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1 **Interspecific shape divergence in Aphodiini dung beetles: the case of *Amidorus obscurus* and *A.***  
2 ***immaturus* (Coleoptera: Scarabaeoidea)**

3

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10

11 **Abstract** The dung beetles *Amidorus obscurus* and *A. immaturus* are nearly indistinguishable, being  
12 characterized by a marked constancy in external morphological traits and little sexual dimorphism in  
13 adults. We studied two syntopic populations from the Italian Alps by means of geometric  
14 morphometric analyses. To identify eventual undetected shape differences between species, we  
15 focused upon the head, pronotum and scutellum (three external traits) and the epipharynx. Results  
16 indicate that the external traits are rather similar in the two species, whereas the epipharynx is clearly  
17 different. Interspecific differences in the aedeagus were also taken into account; they are noteworthy  
18 because parameres of *A. immaturus* are different in shape and at least three times longer than those of  
19 *A. obscurus*. If it is assumed that the diversification of the two species took place during quaternary ice  
20 ages, *A. immaturus* would have evolved marked differences rather quickly, in keeping with the  
21 hypothesis of rapid genital evolution. In an ontogenetic trajectory framework, we also considered the  
22 morphology of larvae. Interspecific divergence in the shape of the epipharynx is already evident at the  
23 preimaginal stage, whereas that of the genital disc is not. Accordingly, we hypothesise that feeding  
24 and reproductive traits of the two species diverge morphologically when they become functional.  
25 Finally, by considering recent advances in ecological and evolutionary knowledge of dung beetles, the  
26 pattern of relative constancy in external morphology exhibited by the tribe Aphodiini, and that of great  
27 morphological diversification displayed by Onthophagini, were compared and hypotheses about the  
28 origins of these differences discussed.

29

30 **Keywords:** constancy in external morphology; divergent evolution; larval instars; geometric  
31 morphometrics; trait functionality.

32

### 33 **Introduction**

34 The subfamily Aphodiinae is a very speciose group (about 2000 species) that includes the dung beetle  
35 tribe Aphodiini, which, with about a thousand species being described so far (Scholtz et al. 2009), is  
36 characterized by a worldwide distribution, with a focus on temperate regions. Aphodiini display a  
37 rather uniform foraging behaviour, with both adults and preimaginal instars of most species being  
38 coprophagous and dung-dwelling (Dellacasa and Dellacasa 2006).

39 The remarkable taxonomic diversity of Aphodiini shows a certain constancy in the phenotypes of  
40 adults, which are characterized by a small body size (2-20 mm), a rather extended clypeus which  
41 covers the mouthparts, a pygidium largely overlapped by the elytra, untrimmed basal margins of  
42 elytra, nine-segmented antennae and the posterior tibiae with two apical spurs (Dellacasa and  
43 Dellacasa 2006). A previous phylogenetic analysis based on morphology suggested that in this group  
44 variation in morphological characters is small (Cabrero-Sañudo 2006). Aphodiini lack evident sexual  
45 dimorphism and alternative phenotypes in males, both potentially promoting diversification and  
46 speciation, and which are widespread in other dung beetle groups (Moczek 2008, 2010; Pfennig et al.  
47 2010). In the subfamily Scarabaeinae, the tribe Onthophagini (2500 taxa or species, Scholtz et al.  
48 2009, Philips 2011), for instance, displays a tremendous diversity of horn structures with many species  
49 developing horns or horn-like structures of some kind, which represent evolutionary modification of  
50 the same, original ancestral structure (Moczek 2005, 2008, 2011). In many species of the genus  
51 *Onthophagus*, there are large *major* males producing horns and smaller *minor* males which are  
52 hornless (a case of male polyphenism; Emlen et al. 2005; Macagno et al. 2009; Tomkins and Moczek  
53 2009, Knell 2011). Phylogenetically close species with similar horns may diverge in static allometries,  
54 males of each species developing horns at different body sizes (Emlen et al. 2005; Lukasik et al. 2006;  
55 Moczek 2002; Tomkins and Simmons 2002).

56 In the pattern of uniformity of phenotypes exhibited in Aphodiini, interspecific differences in external  
57 morphology may be very subtle and certain species pairs may be virtually indistinguishable. To  
58 overcome these difficulties, a geometric morphometric approach can be employed. Geometric  
59 morphometrics has proved to be a useful technique for solving a variety of biological problems, and is  
60 more powerful than traditional morphometrics, having the ability to identify very subtle differences in  
61 shape (Lawing and Polly 2010; Slice 2007).

62 In northern Europe, dung beetle communities are dominated by Aphodiinae (Errouissi et al. 2004; Jay-  
63 Robert et al. 1997, 2008) and the same is true for high-altitude communities of the Alps and Pyrenees  
64 (Cabrero-Sañudo and Zardoya 2004; Cabrero Sañudo 2006; Cabrero Sañudo and Lobo 2006).

65 In the present study, we focused upon two species of the genus *Amidorus* (systematics according to  
66 Dellacasa and Dellacasa 2006 used), inhabiting high-altitude alpine pastures of the Ferret Valley  
67 (north western Italian Alps): *Amidorus obscurus* (Fabricius, 1792) and *A. immaturus* (Mulsant, 1842).  
68 Both species are Palaearctic (Dellacasa and Dellacasa 2006; Palestrini et al. 2008; Tagliaferri 2000,

69 Tarasov 2008), although *A.obscurus* is widespread from Spain to Syria and Transcaucasus, whereas *A.*  
70 *immaturus* is restricted to a smaller area (SE France, NW Italy and Austria), included within the range  
71 of *A. obscurus*.

