilla (*pa*) is indistinct or at most, forming a group of spines  
291 on the same level as the adjacent cuticle (Fig. 6F, G). The subanal papilla is large, bulge-like  
292 and closely appose *ap* (Fig. 5G). Each *sa* is devoid of spines, equipped with a sensillum  
293 basiconicum and two sensilla resembling sensilla ampullacea. In *P. longicornis*, para-anal  
294 papillae (*paa*) are indistinguishable from the adjacent cuticle (Fig. 5G). In *P. indecora*, the *paa*  
295 are in the form of a fold, laterally to the *sa* (Fig. 6G), while in *P. heterochaeta* they form distinct  
296 cones.  
297 The spiracular field carries posterior spiracles and is surrounded by seven pairs of sensilla (Figs.  
298 2C; 5H; 6G). The first instar has papillae *p1*, *p3*, *p5* in the form of long filamentous processes  
299 (Figs. 2C; 5H). In the second and third instars, these processes are present in the form of short,  
300 yet distinct cones (Fig. 6F, G). The remaining papillae (*p2*, *p4*, *p6* and *p7*) are indistinguishable  
301 from the adjacent body surface on all instars.

302 Posterior spiracles (*ps*) are slightly raised above the surface of the *ad* in the first and second  
 303 instars (Fig. 5H). In the third instar *ps* are in a depression (Fig. 6G). A spiracular scar (*ss*) is  
 304 placed in a dorsal position and the respiratory slits (*rs*) are bow-shaped to sinuate (Fig. 6G).  
 305 The peritreme is complete and dark (Fig. 2C).

306

307 **Key to the parasitic third instar larvae of *Passeromyia* (excluding *P. steini* and *P. veitchi*)**

308

309 1. Dark spines/wart-like protuberances over entire body surface including *a6–7*. Para-anal  
 310 papillae (*paa*) distinctly cone-shaped. [Afrotropical, Palearctic and  
 311 Oriental].....*P. heterochaeta*

312

313 Anterior spinal bands (*asb*) on *a6–7* indistinct and *a4–5* light brown or colourless  
 314 [Australasian/Oceanian].....2

315

316 2. Anterior spinal bands on *a3* dark and light brown on *a4–5*. Para-anal papillae forming  
 317 lateral fold to subanal papillae (*sa*). [Australia (mainland), Fiji].....*P. indecora*

318

319 Anterior spinal bands on *a3* light brown and colourless on *a4–5*. Para-anal papillae  
 320 indistinguishable from adjacent cuticle. [Australia (mainland), Tasmania].....*P. longicornis*

321

322 **Discussion**

323 Despite the impact on nestling health and survival, the morphology of *Passeromyia* larvae has  
 324 not been comprehensively studied. Limited to two early works (Zumpt, 1965; Skidmore, 1985),  
 325 previous descriptions are incomplete, which is the case particularly for the finer sclerites of the  
 326 cephaloskeleton we present here, such as the rami and epistomal and labial sclerites. Skidmore's

327 (1985) examination of the third instar cephaloskeleton attached to the puparium of *P. indecora*  
328 failed to recognise an accessory stomal sclerite and oral bar, most likely due to the preparation  
329 method. Previous interpretations of the anal region (Skidmore, 1985) also differ from our  
330 observations. The application of SEM enabled the identification of a sensillum basiconicum on  
331 the surface of each bulge (*sa*) lateral to the anal opening. Sensory sensilla are characteristic of  
332 subanal papillae and have not been observed in any other anal papillae (Grzywacz *et al.*, 2015).  
333 Thus, we conclude that the anal region of *Passeromyia* larvae protrudes into a tube-like  
334 structure and carries apically both an anal plate and subanal papillae.

