



BRUNA DE CÁSSIA MENEZES RAMOS

Alometria ontogenética e valor adaptativo de estruturas corporais exageradas em larvas
de *Gratiana spadicea* (Klug) (Coleoptera: Chrysomelidae)

Dissertação apresentada ao Programa de Pós-Graduação
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...I see hardly any way of distinguishing between the effects, on the one hand, of a part being largely developed through natural selection and another and adjoining part being reduced by this same process..., and, on the other hand, the actual withdrawal of nutriment from one part owing to the excess of growth in another and adjoining part.

(Darwin, 1859. *On the origin of species*, p.147)

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RESUMO

Características morfológicas consideradas “exageradas”, geralmente, estão associadas a padrões alométricos e evolução de comportamentos altamente especializados moldados por pressões seletivas (*e.g.* advindas da seleção sexual, competidores, predadores, nutrição). Larvas de Cassidinae (Coleoptera: Chrysomelidae) apresentam tais características, como, por exemplo, a presença de escolos posicionados lateralmente no corpo e um processo caudal, localizado dorsalmente no nono segmento abdominal. Esta estrutura é responsável pelo ancoramento do escudo larval, composto por fezes e/ ou exúvias, que as larvas constroem e mantêm ao longo de seu desenvolvimento. A função do escudo é controversa, sendo atribuída principalmente em relação à defesa frente a inimigos naturais. O papel frente a fatores abióticos tem sido escassamente investigado. Neste estudo, investigamos a trajetória de crescimento dessas estruturas em larvas de *G. spadicea* (Klug) (Chrysomelidae: Cassidinae) e o valor adaptativo associado ao escudo. Por meio de medidas lineares comparamos o padrão de crescimento ontogenético das estruturas morfológicas e verificamos a ocorrência de alometria ontogenética. Ainda, avaliamos o efeito da privação do alimento no crescimento. E finalmente, testamos, em condições naturais, a influência do escudo em relação à mortalidade e à temperatura corporal das larvas. Foi constatada a existência de alometria ontogenética e investimento diferenciado no crescimento das estruturas mensuradas. E, confirmamos que o valor adaptativo atribuído ao escudo está relacionado à proteção contra predadores. Ainda de forma inédita, demonstramos que essa estrutura proporciona também uma diminuição da exposição corporal das larvas à radiação solar.

CAPÍTULO I

INTRODUÇÃO GERAL

A origem e a evolução da diversidade de características morfológicas têm sido estudadas há décadas (*e.g.* Huxley 1924; Gould 1966; Klingenberg 1996; Daly 1985; Klingenberg 2002; Emlen & Nijhout 2000; Frankino *et al.* 2005). A diversidade de formas dos organismos é, em parte, consequência de vários componentes morfológicos, cujas diferenças nas taxas de crescimento entre estruturas corporais relativas devem-se a um processo denominado de “alometria”, cunhada pela primeira vez por Huxley & Teissier (1936) e descrita através da equação exponencial:

$$y = \alpha + x^\beta$$

onde x e y são dimensões de duas estruturas corporais, e α e β são constantes (Huxley 1924; Huxley & Teissier 1936). Esta equação é referida como equação alométrica e pode ser escrita na forma log-transformada que nesta escala torna-se linear:

$$\log(y) = \log(\alpha) + \beta \log(x)$$

onde o logaritmo de α é o intercepto e β é o coeficiente de inclinação (Figura 1). Se $\beta=1$, a relação de crescimento entre x e y é dita isométrica, ou seja, é constante; se $\beta \neq 1$ a relação é dita alométrica e há crescimento desproporcional entre x e y (Huxley & Teissier 1936; Reiss 1989). Os interceptos e, em especial, os coeficientes de inclinação podem ser usados para testar hipóteses de pressões seletivas (Gould 1966; Emlen & Nijhout 2000).

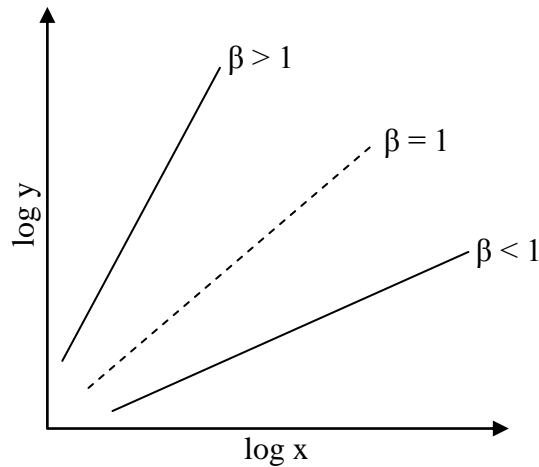


Figura 1. Representação gráfica de $\log y$ em função de $\log x$ e variações do coeficiente alométrico β . $\beta > 1$, alometria positiva; $\beta = 1$, isometria e $\beta < 1$, alometria negativa (Fonte: Reiss 1989).

De acordo com Klingenberg (1996), há três níveis aos quais a alometria ocorre: estática, que se refere à covariação entre indivíduos dentro de um estágio ontogenético de uma única espécie; filogenética (evolutiva), que representa a variação entre linhagens em um único estágio ontogenético e, por fim, quando avaliamos a covariação entre estruturas corporais de indivíduos de uma mesma espécie ao longo da ontogênese, a alometria é definida como ontogenética (Figura 2).

Características morfológicas exageradas (*sensu* Emlen & Nijhout 2000), geralmente, estão associadas a padrões alométricos e evolução de comportamentos altamente especializados moldados por pressões seletivas (Gould 1974; Dial *et al.* 2008; Emlen 2008; Frankino *et al.* 2009). Morfologias exageradas, resultantes de seleção sexual, são evidentes em machos de muitas espécies de animais que apresentam estruturas corporais elaboradas utilizadas como ornamentos para atrair parceiras ou como “armas” em lutas entre machos competidores (Baker & Wilkinson 2001; Emlen & Nijhout 2000; Kodric-Brown *et al.* 2006; Cuervo & Møller 2009). Um exemplo disso é

o besouro *Trypoxylus dichotomus* (Linnaeus) (Coleoptera: Scarabaeidae) que possui projeções cefálicas (chifres), cuja alometria é positiva, e são utilizados como “armas” na competição com outros machos pelas fêmeas (Emlen *et al.* 2012). Ainda, em insetos sociais há polimorfismos associados a distintas castas, como resultado de diferenças alométricas durante o desenvolvimento dos estágios imaturos, que podem ser influenciadas por fatores ambientais como nutrição, temperatura, feromônios, e/ou por fatores genéticos (Stern *et al.* 1996; Schwander *et al.* 2005; Fjerdingstad & Crozier 2006; Jaffé *et al.* 2007; Perrard *et al.* 2012). Por outro lado, a predação também pode ser um fator importante na expressão de características morfológicas das presas, como por exemplo, as larvas de *Leucorrhinia dubia* (Odonata: Libellulidae), cuja presença de predadores afeta as taxas de crescimento dos espinhos abdominais (Dodson 1989; Arnqvist & Johansson 1998; Johansson 2002).

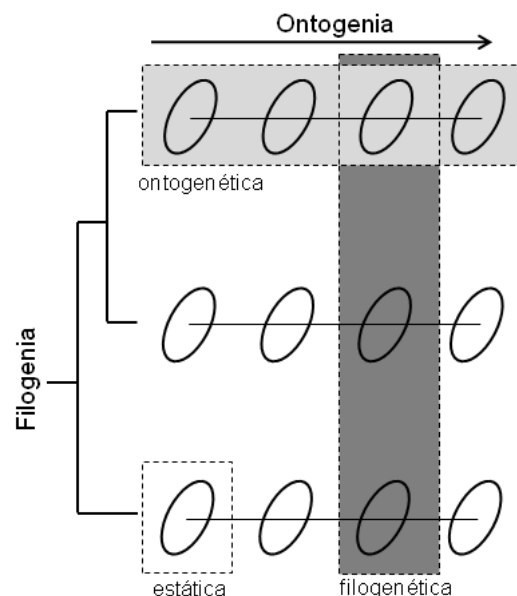


Figura 2. Diagrama sobre alometria estática, filogenética (evolutiva) e ontogenética, mostrando três espécies com quatro estágios ontogenéticos diferentes, considerados homólogos. Cada retângulo compreende uma análise alométrica distinta. (Modificado de Klingenberg 1996).