72 In the pattern of uniformity of phenotypes exhibited in Aphodiini, interspecific differences in external  
73 morphology may be very subtle and certain species pairs may be virtually indistinguishable. *Amidorus*  
74 *obscurus* and *A. immaturus* provide just such an example of strong morphological uniformity due to  
75 low phenotypic plasticity. The two species (and the two sexes) are almost indistinguishable on the  
76 basis of the external morphology, and the external traits typically employed in taxa identification (e.g.,  
77 the clypeal margin of head, the pronotum punctuation, the elytral striae and interstices, the scutellum  
78 features) are in fact virtually useless. Instead, males of the two species can be identified on the basis of  
79 the shape of the aedeagus, which shows clear differences in the two species (Dellacasa 1983). (It must  
80 be underlined, however, that females of both species cannot be identified on the basis of genitalia  
81 (Dellacasa and Dellacasa 2006).) As a consequence, the taxonomic history of *A. immaturus* has been  
82 troubled (Tagliaferri 2000), being correctly identified only in recent times (Nicolas and Riboulet  
83 1967). This may partly explain why the state of the knowledge of the life history of the two species is  
84 insufficient. It is only known that their general ecological habits are alike: both species are montane,  
85 oligotopic, with a preference for exposed pastures, and may be collected from spring to autumn in  
86 different types of dung (Dellacasa and Dellacasa 2006).

87 To identify eventual undetected shape differences between species, we focused at first on adults and  
88 took into account three external traits (head, pronotum, scutellum) and two internal traits (epipharynx  
89 and male genitalia). We examined the overall shape variation of the anatomical parts listed above  
90 (with the exception of the male genitalia, see Method for justification) using geometric  
91 morphometrics. This method has proven to be a useful technique that is more powerful than traditional  
92 morphometrics in identifying very subtle differences in shape (Lawing and Polly 2010; Slice 2007)  
93 and is a robust and reliable technique to study morphological variation in invertebrates (Andrade et al.  
94 2009; Becerra and Valdecasas 2004; Crews 2009; Holwell and Herberstein 2010; Holwell et al. 2010).  
95 To detect the developmental origin of interspecific differentiation, two larval stages (1<sup>st</sup> instar and 2<sup>nd</sup>  
96 instar) were also examined by geometric morphometrics, focusing on the shape of the larval  
97 epipharynx and on the male genital disc (a precursor of male genitalia).

98 The pattern of relative constancy in external morphology exhibited by Aphodiini was compared with  
99 the pattern of great morphological diversification exhibited by the Onthophagini, which we also  
100 studied using geometric morphometrics (Macagno et al. 2009; Pizzo et al. 2006, 2008), with the main  
101 aim to identify possible determinants of the evolution of the two divergent morphological patterns.

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## 105 **Material and Methods**

### 106 **Sampling**

107 About two hundred (N= 242) unidentified and unsexed *Amidorus* adults were collected in the Ferret  
108 Valley, an alpine valley on the Italian side of the Monte Bianco massif, at the top end of the Aosta  
109 Valley, north-western Italy (45°50'60" N; 7°01'00"), in summer 2008. Individuals were reared in  
110 isolation in covered plastic containers (9.5 cm diameter, 12.5 cm height) under laboratory conditions  
111 for about two months. Then, they were dissected and unequivocally assigned to one species using the  
112 aedeagus (males) and the epipharynx (both sexes) as specific diagnostic characters (Roggero, Tocco  
113 and Palestrini, unpublished results). Larvae found in the containers of females were therefore  
114 identified *a posteriori*, i.e. after the identification of their mothers.

115

### 116 **Morphological preparations**

117 Adults and larvae were cleaned in 70% ETOH , stored in vials filled with 99% ETOH and dissected  
118 without boiling.

#### 119 *a) Adults*

120 For morphological analyses, we used all *A. immaturus* we collected (N= 24, i.e. 9 males and 15  
121 females) and only a part of the *A. obscurus* sample (N= 66, 32 males and 34 females, randomly  
122 chosen). Preparations of male genitalia and epipharynges followed the well known, standardized  
123 methods usually employed for Coleoptera (Dellacasa 1983; Dellacasa et al. 2010; Skelley 1993).

124 The aedeagus of the two species diverges in the shape of the apices of paramers. However, as pointed  
125 out by Nicolas and Riboulet (1967), the apices of paramers are membranous and their definite shape  
126 can be fully appreciated only when preparations are re-hydrated; this prevented us from using  
127 geometric morphometric analysis on this trait.

128 The epipharynx of adults is a complex, asymmetrical structure on the inner surface of the clypeus with  
129 a pubescence that is variously-shaped and developed. It is constituted by a membranous lamina and  
130 many sclerotized support structures (Dellacasa 1983; Dellacasa and Dellacasa 2006; Dellacasa et al.  
131 2010), which we used for the geometric morphometric analyses.

132 Epipharynx, head, scutellum and pronotum images were taken using a stereoscopic dissecting  
133 microscope Leica Z16Apo (Leica Microsystems AG, Wetzlar, Germany).

#### 134 *b) Larvae*

135 For the analysis of the epipharynx we used 40 individuals of *A. immaturus* (i.e. 12 male and 7 female  
136 1<sup>st</sup> instar larvae; 8 male and 13 female 2<sup>nd</sup> instar larvae) and 38 of *A. obscurus* (i.e. 8 male and 10  
137 female 1<sup>st</sup> instar larvae; 9 male and 11 female 2<sup>nd</sup> instar larvae).

138 In coleopteran larvae, the epipharynx is an external, relatively simple structure (Steinmann and  
139 Zombori 1984), in which certain parts (i.e. the tormae) are not entirely developed as in adults. Unlike  
140 adults, in the larvae the epipharynx surface is glabrous for the most part.

141 To identify the preimaginal instars, we used the measures of head width as proposed by Daly (1985),  
142 the 1<sup>st</sup> instar being smaller than the 2<sup>nd</sup> instar.

143 To identify the sex of the larvae, we used the genital disc, an unpaired embryonic structure located on  
144 the ventral side of the abdomen, which is well-developed and evident in males only (Martinez and  
145 Lumaret 2003, 2005; Moczek and Nijhout 2002; Roggero, Tocco and Palestini, unpublished results.).  
146 For the analysis of the genital disc we employed 33 larvae of *A. immaturus* (i.e., 18 individuals of 1<sup>st</sup>  
147 instar and 15 individuals of 2<sup>nd</sup> instar) and 41 larvae of *A. obscurus* (19 individuals of 1<sup>st</sup> instar and 22  
148 individuals of 2<sup>nd</sup> instar).

