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# Does plasticity in thermal tolerance trade off with inherent tolerance? The influence of setal tracheal gills on thermal tolerance and its plasticity in a group of European diving beetles.

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1 Title:

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4

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20

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23

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26 Abstract

27 In the face of global warming, both the absolute thermal tolerance of an ectotherm, and its  
28 ability to shift its tolerance level via acclimation, are thought to be fundamentally important.  
29 Understanding the links between tolerance and its plasticity is therefore critical to accurately  
30 predict vulnerability to warming. Previous studies in a number of ectotherm taxa suggest  
31 trade-offs in the evolution of thermal tolerance and its plasticity, something which does not,  
32 however, apply to *Deronectes* diving beetles, where these traits are instead positively  
33 correlated. Here we revisit the relationship between thermal tolerance and plasticity in these  
34 beetles, paying attention to a recently discovered morphological adaptation supporting under  
35 water respiration - setal tracheal gills. Hollow setae on the elytra interconnect with the  
36 beetle's tracheal system, providing a gas exchange surface that allows oxygen to be extracted  
37 directly from the water. This enables individuals to stay submerged for longer than their  
38 subelytral air stores would allow. We show that hypoxia reduced heat tolerance, especially  
39 when individuals were denied access to air, forcing them to rely solely on aquatic gas  
40 exchange. Species with higher densities of these gas-exchanging setae exhibited improved  
41 cold tolerance, but reduced heat tolerance and lower plasticity of heat tolerance. Differences  
42 in setal tracheal gill density across species were also related to habitat use: species with low  
43 gill density were found mainly in intermittent, warmer rivers, where underwater gas exchange  
44 is more problematic and risks of surfacing may be smaller. Moreover, when controlling for  
45 differences in gill density we no longer found a significant relationship between heat  
46 tolerance and its plasticity, suggesting that the previously reported positive relationship  
47 between these variables may be driven by differences in gill density. Differences in  
48 environmental conditions between the preferred habitats could simultaneously select for  
49 characteristic differences in both thermal tolerance and gill density. Such simultaneous  
50 selection may have resulted in a non-causal association between cold tolerance and gill  
51 density. For heat tolerance, the correlations with gill density could reflect a causal  
52 relationship. Species relying strongly on diffusive oxygen uptake *via* setal tracheal gills may  
53 have a reduced oxygen supply capacity and may be left with fewer options for matching  
54 oxygen uptake to oxygen demand during acclimation, which could explain their reduced heat  
55 tolerance and limited plasticity. Our study helps shed light on the mechanisms that underpin  
56 thermal tolerance and plasticity in diving air-breathing ectotherms, and explores how  
57 differences in thermal tolerance across species are linked to their selected habitat,  
58 morphological adaptations and evolutionary history.

59

60 Introduction

61 Global warming is recognized to have profound effects on ectothermic animals. For these  
62 organisms, temperature can be considered a master control variable, as it directly affects their  
63 metabolism, growth, fecundity and survival, which in turn affects population growth rates,  
64 biodiversity, and biogeography. To respond to global warming, both the overall level of  
65 tolerance to thermal extremes (i.e. inherent thermal tolerance) and the ability to shift this in  
66 response to acclimation (i.e. plasticity of thermal tolerance) are considered fundamentally  
67 important (Stillman, 2003; Somero 2010; Huey et al., 2012; Gunderson & Stillman, 2015).  
68 Stillman (2003) compared different species of porcelain crabs and found that species with  
69 high inherent heat tolerance exhibited reduced plasticity in heat tolerance. This led him to  
70 suggest that these two traits are connected *via* an evolutionary trade-off. A similar

71 relationship was more recently documented for caridean shrimps, another group of  
72 crustaceans (Magozzi & Calosi, 2015). In contrast, no such trade-off was found within  
73 *Deronectes* diving beetles, where heat tolerant species actually showed greater plasticity, i.e.  
74 the opposite pattern (Calosi et al., 2008a, see Fig. 1). There may be more than one reason for  
75 this difference amongst arthropod groups. Rather than there being a direct trade-off between  
76 thermal tolerance and plasticity, both traits could have evolved in response to the thermal  
77 regime of the habitat a species occupies. Southwood (1977) proposed that the habitat  
78 provides a templet on which evolution acts to forge the characteristic traits of an organism, so  
79 that it can effectively deal with the conditions experienced. In this case, species experiencing  
80 more variable temperatures could be expected to display greater plasticity of thermal  
81 tolerance (e.g. Janzen, 1967). In a related vein, it has been suggested that the difference could  
82 be related to the direction of colonization from one habitat to another and the associated  
83 change in thermal regime (Bozinovic et al., 2011). Indeed, the ancestral habitat of the  
84 porcelain crabs was cool and stable, but for the beetles this explanation requires a  
85 consideration of the timescales, since the original habitat of dytiscids was probably lentic  
86 (relatively warm and variable), but *Deronectes* have radiated in relatively cold and stable  
87 stream habitats. Also, this explanation requires that an evolutionary trajectory away from  
88 their ancestral thermal regime is coupled with a reduction in the plasticity of thermal  
89 tolerance, irrespective of whether the trajectory is towards warmer or cooler habitats.

90 Alternatively, the contrasting patterns between diving beetles and crustaceans may be related  
91 to differences in respiratory capacity, as capacity limitations on oxygen uptake and delivery  
92 have been shown to be linked to thermal tolerance (Winterstein, 1905, Pörtner, 2006),  
93 especially in aquatic taxa (Woods, 1999; Verberk and Atkinson, 2013; Verberk et al., 2016a).  
94 Variation in heat tolerance has been linked to mode of respiration in aquatic insects (Verberk  
95 & Bilton, 2013; 2015), to evolutionary innovations in respiration in crabs (Giomi et al.,  
96 2014). In porcelain crabs, as in other malacostracans such as caridean shrimps, gills have  
97 multiple functions, being important for osmotic and ionic regulation, acid-base balance, and  
98 ammonia excretion in addition to being a site for gas exchange (Freire et al., 2008; Henry et  
99 al., 2012). Thus it is possible that these other functional demands place constraints on the  
100 capacity for gas exchange (e.g. larger gills allow faster rates of oxygen uptake, but may also  
101 increase the need for osmoregulation). If such constraints are stronger in species that already  
102 have a high capacity for gas exchange, this could generate a negative relationship between  
103 inherent thermal tolerance and its plasticity. Adult *Deronectes* beetles, like most insects, use  
104 trachea exclusively for gas exchange. There are, therefore, arguably fewer constraints in their  
105 function in this regard. Observed differences in thermal biology between arthropod groups  
106 could simply reflect a fundamental difference in how crustaceans and insects breathe.

107 Here we revisit the relationship between thermal tolerance and its plasticity in *Deronectes*  
108 diving beetles (see Calosi et al., 2008a), examining the possible impact of the recently  
109 discovered setal tracheal gills (Kehl & Dettner, 2009). *Deronectes* diving beetles live in fast-  
110 flowing waters and are relatively poor swimmers (Ribera et al., 1997). Hence, surfacing to  
111 replenish air stored in their subelytral space carries the risk of being swept away by currents  
112 as well as exposing beetles to predation. Furthermore, beetles would be predicted to  
113 maximize time spent submerged where feeding and mating are carried out (Calosi et al.  
114 2012). *Deronectes* and some associated clades have evolved a unique solution to deal with  
115 this challenge. The surface of their elytra is densely covered with setae that are tracheated and  
116 link up to channels that transverse the cuticle and connect to the longitudinal tracheal trunks

117 embedded in the elytra. These setal tracheal gills enable beetles to extract and transport  
118 oxygen from the water directly into their tracheal system (Kehl & Dettner, 2009; Madsen,  
119 2012). The gills allows the beetles to circumvent the diffusion barrier inherent to their thick  
120 exoskeleton and enables them to perform underwater gas exchange which is functionally  
121 similar to integumental respiration seen in many other aquatic insects (Mill, 1974).  
122 Experiments covering the elytra of *Deronectes aubei aubei* with a synthetic resin to negate  
123 oxygen uptake *via* setal tracheal gills greatly reduced their ability to extract oxygen from the  
124 water, whereas non-covered animals survived submerged for over six weeks (Kehl & Dettner,  
125 2009).

126 To investigate the relationships between capacity for oxygen uptake and thermal tolerance  
127 and its plasticity, we first tested whether there was a link between oxygen-limited heat  
128 tolerance and respiratory mode by comparing individuals of *D. latus* that were forced to rely  
129 exclusively on aquatic gas exchange using tracheal respiration with individuals that could  
130 also employ aerial gas exchange using surfacing. Next, within a phylogenetically controlled  
131 framework, we (i) explored the extent to which *Deronectes* species differ in the density of  
132 setal tracheal gills as a proxy for their reliance on diffusive gas exchange, and (ii) determined  
133 whether this relates to their inherent thermal tolerance and its plasticity. Building on the  
134 habitat templet concept of Southwood (1977), we also explored whether patterns in thermal  
135 tolerance and plasticity in thermal tolerance could be related to the thermal regime of the  
136 habitat individual species occupy. Whilst all the species in this study inhabit running waters,  
137 there are differences in stream temperature and flow/permanence regimes across taxa.