Larvas de Cassidinae (Coleoptera: Chrysomelidae) apresentam algumas características morfológicas exageradas, como, por exemplo, a presença de escolos posicionados lateralmente no corpo e a presença de um processo caudal, localizado dorsalmente no nono segmento abdominal (Chaboo 2007; Swietojanska 2009). Também denominado de furca, esse processo é responsável pelo ancoramento de um escudo composto por fezes e/ou exúvias que as larvas constroem e mantêm ao longo do seu desenvolvimento através da movimentação do telescópio anal (Figura 3) (Chaboo & Nguyen 2004; Chaboo 2007; Swietojanska 2009). Quando o escudo é composto somente por fezes, estas são adicionadas à furca pelo telescópio anal; por outro lado, quando composto somente por exúvias, a cada ecdise a exúvia do instar anterior é “empurrada” através de movimentos do corpo (pressão hidrostática interna) até a furca, onde fica encaixada na furca do instar subsequente; e, por fim, quando o escudo é composto por fezes e exúvias, os comportamentos descritos acima ocorrem em conjunto, se repetindo a cada ecdise. Além da composição, o escudo pode variar quanto a sua arquitetura, cobrindo parcialmente ou completamente o corpo da larva (Eisner & Eisner 2000; Chaboo 2007). Para essas estruturas, trabalhos de cunho morfológico já foram desenvolvidos (McBride *et al.* 2000; Chaboo & Nguyen 2004; Swietojanska 2009), no entanto carecem de análises morfométricas com enfoque alométrico adaptativo.

A função do escudo dos cassidíneos tem sido estudada, principalmente, no que se refere à proteção contra inimigos naturais. De forma geral, o papel do escudo frente a predadores tem sido considerado controverso, pois a eficácia do mesmo em dificultar o acesso do predador à larva pode variar de acordo com a espécie do predador, ou ainda, a proteção da larva pode ser devida a compostos químicos presentes em seu corpo e não ao escudo em si (Eisner *et al.* 1967; Olmstead & Denno 1993; Eisner & Eisner 2000;

Nogueira-de-Sá & Trigo 2002; Müller & Hilker 2003; Bottcher *et al.* 2009). Outra hipótese a cerca da função do escudo é proteção contra fatores abióticos. Entretanto, somente Bacher & Luder (2005) testaram, em condições de laboratório, se o escudo das larvas de *Cassida rubiginosa* Müller as protege de radiação UVB e, como resultado, obtiveram alta mortalidade das larvas com e sem escudo. De forma geral, os estágios imaturos de insetos estão mais expostos aos efeitos de fatores como temperatura, insolação e umidade, uma vez que apresentam pouca mobilidade (Heming 1999; Müller & Hilker 2003; Chaboo 2011). A função termorregulatória dos escudos em Cassidinae, pelo que conhecemos, não foi explorada até então.

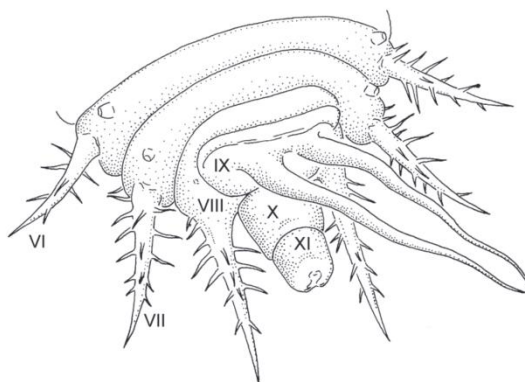


Figura 3. Representação esquemática da região posterior do corpo de uma larva de Cassidinae. VI–VIII - últimos escolos abdominais; IX- segmento abdominal modificado em processo caudal (=furca); X e XI segmentos abdominais formando o telescópio anal. (Modificado de Swietojanska 2009).

Nossa espécie de estudo é *Gratiana spadicea* (Klug), um cassidíneo monófago (*sensu* Bernays & Chapman 1994) de *Solanum sisymbriifolium* Lam. (Solanaceae). Esta planta é considerada invasora e ocorre principalmente em ambientes alterados e ensolarados (Groth 1989; Mentz & Oliveira 2004). A interação dessa espécie com sua

planta-hospedeira tem sido amplamente estudada em relação à performance, preferência, desenvolvimento, comportamento alimentar, deslocamento e interação com tricomas da planta hospedeira (Medeiros & Moreira 2002; Medeiros & Moreira 2003; Medeiros & Moreira 2005; Boligon *et al.* 2009), e devido sua especificidade alimentar, tem grande potencial como agente de controle biológico (Hill & Hulley 1995; Hill *et al.* 1997).

Neste estudo, investigamos a trajetória de crescimento de estruturas morfológicas exageradas (escolos e furca) das larvas de *G. spadicea* (Klug) (Chrysomelidae: Cassidinae) e o valor adaptativo associado ao escudo. Primeiro, através de medidas lineares comparamos o padrão de crescimento ontogenético das estruturas morfológicas. Segundo, verificamos o efeito da variação quantitativa da dieta no crescimento dessas estruturas corporais. Terceiro, calculamos e comparamos os coeficientes alométricos ao longo da ontogênese larval (alometria ontogenética). E finalmente, testamos a influência do escudo em relação à mortalidade e à temperatura corporal das larvas em experimentos realizados sob condições naturais.

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CAPÍTULO II

ARTIGO A SER SUBMETIDO À REVISTA *JOURNAL OF EVOLUTIONARY*

BIOLOGY EM MARÇO DE 2014.

Ramos, B. C. M., Boligon, D. S. & Moreira, G. R. P. 2014. Allometry of growth and adaptive value of exaggerated corporal structures in *Gratiana spadicea* (Klug) (Coleoptera: Chrysomelidae) larval stage

1 **Article title:** Allometry of growth and adaptive value of exaggerated corporal structures
2 in *Gratiana spadicea* (Klug) (Coleoptera: Chrysomelidae) larval stage

3

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16 **Short running title:** Allometric growth in Cassidinae larvae

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26 **Abstract**

27 *Gratiana spadicea* (Klug) (Coleoptera: Chrysomelidae) larvae present
28 exaggerated body parts in association with an abdominal shield, made of faeces and
29 exuviae what are deposited on caudal furca throughout ontogeny. Growth trajectories
30 and scale relationships between such structures and function associated to the shield, if
31 any, are unknown. In this study, we investigate first, under laboratory conditions, the
32 growth trajectory and allometric relationship among body parts in *G. spadicea*,
33 including scoli, furca and furcal shield (= furca + attached exuvia and faeces). Also, we
34 determined the influence of food quantity on structural development on such structures.
35 In addition, we tested under field conditions whether there is adaptive value associated
36 to the furcal shield regarding protection to predation and sunlight. Data showed that the
37 furca and some scoli are exaggerated structures, extremely specialized of *G. spadicea*,
38 the development of which involving allometric growth and greater energetic investment
39 (positive allometry) during ontogeny. There was an indirect gain, and energy saving for
40 somatic construction of the furca, due the accumulation of the exuviae and faeces. We
41 confirmed that the adaptive value assigned to furcal shield is related to protection
42 against predators. Furthermore, we unprecedentedly demonstrated that the furcal shield
43 acts like a parachoot, decreasing the exposure of larval body to sunlight. We discuss the
44 possible consequences in terms of developmental costs and survivorship benefits
45 associated to evolution of such exaggerated structures.

46

47 **Key Words:** allometric growth, furcal shield, defense mechanisms, cassidine beetles

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51 **Introduction**

52 Exaggerated traits (*sensu* Emlen & Nijhout, 2000) are usually associated to
53 allometric models and evolution of highly specialized behaviors raised by selective
54 pressures (Gould, 1974; Emlen, 1997; Emlen *et al.*, 2005; Dial *et al.*, 2008; Frankino *et*
55 *al.*, 2009; Nijhout & Germán, 2012). Exaggerated morphologies resulting by sexual
56 selection are evident for males among several animal species that present body parts
57 used as ornaments to attract females and/or weapons to compete for a mate (Baker &
58 Wilkinson, 2001; Emlen & Nijhout, 2000; Kodric-Brown *et al.*, 2006; Cuervo &
59 MØller, 2009). Also, on social insects there are polymorphisms associated to different
60 castes (*e.g.* those involved with defense) that result from allometry during immature
61 development, and that can also be affected by nutrition, temperature, pheromones and
62 genetic factors (Stern *et al.*, 1996; Schwander *et al.*, 2005; Fjerdingstad & Crozier,
63 2006; Jaffé *et al.*, 2007; Perrard *et al.*, 2012). On the other hand, predation can be an
64 important factor on expression of prey morphological traits, as for example in Odonata
65 larvae, which have abdominal spines that exhibit different growth trajectories under
66 variation of predation risk (Dodson, 1989; Arnqvist & Johansson, 1998; Johansson,
67 2002).