### 149 **Landmarks and measurements**

150 In landmark-based morphometric analyses, the morphology of an object is represented by coordinates  
151 of sets of landmarks (Bookstein 1991). The landmark points (as defined in Appendix 1) were chosen  
152 for their ease of identification, homology in the two species and ability to capture the general shape of  
153 each morphological structure, and were digitized using tpsDig 2.16 (Rohlf 2010a).

154 In adults, landmarks on the head (N = 6), scutellum (N = 5), and epipharynx (N = 9) were digitized as  
155 shown in Fig. 1. For the pronotum (N = 16) we used the sliding semi-landmarks method (Bookstein  
156 1997; Perez et al. 2006; Mitteroecker & Gunz 2009), as implemented in tpsRelw v1.49 (Rohlf 2010b)  
157 (Fig. 1). In larval instars, landmarks were digitized on the epipharynx (N = 9) and genital disc (N = 7),  
158 as shown in Fig. 2.

### 159 **Statistical analyses**

160 To test whether the variation in shape in each dataset was small enough to have an adequate  
161 approximation of the linear tangent space to the non-linear Kendall shape space, we employed  
162 tpsSmall v1.20 (Rohlf 2003) on the different landmark configurations.

163 Generalized Procrustes analysis (GPA) was performed by tpsRelw v1.49 (Rohlf 2010b), retaining the  
164 centroid size and relative warp values for further analyses. For each anatomical structure, we drew  
165 scatterplots of the shape variation within the sample summarized by the relative warps (SPSS Statistics  
166 v18.0, SPSS<sup>®</sup> Inc.).

167 Significance of group membership of specimens for each configuration of points was tested by the  
168 Multivariate Test of Significance (equivalent to the Hotelling Generalized T<sup>2</sup> test) as implemented by  
169 tpsRegr v1.37 (Rohlf 2009), in which the Goodall's F-test (Goodall 1991) is employed to test whether  
170 the mean shapes would differ more than expected by chance (Hallgrímsson et al. 2007). Goodall's F  
171 test compares the difference in mean shape between two samples relative to the shape variation found  
172 within the samples and is the most powerful approach to test whether the mean shapes differ more  
173 than expected by chance. The proportion of Goodall's F statistics from randomly permuted data sets (n  
174 = 1000 here) as great or greater than the Goodall's statistic on the original data set is given as the  
175 significance probability. Use of the permutation test relaxes some of the restrictive assumptions of  
176 Goodall's F test (Goodall 1991; Rohlf 2000). Goodall's F test only considers the total amount of shape

177 variation, and does not consider the directionality of the variation. With small samples (relative to the  
178 number of landmark coordinates), this is a useful property.

179 Discriminant Function Analysis was carried out on the whole data set of relative warp scores to obtain  
180 a classification matrix based on shape variation (in keeping with Janžekovic and Kryštufek 2004;  
181 Kryštufek and Janžekovic 2005; Pizzo et al. 2006, 2008) using SPSS v18. We used the percentage of  
182 correct classifications of cross-validation to further evaluate interspecific differences in shape (in  
183 cross-validation, each case is classified by the functions derived from all cases other than the case in  
184 question).

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## 188 **Results**

### 189 **Adults**

190 A very good correspondence between shape and tangent space was found for all anatomical traits  
191 (slope = 0.99; correlation = 1.0), thus indicating that the geometrical heterogeneity of the sample was  
192 small enough to allow subsequent analyses.

#### 193 *External traits*

194 Plots of the first two RW scores (Fig. 3) suggested that external traits (head, pronotum and scutellum)  
195 were rather similar in shape. Interspecific differences in the shape of the head, pronotum and  
196 scutellum tested through the Multivariate Test of Significance are given in Table 1. Results of  
197 permutation tests again suggested a poor interspecific differentiation in all the external anatomical  
198 traits (Table 1). In the Discriminant Function Analysis, percentages of correct classification in cross-  
199 validation were poor, all being below 70% (head: 64.4%; pronotum: 64.4%; scutellum: 65.6%).

#### 200 *Aedeagus and epipharynx*

201 Interspecific differences in the shape of the aedeagus were visually apparent because of the peculiar  
202 shape of the apices of paramers, that are unmistakably different in the two species (Nicolas and  
203 Riboulet 1967; Dellacasa 1983). They are short, arched, tapered and pointed in *A. obscurus*, and at  
204 least three times longer, mostly rectilinear, evenly broad and rounded distally in *A. immaturus*. The  
205 interspecific difference in the shape of epipharynx was also highly significant (Table 1). The plot of  
206 the first two RW scores confirmed this pattern because it displayed two distinct, non-overlapping,  
207 clusters, clearly demonstrating this trait does vary between species (Fig. 3, bottom, on the right).

208 The results of the Generalized Goodall F-test and the Permutation Tests (Table 1) were significant,  
209 suggesting specimens can be correctly classified on the grounds of the shape of the epipharynx.

210 Finally, in the Discriminant Analysis most cross-validated grouped cases were correctly classified  
211 (96.7%).

### 212 **Larvae**



213 A very good correspondence between shape and tangent space was obtained for all traits (genital disc  
214 slope = 0.99, correlation = 0.99 for both instars; epipharynx slope = 0.99, correlation = 1.00 for both  
215 instars), thus indicating that the geometrical heterogeneity of the sample was small enough to allow  
216 subsequent analyses.

#### 217 *Genital disc*

218 Plots of the RW values evidenced a marked superimposition of the two species (Fig. 4, top).  
219 Interspecific differences in the shape of the genital discs tested through the Multivariate Test of  
220 Significance confirmed a marked species similarity (Table 2), suggesting that they cannot be  
221 distinguished by the genital disc in either preimaginal stage. The Discriminant Function Analysis  
222 correctly classified 64.9% (1<sup>st</sup> instar) and 75.7 % (2<sup>nd</sup> instar) of individuals, respectively.