138

## 139 Methods

### 140 *Study species*

141 Full data on thermal tolerance, changes in thermal tolerance following exposure to elevated  
142 temperature (thermal tolerance plasticity), density of setal tracheal gills and phylogeny were  
143 obtained for 15 *Deronectes* species (see Table S1 for an overview of the species and their  
144 morphological and physiological traits). For 13 species, thermal tolerance data have been  
145 reported elsewhere (Calosi et al. 2008a; Calosi et al. 2010). In addition, in this study we  
146 included previously unpublished data for *D. brannanii* and *D. lareynii*. Data for these two  
147 species were excluded from the previous work dealing with the relationship between thermal  
148 tolerance and geographical range size as both species are island endemics with hard dispersal  
149 barriers setting geographical range limits. Specimen collection, maintenance in the laboratory  
150 and preparation for thermal trials are described in detail elsewhere (Calosi et al. 2010).  
151 Briefly, adult *Deronectes* were collected during spring and summer 2006. By collecting  
152 species from higher latitudes later in the season we standardized as much as possible for  
153 phenological differences. All individuals collected were early post-teneral adults, minimizing  
154 any possible confounding effects due to age variation. In these beetles, adults are the longest  
155 life-history stage ( $\geq 1$  year), whilst larvae are short lived (ca. 1–2 months). As a result, adult  
156 beetles typically overwinter, and/or survive periodic droughts (Nilsson & Holmen 1995), and  
157 likely experience the greatest thermal challenges. All species were collected as close as  
158 possible to the central point of their latitudinal ranges, to avoid the possible confounding  
159 effects of local adaptation in range edge populations, and to ensure data were comparable  
160 across species (Thompson et al. 1999).

161 After collection individuals were transported to the laboratory where they were maintained in  
162 aquaria (vol. = 5 L, maximum 20 indiv. *per* aquarium) with aerated artificial pond water  
163 under a 12 : 12 h L/D regime, and fed chironomid larvae *ad libitum*. For each species,  
164 specimens were equally divided haphazardly into two equal groups, exposed for 7 d to either  
165 14.5 or 20.5 °C respectively before experiments were conducted. Extreme exposure  
166 temperatures were avoided and acclimation was in most cases not stressful (see Calosi et al.  
167 2010), and indeed no mortality occurred in any species during the exposure period.

168

#### 169 *Thermal tolerance and its plasticity*

170 After the exposure period, individuals from each acclimation temperature were haphazardly  
171 assigned to two equal subgroups used to determine their tolerance to heat and cold. Full  
172 methodology is described in Calosi et al. (2008a; 2008b; 2010). In short, thermal tolerance to  
173 cold (CT<sub>min</sub>) and heat (CT<sub>max</sub>) were determined using a dynamic method, by heating or  
174 cooling individuals, *via* a ramping program ( $\pm 1$  °C min<sup>-1</sup>). Ramping trials commenced at the  
175 temperature to which individuals had been acclimated. Up to 12 individuals were placed in  
176 24 well (diam. = 12 mm, depth = 18 mm) plastic culture plates, and in turn these were placed  
177 in the water baths. Temperature within wells was measured using a digital thermometer  
178 (Omega® HH11; Omega Engineering Inc., CT, USA) with a precision fine wire  
179 thermocouple (accuracy of 0.1 °C). The wells did not contain water and hence the animals  
180 did not have to exhibit surfacing behavior for aerial gas exchange. In our analyses we  
181 employed lethal endpoints, since these showed the lowest variance amongst all end-points  
182 recorded. When animals lost responsiveness they were considered to have entered a heat or  
183 chill coma and eventually died. Plasticity in upper and lower thermal tolerance were  
184 estimated following Stillman (2003) as the absolute difference in tolerance (CT<sub>max</sub> or  
185 CT<sub>min</sub>) between both acclimation temperatures. A positive value for either plasticity of cold  
186 or heat tolerance indicates an improved critical temperature (higher CT<sub>max</sub> following  
187 acclimation at the higher temperature and lower CT<sub>min</sub> following acclimation at the lower  
188 temperature). Inherent, or overall level of tolerance against thermal extremes is given by the  
189 absolute critical temperatures and so we have two measures of inherent tolerance, one for  
190 each acclimation temperature. In our results we focused on the inherent thermal tolerance for  
191 both CT<sub>max</sub> and CT<sub>min</sub> displayed by animals acclimated to a common temperature of 20.5  
192 °C, but results for both acclimation temperatures are reported in full (Table 1).

193

#### 194 *Effect of oxygen availability on heat tolerance in D. latus*

195 We assessed the impact of mode of respiration on heat tolerance under different oxygen  
196 conditions in one of the 15 species: *D. latus*, the most tolerant species in our comparison,  
197 using previously described methods (Verberk and Calosi 2012; Verberk and Bilton, 2015).  
198 Briefly, individuals were placed in flow-through chambers, whose water supply could be  
199 heated. For one group of animals, we used chambers where the animals were completely  
200 submerged and had no access to air, while for a second group of animals chambers were used  
201 with a small head space holding a layer of air, meaning that these animals could obtain  
202 oxygen either from the air compartment by surfacing or from the water with oxygen diffusing  
203 directly into their tracheal system *via* the setae or oxygen diffusing into their subelytral air  
204 reservoir *via* their physical gill. Individuals were left to settle for 1 h at the equilibration

205 temperature of 10 °C, after which the temperature was ramped up at 0.25 °C min<sup>-1</sup>. The  
206 CTmax was defined as the point at which animals lost coordinated swimming, hence losing  
207 their ability to escape from the conditions that will lead to their death (Lutterschmidt and  
208 Hutchison, 1997). The heating rate, endpoint and starting temperature all therefore differed  
209 from the methodology described above, meaning that the critical thermal temperatures from  
210 both methods cannot be compared directly. CTmax was assessed under normoxia, hypoxia  
211 and hyperoxia conditions (5, 20, 60 kPa O<sub>2</sub> respectively) and adults were assessed with and  
212 without access to air. Oxygen tension of both the water and the air in the headspace was  
213 altered to produce hypoxia and hyperoxia, as described by Verberk and Bilton (2015).

214

#### 215 *Setal tracheal gill enumeration*

216 Species of the genus *Deronectes* possess different types of setae. The setae for which a  
217 respiratory function has been demonstrated are spoon-shaped, with an enlarged base, situated  
218 in simple punctures. In addition, beetles possess long sensory setae in punctures encircled by  
219 concentric ridges, and rod-like setae associated with deep punctures or craters (see Fig. 2).  
220 Only the spoon-like, setal tracheal gills, which were by far the most dominant type on  
221 *Deronectes* elytra, were enumerated.  
222 Density of setal tracheal gills was determined from digital images of the elytra, using light  
223 microscopy. With the use of image acquisition software (Olympus software package  
224 “Cell^A”), the number of setae were counted in four regions of the elytra; the posterior  
225 section of the elytra (at 100x magnification, on average 0.074 mm<sup>2</sup>), the middle section (at  
226 40x magnification, ca. 0.155 mm<sup>2</sup>) the anterior section (at 200x magnification, ca. 0.040  
227 mm<sup>2</sup>), and lastly setae were enumerated in an anterior section without the deep punctation  
228 that is associated with the rod-like setae (at 200x magnification, ca. 0.0050 mm<sup>2</sup>). In each  
229 section, the largest relatively flat area was chosen to count setae as this ensured they all  
230 appeared in focus. The size of this area was automatically calculated by the Olympus cell  
231 program. Setal density is expressed as the number of setae *per* mm<sup>2</sup>. More than 100,000 setae  
232 were counted on a total of 74 individuals, five for each species, with the exception of *D.*  
233 *angusi* (n = 3) and *D. moestus* (n = 6).

#### 234 *Data analysis*

235 In order to investigate the effect of oxygen tension on the CTmax of *D. latus*, we used linear  
236 models with ‘oxygen conditions’ (hypoxia, normoxia or hyperoxia) and ‘access to air’  
237 (access or no access to air) as fixed factors. We also included the interaction between these  
238 two terms to test whether effects of oxygen on CTmax differed when individuals exposed to  
239 different oxygen levels had access to air or not. Data from these trials showed small  
240 deviations from normality (visually assessed from Q-Q plots) and homogeneity of variances  
241 (formally tested using Levene’s test), which were due to large variability in CTmax observed  
242 under hypoxia in the treatment without access to air. A conservative analysis, which excluded  
243 the three lowest values to meet test assumptions, yielded qualitatively similar results, flagging  
244 the same contrasts as being significant. We therefore deemed the analysis robust to these  
245 small deviations and present the complete results.