68 Cassidinae larvae (Coleoptera: Chrysomelidae) shows some exaggerated traits,
69 as presence of lateral scoli in the body and a caudal process, located on terga of the
70 ninth abdominal segment (Fig. 1b; Chaboo, 2007; Swietojanska, 2009). This caudal
71 process, also called furcae, is responsible for anchoring a shield made of faeces and / or
72 exuviae (Figs. 1a,c), constructed throughout larval development by elongation and
73 movement of the telescoped anus (Chaboo & Nguyen, 2004; Chaboo, 2007;
74 Swietojanska, 2009). When the furcal shield is composed entirely by faeces, these are
75 added by the telescoped anus; on the other hand, when the shield is composed just by

76 exuviae, on each molt, the shedding exuvia is pushed out through body movement into
77 the furca, where it remains attached in the larval stage; and, finally, when the shield is
78 formed by both faeces and exuviae, the two behaviors above described occurs in parallel
79 throughout ontogeny. Besides composition, the furcal shield is known to vary in relation
80 to size, shape and architecture, covering for example either totally or partially the larval
81 body (McBride *et al.*, 2000; Chaboo & Nguyen, 2004; Swietojanska, 2009). However,
82 there is a lack of morphometric analysis focusing on existence of adaptive allometry
83 associated to such structures.

84 Function of the furcal shield in cassidines has been studied, especially in relation
85 to protection against natural enemies. The role of the shield on defence against predators
86 has been controversial, however, as the corresponding effectiveness may vary as
87 function of predator species, and also, because protection can be due to any body
88 chemical composition and not to the shield (Eisner *et al.*, 1967; Olmstead & Denno,
89 1993; Eisner & Eisner, 2000; Nogueira-de-Sá & Trigo, 2002; Müller & Hilker, 2003;
90 Bottcher *et al.*, 2009). Other hypothesis about role of shield is related to protection
91 against stress caused by abiotics factors. For example, Bacher & Luder (2005) tested,
92 under laboratory conditions whether the furcal shield protects larvae of *Cassida*
93 *rubiginosa* Müller from UVB radiation, but did not find effects on mortality when
94 compared larvae exposed with and without furcal shield. In general, the immature stages
95 of insects are more exposed to effects such as those of temperature, insolation and
96 humidity, because of low mobility (Heming, 1999; Müller & Hilker, 2003; Chaboo,
97 2011). To our knowledge, the thermoregulatory function of furcal shields in Cassidinae,
98 if any, was not explored until now.

99 In this study, we investigated the growth trajectories of exaggerated body parts
100 in *Gratiana spadicea* (Klug) (Chrysomelidae: Cassidinae) larvae, including scoli, furca

101 and furcal shield. We also determined the existence of adaptive value for the furcal
102 shield, regarding predation and incidence of sunlight. First, we compared ontogenetic
103 growth pattern of such structures with other body parts not directly associated with
104 defense. Second, we quantify in a comparative way the effect of quantitative variation
105 of food offered on growth rates of these morphological structures. Third, we determined
106 and compared the corresponding allometric coefficients (ontogenetic allometry). And
107 finally, by manipulating the furcal shield composition we tested the corresponding
108 influence on mortality due to predation and on thermoregulation under natural
109 conditions.

110

111 **Materials and methods**

112 *Insect and plants*

113 *Gratiana spadicea* is a monophagous cassidine (*sensu* Bernays & Chapman,
114 1994) that feeds on *Solanum sisymbriifolium* Lam. (Solanaceae). This plant is
115 considered a weed in some regions of Brazil, occurring mainly on open grasslands and
116 growing expontaneously on altered environments (Groth, 1989; Mentz & Oliveira,
117 2004).

118 *Gratiana spadicea* adults were field-collected from leaves of *S. sisymbriifolium*
119 plants in the vicinity areas of Porto Alegre city, and reared in transparent plastic pots
120 (11 cm x 11 cm x 3,2 cm) kept in a laboratory chamber (25 ± 1 °C; L14:D10), at the
121 Departamento de Zoologia da Universidade Federal do Rio Grande do Sul (UFRGS),
122 Porto Alegre, RS, Brazil. *Solanum sisymbriifolium* leaves coming from plants grown
123 outdoors in flower pots were offered *ad libitum*, and replaced every two days. Leaves
124 were checked daily for the presence of oothecae, which were placed on Petri dishes
125 covered with moistened filter paper until larvae hatching. Additional plants (n = 100)

126 used in the mortality experiment were grown from seedlings that were field-collected in
127 the same locality. They were transplanted to five flowerbeds (20 plants each),
128 containing enriched organic soil, and watered regularly.

129

130 *Body party measurements*

131 Linear measurements were performed on seven morphological structures of all
132 larval instars (n= 20 per instar) (Fig. 2). We considered an individual furca, the furca
133 growth in a given instar, and an apparent furca the set of one or more exuvia attached to
134 the furca every molt during larval development. Larvae were previously fixed with
135 Dietrich's fluid, clarified in a 10% KOH solution, and slide-mounted in liquid glycerin.
136 The measures were performed with the aid of Leica® M125 stereomicroscope that was
137 equipped with an ocular micrometer. To obtain measures of body and shield area all
138 instars larvae were immobilized under - 20°C and photographed after 24h of molt.
139 Larvae were photographed with and without shield. We removed the shield carefully
140 under stereomicroscope with help of small moistened brush. Individuals were
141 photographed with a Sony® Cyber-shot DSC-H10 camera attached to a Leica® M125
142 stereomicroscope and measured by Axio Vision 40 V4.8.2.0 software. Data were tested
143 regarding normality and homoscedasticity through Kolmogorov-Smirnov and Bartlett
144 tests, respectively. To test the covering area of shield in relation do body, we calculated
145 the corresponding ratio. Then, all data were compared using a one-way ANOVA,
146 followed by Tukey's multiple comparison tests.

147

148 *Growth trajectories*

149 Data on size of body parts, including scoli and furcal shield that were obtained
150 for all larval instars (I to V) were adjusted to an exponential equation ($y = \alpha e^{\beta X}$).

151 To verify growth relationship between body parts during ontogeny we used the
152 linear version of Huxley's allometric equation: $\ln (y) = \ln (\alpha) + \beta (x)$, taking head
153 capsule as a covariate. To compare growth rates we used the log-transformed data; the
154 parallelism of slopes and homogeneity between intercepts were compared using one-
155 way ANCOVA (Sokal & Rohlf, 1996).

156

157 *Effect of starvation*

158 Insects used in these experiments were obtained from laboratory rearings and
159 individualized in plastic containers (11 cm x 11 cm x 3, 2 cm). The experiment
160 consisted in two treatments: 1) larvae fed *ad libitum* from hatching until fifth instar, and
161 2) larvae starved during third instar for 12h until molt to fifth instar, when again started
162 feeding *ad libitum*. After molting to fifth instar, they were fixed in Dietrich's fluid.
163 Structures to be measured were previously clarified in KOH solution and the slide-
164 mounted in glycerin jelly. Measurements were performed as described above. Data were
165 tested similarly regarding normality and homoscedasticity, and compared between the
166 two treatments using unpaired *t* Student's tests.

167

168 *Field mortality*

169 The experiment was carried out in Viamão Municipality (RS), in open field area
170 covered by herbaceous vegetation, mainly grasses and Asteraceae (Appendix S1) from
171 January to February in 2013. Experimental units (n = 100) were cultivated plants that
172 were transplanted to the field, at 1.5 m intervals. The experiment was replicated in five
173 occasions, always on sunny days with temperature greater than 25°C, and lasted for
174 two days. To verify the influence of the furcal shield on mortality, individuals were
175 randomly selected from laboratory rearings, forming pairs within instars and distributed

176 between treatments as follows: 1) larvae with intact furcal shields and 2) with furcal
177 shields removed. Furcal shield were removed by gently using a small, soft brush. Larvae
178 from different treatments were placed on opposite leaves of each host plant (n=50 larvae
179 per treatment/occasion). Plants were checked after 24 hours, to determine number of
180 survivors and to remove eventual exuvia and/or faeces deposited on furca of those
181 belonging to treatment two (furcal shield removed). At the same time, larvae with intact
182 furcal shields had their abdomen gently touched with the brush mentioned above, in
183 order to provoke a perturbation similar to that made on larvae subjected to the other
184 treatment (Nogueira-de-Sá & Trigo, 2002). At the end of the experiment, the mortality
185 of larvae with and without shield were compared through Binomial proportion test
186 (Snedecor & Cochran, 1980).

187 To verify whether remotion of the shield influenced on larval mortality, we
188 conducted similarly an additional experiment, during two occasions (n = 30 individuals
189 per treatment per occasion). In this case, plants were covered with a fine mesh, plastic
190 protection and Tanglefoot® was applied at their stem base to prevented for attack by
191 natural enemies. On both occasions, mortality was null. Therefore, we assume that the
192 absence of larvae on experiments was due to predation, and not related to manipulation
193 of the furcal shield.

194

195 *Body temperature*

196 To evaluate the effect of the furcal shield on body temperature, we used fifth
197 instar larvae that were randomly distributed into two treatments: 1) larvae with furcal
198 shield and 2) larvae without furcal shield; different pairs were concomitantly exposed to
199 sunlighth (average \pm SE = 1082.68 \pm 1.815 lux) and shadow (53.94 \pm 6.461 lux) conditions,
200 on opposite apical leaves of *S. sisymbriifolium* (Appendix S2). Body temperatures were

201 measured using a laser thermometer Raytek®, model MT4, straightly directed to dorsal
202 view of larval body and to the shield (n=25 larvae per treatment/light regime). For each
203 individual we took the temperature five times, and corresponding values been expressed
204 as means. The experiment was conducted on sunny days with temperature higher than
205 25°C.