335 Larval morphology of Muscidae, particularly details of the cephaloskeleton, provides valuable  
336 phylogenetic information and is also a rich source of information on larval feeding habits  
337 (Skidmore, 1985; Grzywacz *et al.*, 2021). Our results confirm the position of the genus  
338 *Passeromyia* within Reinwardtiinae on the basis of features shared with other members of the  
339 subfamily. These include massive, strongly sclerotized cephaloskeleton, well separated and  
340 symmetrical mouthhooks, broad intermediate sclerite with rod-like paired rami, robust basal  
341 sclerite and the distribution and size of accessory oral sclerites and dental sclerites (Skidmore,  
342 1985; Velásquez *et al.*, 2013; Grzywacz *et al.*, 2015). Other features of the larval morphology  
343 corroborate the monophyly of *Passeromyia*, and the most conspicuous may be the three pairs  
344 of filamentous processes on the anal division of the first instar, which is a unique character state  
345 among muscid flies and even within the entire cyclorrhaphan Diptera (Ferrar, 1987). Previous  
346 authors did not recognise these processes and their concomitant sensory papillae as homologous  
347 with *p1*, *p3* and *p5* of other calyptrate flies (Grzywacz *et al.*, 2015). The massive mouthhooks  
348 of first instar *Passeromyia* is another unique character state within Muscidae (Keilin & Tate,  
349 1930; Schumann, 1954; Skidmore, 1985; Velásquez *et al.*, 2013; Grzywacz & Pape, 2014). The  
350 vestigial first instar cephaloskeletal labrum is diagnostic for species of *Passeromyia*, but is not  
351 unique among calyptrate flies. A similar reduction of the first instar labrum is observed in all

352 species of the megadiverse flesh fly subfamily Sarcophaginae, in all species of the  
353 Rhinophorinae (now to be considered a blow fly subfamily, see Yan *et al.*, 2021) and in  
354 calliphorine blowflies of the genera *Bellardia* Robineau-Desvoidy, 1863 and *Onesia* Robineau-  
355 Desvoidy, 1830 (Ferrar, 1987). It is therefore difficult to claim a correlation between this  
356 character state and a specific feeding habit given the diverse life histories exhibited by the above  
357 taxa (Ferrar, 1987; Pape, 1996; Cerretti *et al.*, 2020).

358 The accurate diagnosis of myiasis-causing agents such as *Passeromyia* spp. optimises health  
359 and disease monitoring in avian nestling populations. Until now, differentiation of *Passeromyia*  
360 species on the basis of larval morphology has been challenging due to the lack of descriptions  
361 of distinguishing features, visible by light microscope. However, we propose that the  
362 arrangement and colouration of spines on the body segments form species-specific patterns,  
363 allowing identification of *P. indecora*, *P. heterochaeta*, *P. longicornis* and possibly *P. steini*,  
364 pending examination of additional material. Spinal arrangement has been utilised in taxonomic  
365 studies of *Muscina* Robineau-Desvoidy, 1830 (Grzywacz *et al.*, 2015), although this character  
366 is not always easily observed.

367 According to the literature, larvae of the different species of *Passeromyia* differ in food  
368 acquisition strategy (saprophagy, haematophagy, necrophagy), as well as preferred food type  
369 (excreta, food particles, blood, dead nestlings) (Skidmore, 1985). Despite the frequently  
370 observed relationship between cephaloskeletal structure and feeding habit among dipteran  
371 larvae (Skidmore, 1985; Ferrar, 1987), we found that the cephaloskeletons of species of  
372 *Passeromyia* are largely indiscernible, even comparing the saprophagous *P. steini* with the  
373 hematophagous species. For example, unlike the mouthhooks of other saprophagous Muscidae,  
374 the second instar of *P. steini* is equipped with teeth similar to those used for piercing host skin  
375 by obligate parasitic members of the genus (Skidmore 1985), and this feature is therefore best  
376 interpreted as part of the ground plan for the genus. Two alternative hypotheses may explain

377 the lack of morphological diversity among *Passeromyia* species that occupy different  
378 ecological and feeding niches: 1) reported differences in feeding strategy are real and are  
379 manifested in either physiological or behavioural adaptations, while cephaloskeleton structures  
380 did not differentiate during speciation; 2) reported differences are premature conclusions  
381 resulting from a scarcity of field observations. We find the second hypothesis most likely and  
382 suggest that larvae of *P. steini* may be facultative or opportunistic parasites of nestling birds.  
383 Observations in the field are required to substantiate this assertion.