246 Differences in setal tracheal gill density across species were analyzed using a linear model  
247 with ‘species’ as a fixed factor, followed by Tukey post-hoc tests. Preliminary analysis  
248 showed that the three measures of seta on the anterior, middle and posterior region were  
249 highly correlated across the 15 species ( $R^2 > 0.923$ ,  $t_{1,13} > 12.97$ ;  $P < 0.0001$ ) and also across

250 all 74 individuals, accounting for species differences in a mixed effect model ( $R^2 > 0.80$ ;  $t_{1,72}$   
251  $> 11.51$ ;  $P < 0.0001$ ). Therefore these three measures of gill density were averaged to  
252 produce a composite measure (hereafter referred to as gill density in punctated sections). The  
253 fourth measure of gill density in sections without punctuation was found to be correlated much  
254 less strongly to this composite measure in a mixed effect model ( $R^2 > 0.13$ ;  $t_{1,72} > 3.707$ ;  $P =$   
255  $0.00021$ ) and was therefore analyzed separately (hereafter referred to as gill density).  
256 Preliminary analyses also showed that variation in setal density of individuals was mainly  
257 due to interspecific differences rather than body size and sex. When included in a mixed  
258 effect model to explain differences in gill density, elytra length (as a measure of body size)  
259 was not significant ( $t_{1,72} = 0.145$ ;  $P = 0.885$ ). Similar results were obtained for the composite  
260 measure of gill density in punctated sections ( $t_{1,72} = -0.080$ ;  $P = 0.936$ ). Furthermore, across  
261 the 15 species, body size was not significantly related to gill density ( $t_{1,13} = -0.700$ ;  $P =$   
262  $0.496$ ) nor gill density in punctated sections ( $t_{1,13} = -0.899$ ;  $P = 0.385$ ). Similar non-  
263 significant results were found when including sex in a mixed effect model on individuals ( $t_{1,72}$   
264  $> -1.505$ ;  $P > 0.13$ ), indicating that gill density did not differ between males and females.

265 Relationships between a species mean gill density, mean thermal tolerance (CTmax and  
266 CTmin) and plasticity in thermal tolerance were analyzed using linear regressions across the  
267 15 *Deronectes* species. To test whether the same outcome was obtained within a  
268 phylogenetically controlled framework, we also analyzed the relationships between gill  
269 density, CTmax, and plasticity in CTmax using phylogenetic independent contrasts, in the R-  
270 package {ape} (Paradis et al. 2004). Independent contrasts were derived from DNA based  
271 phylogenies (García-Vázquez et al., 2016). Preliminary analyses showed that none of the  
272 thermal tolerance traits exhibited a strong phylogenetic signal ( $K < 0.37$ ; see Blomberg et al.,  
273 2003). The same was true for both measures of gill density ( $K < 0.39$ ). We therefore opted to  
274 rescale the tree using a lambda of 0.5, representing the intermediate between a Brownian  
275 evolution model and a star phylogeny. Diagnostic tests were performed using the function  
276 {caic.diagnostics} from the R-package {caper} (Orme et al., 2011). These diagnostics  
277 showed that the estimated nodal values correlated with the magnitude of the estimated  
278 contrasts, a problem that was solved by log-transformation of the data on gill density in the  
279 phylogenetic independent contrast analyses.

280 Variation in habitats used by *Deronectes* species was condensed into two categories:  
281 permanent streams, often at high altitudes, which tend to be cooler, often faster flowing and  
282 thermally more constant (constant streams) and streams which may be intermittent, have  
283 lower flow and exhibit higher and more widely fluctuating temperatures (fluctuating  
284 streams). Species primarily inhabiting the permanent streams are *D. angusi*, *D. aubei aubei*,  
285 *D. bicostatus*, *D. depressicollis*, *D. lareynii*, *D. platynotus platynotus*, *D. semirufus* and *D.*  
286 *wewalkai*. Species primarily inhabiting the warmer, mostly intermittent, streams are *D.*  
287 *algibensis*, *D. brannanii*, *D. latus*, *D. fairmairei*, *D. hispanicus*, *D. moestus* and *D. opatrinus*.  
288 Differences in gill density between species occupying the two habitat types were assessed  
289 using a t-test.

290

291 Results

292 *Heat tolerance of Deronectes latus in relation to respiratory mode*

293 In *Deronectes latus*, CTmax was reduced by 1.8 °C in hypoxia (5 kPa), compared to  
294 normoxia (20 kPa). This reduction increased to 6.2 °C for individuals denied access to air  
295 (Fig. 3).

296

#### 297 *Density of setal tracheal gills*

298 There were clear differences between species in mean gill density (Fig. 4A). These  
299 differences were found to be significant (GLM: Species  $F_{15,74} = 33.74$ ;  $P < 0.0001$ ), with  
300 average densities (# seta per mm<sup>2</sup>) varying from 3,444 in *D. hispanicus* to 6,680 in *D.*  
301 *wewalkai*. Differences across species in gill density in punctated sections were smaller (Fig.  
302 4B;  $F_{15,74} = 23.18$ ;  $P < 0.0001$ ) and this measure of gill density had a higher coefficient of  
303 variation (7.2% vs 5.9%). Differences between density in punctated and non-punctated  
304 regions were greatest in *D. bicostatus*, *D. angusi* and *D. wewalkai*. As variation in punctation  
305 is likely related to differences in flow sensory ability, we focus subsequent analyses on non-  
306 punctated sections (see Table 1), which was not confounded by the degree of punctation and  
307 report results on setal density in punctated regions in Table S2.

308

#### 309 *Thermal tolerance in relation to setal tracheal gill density*

310 In beetles acclimated to 20.5 °C, species with high gill density had significantly reduced  
311 CTmax ( $F_{1,13} = 38.72$ ;  $P < 0.0001$ ;  $R^2 = 0.75$ ; Fig. 5A), and improved (*i.e.* lower) CTmin  
312 ( $F_{1,13} = 5.79$ ;  $P = 0.0318$ ;  $R^2 = 0.308$ ; Fig. 5B). There was no significant relationship between  
313 gill density and the difference between CTmin and CTmax ( $P = 0.505$ ), indicating that the  
314 thermal window shifted with gill density, rather than widening or narrowing. The  
315 relationships between gill density and thermal tolerance were upheld when phylogenetic non-  
316 independence was accounted for (Table 1). No significant relationship was detected between  
317 thermal tolerance and gill density for individuals acclimated to 14.5 °C, neither for heat  
318 tolerance ( $P = 0.169$ ) nor cold tolerance ( $P = 0.125$ ). Also, no significant relationship was  
319 detected between thermal tolerance and the composite measure of gill density in punctated  
320 regions (Table S2;  $P > 0.31$ ). Furthermore, analyses accounting for differences in body size  
321 by including body size as a covariate showed that size did not have a significant effect ( $P >$   
322  $0.063$ ). When gill density was expressed on a size-specific basis, we found a significant  
323 relationship for CTmax only ( $P = 0.0112$ ), where species with a relatively high gill density  
324 had reduced CTmax.

325

#### 326 *Plasticity in thermal tolerance in relation to gill density*

327 Plasticity in thermal tolerance (*i.e.* the change in critical temperatures with acclimation) was  
328 related to gill density. Beetles with a higher gill density showed greater plasticity in CTmax  
329 ( $F_{1,13} = 17.48$ ;  $P = 0.0011$ ;  $R^2 = 0.574$ ; Fig. 6A), but not CTmin ( $P = 0.412$ ; Fig. 6B). As  
330 noted in the introduction, inherent heat tolerance and plasticity in heat tolerance were also  
331 correlated across these *Deronectes* species (Fig. 1). Therefore, we used partial regressions to  
332 factor out any confounding influences. This analysis still revealed an effect of gill density on  
333 plasticity in CTmax, when controlling for CTmax ( $P = 0.046$ ). In contrast, we found no  
334 significant relationship between CTmax and plasticity in CTmax after controlling for the  
335 effect of gill density ( $P = 0.98$ ). When applying phylogenetic independent contrasts, an even

336 stronger relationship between gill density and plasticity in thermal tolerance was found for  
337 CTmax ( $P = 0.00023$ ;  $R^2 = 0.659$ ), but the relationship remained non-significant for CTmin  
338 ( $P = 0.220$ ) (Fig. S1). Plasticity in thermal tolerance was found to be unrelated to the  
339 composite measure of gill density in punctated regions (Table S2;  $P > 0.282$ ). Also,  
340 accounting for differences across species in body size did not reveal an effect on plasticity in  
341 CTmax ( $P = 0.679$ ), but larger species did show lower plasticity in CTmin ( $P = 0.0480$ ).  
342 When gill density was expressed on a size specific basis, we found no significant effects on  
343 plasticity in CTmax or CTmin ( $P > 0.0771$ ).

344

#### 345 *Habitat use*

346 Species of the two habitat categories differed in their gill density (Fig. 7;  $t_{1,13} = -3.034$ ;  $P =$   
347  $0.0096$ ); taxa associated with thermally constant streams having higher gill densities than  
348 those from thermally fluctuating habitats (see methods for habitat categorization).