206 Data were also tested regarding normality and homoscedasticity, as described
207 above. Body temperatures were compared by Mann-Witney *U* test, since they did adjust
208 to a normal curve. All analyses were conducted with alpha=0.05.

209

210 **Results**

211 *Growth trajectories*

212 The ontogenetic growth of the larval body parts of *G. spadicea* adjusted
213 significantly to an exponential curve (Fig. 3). The coefficient values indicated that head
214 capsule, mesothoracic legs, telescoped anus and furcal shield intervals increase similarly
215 as the larvae develop in comparison with other structures evaluated (Fig. 3a-c, i).
216 Otherwise, lateral scoli increased more than others, mainly when compared to third
217 mesothoracic and first abdominal scoli, which grew proportionally greater than eight
218 abdominal scolus (Fig. 3d-f). The individual furcae grew less than apparent furca (Fig.
219 3g,h). The body and shield area present exponential growth during ontogeny (Fig. 4a,b).
220 However, the ratio of covered area in relation to body area increased until third instar,
221 and decreased on last instars (Fig. 4c).

222 Among all structures evaluated, mesothoracic legs and eighth pair of abdominal
223 scoli present similar slopes, but the intercept is different, greater on eighth pair of
224 abdominal scoli (Table 1; Fig. 5a,d). The structures which present greater slopes are
225 third pair of mesothoracic scoli and first pair of abdominal scoli, with similar growth

226 rate and different intercept (Fig. 5b,c; Table 1); and, also, they grow in greater rate than
227 eighth pair of abdominal scoli (Fig. 5b-d; Table 1). In other hand, the individual furcae,
228 the intervals are the only one to present negative allometry, with similar slopes (Fig.
229 5e,g; Table 1). Apparent furcae grows proportionally more than individual furcae and
230 intervals and presents a positive allometric coefficient (Fig. 5f). Apparent furcae has the
231 same slope as mesothoracic legs, eighth abdominal scoli and telescoped anus, but the
232 intercepts are different (Table 1).

233

234 *Effect of starvation*

235 The starvation during third larval instar changed significantly the length of body,
236 scoli and individual furcae of the fifth instar larvae. The starved larvae were smaller
237 than larvae fed *ad libitum* (Table 2). The head capsule width and legs length did not
238 differ between two treatments (Table 2). Also, developmental time from third until
239 fourth instar to starved larvae was significantly shorter than larvae fed *ad libitum*, but
240 not to total development time (from first to fourth instar), when there was no difference
241 ($P < 0.001$ e $P = 0.251$, respectively).

242

243 *Field mortality*

244 Overall, mortality rates under field conditions varied from 22 to 70%. It was
245 significantly greater for larvae without furcal shield compared to those with furcal
246 shield, in all occasions (Fig. 6a-e).

247

248 *Body temperature*

249 The furcal shield influenced body temperature and its own temperature, when
250 larvae under shadow and sunlight conditions are compared. The mean temperature of

251 larval body without furcal shield under sunlight was greater than on larvae with shield
252 (average \pm SE = 34.38 ± 0.93 and 32.99 ± 0.76 , respectively) (Fig. 7a). However, under
253 shadow conditions, body temperature was similar to larvae with and without shield
254 (30.25 ± 0.85 ; 30.58 ± 0.71 , respectively) (Fig. 7c). The shield temperature for larvae
255 under sunlight was greater than that for larvae under shadow conditions (34.26 ± 0.69
256 and 30.40 ± 0.72 , respectively) (Fig. 7b).

257

258 **Discussion**

259 As it has been known for several insect species, *G. spadicea* larvae grow
260 exponentially throughout the larval instars (Dyar, 1890; Daly, 1985; Heming, 1999).
261 However, we found herein first a greater growth on scoli and apparent furca when
262 compared to other body parts (with rates more stable), and second, that such a pattern is
263 related to protection offered by such structures to larvae when under both high
264 temperatures and predation risk.

265 Such evidences came from different allometric relationships found among body
266 parts, mainly in relation to scoli, individual and apparent furcae. There was a greater
267 investment on growth rate of anterior scoli, probably to maximize the protected area and
268 reduce the predation, since the shield does not cover the body anterior portion. The
269 opposite occurred on the caudal pair of scoli which grew proportionally less. We thus
270 associated it to the fact this body region does not need more such kind of protection,
271 since it has full protection of the furcal shield. One example of pressure over growth
272 expression of body parts was provided by Arnqvist & Johansson (1998), who verified a
273 greater growth rate on abdominal spines of *Leucorrhinia dubia* (Vander Linden)
274 (Odonata: Libellulidae) larvae, when exposed to predators. Accumulation of exuviae on
275 the furca, resulting in what we called here apparent furca, provides a greater area,

276 suitable to anchoring faeces, thus requiring less investment on growth of individual
277 furca during ontogeny; for this structure, in fact, growth was negatively allometric. In
278 other words, we infer that the energy saving regarding somatic construction of the furca
279 is related to recycling of developmental wastes (exuvia + faeces).

280 Similar growth rates were found for the corporal area and that of the furcal
281 shield, during early ontogeny. The correspondent decrease in such ratio later in
282 ontogeny is explained by the fact that the furcal shield surface grows at lower rate.
283 Although not measured, we suspect that specially in the last instars, when feeding rates
284 are the greatest (Medeiros & Moreira, 2003), faeces exceed the space available on furcal
285 shield surface, and are then deposited in layers. This should not invalidate the
286 importance of such structure as an effective defense mechanism, since early instar of
287 insects are in general more vulnerable, especially those of *G. spadicea* (Medeiros &
288 Moreira, 2005).

289 There was a strong correspondence (isometry) between growth rates of the
290 telescoped anus and the apparent furca. This was expected, since there is a functional
291 correlation between such structures; they have to have similar length, since the former is
292 used to deposit faeces on the latter.

293 Results have also showed clearly that amount of ingested food affects body parts
294 of *G. spadicea* differently in terms of growth rates. A corresponding decrease in length
295 achieved by scoli and individual furca indirectly demonstrated first that there is an
296 energetic cost to produce such structures. Second, that they have lower priority in terms
297 of somatic investment compared to other body parts, such as the head capsule and legs.
298 These structures, however, have fundamental functions associated to them, as for
299 example feeding, cognition and locomotion, among others. In insects, the size of a given
300 body structure depends on the prevailing conditions, and in fact is related to

301 development of others structures essentials to life maintenance (*e.g.* antennae, eyes and
302 wings) (Heming, 1999). On the other hand, selection can not occur independently in
303 relation to all traits, because they might be linked through ontogenetic development.
304 The nutritional factor acts as an important selective pressure on growth of body parts, as
305 demonstrated with horns of males in *Onthophagus acuminatus* Har. (Coleoptera:
306 Scarabaeidae), head capsule in larvae of *Agabus disintegratus* (Crotch) (Coleoptera:
307 Dytiscidae) and mouth parts of *Pseudaletia unipuncta* (Haworth) (Lepidoptera:
308 Noctuidae), among others (Bernays, 1986; Emlen, 1997; Nijhout & Emlen, 1998;
309 Brannen *et al.* 2005; Tobler & Nijhout, 2010). Cotton *et al.* (2004) demonstrated that a
310 quantitative variation of food available to *Cyrtodiopsis dalmanni* (Wiedemann)
311 (Diptera: Diopsidae) influences the growth rate of head ornaments in the larval stage
312 and body size of adults. Males of *O. acuminatus* Harold (Coleoptera: Scarabaeidae)
313 change their growth pattern in response to variation in nutritional quality during larval
314 development. Such a nutritional effects alters the ratio between horn length and body
315 size, traits that in turn are important in sexual selection (Emlen, 1997). In this study, on
316 the other hand, we demonstrated that variation in feeding leads to different allocation of
317 resources among *G. spadicea* other body parts, which are subjected to natural selection.

318 The size of a given organism generally has important implications regarding
319 interaction with others (*e.g.*, predators, competitors) and also the environment. Thus,
320 allometric patterns may have consequences in terms of evolution of adaptations (Dial *et*
321 *al.*, 2008). In the present study, we showed the existence of adaptive value associated to
322 the furcal shield in *G. spadicea*, regarding influence of both biotic and abiotic mortality
323 factors. Our results support the hypothesis that the furcal shield acts as a barrier against
324 natural enemies (Eisner *et al.*, 1967; Olmstead & Denno, 1993; Müller & Hilker, 1999;
325 Eisner & Eisner 2000; Müller, 2002; Müller & Hilker, 2003; Nogueira-de-Sá & Trigo,

2005). In addition, that it reduces the larval exposure to the sun radiation, working like an umbrella. This should be important, in terms of thermoregulation of body temperature and dehydration avoidance, since *S. sisymbriifolium* plants are commonly found in sunny areas and larvae stay directly exposed to sunlight on the adaxial surface of leaves (Becker & Freire, 1996).