#### 223 *Epipharynx*

224 Plots of the two first RW scores of the 1<sup>st</sup> instar showed a relative overlap between the two species,  
225 whereas that of 2<sup>nd</sup> instar displayed two distinct clusters, although partly superimposed (Fig. 4, bottom,  
226 on the right). Interspecific differences in the shape of epipharynx, however, suggested differences  
227 were significant in both larval instars (table 2). Discriminant analysis indicated interspecific  
228 differences were more conspicuous in the 2<sup>nd</sup> instar (97.5%) than in the 1<sup>st</sup> instar (89.5%).

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## 232 **Discussion**

### 233 **Interspecific divergence between *A. obscurus* and *A. immaturus***

234 Geometric morphometrics was used to describe interspecific morphological variation and to depict  
235 subtle interspecific differences. Plots of relative warp scores, Multivariate Tests of Significance and  
236 Discriminant function analyses gave rise, generally, to complementary results: in adults, the shape of  
237 external traits (head, pronotum and scutellum) were rather similar in the two species, whereas the  
238 epipharynx was clearly different. The aedeagus was not analysed morphometrically because  
239 interspecific differences in shape and length of parameres were quite apparent. Therefore, the  
240 interspecific shape divergence pattern evidenced in adults was similar to that observed in two  
241 *Onthophagus* sister species (*O. taurus* and *O. illyricus*), which were poorly distinguishable on the  
242 basis of morphological external traits, but revealed significant differences in the aedeagus and  
243 epipharynx (Pizzo et al. 2006, 2009).

244 The two species examined in this study are phylogenetically very closely related. Piau et al. (1999), on  
245 the grounds of the genetic differences observed through mtDNA and PCR-RFLP analyses, suggested  
246 the mitotypes of the two species derived from one common mitotype ancestor. Jay-Robert et al.  
247 (1997), mostly on the grounds of the characteristic distribution of the two species of *Amidorus*,

248 assumed that certain populations remained isolated within the Alps during quaternary glaciations and  
249 produced local speciation in a mountain system.

250 If so, and with reference, in particular, to the last glaciation (15-20000 years ago), *A. immaturus* would  
251 have developed an exaggerated trait, the elongated aedeagus, rather quickly. Within this framework,  
252 the evolution of an exaggerated and complex primary sexual trait may have been an adaptation to  
253 avoid hybridization during and after the speciation event. All the above is in keeping with the tenet  
254 which assumes reproductive organs evolve quickly in insects (rapid genital evolution), and also with  
255 the results of several studies regarding the role of genital divergence in speciation (Dapporto 2010a,  
256 2010b; Gilligan and Wenzel 2008; House and Simmons 2003; Mutanen and Pretorius 2007, Mutanen  
257 et al. 2007; Parzer and Moczek 2008; Pizzo et al. 2008).

258 After having detected this pattern in adults, we focused upon interspecific differences in the genital  
259 disc and epipharynx of larvae, looking for the ontogenetic stage at which interspecific differentiation  
260 arises. It worked out that the male genital disc is neither differentiated in 1<sup>st</sup> nor in 2<sup>nd</sup> instars, whereas  
261 the epipharynx is significantly differentiated in the 2<sup>nd</sup>, but not the 1<sup>st</sup>, instar. It should be taken into  
262 account that early larval stages are smaller and therefore less differentiated than later ones, hence  
263 probably making interspecific differences in shape morphology less apparent. We believe the low  
264 morphological divergence in the epipharynx of the 1<sup>st</sup> instar (Multivariate Test of Significance gave  
265 significant results, Discriminant Function Analysis correctly classified 89.5% of individuals, but the  
266 first two relative warp scores were largely overlapping) may be interpreted in this context. It can be  
267 hypothesized that evolutionary changes that produced the great interspecific difference in the shape of  
268 the aedeagus discussed above took place in the latest preimaginal instar, as in most hemimetabolous  
269 insects. The preimaginal instars, in fact, are more prone to changes than adult stages, whose  
270 morphology is somehow frozen in the invariance of its exoskeleton (Minelli 2007).

271 Finally, if we consider in the same context both the imaginal and the preimaginal data, a correlation  
272 between interspecific divergence and functionality of the trait seems to arise. The adult male aedeagus  
273 is functional (adults are able to reproduce), whereas the larval male genital disc, an aggregate of  
274 undifferentiated cells that are the precursor of adult genitalia, is not. Accordingly, the former trait is  
275 different in the two species, while the latter is not (although it must be admitted that, at this stage, an  
276 eventual diverging trait may not have shown a size or a developmental status accessible to our  
277 geometric morphometric analyses). In a similar way, the shape of the epipharynx is functional both in  
278 adults and in larvae (that, in fact, spend most of the time feeding on dung) and, accordingly, it is  
279 significantly divergent in the two species both in adults and in larvae (in the 2<sup>nd</sup> instar at least). Our  
280 results indicate that interspecific divergence in the shape of the trait may be correlated with its  
281 reproductive or feeding functionality, i.e. feeding and reproductive traits of the two species diverge  
282 morphologically when, during the ontogenetic development, they become functional.

283 This study focused on specimens from both species sampled from the same locality. In syntopic  
284 conditions, in theory, there could either be hybridisation or increased contrast between species due to  
285 selection against hybridisation. However, the morphological differences of genitalia suggest the two  
286 species are now reproductively isolated and we are therefore confident the distinct inter-specific  
287 divergence found here for some traits could also be recovered in populations where either of the  
288 species occurs alone (Piau et al 1999).

### 289 **Divergent patterns of evolution in Aphodiini and Onthophagini**

290 Aphodiini and Onthophagini display dramatically divergent patterns of diversification in external  
291 morphology. Against the relative constancy in external phenotype exhibited by the Aphodiini lineage  
292 with lower levels of phenotypic plasticity, Onthophagini present a large interspecific diversity of  
293 external morphologies (Parzer and Moczek 2008; Tomkins and Moczek 2009). This diversification  
294 pattern still holds at the intra-specific scale. Aphodiini do not exhibit sexual dimorphism, whereas  
295 Onthophagini exhibit both sexual dimorphism and male polyphenism (Moczek 2010; Simmons et al.  
296 2007).