349

#### 350 Discussion

351 Southwood (1977) highlighted habitat as a driving force for evolutionary adaptations, acting  
352 as a templet on which evolution acts to forge characteristic traits enabling an organism to  
353 survive in its environment. Here we show how differences in gill density are associated with  
354 ecological differences in habitat use in *Deronectes* diving beetles, and that these are  
355 correlated to physiological differences in thermal tolerance and plasticity. Correlation does  
356 not, of course, equate to causation, and the associations demonstrated in this study could arise  
357 in a number of ways. Differences in environmental conditions could simultaneously select for  
358 differences in thermal tolerance and gill density, resulting in a non-causal association  
359 between these traits. Alternatively, gill density could shape thermal tolerance directly,  
360 reinforcing any correlation between these two characteristics.

361 Differences in thermal tolerance between *Deronectes* species have been related to aspects of  
362 their geographic range (extent, northern and southern limits - see Calosi et al., 2010),  
363 suggesting that the thermal regime of the habitat is indeed related to thermal tolerance. The  
364 CTmax and CTmin observed in short-term ramping experiments are unlikely to match the  
365 temperatures that the beetles would normally experience in the field, but are best viewed as  
366 proxies for the temperatures that species can tolerate *in situ*. Indeed, a recent study on  
367 mayflies showed interactive effects between warming and hypoxia for both lethal  
368 temperatures in short-term laboratory ramping experiments and sublethal temperatures  
369 experienced in the field, suggesting a commonality of the physiological mechanisms involved  
370 in both lethal and sublethal thresholds (Verberk et al., 2016b).

371 Habitat conditions may also directly select for differences in gill density. The evolution of  
372 high densities of setal tracheal gills within the *Deronectes* group suggests that staying  
373 submerged is an adaptive strategy in these largely lotic aquatic insects. Presence/absence and  
374 variation in gill density may capture a gradient from beetles relying completely on aerial gas  
375 exchange *via* surfacing to beetles relying on diffusive oxygen uptake, enabled by dense setal  
376 tracheal gills which allow beetles to remain submerged for longer (Kehl & Dettner, 2009).  
377 Higher gill densities, enabling more oxygen uptake, could be argued to be more important in  
378 warmer habitats where beetles require more oxygen, yet we found high densities to be

379 associated with cold, stable, permanent flowing waters (Fig. 7). Being more reliant on  
380 diffusive oxygen uptake carries the disadvantage of reduced capacity to regulate oxygen  
381 uptake, making beetles more prone to oxygen limitation (Verberk & Bilton, 2013; Verberk &  
382 Atkinson, 2013). Both fast flow leading to thinner boundary layers and cool water reduce the  
383 risk of asphyxiation. Warm, intermittent streams are often reduced to isolated pools of  
384 standing water in the summer, which can warm up dramatically. Under these conditions,  
385 aerial gas exchange by surfacing represents a more convenient respiratory strategy when  
386 compared to under water gas exchange. Other aquatic insects that rely on diffusive oxygen  
387 uptake *via* a plastron likewise depend on cold, flowing water (Jones et al., 2017) and are  
388 more prone to oxygen limitation (Verberk & Bilton, 2015).

389 Given that habitat conditions likely influence both thermal tolerance and gill density, the key  
390 question is whether these two characteristics are mechanistically linked. This could be  
391 different for cold and heat tolerance, as the underlying mechanisms may differ with  
392 mechanisms other than oxygen limitation being more important in cold tolerance (Hoffmann  
393 et al., 2002; Stevens et al. 2010; Verberk et al., 2016a). A relationship between CT<sub>min</sub> and  
394 respiratory structures is therefore less likely and indeed the observed relationships with gill  
395 density are weaker for CT<sub>min</sub> than for CT<sub>max</sub> (Table 1). Thus, we believe that differences in  
396 cold tolerance may predominantly reflect selection pressures originating from the different  
397 habitat conditions and that the correlation between cold tolerance and gill density is non-  
398 causal. The concordant differences between, on the one hand, gill density and on the other  
399 heat tolerance and plasticity for heat tolerance, could reflect selection pressures originating  
400 from the different habitat conditions, similar to the situation for cold tolerance. Alternatively,  
401 thermal tolerance traits may be directly linked to the reliance of species on diffusive gas  
402 exchange. We found that hypoxia reduced heat tolerance in *Deronectes latus*, especially  
403 when individuals were denied access to air, forcing them to solely rely on aquatic gas  
404 exchange. This indicates that aerial gas exchange by surfacing is important for *D. latus* when  
405 faced with warmer waters. In aquatic hemipterans we have similarly shown that oxygen  
406 limitation of thermal tolerance can be induced in a bimodal breather by negating aerial  
407 respiration (Verberk & Bilton 2015).

408 Our observations on *D. latus* point to a role of oxygen and mode of respiration in setting  
409 CT<sub>max</sub>, but cannot explain the observed patterns in thermal tolerance across all species  
410 investigated, since thermal tolerance trials were conducted under aerial, normoxic conditions.  
411 It is possible that species which are more reliant on underwater gas exchange have lower  
412 tracheal conductance, and a reduced capacity for aerial breathing, but this has not yet been  
413 verified experimentally. The strong negative correlation between plasticity of heat tolerance  
414 and gill density is suggestive of a direct relationship, although it is not immediately obvious  
415 how plasticity of heat tolerance and gill density would be linked mechanistically. It is  
416 possible that high reliance on diffusive oxygen uptake *via* setal tracheal gills provides fewer  
417 options for matching oxygen uptake to oxygen demand, which could in turn limit plasticity  
418 for heat tolerance. One way to increase diffusive oxygen uptake is to maintain steeper  
419 gradients in  $pO_2$  but obviously there are limits to how far internal  $pO_2$  can be lowered in  
420 practice. Lane et al. (2017) show that such limits can explain maximum body sizes in  
421 pycnogonids, which also rely on gas exchange across their cuticle. As individual species may  
422 differ in the thermal windows over which they can effectively acclimate (Calosi et al., 2010),  
423 it is also possible that using the same acclimation temperatures across all species may have

424 underestimated plasticity of heat tolerance in species with high gill density, which typically  
425 occupy cooler habitats.

426 Strong relationships were found for gill density in non-punctated sections of the elytra, but  
427 not in punctated regions (Table S2). Punctures and associated setae may have a sensory  
428 function, meaning that their densities and distribution are driven by selection pressures  
429 unrelated to gas exchange. Punctures take up surface area that cannot be covered by setal  
430 tracheal gills and the density of gills in punctated sections may be driven largely by non-  
431 respiratory factors. Gill density in non-punctated sections of the elytra may better reflect  
432 selection to increase capacity for underwater gas exchange, and could be accompanied by  
433 other physiological changes to further increase supply capacity (e.g. a lower internal  $pO_2$ ).  
434 Since the coldest habitats are also characterised by faster flow and more stable discharge, it is  
435 difficult to disentangle the selection pressures on gill density and heat tolerance. Seebacher  
436 (2015) reported greater plasticity in freshwater species from more thermally variable, warmer  
437 habitats, which would support the explanation that variation in heat tolerance across species  
438 is driven by the thermal regime of their preferred habitat (see also Gaston et al. 2009,  
439 Bozinovic et al. 2011). What is clear though is that beetles with high gill density prefer cold,  
440 fast flowing waters. Here, underwater gas exchange by diffusion can be sufficient to sustain  
441 the low metabolic demands and enable prolonged submergence. The thermal regime of the  
442 preferred habitat of these beetles matches their relatively low heat tolerance and plasticity.

443 Our study contributes to our overall understanding of thermal tolerance and plasticity in  
444 ectotherms by linking such differences across species to their morphological adaptations,  
445 whilst controlling for their evolutionary history. Our work on *Deronectes* shows that heat  
446 tolerance and plasticity need not be negatively correlated, suggesting that the postulated  
447 trade-off does not exist or can at least be circumvented. The previously reported positive  
448 relationship between inherent heat tolerance and plasticity in *Deronectes* beetles may be  
449 driven by differences in gill density, as no relationship between inherent heat tolerance and  
450 plasticity remained after accounting for differences in these structures. This suggests that the  
451 positive relationship observed in *Deronectes* may be an exception (Stillman, 2003; Magozzi  
452 & Calosi 2015). As a prediction, we would not expect a positive relationship between  
453 inherent heat tolerance and plasticity in beetles that do not possess setal tracheal gills and  
454 instead use aerial respiration. Indeed using published and unpublished data for 13 species of  
455 the dytiscid tribe Agabini (see Calosi et al., 2008a), no relationship was detected between  
456 plasticity and inherent heat tolerance either in individuals acclimated at 20.5 °C ( $\beta = 0.451$ ;  
457  $F_{1,11} = 4.30$ ;  $P = 0.062$ ;  $R^2 = 0.22$ ) or 14.5 °C ( $\beta = -0.332$ ;  $F_{1,11} = 1.57$ ;  $P = 0.236$ ;  $R^2 = 0.05$ ).

458

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464 beetle ecophysiology.

465

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580

581 Figure captions

582

583 **Fig. 1.** Plasticity of CTmax in relation to inherent CTmax across the 15 *Deronectes* species  
584 investigated in this study. Data for 13 of these species were previously reported by Calosi *et*  
585 *al.* (2008a). Plasticity in heat tolerance is higher for species with high inherent heat tolerance  
586 ( $F_{1,13} = 9.69$ ;  $P = 0.0082$ ).