There are a variety of adaptive behavioral mechanisms that allow effect response from organisms to variation abiotic factors, as for example the construction of shelter, avoiding stress caused by environmental intense heat and/or radiation (May, 1979; Heinrich, 1993). For example, larvae of *Cicindela willistoni* LeConte (Coleoptera: Carabidae) that are typical of environments where high temperature prevail, build vertical turrets in the soil where temperature are lower inside than outside, this behavior allows them to thermoregulate and keep an active predation throughout the day (Knisley & Pearson, 1981). As far as we are concerned, however, the case of *G. spadicea* is the first one reported in insects, where body parts are directly used in such way. But it is not only insects that have thermoregulatory behaviors associated to body parts. For example, the cape ground squirrel of the desert *Xerus inauris* (Zimmermann) (Rodentia: Sciuridae) uses its own tail by curving it anteriorly over the body as a parasol, to protect from the sunlight (Bennett *et al.*, 1984).

In summary, we provided information in this study in the sense that, by using wasted exuvia and faeces that are deposited on a caudal process (named furca) forming a furca shield, larvae of *G. spadicea* may attenuate stress related to sunlight and improve survivorship. This is achieved by positive allometric growth of the furca, which is also the case of scoli not located under the protection of the furcal shield. Development of such exaggerated structures have energetic costs, since they have

350 reduced sizes in case of starvation. In this case, development of other body parts that are
351 essential to life maintenance is favored.

352 Future studies should approach the efficiency of the furcal shield for different
353 groups of predators, as well as verify whether the corresponding sources of protection
354 are physical and/or chemical. Additional studies should be carried out to test for
355 changes in behavior in this systems, such as the larval movement among different plant
356 parts (*e.g.* from adaxial to abaxial leaf surfaces) under variation in light intensity, taking
357 into account the presence or absence of the faecal shield. Also, we suggest to evaluate
358 the existence of phylogenetic allometry regarding such body parts in others species of
359 Cassidinae including those having different patterns to build their faecal shield.

360

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SUPPORTING INFORMATION

552

553 Additional Supporting Information may be found in the online version of this article at
554 the publisher's web-site:

555 Appendix S1. Field experimental area (900 m²) located in Viamão municipality, state of
556 Rio Grande do Sul, Brazil, where experiments on mortality were conducted.

557 Appendix S2. Thermoregulation experiment set located in Viamão municipality, state of
558 Rio Grande do Sul, Brazil, showing how sunlight (a) and shadow (b) regimes were
559 simulated.

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561

562 Table 1. Comparison of allometric parameters among body parts of *Gratiana spadicea*
 563 larvae throughout ontogeny (one-way ANCOVA, alpha= 0.05).

Dimension x Dimension	564			
	B		α	
	F	P	F	P
Legs mesothoracics				
<i>Scoli</i>				
3rd pair mesothoracic	258.358	<0.001	-	-
1st pair abdominal	226.278	<0.001	-	-
8th pair abdominal	0.435	0.510	221.868	<0.001
<i>Furcae</i>				
individual	48.955	<0.001	-	-
apparent	0.019	0.891	6238.910	<0.001
intervals	22.657	<0.001	-	-
<i>Telescoped anus</i>	3.422	0.066	282.731	<0.001
3rd pair mesothoracic scoli				
<i>Scoli</i>				
1st pair abdominal	0.005	0.945	104.611	<0.001
8th pair abdominal	197.269	<0.001	-	-
<i>Furcae</i>				
individual	359.116	<0.001	-	-
apparent	141.777	<0.001	-	-
intervals	202.076	<0.001	-	-
<i>Telescoped anus</i>	165.729	<0.001	-	-
1st pair abdominal scoli				
<i>Scoli</i>				
8th pair abdominal	176.314	<0.001	-	-
<i>Furcae</i>				
individual	327.908	<0.001	-	-
apparent	126.139	<0.001	-	-
intervals	184.862	<0.001	-	-
<i>Telescoped anus</i>	148.435	<0.001	-	-
8th pair abdominal scoli				
<i>Furcae</i>				
individual	47.048	<0.001	-	-
apparent	0.352	0.554	3211.150	<0.001
intervals	22.381	<0.001	-	-
<i>Telescoped anus</i>	1.139	0.287	4.567	0.034
Individual furcae				
<i>Furcae</i>				
apparent	25.375	<0.001	-	-
intervals	0.293	0.588	781.963	<0.001
<i>Telescoped anus</i>	59.299	<0.001	-	-
Apparent furcae				
<i>Furcae</i>				
intervals	12.779	<0.001	-	-
<i>Telescoped anus</i>	2.104	0.149	2815.470	<0.001
Intervals				
<i>Telescoped anus</i>	29.120	<0.001	-	-

587 Table 2. Effect of deprivation of food on fifth instar larvae of *Gratiana spadicea* (*t*
 588 Student' tests; alpha=0.05) regarding size of body parts

Dimension (mm)	Treatment (mean±SE)		<i>t</i> -value	P
	<i>ad libitum</i>	deprivation		
Body length	5.238±0.142	4.659±0.221	2.200	0.036
Head capsule width	1.031±0.009	1.005±0.009	1.955	0.060
Leg length				
prothoracic	1.026±1.016	0.983±0.015	1.906	0.066
mesothoracic	1.046±0.015	1.001±0.021	1.692	0.101
metathoracic	1.071±0.013	1.042±0.016	1.345	0.189
Scolus length				
3rd pair mesothoracic	1.831±0.030	1.565±0.040	5.263	< 0.001
1st pair abdominal	1.501±0.023	1.322±0.025	5.153	< 0.001
8th pair abdominal	1.859±0.042	1.485±0.057	5.225	< 0.001
Individual furcae length	2.789±0.113	2.418±0.128	2.171	0.038

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593 **Legend of figures:**

594 Fig. 1. *Gratiana spadicea* fifth larval instar, under dorsal view. Larva with complete
595 furcal shield (faeces and exuviae) (a), without furcal shield (showing only the furca),
596 and (b) a furcal shield with faeces removed (c). Bars= 1 mm

597

598 Fig. 2. Schematic representation of a *Gratiana spadicea* larva, under dorsal (a) and
599 ventral (c) views, and apparent furca in detail (b). Bars represent length measurements
600 as follows: third pair of mesothoracic scoli, A; first pair of abdominal scoli, B; eighth
601 pair of abdominal scoli, C; individual furca, D; interval between furcae, E; apparent
602 furca, F; head capsule width, G; mesothoracic legs, H; telescoped anus length, I.

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604 Fig. 3. Growth trajectories (median and corresponding quartiles) of body parts of
605 *Gratiana spadicea* during larval development. Head capsule (a), mesothoracic legs (b),
606 telescoped anus (c), third pair of mesothoracic scoli (d), first pair of abdominal scoli (e),
607 eighth pair of abdominal scoli (f), individual furca (g), apparent furca (h) and
608 corresponding intervals (i) ($P < 0.0001$; $n = 20$ per instar).

609

610 Fig. 4. Growth trajectories of body area (a), shield area (b) and corresponding ratio
611 during *Gratiana spadicea* larval development. Different letters indicate statistical
612 significance between instars ($n = 20$ per instar; one-way ANOVA, followed by Tukey's
613 multiple comparison tests, $\alpha = 0.05$).

614

615 Fig. 5. Allometric relationships between body parts of *Gratiana spadicea* larvae during
616 ontogeny (n=20 per instar). Mesothoracic legs (a), third pair of mesothoracic scoli (b),
617 first pair of abdominal scoli (c), eighth pair of abdominal scoli (d), individual furca (e),
618 apparent furca (f), and corresponding intervals (g), and telescoped anus (h). The dotted
619 line represents isometry. In schematic representation located in the right bottom of each
620 graph are shown larvae with corresponding measurement.

621

622 Fig. 6. Effect of faecal shield on mortality of *Gratiana spadicea* larvae in five occasions
623 (January- February in 2013); n=50 per instar/ treatment/ occasion) in Viamão
624 municipality, state of Rio Grande do Sul, Brazil. Asterisk indicates statistical
625 significance between bars in each occasion (Binomial proportion test, $\alpha=0.05$).