297 Species ecology is known to influence the evolution of certain morphological traits. Stebnicka and  
298 Howden (1995), for instance, have identified two typologies of epipharynges in adults (divergent as  
299 for pubescence and general shape) that correspond to coprophagous and saprophagous beetles.

300 Aphodiini and Onthophagini display contrasting habitat/climate requirements, the former being typical  
301 of habitats in cold/temperate climates whereas the latter is typical of habitats in warm/temperate  
302 climates. Onthophagini, in fact, are mainly distributed in the Mediterranean and other temperate or  
303 warm areas (Scholtz et al. 2009), whereas Aphodiini dominate northern European dung beetle  
304 communities (Jay-Robert et al. 1997) as well as high-altitude communities of the Alps and the  
305 Pyrenees (Cabrero-Sañudo and Zardoya 2004; Cabrero Sañudo 2006; Cabrero Sañudo and Lobo  
306 2006). Most of Onthophaginae are considered to be warm-adapted, whereas many Aphodiinae are  
307 considered to be cold-adapted (Lobo et al. 2007a, 2007b; Scholtz et al. 2009; Verdù et al. 2007). These  
308 differences are reflected in the local distribution of species; in the Alps Scarabaeinae dominate during  
309 spring and summer on south-facing slopes, whereas Aphodiinae dominate from spring to autumn on  
310 north-facing slopes (Jay-Robert et al. 2008; Zamora et al. 2007).

311 We suggest habitat/climate factors have acted to set divergent breeding and thermoregulatory  
312 strategies in the two groups. Within these different strategies, sexual selection (breeding strategies)  
313 and natural selection (thermoregulatory strategies) may have contributed to drive the divergent  
314 morphological evolution of the two groups producing medium-size horned species (Onthophagini) on  
315 the one hand and small-sized unhorned species (Aphodiini) on the other.

316

### 317 **i) Breeding ecology**

318 Except for a few cases, Aphodiini adults live and breed in the dung mass where they do not construct  
319 nests (dwellers) (Dellacasa and Dellacasa 2006; Hanski and Cambefort 1991). Conversely,  
320 Onthophagini dig burrows under the dung mass that are provisioned with dung to form a ball (the  
321 brood ball, destined for larval food) where an egg is laid (tunnelers). In Onthophagini, horns of large  
322 males are used to defend the nest and prevent other males from copulating with females, which may  
323 spend long periods underground caring for the brood (Hunt and Simmons 2002, Knell 2011). The  
324 different expression of sexually dimorphic ornamentation is the major form of interspecific variation  
325 in Onthophagini. Aphodiini males do not need to defend the nest and, therefore, they do not need to  
326 develop horns. It has been suggested that nesting behavior has been mediated more by environmental  
327 conditions than phylogeny (Scholtz et al. 2009). We hypothesized therefore that these two divergent  
328 breeding strategies may have arisen as an answer to the different ecological and climatic conditions  
329 experienced. The non-nesting strategy, in particular, seems to be the best one for species living in cold  
330 habitats by enabling individuals to breed several times within the short breeding period characteristic  
331 of the habitat (Scholtz et al. 2009).

332 **ii) Thermoregulatory ecology.** Species of both Aphodiini and Onthophagini are usually smaller than  
333 the body size above which the regulation of body temperature through internal processes is thought to  
334 be physiologically possible (Bartholomew and Heinrich 1978; Prange 1996; Roxburgh et al. 1996,  
335 Chow and Klok 2011) and therefore have to rely on other mechanisms to respond to changes in  
336 external temperature. One mechanism to respond to such changes is to actively seek out locations with  
337 appropriate temperatures, or to move between warm and cold temperature locations so as to achieve a  
338 preferred average temperature. Shepherd et al. (2008) found that horn possession in two Onthophagini  
339 species dramatically alters this thermal preference behavior. They also hypothesized that the formation  
340 of long horns diverts resources away from structures crucial for active heat transfer, such as thoracic  
341 muscles, and compromises the later ability to effectively thermoregulate in the face of temperature  
342 fluctuations. To our knowledge, no thermoregulatory data are available for Aphodiini, but it is  
343 reasonable to think that an efficient thermo-regulatory behavior is needed to secure survival in cold  
344 climates. This might also explain why Aphodiini do not develop horns. Moreover, Shepherd et al.  
345 (2008) also demonstrated that body size significantly affects thermoregulation and suggested that  
346 larger individuals, by virtue of their size, can operate at higher temperatures, whereas smaller  
347 individuals prefer to operate at lower temperatures. This may also serve to explain why, as a rule,  
348 Aphodiini are smaller than Onthophagini.

349 Summing up, we suggest habitat/climate factors may have underpinned divergent reproductive and  
350 thermoregulatory strategies in the two groups and that strategies of the Onthophagini enabled the  
351 development of horns (and other forms of sexual dimorphism), whereas those of Aphodiini were not.

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551

552 **Appendix 1.**

553 Landmark setting. Nomenclature of the different parts of traits where landmarks were set follow  
 554 Dellacasa and Dellacasa 2006, and Steinmann and Zombori 1984.

**ADULT****Epipharynx**

- 1 Along midline, on tylus
- 2 Apex of left chaetoparia
- 3 At proplegmatium
- 4 At base of laeotorma
- 5 On first apex of crepis
- 6 On longer apex of crepis
- 7 At midline, on nesium
- 8 At joining point of proplegmatium and left chaetoparia
- 9 Apex of left apotorma

**Pronotum**

- 1 Along midline, on fore margin
- 2-3 Semilandmarks
- 4 At base of lateral angle of fore margin
- 5 At side angle of fore margin
- 6-10 Semilandmarks
- 11 On hind side angle of pronotum
- 12-15 Semilandmarks
- 16 Along midline, on hind margin

**Head**

- 1 Midline, on fore margin
- 2 Lateral apex of fore margin
- 3 Lateral carina, on margin
- 4 Anterior base of left gena
- 5 Apex of gena
- 6 Posterior base of gena

**Scutellum**

- 1 Left apex
- 2 Right apex
- 3 Medial point of right side
- 4 Hind apex
- 5 Medial point of left side