384 Our examination of *Passeromyia* larvae revealed ambiguities in the interpretation of some  
385 sclerites of the cephaloskeleton. During preliminary screening under the light microscope, rod-  
386 like extensions of the basal sclerite directed towards the dorsal surface of the intermediate  
387 sclerite were observed in the second and third instars of some individuals (Figs. 3G–I).  
388 Simultaneously, the dorsal surface of the intermediate sclerite was seen to be equipped with a  
389 distinct anterodorsal extension and a faint posterodorsal extension in some specimens. Rod-like  
390 extensions of the basal sclerite and posterodorsal extensions of the intermediate sclerite in some  
391 specimens appeared as a single structure, while in others a fracture was present. Due to the  
392 shape and position, we initially interpreted these structures as a parastomal bar fused dorsally  
393 with the intermediate sclerite with the occasional basal rupture. However, the lack of parastomal  
394 bars has been generally accepted as the single larval synapomorphy for Muscidae (Roback,  
395 1951; Ferrar, 1979; Skidmore, 1985; Grzywacz *et al.*, 2017). Skidmore (1985) observed the  
396 presence of slender rods between the upper edges of the intermediate sclerite in the third instar  
397 of *P. heterochaeta* and considered these atrophied parastomal bars. Siddons & Roy (1942)  
398 illustrated a similar rod-like structures to those in the current study in their work on the second  
399 instar of *Synthesiomyia nudiseta* (Wulp, 1883) (Siddons & Roy, 1942, fig. 7). An extension of  
400 the basal sclerite, apparently fused apically with the intermediate sclerite, was recently observed  
401 in some specimens of *Muscina* (Grzywacz *et al.*, 2015). Our re-examination of third instar

402 larvae of *Muscina prolapsa* (Harris, 1780) with CLSM revealed occurrence of two pairs of rod-  
403 like structures associated with the intermediate sclerite (Fig. 7A, B). The first pair emerge from  
404 the lateral arms of the intermediate sclerite, and the second pair lie freely between the lateral  
405 arms of the intermediate sclerite. Additionally, in a single specimen we observed a distinct  
406 connection between the basal sclerite and bars emerging from the lateral arms of the  
407 intermediate sclerite (Fig. 7A, B). Furthermore, frame-by-frame analysis of CLSM stack  
408 images confirmed that these bars are fragmentarily fused with the intermediate sclerite. These  
409 observations allowed us to conclude, that parastomal bars, considered to be absent in Muscidae,  
410 are present in the second and third instar larvae, yet in a modified form. The apical part of the  
411 parastomal bar is fused with the intermediate sclerite, forming a rod-like extension of the  
412 intermediate sclerite, while the basal part of the parastomal bar is reduced, and only rarely  
413 connected with the basal sclerite. The apical part of the parastomal bar in the form of a distinct  
414 upwardly directed extension is characteristic of Reinwardtiinae (e.g. *Philornis* Meinert, 1890,  
415 *Muscina*, *Synthesiomyia*). Nevertheless, more research is necessary to further investigate the  
416 shape and limits of the parastomal bar in the remaining muscid larvae. Consequently, paired  
417 rods lying freely between the arms of the intermediate sclerite we considered not homologous  
418 to parastomal bars. To our knowledge, no studies to date have shown the presence of a structure  
419 with similar characteristics in the Muscidae nor in any other calyptrate family. However, an  
420 extensive literature search showed that similar rods, named “rami”, have been reported in the  
421 second and third instars of some Lauxaniidae (Semelbauer & Kozánek, 2011, 2012, 2014). To  
422 provide greater certainty on these structures, we analysed additional material including some  
423 species from the muscid genera *Alluaudinella* Giglio-Tos, 1895, *Australophyra* Malloch, 1923,  
424 *Coenosia* Meigen, 1826, *Hydrotaea* Robineau-Desvoidy, 1830, *Lispe* Latreille, 1796, *Musca*  
425 Linnaeus, 1758, *Muscina*, *Potamia* Robineau-Desvoidy, 1830, *Stomoxys* Geoffroy, 1762,  
426 *Synthesiomyia* Brauer & Bergenstamm, 1893 and *Thricops* Rondani, 1856. Our preliminary

427 analysis suggests that rami are a common feature of late instars of Muscidae (Fig. 7A–C) with  
428 the exception of *Stomoxys* and the subfamily Coenosiinae (Fig. 7D, E). Rami always occur  
429 between the lateral arms of the intermediate sclerite, are slender or somewhat flattened, lying  
430 freely or tightly appressed to the inner part of the intermediate sclerite. Beyond Muscidae, in  
431 calyptrate families known to possess parastomal bars we also observed rami. For instance,  
432 Anthomyiidae have two pairs of rod-like sclerites, that is, the rami lying freely between the  
433 arms of the intermediate sclerite, and well-developed parastomal bars, which may be free lying  
434 or as a rod-like extension of the anterior margin of the intermediate sclerite (Fig. 7F; KW &  
435 AG, unpublished data).