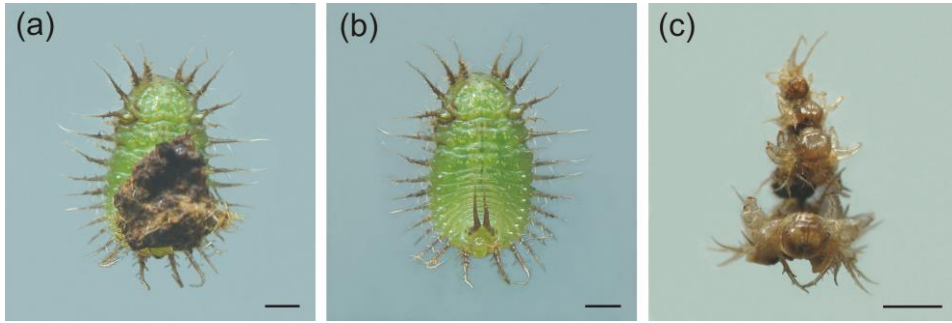
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627 Fig. 7. Effect of faecal shield on body temperature (median and corresponding quartiles)
628 in fifth-instar *Gratiana spadicea* larvae under sunlight (a) and shadow (c), and variation
629 of temperature on the shield (b). Asterisk indicates statistical significance between
630 treatments (n= 25 per instar/ treatment/ light regime; Mann-Whitney *U* test, $\alpha=0.05$).

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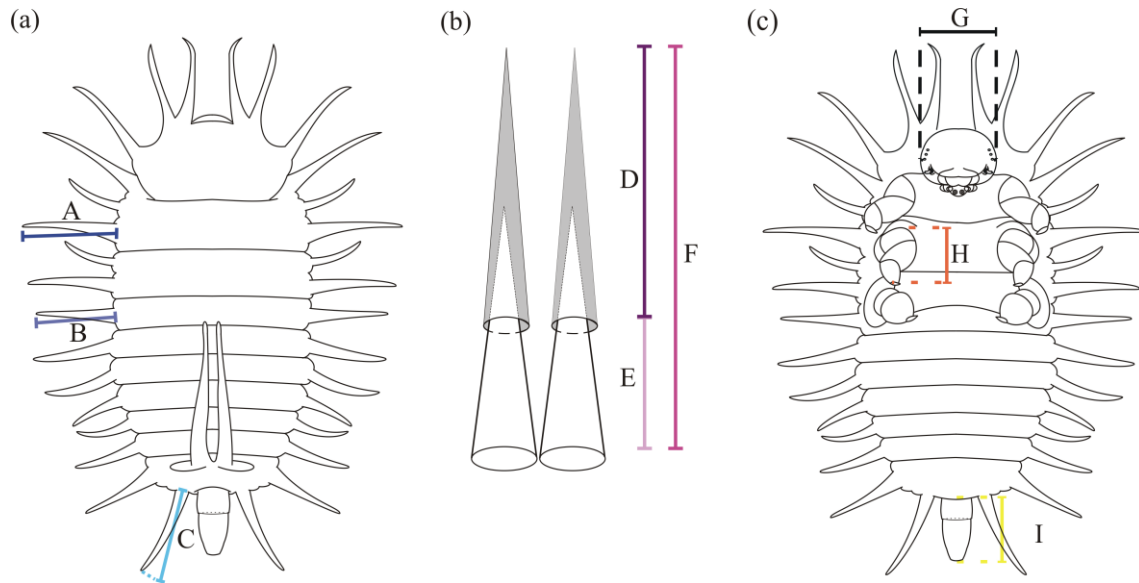
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Ramos *et al.* - Fig. 1

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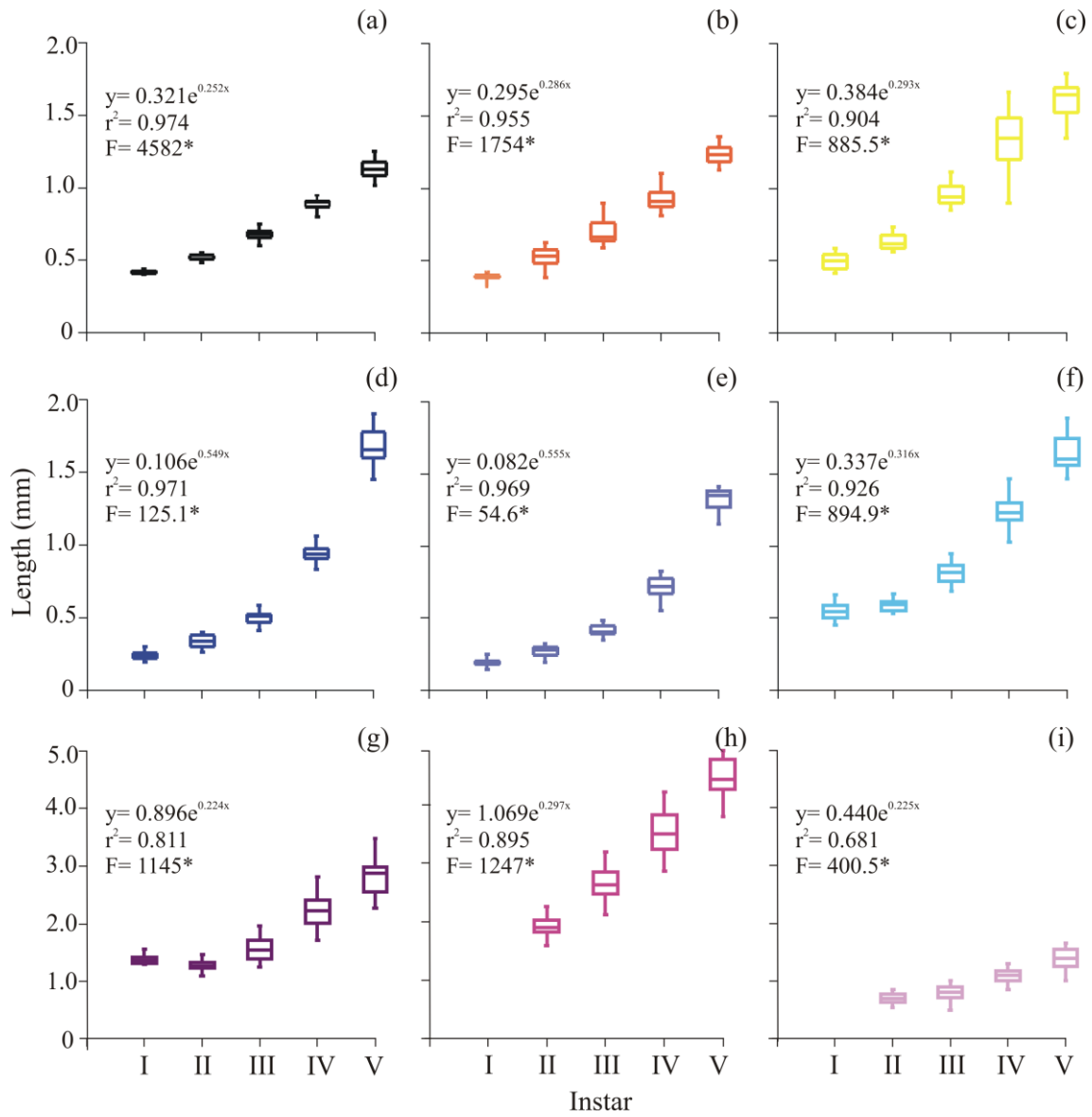
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Ramos *et al.* – Fig. 2



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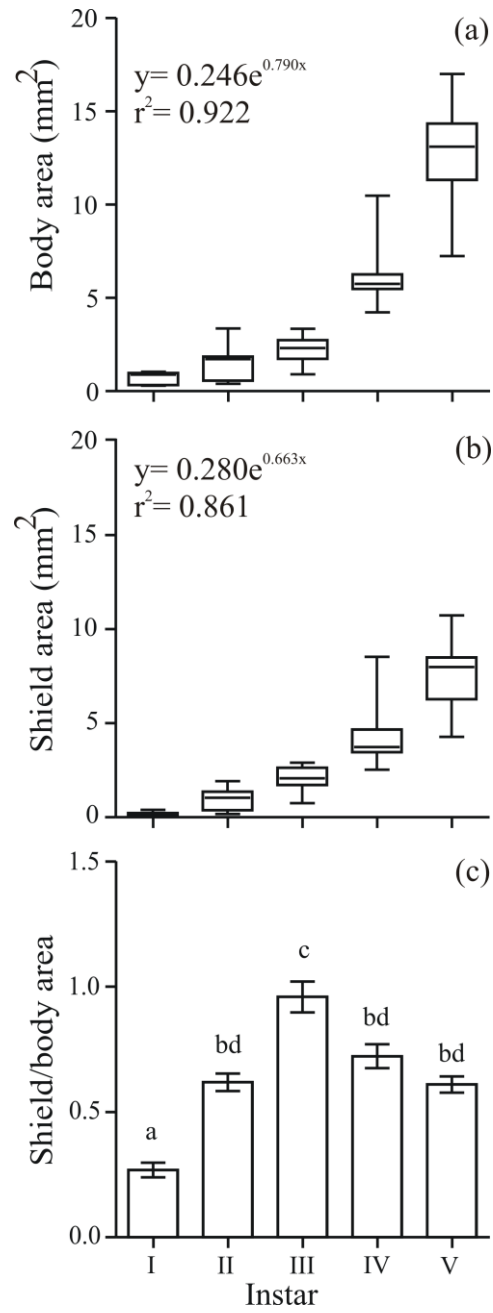
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Ramos *et al.* – Fig.3