**LARVA****Epipharynx**

- 1 Midline, on fore margin
- 2 On left clythra
- 3 On side margin of left lobe
- 4 At superior margin of laeotorma
- 5 At inner base of laeotorma
- 6 At base of anterior epitorma
- 7 At base of apotorma
- 8 At apex of apotorma
- 9 At apex of anterior epitorma

**Genital Disc**

- 1 Right base of disc
- 2 Right base of disc, anterior angle
- 3 Right apex of disc
- 4 Midline, anterior margin
- 5 Left apex of disc
- 6 Left base of disc, anterior angle
- 7 Left base of disc

555

556

557 **Table 1.** Results of Multivariate Test of Significance regarding external traits (head, pronotum and  
 558 scutellum) and the epipharynx in adults of *A. immaturus* and *A. obscurus*. The proportion of Goodall's  
 559 F statistics from randomised data sets as great or greater than the Goodall's statistic from the original  
 560 data set is given as the significance probability (1000 random permutations were used).

561

	Generalized Goodall F-test			Permutation
	F	df	P	tests*
Head	2.2128	8, 680	0.0248	5.30%
Pronotum	1.8078	28, 2464	0.0059	12.90%
Scutellum	1.1568	8, 704	0.3230	31.60%
Epipharynx	33.8985	20, 1760	0.0000	0.10%

\*Percent of Goodall F values  $\geq$ observed

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563

564

565 **Table 2.** Results of Multivariate Tests of Significance regarding the genital disc and the epipharynx in  
 566 larvae of *A. immaturus* and *A. obscurus*. The proportion of Goodall's F statistics from randomised data  
 567 sets as great or greater than the Goodall's statistic from the original data set is given as the significance  
 568 probability (1000 random permutations were used).

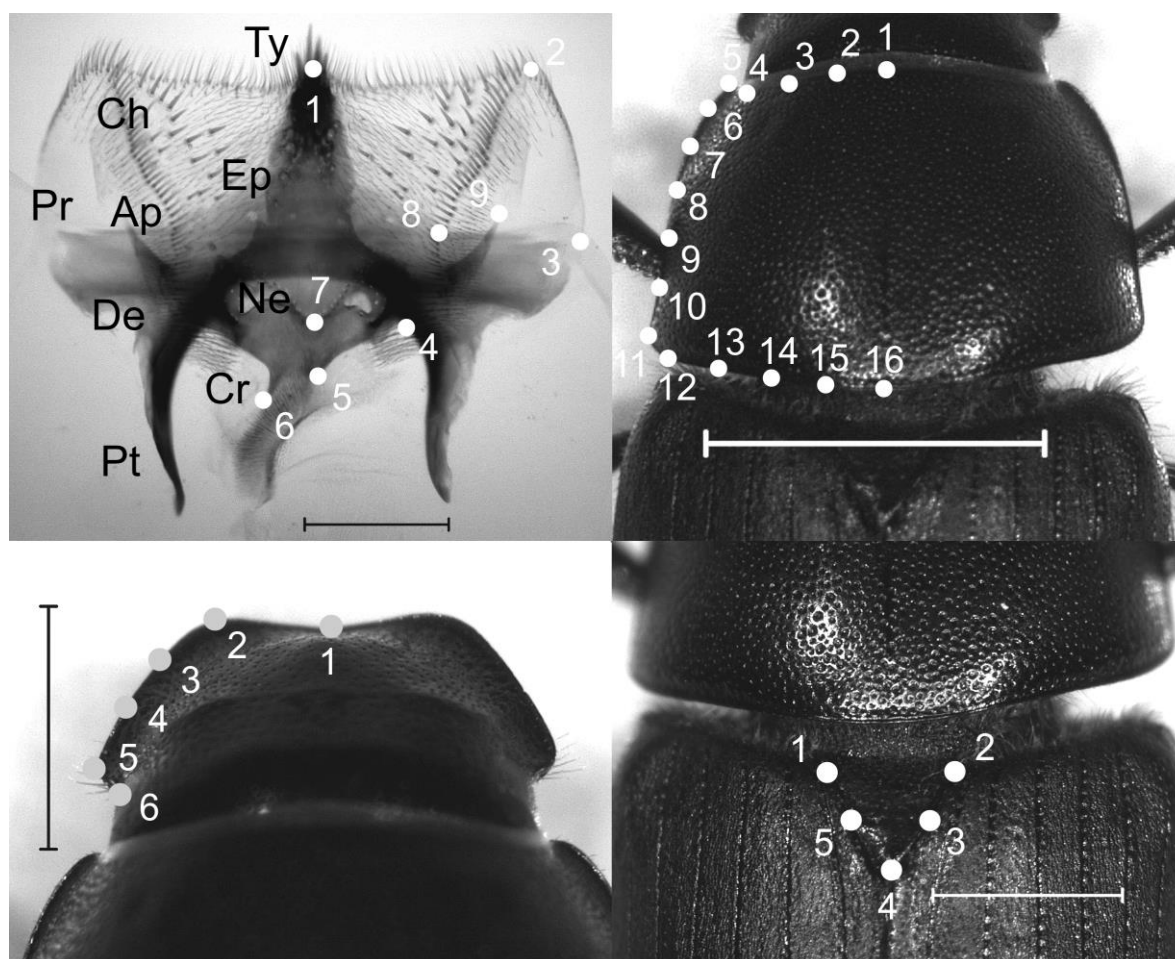
569

	Instar	Generalized Goodall F-test			Permutation
		F	df	P	tests
Genital disc	1	2.0632	10, 350	0.0268	11.0%
	2	1.1339	10, 350	0.3357	33.70%
Epipharynx	1	3.1417	14, 504	0.0001	0.10%
	2	5.6075	14, 532	0.0000	0.10%