587

588 **Fig. 2.** Elytral punctation and setation in *Deronectes aubei aubei*. Setal tracheal gills are  
589 spoon-shaped and flattened (A), corresponding to a form of sensillum trichoideum type 2 of  
590 Wolfe & Zimmermann (1984). Also visible are scattered examples of sensillum trichoideum  
591 type 1 (B) and rod-like setae associated with large punctures (C – also a form of sensillum  
592 trichoideum type 2). Only setae of type (A) were enumerated, the rectangle highlighting an  
593 area without punctures (see text). Scale bar = 10  $\mu\text{m}$ .

594

595 **Fig. 3.** Mean heat tolerance of *Deronectes latus* at different oxygen tensions. Treatment  
596 differences with (blue) and without (red) access to air are shown separately. Letters indicate  
597 differences between oxygen levels within treatment and asterisks indicate differences  
598 between treatments within oxygen levels. Error bars indicate SEs ( $n = 9$  in all cases except  
599 for the normoxia and hyperoxia treatments without access to air where  $n = 10$ ).

600

601 **Fig. 4.** Setal tracheal gill densities in *Deronectes* species. A) Density measured in a section  
602 without punctation, and B) Density in sections with punctation, averaged across anterior,  
603 middle and posterior sections of the beetle's elytra (see methods). Different letters indicate  
604 significant differences between species ( $P < 0.05$ ). Individuals are indicated by blue circles to  
605 illustrate the spread and distribution of data.

606

607 **Fig. 5.** Relationship between gill density and mean CTmax (A) and CTmin (B) in 15  
608 *Deronectes* species, acclimated to 20.5  $^{\circ}\text{C}$ . A) Trendlines indicate significant relationships ( $P$   
609  $< 0.05$ ).

610

611 **Fig. 6.** Relationship between gill density and mean plasticity of thermal tolerance for CTmax  
612 (A) and CTmin (B) in 15 *Deronectes* species. Trendlines indicate significant relationships ( $P$   
613  $< 0.05$ ).

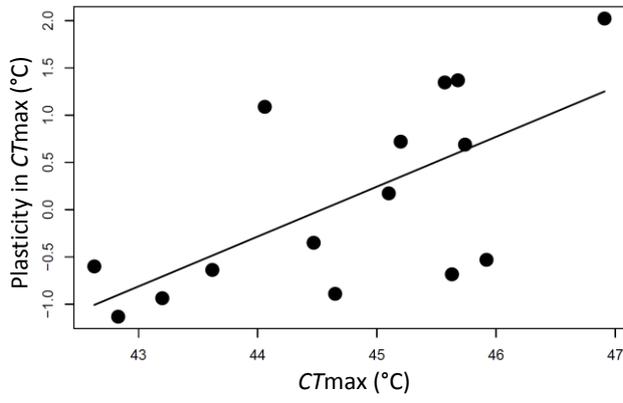
614

615 **Fig. 7.** Difference in mean setal tracheal gill density between *Deronectes* species inhabiting  
616 cool, thermally constant streams and warmer, thermally variable streams. Species are  
617 indicated by blue circles to illustrate the spread and distribution of the data.

618

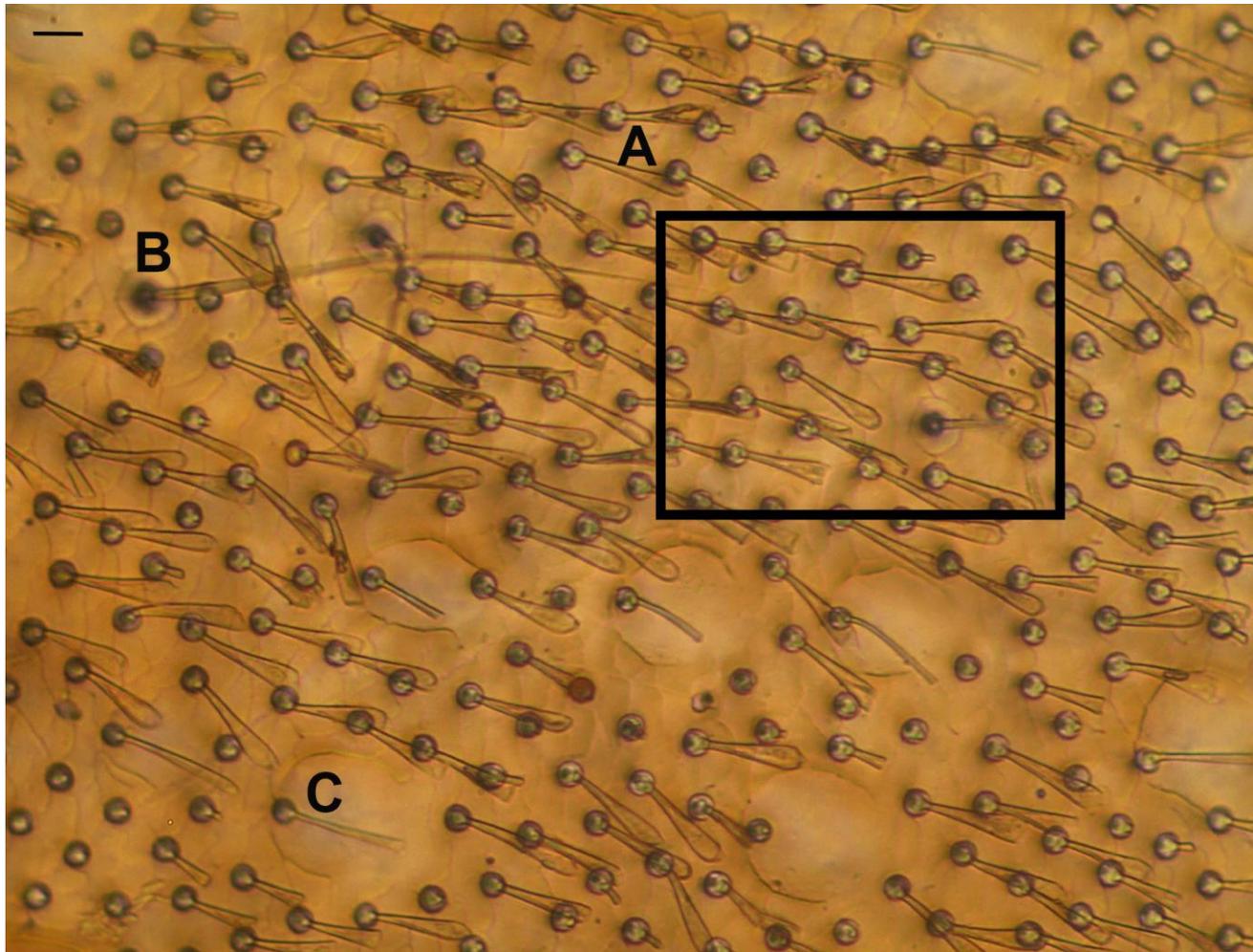
619 **Figures**

620



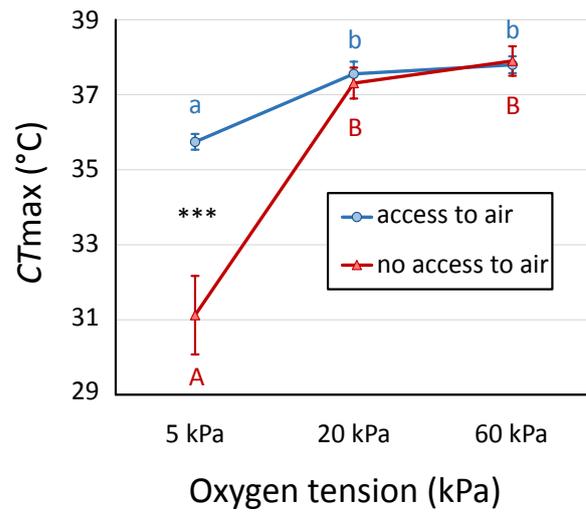
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622 **Figure 1**



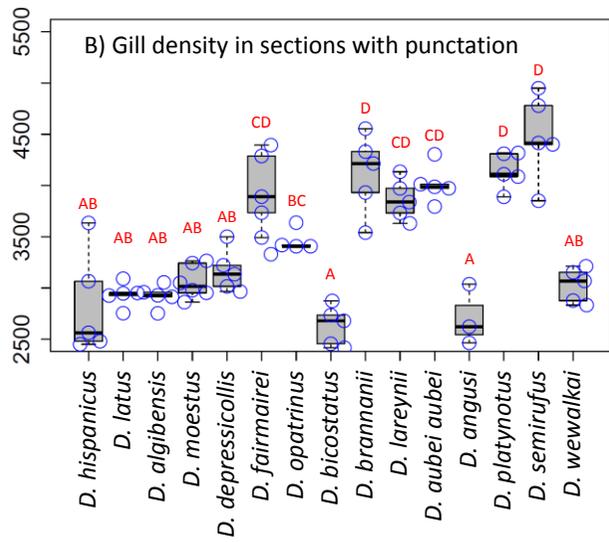
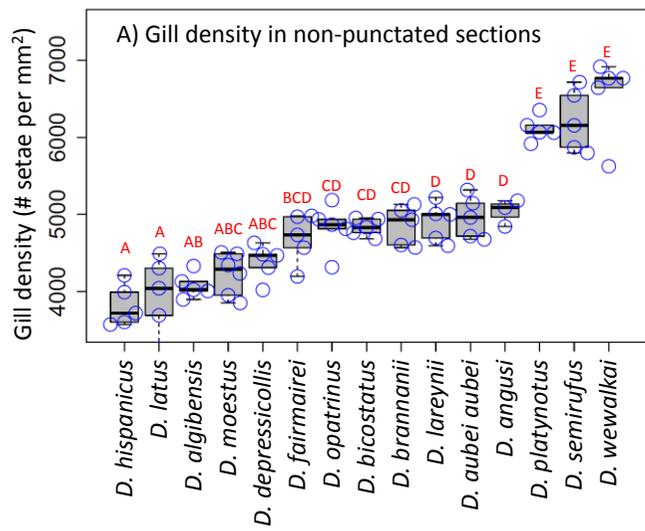
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624 Figure 2