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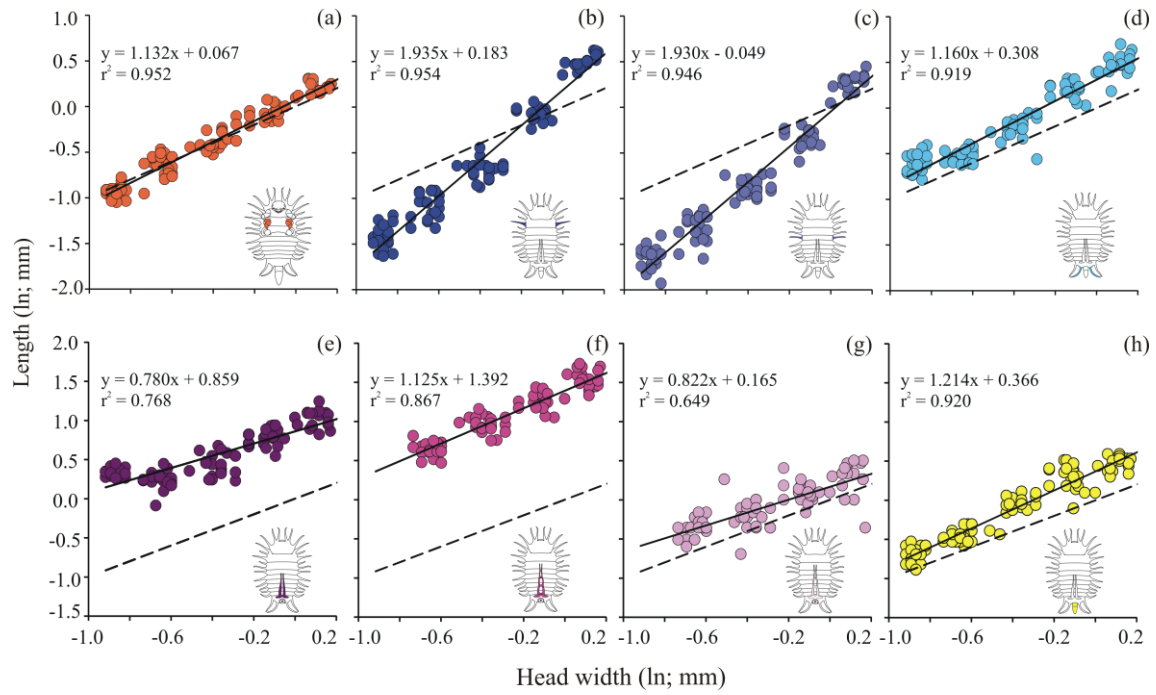
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Ramos *et al.* – Fig. 4

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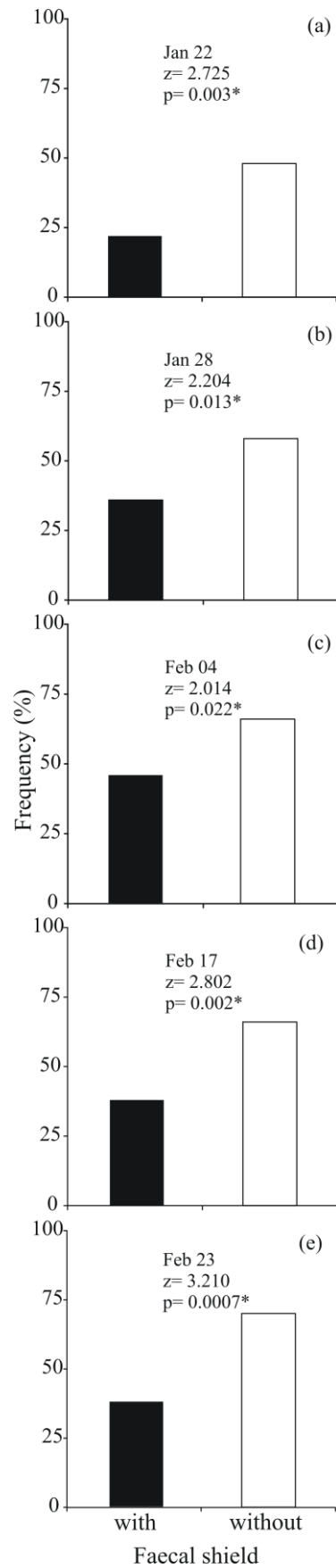
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Ramos *et al.* – Fig.5

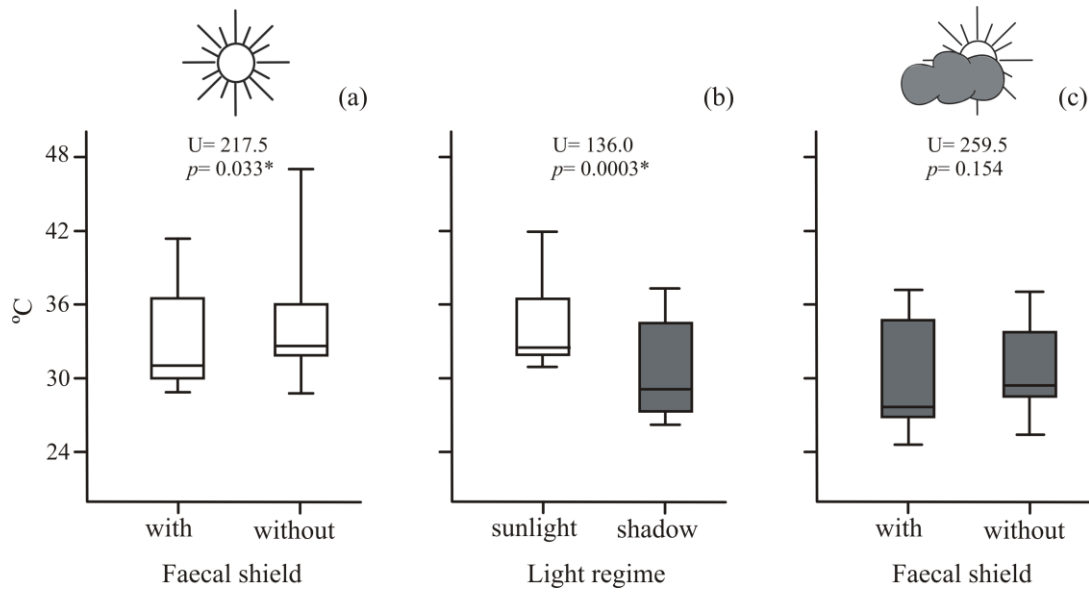
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Ramos *et al.* – Fig. 6



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Ramos *et al.* – Fig. 7

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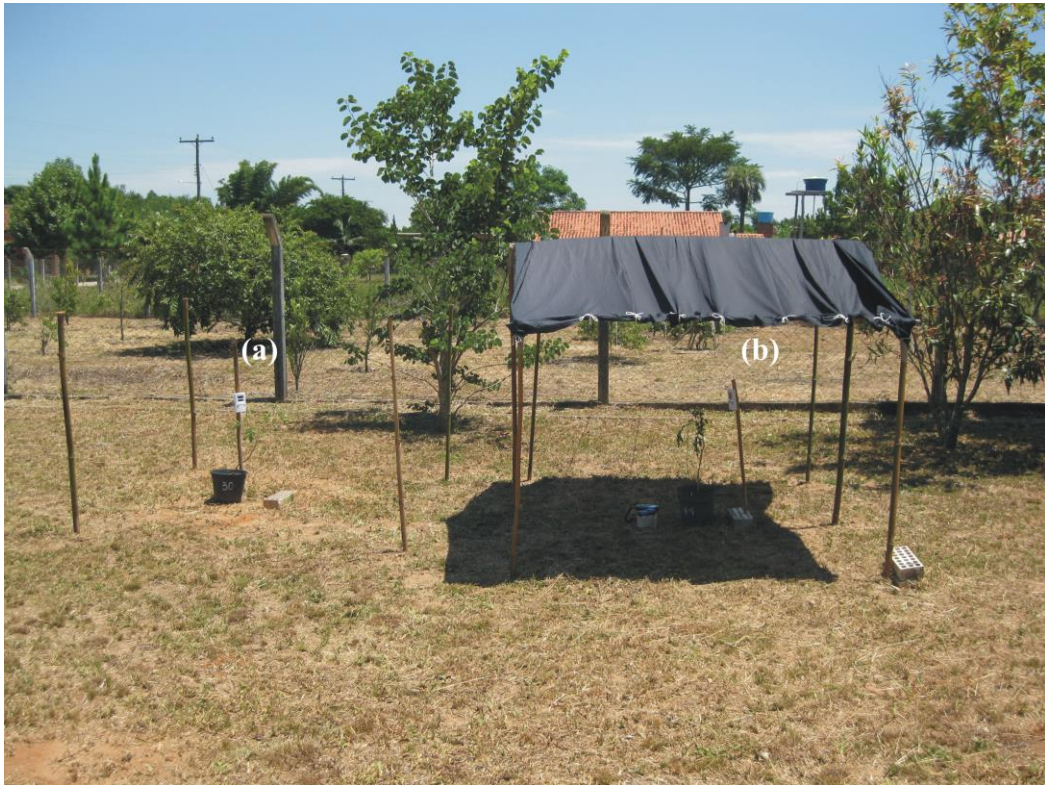
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Ramos *et al.* – S1

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CAPÍTULO III

CONSIDERAÇÕES FINAIS

As estruturas corporais das larvas de *Gratiana spadicea* (Klug) (Coleoptera: Chrysomelidae) aqui avaliadas apresentaram crescimento exponencial ao longo da ontogênese. Houve um crescimento significativamente mais pronunciado do terceiro par de escolos mesotorácicos, primeiro par de escolos abdominais e furca aparente, quando comparados com as demais estruturas corporais. A área do corpo e do escudo cresceu exponencialmente ao longo dos instares, sendo que nos três primeiros instares verificou-se maior cobertura do escudo em relação ao corpo.