436 Variation in shape and location of the rami among genera and individual specimens probably  
437 explains why the feature has been previously overlooked. These rods are arranged either above  
438 the intermediate sclerite and visible in lateral view, or positioned between the arms of the  
439 intermediate sclerite and poorly visible laterally. We conclude from our observations that rami  
440 are likely to occur widespread within Schizophora and then possibly within all cyclorrhaphan  
441 Diptera, but additional data from multiple taxa are required.

442 This study provides the first comprehensive documentation of the cephaloskeleton of muscid  
443 species obtained by confocal laser scanning microscopy (CLSM). Despite earlier evidence of  
444 the utility of CLSM in visualizing morphological structures, this powerful tool has rarely been  
445 used on immature stages of Diptera (Grzywacz *et al.*, 2014a; Szpila *et al.*, 2016, 2021; Li *et al.*,  
446 2021). The main obstacles are high costs, equipment availability and lack of standard protocols  
447 for the preparation and visualization of specimens. The available protocols for the preparation  
448 of material for CLSM turn out to be only basic guidelines, and modifications are often required  
449 for individual preparations (Grzywacz *et al.*, 2014a; Szpila *et al.*, 2021). Throughout this study,  
450 we found that the condition of material, the presence of impurities, previous storage conditions  
451 and the time of specimen maceration are critical to a successful CLSM analysis. Nonetheless,

452 CLSM is an innovative tool that allows visualisation of the position and shape of fine, complex  
453 morphological structures without the requirement for additional staining of specimens  
454 (Grzywacz *et al.*, 2014a). Here, the application of light microscopy provided insufficient  
455 resolution to visualize fine, tightly arranged and multi-layered cephaloskeletal structures of the  
456 larvae. The correct interpretation of rami and parastomal bars was possible only thanks to the  
457 application of CLSM. Most importantly, the ability to generate 3D visualizations revealed  
458 interactions between individual sclerites that could not be obtained with light microscopy alone.

459

#### 460 **Acknowledgments**

461 We would like to express our appreciation to Dr. Dejan Stojanovic (Canberra, ACT, Australia)  
462 for help in obtaining material. K.W. and A.G. received support from the European Union's  
463 Horizon 2020 research and innovation programme under grant agreement No 823827  
464 SYNTHESYS+, and the National Science Centre of Poland (grant no. 2019/33/B/NZ8/02316).  
465 F.A. received support from The Holsworth Wildlife Research Endowment & the Ecological  
466 Society of Australia; the Mohamed bin Zayed Species Conservation Fund [172516602], and  
467 the Australian National University Research School of Biology.

468

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573

574 **Figure captions**

575 Fig. 1. Cephaloskeleton of *Passeromyia* spp. larvae. (A) *P. longicornis*, first instar larva, lateral  
576 view and (B) dorsal view; (C) *P. longicornis*, second instar larva, lateral view; (D) *P.*  
577 *longicornis*, third instar larva, lateral view; (E) *P. indecora*, first instar larva, lateral view; (F)  
578 *P. indecora*, third instar larva, lateral view; (G) *P. heterochaeta*, young third instar larva, lateral  
579 view; (H) *P. heterochaeta*, mature third instar larva, lateral view; (I) *P. steini*, second instar  
580 larva, lateral view. Abbreviations: acc, accessory stomal sclerite; crs, crossbeam; db, dorsal  
581 bridge; dc, dorsal cornu; de, dorsal extension; ds, dental sclerite; es, epistomal sclerite; is,  
582 intermediate sclerite; la, lateral arm; lb, labrum; ls, labial sclerite; mh, mouthhook; ob, oral bar;  
583 pb, parastomal bar; r, rami; sub, suprabuccal teeth; vb, ventral bridge; vc, ventral cornu; vp,  
584 vertical plate. Scale bars: 0.05 mm.

585  
586 Fig. 2. Details of *Passeromyia* spp. larvae, material in Hoyer's medium (A–F) and methyl  
587 salicylate (G–I). (A) *P. indecora*, cephaloskeleton of the first instar larva, lateral view; (B) *P.*  
588 *longicornis*, cephaloskeleton of the first instar larva, lateral view; (C) *P. longicornis*, anal  
589 division of the first instar larva, posterior view; (D) *P. longicornis*, cephaloskeleton of the  
590 second instar larva, lateral view; (E) *P. indecora*, cephaloskeleton of the third instar larva,  
591 lateral view; (F) *P. longicornis*, cephaloskeleton of the third instar larva, lateral view; (G) *P.*  
592 *steini*, cephaloskeleton of the second instar larva, lateral view; (H) *P. heterochaeta*,  
593 cephaloskeleton of the young third instar larva, lateral view; (I) *P. heterochaeta*,  
594 cephaloskeleton of the mature third instar larva, lateral view. Scale bars: (A, B, D–I) 0.05 mm;  
595 (C) 0.1 mm.