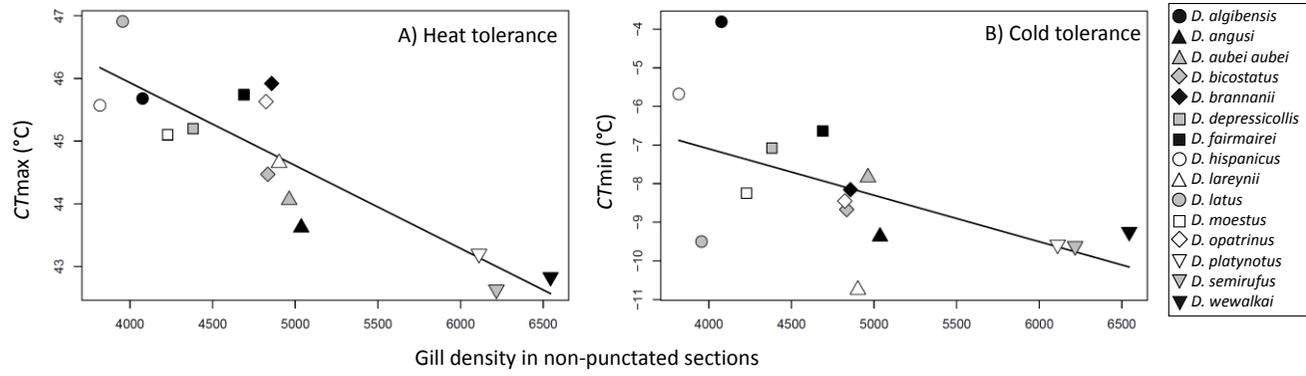
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626 Figure 3.



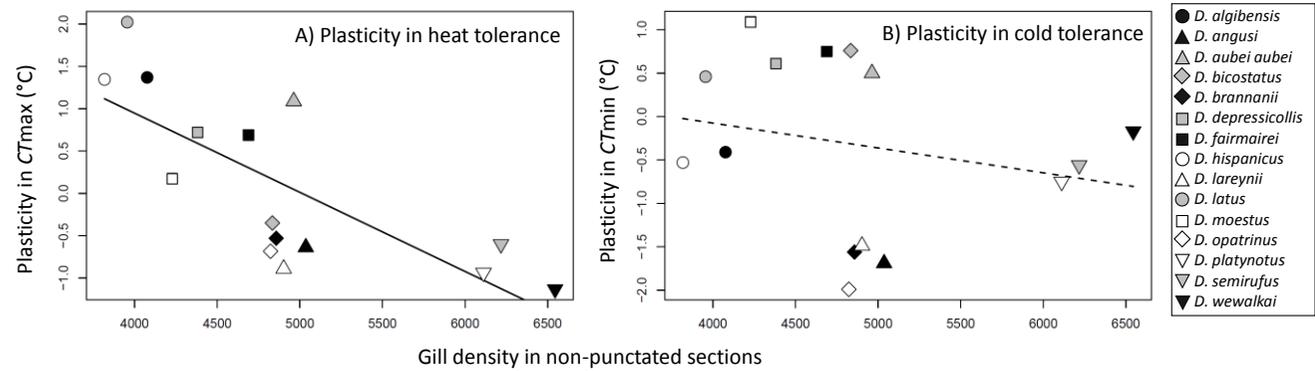
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628 Figure 4.



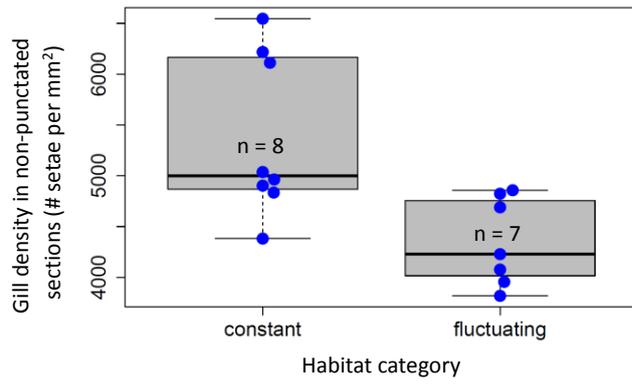
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630 Figure 5



631

632 Figure 6.



633

634 Figure 7

635 Table 1. Summary of OLC and PGLS analyses for *CT*max in individuals acclimated either to  
 636 20.5 °C (A), or 14.5 °C (B), *CT*min in individuals acclimated either to 20.5 °C (C), or  
 637 14.5 °C (D), plasticity in *CT*max (E) and plasticity in *CT*min (F). Gill density was  
 638 measured in non-punctate regions.

Models	Coefficients	df (num, den)	estimate	SE	p	R2
<b>A) <i>CT</i>max in 20 C acclimated animals</b>						
OLS: gill density	intercept	1,13	51.22	1.05	<b>0.0000</b>	74.9%
	gill density	1,13	-0.00132	0.00021	<b>0.0000</b>	
OLS: gill density + body size	intercept	1,12	50.81	1.56	<b>0.0000</b>	75.1%
	gill density	1,12	-0.00131	0.00022	<b>0.0001</b>	
	body size	1,12	0.0419	0.114	0.7186	
OLS: gill density/body size	intercept	1,13	47.41	0.94	<b>0.0000</b>	40.2%
	gill density/body size	1,13	-0.00414	0.00140	<b>0.0112</b>	
PIC: gill density	gill density	1,13	-6.321	1.202	<b>0.0002</b>	68.0%
PIC: gill density + body size	gill density	1,12	-6.313	1.239	<b>0.0003</b>	68.6%
	body size	1,12	0.450	0.937	0.6400	
PIC: gill density/body size	gill density/body size	1,13	-2.593	1.052	<b>0.0284</b>	31.8%
<b>B) <i>CT</i>max in 14.5 C acclimated animals</b>						
OLS: gill density	intercept	1,13	46.32	1.30	<b>0.0000</b>	14.0%
	gill density	1,13	-0.00038	0.00026	0.1689	
OLS: gill density + body size	intercept	1,12	45.66	1.92	<b>0.0000</b>	15.6%
	gill density	1,12	-0.00036	0.00028	0.2195	
	body size	1,12	0.0670	0.140	0.6407	
OLS: gill density/body size	intercept	1,13	45.36	0.77	<b>0.0000</b>	10.6%
	gill density/body size	1,13	-0.00142	0.00115	0.2370	
PIC: gill density	gill density	1,13	-1.132	1.394	0.4313	4.8%
PIC: gill density + body size	gill density	1,12	-1.125	1.440	0.4501	6.2%
	body size	1,12	0.456	1.090	0.6828	
PIC: gill density/body size	gill density/body size	1,13	-0.701	0.835	0.4164	5.1%
<b>C) <i>CT</i>min in 20 C acclimated animals</b>						
OLS: gill density	intercept	1,13	-2.29	2.48	0.3714	30.8%
	gill density	1,13	-0.00120	0.00050	<b>0.0317</b>	
OLS: gill density + body size	intercept	1,12	-3.99	3.63	0.2925	33.2%
	gill density	1,12	-0.00114	0.00052	<b>0.0495</b>	
	body size	1,12	0.1728	0.264	0.5250	
OLS: gill density/body size	intercept	1,13	-5.55	1.54	<b>0.0032</b>	19.5%
	gill density/body size	1,13	-0.00409	0.00230	0.0990	
PIC: gill density	gill density	1,13	-6.425	2.386	<b>0.0184</b>	35.8%
PIC: gill density + body size	gill density	1,12	-6.407	2.449	<b>0.0226</b>	37.5%
	body size	1,12	1.070	1.853	0.5743	