\*Percent of Goodall F values  $\geq$ observed

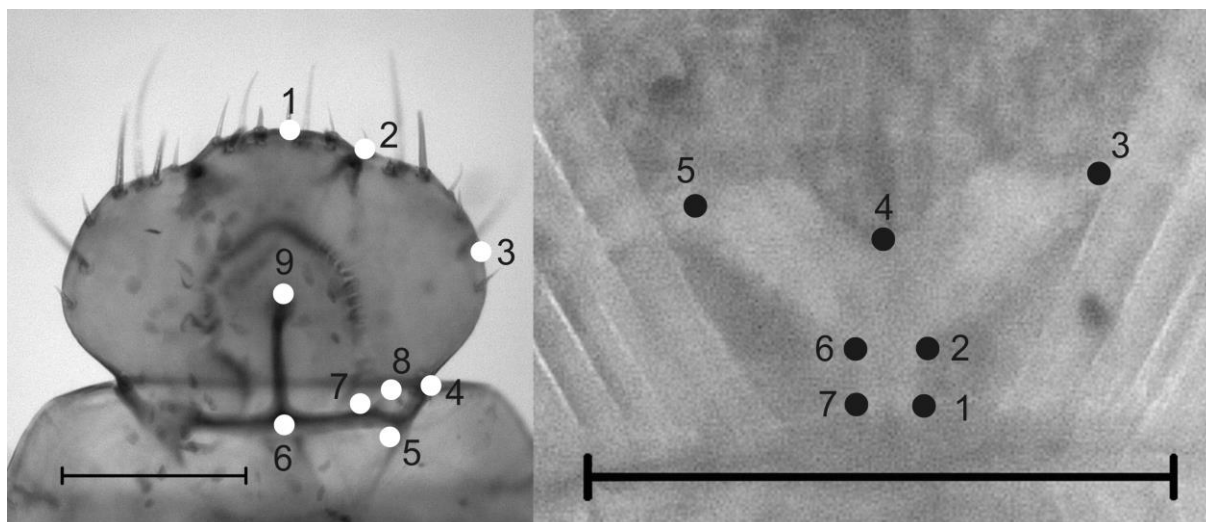
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572 **Figures**

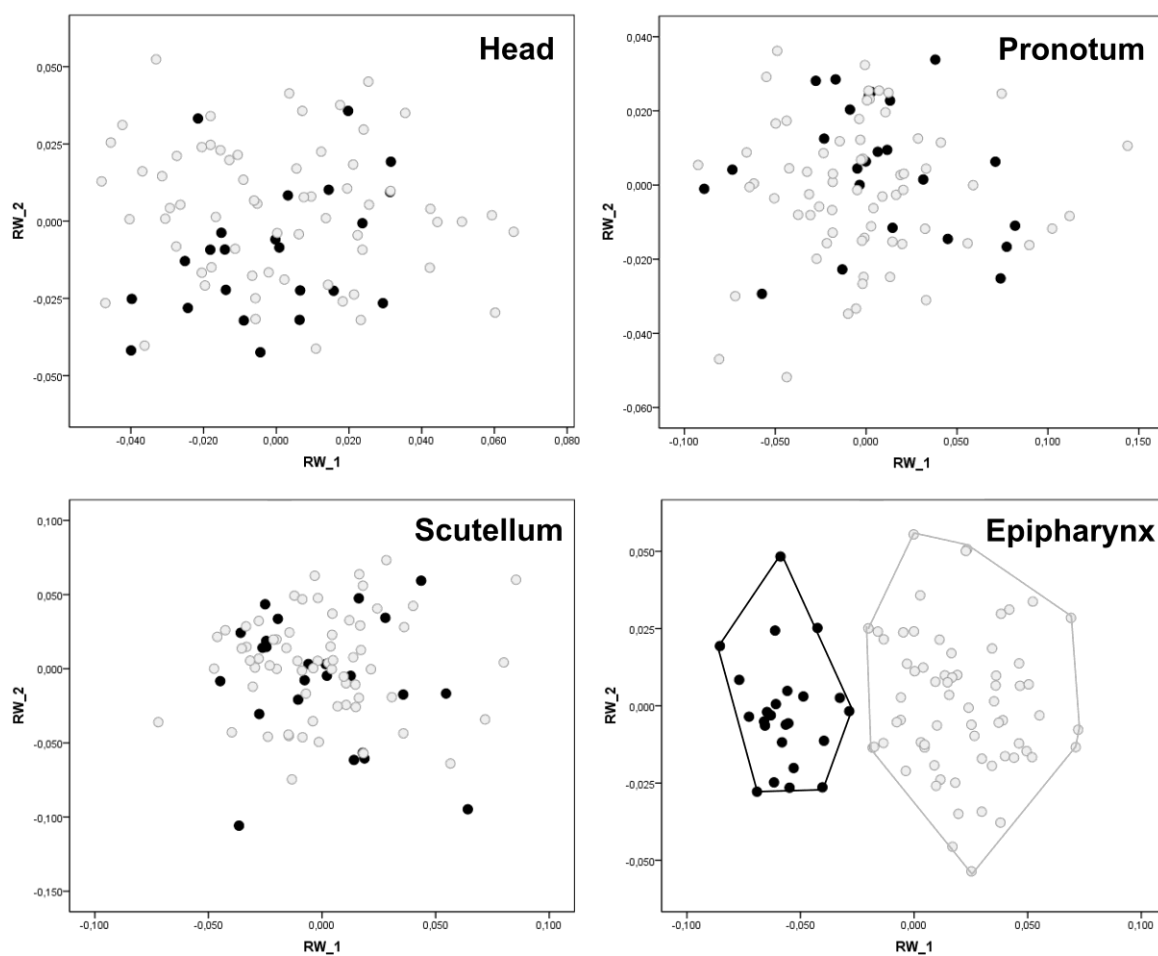
573  
 574 Fig. 1. *Amidorus obscurus*, for each anatomical structure the number ( $N_L$ ) and position of landmarks  
 575 are shown. **A:** Epipharynx, scale bar = 2 mm. On the left: the acronyms of the parts of the structure.  
 576 On the right: the position of the chosen landmarks ( $N_L = 9$ ); on left the acronyms of the different parts  
 577 are shown: tylus (Ty), chaetopariae (Ch), epitorma (Ep), proplegmatium (Pr), apotorma (Ap),  
 578 dexiotorma (De), nesium (Ne), crepis (Cr), and pternotorma (Pt). **B:** Pronotum ( $N_L = 16$ ), scale bar = 2  
 579 mm. **C:** Head ( $N_L = 6$ ), scale bar = 1 mm. **D:** Scutellum ( $N_L = 5$ ), scale bar = 1 mm. In the pronotum  
 580 configuration (B), the points 2, 3, 6, 7, 8, 9, 10, 12, 13, 14 and 15 were treated as semi-landmarks, the  
 581 others as landmarks.