596  
597 Fig. 3. CLSM images of *Passeromyia longicornis*. (A) cephaloskeleton, first instar larva, dorso-  
598 lateral view and (B) dorsal view; (C) intermediate sclerite with rami, second instar larva, lateral

599 view and (D) dorsal view; (E) mouthhooks, second instar larva, antero-lateral view; (F)  
600 intermediate sclerite with rami, third instar larva, lateral view. Abbreviations: bs, basal sclerite;  
601 crs, crossbeam; ds, dental sclerite; es, epistomal sclerite; is, intermediate sclerite; lb, labrum; ls,  
602 labial sclerite; mh, mouthhook; pb, parastomal bar; r, rami; sub, suprabuccal teeth. Scale bars:  
603 0.05 mm.

604

605 Fig. 4. CLSM images of *Passeromyia indecora*. (A) cephaloskeleton, first instar larva, lateral  
606 view of anterior part; (B) dorsal view of anterior part and (C) ventral view of intermediate  
607 sclerite; (D) mouthhooks, third instar larva, lateral view; (E) intermediate sclerite with rami,  
608 third instar larva, lateral view; (F) apical part of basal sclerite, third instar larva, dorsal view.  
609 Abbreviations: db, dorsal bridge; ds, dental sclerite; is, intermediate sclerite; lb, labrum; ob,  
610 oral bar; pb, parastomal bar; r, rami; vb, ventral bridge. Scale bars: (A–C, E, F) 0.05 mm; (D)  
611 0.1 mm.

612

613 Fig. 5. First instar of *Passeromyia longicornis* [SEM]. (A) anterior end of body with anterior  
614 spinose band, lateral view; (B) anterior spinose band on the second thoracic segment; (C)  
615 posterior spinose band on the seventh abdominal segment; (D) maxillary palpus; (E) antennal  
616 complex; (F) seventh abdominal segment, ventral view; (G) posterior end of body, ventral view;  
617 (H) posterior end of body, lateral view. Abbreviations: a6–7, abdominal segments 6–7; ap, anal  
618 plate; cr, transverse crevice; ns1–2, first and second additional sensillum coeloconicum; p1, p3,  
619 p5, papillae 1, 3, 5 surrounding spiracular field; sa, subanal papillae; sb1–3, sensilla basiconica  
620 1–3; sc1–3, sensilla coeloconica 1–3; vcw, ventral creeping welt; vm, ventromedian process.

621

622 Fig. 6. Third instar of *Passeromyia indecora* [SEM]. (A) anterior end of body, ventral view;  
623 (B) anterior end of body, lateral view; (C) second abdominal segment, lateral view; (D) second

624 abdominal segment, ventral view; (E) posterior spinose band on the second abdominal segment;  
625 (F) posterior end of body, lateral view; (G) anal division, posterior view; (H) ventral creeping  
626 welt on the third abdominal segment. Abbreviations: an, antennal complex; asb, anterior  
627 spinose band; mh, mouthhook; mp, maxillary palpus; ob, oral bar; paa, para-anal papilla; sa,  
628 subanal papilla.

629

630 Fig. 7. CLSM images of third instar larvae of some representatives of the family Muscidae (A–  
631 E), Anthomyiidae (F) and Calliphoridae (G) with rami (A–C, F) and without rami (D–E, G).  
632 (A) *Muscina prolapsa*, intermediate sclerite, lateral view; (B) *Muscina prolapsa*, intermediate  
633 sclerite, dorsal view; (C) *Synthesiomyia nudiseta*, intermediate sclerite, dorsal view; (D) *Lispe*  
634 *tentaculata* (De Geer, 1776) (Diptera: Muscidae), intermediate sclerite, dorsal view; (E) *Lispe*  
635 *tentaculata*, intermediate sclerite, dorso-lateral view; (F) *Delia* sp. Robineau-Desvoidy, 1830  
636 (Diptera: Anthomyiidae), intermediate sclerite, dorsal view; (G) *Lucilia sericata* (Meigen,  
637 1826) (Diptera: Calliphoridae), intermediate sclerite, dorsal view. Abbreviations: pb,  
638 parastomal bar; r, rami. Scale bars: 0.05 mm.