PIC: gill density/body size	gill density/body size	1,13	-3.021	1.577	0.0776	22.0%
<b>D) CTmin in 14.5 C acclimated animals</b>						
OLS: gill density	intercept	1,13	-3.37	2.77	0.2459	17.1%
	gill density	1,13	-0.00091	0.00056	0.1252	
OLS: gill density + body size	intercept	1,12	-8.47	3.58	<b>0.0355</b>	37.8%
	gill density	1,12	-0.00072	0.00051	0.1859	
	body size	1,12	0.5190	0.260	0.0692	
OLS: gill density/body size	intercept	1,13	-4.70	1.50	<b>0.0078</b>	26.8%
	gill density/body size	1,13	-0.00489	0.00224	<b>0.0481</b>	
PIC: gill density	gill density	1,13	-3.734	2.858	0.2141	11.6%
PIC: gill density + body size	gill density	1,12	-3.671	2.594	0.1825	32.8%
	body size	1,12	3.818	1.962	0.0755	
PIC: gill density/body size	gill density/body size	1,13	-3.764	1.495	<b>0.0257</b>	32.8%
<b>E) delta CTmax</b>						
OLS: gill density	intercept	1,13	4.69	1.11	<b>0.0010</b>	57.4%
	gill density	1,13	-0.00093	0.00022	<b>0.0011</b>	
OLS: gill density + body size	intercept	1,12	5.18	1.64	<b>0.0082</b>	58.0%
	gill density	1,12	-0.00095	0.00024	<b>0.0016</b>	
	body size	1,12	-0.0505	0.119	0.6794	
OLS: gill density/body size	intercept	1,13	1.70	0.86	0.0704	22.1%
	gill density/body size	1,13	-0.00248	0.00129	0.0771	
PIC: gill density	gill density	1,13	-5.463	1.089	<b>0.0002</b>	65.9%
PIC: gill density + body size	gill density	1,12	-5.471	1.121	<b>0.0004</b>	66.7%
	body size	1,12	-0.436	0.848	0.6163	
PIC: gill density/body size	gill density/body size	1,13	-1.723	1.012	0.1123	18.2%
<b>F) delta CTmin</b>						
OLS: gill density	intercept	1,13	1.07	1.65	0.5268	5.4%
	gill density	1,13	-0.00029	0.00033	0.4038	
OLS: gill density + body size	intercept	1,12	4.48	2.03	<b>0.0481</b>	35.0%
	gill density	1,12	-0.00042	0.00029	0.1788	
	body size	1,12	-0.3462	0.148	<b>0.0375</b>	
OLS: gill density/body size	intercept	1,13	-0.85	0.96	0.3957	2.3%
	gill density/body size	1,13	0.00080	0.00144	0.5876	
PIC: gill density	gill density	1,13	-2.691	1.801	0.1591	14.7%
PIC: gill density + body size	gill density	1,12	-2.736	1.554	0.1037	41.4%
	body size	1,12	-2.748	1.176	<b>0.0375</b>	
PIC: gill density/body size	gill density/body size	1,13	0.743	1.151	0.5297	3.1%

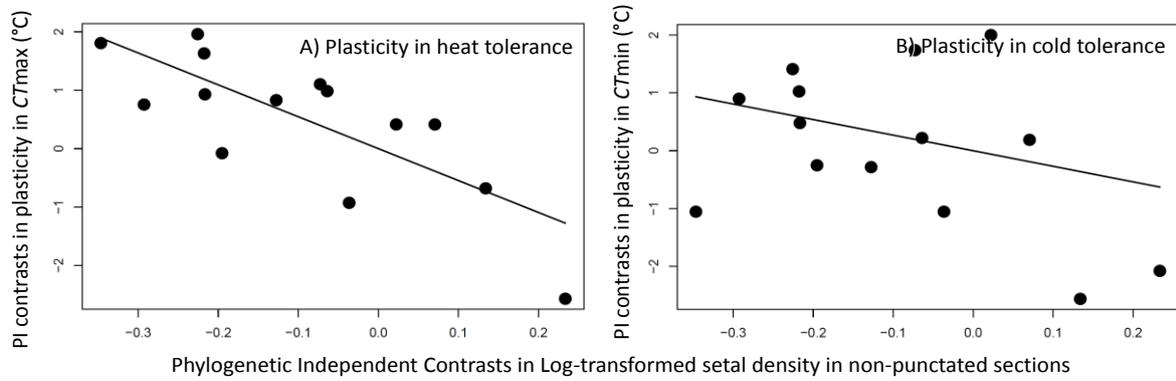
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Figure S1. Relationship between setal tracheal gill density and plasticity in either heat tolerance (A) or cold tolerance (B) evaluated by phylogenetic independent (PI) contrasts. Note that for the plasticity in heat tolerance, the relationship based on PI contrasts is stronger than that based on OLS (see Fig. 6A).

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653 Table S1. Overview of the body size, collection locality, gill density and thermal tolerance  
 654 for the 15 species of *Deronectes* diving beetles investigated in this study.

Species name	Authority	Body size (+SE)
<i>D. aubei aubei</i>	(Mulsant, 1843)	6.34 (+0.12)
<i>D. semirufus</i>	(Germar, 1845)	8.21 (+0.17)
<i>D. platynotus</i>	(Germar, 1834)	5.37 (+0.2)
<i>D. lareynii</i>	(Fairmaire, 1858)	9.177 (+0.19)
<i>D. fairmairei</i>	(Leprieur, 1876)	8.91 (+0.1)
<i>D. moestus</i>	(Fairmaire, 1858)	6.01 (+0.19)
<i>D. brannanii</i>	(Schauffus, 1869)	6.838 (+0.13)
<i>D. bicostatus</i>	(Schaum, 1864)	6.82 (+0.12)
<i>D. wewalkai</i>	Fery & Fresneda, 1988	8.54 (+0.11)
<i>D. algibensis</i>	Fery & Fresneda, 1988	9.89 (+0.22)
<i>D. depressicollis</i>	(Rosenhauer, 1856)	6.28 (+0.14)
<i>D. opatrinus</i>	(Germar, 1824)	10.54 (+0.28)
<i>D. hispanicus</i>	(Rosenhauer, 1856)	9.88 (+0.15)
<i>D. angusi</i>	Fery & Brancucci, 1990	9.14 (+0.21)
<i>D. latus</i>	(Stephens, 1829)	7.96 (+0.13)

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Species name	Habitat type	Gill density (+SE)	Gill density in punctated region (+SE)	n (gill density)
<i>D. aubei aubei</i>	1. cold, fast flowing, permanent streams with more constant temperatures	4963.2 (+122.6)	4013.6 (+82)	5
<i>D. semirufus</i>	1. streams with pools of more constant temperatures and connected by groundwater	6217.4 (+181.2)	4478.6 (+189.1)	5
<i>D. platynotus</i>	1. permanent streams with more constant temperatures	6111.6 (+71.9)	4142 (+79.5)	5
<i>D. lareynii</i>	1. cold, fast flowing, permanent streams with more constant temperatures	4902.2 (+113.9)	3860.9 (+88.7)	5
<i>D. fairmairei</i>	2. warm, intermittent, lowland streams with more fluctuating temperatures	4689.4 (+145.9)	3959.5 (+168.7)	5
<i>D. moestus</i>	2. warm, intermittent, lowland streams with more fluctuating temperatures	4228.2 (+112.3)	3058.1 (+66.5)	6
<i>D. brannanii</i>	2. warm, intermittent, lowland streams with more fluctuating temperatures	4857.2 (+115.2)	4113.9 (+175.1)	5
<i>D. bicostatus</i>	1. cold, fast flowing, permanent streams with more constant temperatures	4834.2 (+51.7)	2631.9 (+86.2)	5
<i>D. wewalkai</i>	1. cold, fast flowing, permanent streams with more constant temperatures	6544.9 (+234.1)	3028.5 (+74.7)	5
<i>D. algibensis</i>	2. warm, intermittent, lowland streams with more fluctuating temperatures	4076.2 (+73.4)	2921.4 (+48.8)	5
<i>D. depressicollis</i>	1. cold, fast flowing, permanent streams with more constant temperatures	4381.4 (+104)	3167.2 (+94.5)	5
<i>D. opatrinus</i>	2. warm, intermittent, lowland streams with more fluctuating temperatures	4823.1 (+142.4)	3439.7 (+51.9)	5
<i>D. hispanicus</i>	2. warm, intermittent, lowland streams with more fluctuating temperatures	3818.1 (+122.5)	2838.9 (+228.1)	5
<i>D. angusi</i>	1. cold, permanent streams with more constant temperatures	5036.4 (+99.9)	2707.8 (+170.7)	3
<i>D. latus</i>	2. warm, permanent, lowland streams	3955.5 (+220.4)	2933.5 (+53.6)	5