582



583  
 584 Fig. 2. Epipharynx of the larval instars with 9 landmarks (A), and male genital disc with 7 landmarks  
 585 (B). Scale bars = 0.2 mm.

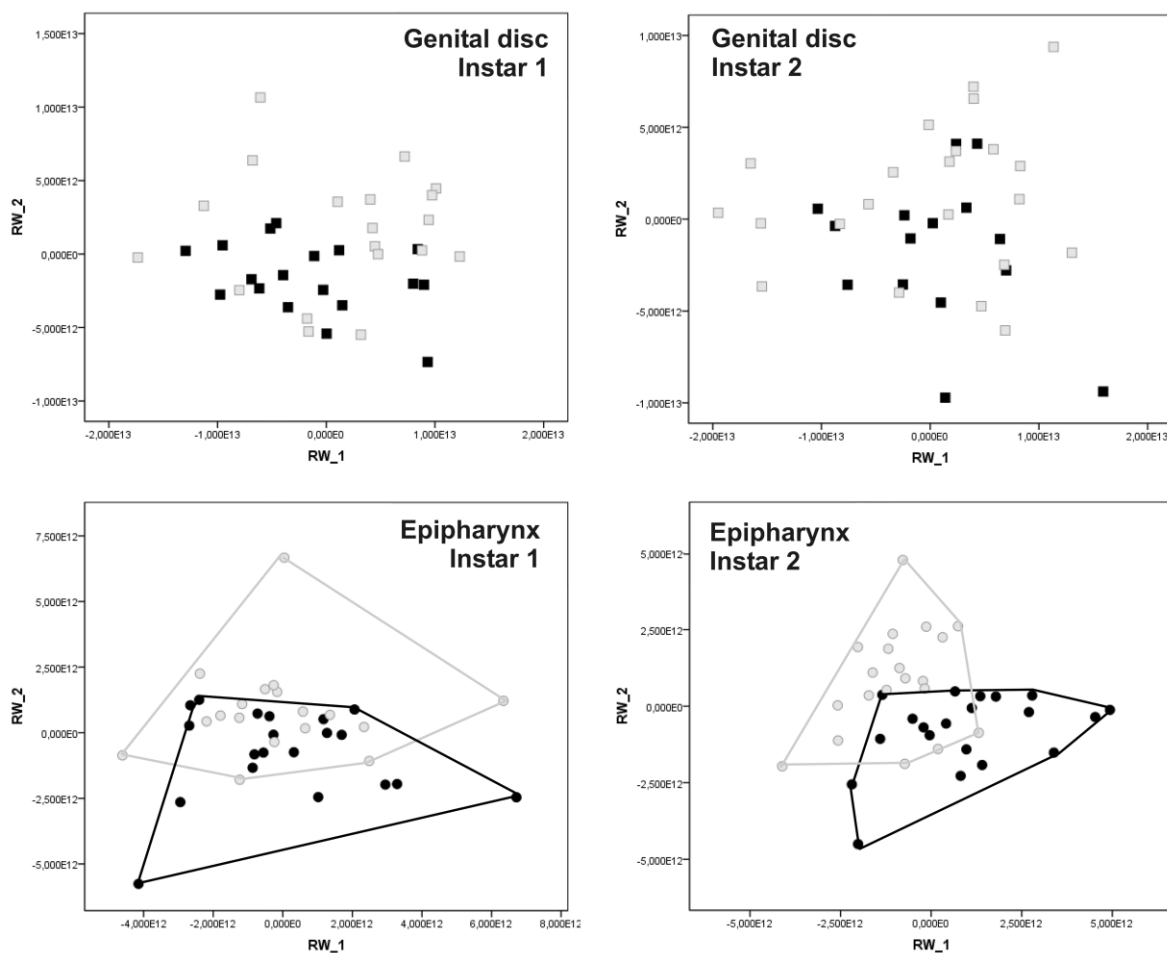
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587  
 588 Fig. 3. Scatterplots of the first two relative warp scores obtained from the Relative Warps Analysis of  
 589 the head (A, percentages of the explained variance: RW\_1 = 35.39%, RW\_2 = 25.63%), pronotum (B,

590 RW\_1 = 69.64%, RW\_2 = 12.32%), scutellum (**C**, RW\_1 = 30.58%, RW\_2 = 19.91%) and  
 591 epipharynx (**D**, RW\_1 = 37.27%, RW\_2 = 10.49%) of adults of *A. immaturus* (black symbols) and *A.*  
 592 *obscurus* (grey symbols). The number of relative warps obtained was 8, 28, 8 and 20 for the head,  
 593 pronotum, scutellum and epipharynx, respectively.

594  
 595



596  
 597 Fig. 4. Scatterplots of the first two relative warp scores obtained from the Relative Warps Analysis of  
 598 male genital discs and epipharynges of larvae of *A. immaturus* (black symbols) and *A. obscurus* (grey  
 599 symbols). Genital disc: 1<sup>st</sup> instar (**A**, percentages of the explained variance: RW\_1 = 60.93%, RW\_2 =  
 600 15.19%) and 2<sup>nd</sup> instar (**B**, RW\_1 = 64.08%, RW\_2 = 16.37%). Epipharynx: 1<sup>st</sup> instar (**C**, RW\_1 =  
 601 28.40%, RW\_2 = 17.72%) and 2<sup>nd</sup> instar (**D**, RW\_1 = 25.60% and by RW\_2 = 20.41%). The number  
 602 of relative warps obtained was 10 for genital discs and 14 for epipharynges of both instars.  
 603