Species name	mean CTmax (+ SE) at 14.5 °C	mean CTmax (+ SE) at 20.5 °C	Plasticity in Ctmax	mean CTmin (+ SE) at 14.5 °C	mean CTmin (+ SE) at 20.5 °C	Plasticity in Ctmin	n (thermal tolerance )
<i>D. aubei</i>							
<i>aubei</i>	42.97 (+0.55)	44.06 (+0.27)	1.0886	-8.34 (+0.54)	-7.84 (+0.45)	0.5014	28
<i>D. semirufus</i>							
<i>semirufus</i>	43.23 (+0.69)	42.63 (+0.2)	-0.6	-9.06 (+0.37)	-9.62 (+0.4)	-0.56	60
<i>D. platynotus</i>							
<i>platynotus</i>	44.14 (+0.78)	43.2 (+0.32)	-0.9364	-8.83 (+0.35)	-9.58 (+0.42)	-0.7497	47
<i>D. lareynii</i>							
<i>lareynii</i>	43.76 (+0.43)	44.65 (+0.25)	-0.89	-9.26 (+0.31)	-10.75 (+0.53)	-1.49	48
<i>D. fairmairei</i>							
<i>fairmairei</i>	45.06 (+0.16)	45.74 (+0.21)	0.6888	-7.39 (+0.61)	-6.64 (+0.29)	0.7449	36
<i>D. moestus</i>							
<i>moestus</i>	44.93 (+0.21)	45.1 (+0.3)	0.1727	-9.34 (+0.44)	-8.25 (+0.52)	1.0867	48
<i>D. brannanii</i>							
<i>brannanii</i>	45.39 (+0.16)	45.92 (+0.15)	-0.53	-6.60 (+0.55)	-8.16 (+0.51)	-1.56	73
<i>D. bicostatus</i>							
<i>bicostatus</i>	44.82 (+0.34)	44.47 (+0.27)	-0.35	-9.43 (+0.72)	-8.67 (+0.66)	0.7623	53
<i>D. wewalkai</i>							
<i>wewalkai</i>	43.96 (+1.01)	42.83 (+0.23)	-1.132	-9.08 (+0.33)	-9.25 (+0.42)	-0.1697	68
<i>D. algibensis</i>							
<i>algibensis</i>	44.3 (+0.36)	45.68 (+0.27)	1.3695	-3.4 (+0.8)	-3.81 (+0.47)	-0.414	52
<i>D. depressicollis</i>							
<i>depressicollis</i>	44.48 (+0.17)	45.2 (+0.26)	0.72	-7.69 (+0.36)	-7.08 (+0.52)	0.6057	57
<i>D. opatrinus</i>							
<i>opatrinus</i>	46.31 (+0.24)	45.63 (+0.23)	-0.6835	-6.46 (+1.08)	-8.45 (+0.35)	-1.9889	54
<i>D. hispanicus</i>							
<i>hispanicus</i>	44.22 (+0.14)	45.57 (+0.09)	1.3465	-5.15 (+0.34)	-5.68 (+0.29)	-0.5288	92
<i>D. angusi</i>							
<i>angusi</i>	44.26 (+0.93)	43.62 (+0.24)	-0.6371	-7.68 (+0.82)	-9.37 (+0.52)	-1.6837	26
<i>D. latus</i>							
<i>latus</i>	44.88 (+0.18)	46.91 (+0.28)	2.0226	-9.96 (+0.33)	-9.5 (+0.43)	0.4591	75

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661 Table S2. Summary of OLC and PGLS analyses for *CT*<sub>max</sub> in individuals acclimated either  
 662 to 20.5 °C (A), or 14.5 °C (B), *CT*<sub>min</sub> in individuals acclimated either to 20.5 °C (C),  
 663 or 14.5 °C (D), plasticity in *CT*<sub>max</sub> (E) and plasticity in *CT*<sub>min</sub> (F). Gill density was  
 664 measured in punctate regions.

Models	Coefficients	df (num, den)	estimate	SE	p	R <sup>2</sup>
<b>A) <i>CT</i><sub>max</sub> in 20 C acclimated animals</b>						
OLS: gill density	intercept	1,13	46.64	1.90	<b>0.0000</b>	7.3%
	gill density	1,13	-0.00055	0.00055	0.3307	
OLS: gill density + body size	intercept	1,12	45.37	2.96	<b>0.0000</b>	9.7%
	gill density	1,12	-0.00047	0.00058	0.4300	
	body size	1,12	0.1250	0.219	0.5788	
OLS: gill density/body size	intercept	1,13	46.00	1.09	<b>0.0000</b>	10.0%
	gill density/body size	1,13	-0.00278	0.00231	0.2505	
PIC: gill density	gill density	1,13	-1.521	2.146	0.4909	3.7%
PIC: gill density + body size	gill density	1,12	-1.491	2.229	0.5162	4.3%
	body size	1,12	0.456	1.639	0.7854	
	gill density/body size	1,13	-0.826	1.245	0.5186	3.3%
<b>B) <i>CT</i><sub>max</sub> in 14.5 C acclimated animals</b>						
OLS: gill density	intercept	1,13	45.63	1.28	<b>0.0000</b>	6.4%
	gill density	1,13	-0.00035	0.00037	0.3620	
OLS: gill density + body size	intercept	1,12	44.88	2.00	<b>0.0000</b>	8.4%
	gill density	1,12	-0.00030	0.00039	0.4570	
	body size	1,12	0.0742	0.148	0.6245	
OLS: gill density/body size	intercept	1,13	45.16	0.74	<b>0.0000</b>	7.2%
	gill density/body size	1,13	-0.00158	0.00157	0.3338	
PIC: gill density	gill density	1,13	-0.652	1.459	0.6625	1.5%
PIC: gill density + body size	gill density	1,12	-0.622	1.510	0.6877	2.8%
	body size	1,12	0.445	1.110	0.6959	
	gill density/body size	1,13	-0.508	0.839	0.5555	2.7%
<b>C) <i>CT</i><sub>min</sub> in 20 C acclimated animals</b>						
OLS: gill density	intercept	1,13	-5.44	2.68	0.0638	7.6%
	gill density	1,13	-0.00080	0.00077	0.3194	
OLS: gill density + body size	intercept	1,12	-7.70	4.15	0.0885	11.5%
	gill density	1,12	-0.00066	0.00081	0.4332	
	body size	1,12	0.2224	0.307	0.4830	
OLS: gill density/body size	intercept	1,13	-6.56	1.56	<b>0.0010</b>	8.3%
	gill density/body size	1,13	-0.00359	0.00331	0.2976	
PIC: gill density	gill density	1,13	-3.149	2.936	0.3030	8.1%
PIC: gill density + body size	gill density	1,12	-3.081	3.034	0.3298	9.7%
	body size	1,12	1.020	2.230	0.6555	

PIC: gill density/body size	gill density/body size	1,13	-1.757	1.706	0.3217	7.5%
<b>D) CTmin in 14.5 C acclimated animals</b>						
OLS: gill density	intercept	1,13	-6.28	2.82	<b>0.0442</b>	2.4%
	gill density	1,13	-0.00046	0.00081	0.5821	
OLS: gill density + body size	intercept	1,12	-12.19	3.84	<b>0.0080</b>	27.6%
	gill density	1,12	-0.00009	0.00075	0.9094	
	body size	1,12	0.5806	0.284	0.0633	
OLS: gill density/body size	intercept	1,13	-5.79	1.55	<b>0.0025</b>	12.9%
	gill density/body size	1,13	-0.00457	0.00329	0.1882	
PIC: gill density	gill density	1,13	-0.885	3.118	0.7810	0.6%
PIC: gill density + body size	gill density	1,12	-0.631	2.881	0.8303	21.9%
	body size	1,12	3.830	2.118	0.0956	
PIC: gill density/body size	gill density/body size	1,13	-2.687	1.651	0.1276	16.9%
<b>E) delta CTmax</b>						
OLS: gill density	intercept	1,13	1.79	1.52	0.2596	8.8%
	gill density	1,13	-0.00049	0.00044	0.2818	
OLS: gill density + body size	intercept	1,12	1.83	2.40	0.4617	8.8%
	gill density	1,12	-0.00049	0.00047	0.3137	
	body size	1,12	-0.0035	0.178	0.9845	
OLS: gill density/body size	intercept	1,13	0.82	0.90	0.3812	4.9%
	gill density/body size	1,13	-0.00158	0.00192	0.4265	
PIC: gill density	gill density	1,13	-1.795	1.854	0.3507	6.7%
PIC: gill density + body size	gill density	1,12	-1.824	1.924	0.3617	7.5%
	body size	1,12	-0.450	1.415	0.7561	
PIC: gill density/body size	gill density/body size	1,13	-0.363	1.107	0.7479	0.8%
<b>F) delta CTmin</b>						
OLS: gill density	intercept	1,13	0.84	1.55	0.5983	4.3%
	gill density	1,13	-0.00034	0.00045	0.4580	
OLS: gill density + body size	intercept	1,12	4.49	2.02	<b>0.0465</b>	35.3%
	gill density	1,12	-0.00057	0.00040	0.1737	
	body size	1,12	-0.3582	0.149	<b>0.0338</b>	
OLS: gill density/body size	intercept	1,13	-0.77	0.92	0.4131	1.9%
	gill density/body size	1,13	0.00098	0.00195	0.6219	
PIC: gill density	gill density	1,13	-2.264	1.905	0.2559	9.8%
PIC: gill density + body size	gill density	1,12	-2.450	1.650	0.1634	37.7%
	body size	1,12	-2.810	1.213	<b>0.0391</b>	
PIC: gill density/body size	gill density/body size	1,13	0.929	1.133	0.4267	4.9%