As relações de crescimento entre as estruturas corporais ao longo da ontogênese apresentaram distintos padrões alométricos. Os escolos e a furca aparente apresentaram alometria positiva, enquanto que a furca individual alometria negativa. Sobre essas relações de crescimento pode-se inferir que há um investimento maior no crescimento dos escolos anteriores, provavelmente, para maximizar a área de proteção dessa região corporal à qual é parcialmente coberta pelo escudo. Por outro lado, o menor investimento na furca individual deve-se, provavelmente, pelo comportamento de acumular exúvias (furca aparente), o que maximiza seu tamanho real e proporciona maior área de apoio para o escudo.

Verificamos que a privação quantitativa da dieta influenciou no tamanho dos escolos e da furca individual, os quais apresentaram-se proporcionalmente menores, o que deve estar associado à manutenção do tamanho da cápsula cefálica e pernas, sugerindo que ambos estão envolvidos em uma alocação diferenciada de recursos favorecida por seleção natural.

Ainda, observamos que a presença do escudo é importante para a sobrevivência das larvas em condições naturais, representando uma barreira contra alguns inimigos naturais, uma vez que na sua ausência a mortalidade é alta. Além disso, fisicamente o escudo reduz a exposição das larvas ao sol, como um tipo de *parasol*, apresentando papel importante na regulação da temperatura corporal, uma vez que larvas com escudo apresentam temperatura de corpo menor quando comparadas com larvas sem escudo.

Em suma, o escudo e o aparato morfológico necessário para sua construção e manutenção representam estruturas morfológicas exageradas altamente especializadas em *G. spadicea*, cujo desenvolvimento envolve o crescimento alométrico e investimento energético diferenciado (alometria positiva) dentre elas ao longo da ontogênese. Em relação à furca, há um benefício indireto pelo acúmulo de ecdises e fezes, aonde o crescimento, ao contrário, é proporcionalmente menor (alometria negativa); isto, indiretamente, implica na economia de recursos na construção desta estrutura ao longo da ontogênese. Confirmamos que o valor adaptativo atribuído ao escudo está relacionado à proteção contra predadores e, de forma inédita, demonstramos que essa estrutura proporciona também uma diminuição da exposição corporal das larvas à radiação solar.

CAPÍTULO III

ANEXOS

1) Normas para publicação na *Journal of Evolutionary Biology*

Scope

The *Journal of Evolutionary Biology* seeks to publish very good papers of broad interest to general evolutionary biologists. Papers that are of narrow interest, or are not original in scope are not suitable for JEB.

Publication charges

There are no page charges for the *Journal of Evolutionary Biology*. Charges apply for colour figures that are published in print (for details see 'Colour charges', below) and for open access publication of your article if you choose this (for details see 'Online Open', below).

Online submission and review

Full instructions for preparing and submitting manuscripts are provided at the submission site: <http://mc.manuscriptcentral.com/jeb>. All submissions should be online through this site.

Manuscript categories

Journal of Evolutionary Biology publishes ordinary Research Articles (typically 6-10 printed pages), Short Communications (typically 3-5 pages; results and discussion may be combined), and Review Articles. Review Articles are in depth examinations of areas, rather than short commentaries or opinion pieces (see recent examples); in rare cases, typically commissioned, reviews are published with commentaries as a Target Review. Manuscripts in all categories (except commentaries on Target Reviews) to include an abstract.

Submission and handling of manuscripts

Manuscripts are allocated to the Editor-in-Chief, Reviews Editor or one of the Deciding Editors by the Managing Editor. Manuscripts are typically reviewed by two reviewers; however, manuscripts can be declined without review by an editor if their contribution to the journal's goals is deemed to be marginal or if it seems unlikely that they would fare well in the review process. Due to increasing number of submissions, these immediate rejections are becoming more frequent.

Manuscripts for the *Journal of Evolutionary Biology* are submitted online through ScholarOne at <http://mc.manuscriptcentral.com/jeb>. Submission online enables the quickest possible review and online manuscript tracking. Manuscripts can be uploaded

as a Word (.doc, .docx), Rich Text Format (.rtf) or LaTeX document. LATEX SUBMISSIONS: Author must also (1) upload a PDF file of the entire manuscript for the reviewers, and (2) compress your LaTeX files into a zip or tar file before uploading and select 'Not for review' when uploading the zip/tar file. Full upload instructions and support are available online from the submission site via the 'Get Help Now' button. You can also email for online Submission support.

All submitted manuscripts must be accompanied by a covering letter stating that the enclosed work is not under consideration for publication in another journal or book; that its submission for publication has been approved by all relevant authors and institutions; and that all persons entitled to authorship have been so named. Submitting authors should also assert that all authors have seen and agreed to the submitted version of the manuscript. The covering letter should give the word count of the full text, excluding abstract, references, tables and figure legends. Authors should always complete the preferred reviewers section when submitting manuscripts. Please identify up to four members of the Editorial Board as potential reviewers. You are welcome to specify non-Board reviewers as well, particularly if they are specialists in your subject area.

In order to expedite production, authors are required to submit their revised manuscripts online. It is in the interests both of the authors and of the journal that amended manuscripts are returned promptly. A revised paper will retain its original date of receipt only if it is received within 8 weeks of the date of return to the author. Revised papers submitted after this interval will be treated as a new submission. When resubmitting your manuscript please ensure you upload your response letter with your other files, so it will form part of the PDF. All final decisions will rest with the Editorial Office and authors will receive decision letters from the Editorial Office.

Artwork will be produced from electronic files if at all possible (see below). On request, authors should provide two sets of the original figures.

Online production tracking is now available for your article through **Author Services**. This enables authors to track their article - once it has been accepted - through the production process to publication online and in print. Authors can check the status of their articles online and choose to receive automated e-mails at key stages of production so they do not need to contact the production editor to check on progress. Visit <http://authorservices.wiley.com/bauthor/> for more details on online production tracking and for a wealth of resources including FAQs and tips on article preparation, submission and more.

Referrals to the Open Access Journal "Ecology and Evolution"

This journal works together with Wiley's Open Access Journal, [Ecology and Evolution](#), to enable rapid publication of good quality research that is unable to be accepted for publication by our journal. Authors will be offered the option of having the paper, along with any related reviews, automatically transferred for consideration by the Editor of

Ecology and Evolution. Authors will not need to reformat or rewrite their manuscript at this stage, and publication decisions will be made a short time after the transfer takes place. The Editor of Ecology and Evolution will accept submissions that report well-conducted research which reaches the standard acceptable for publication. Accepted papers can be published rapidly: typically within 15 days of acceptance. Ecology and Evolution is a Wiley Open Access journal and article publication fees apply. For more information please go to www.ecolevol.org/info.

Presentation of manuscripts

Manuscripts should be written in clear, concise, and grammatically correct English; manuscripts that are inadequately prepared will be returned to the authors. Manuscripts submitted by non-native English speakers must be thoroughly corrected by a native English speaker prior to submission. Manuscripts should be typewritten in double-spacing on one side of 297 mm x 210 mm (11 inch x 8.5 inch) white paper, with 2.5 cm (1 inch) margins on all sides. All pages should be numbered consecutively, and line numbers should be printed on each page to facilitate ease of reference for reviewers.

Length of the manuscripts: Manuscripts should not typically exceed 10 printed pages (including figures and tables). In general, allow 900 words per printed page and subtract 160 words for each figure and table.

Title page: The title page should contain the article title, full name(s) of author(s), affiliation(s), a short running title (abbreviated form of title) less than 45 characters including spaces, and the name and complete mailing address, **including telephone and fax numbers and e-mail address** of the person to whom correspondence should be sent.

Abstract page: The abstract page should contain a short summary not exceeding 250 words and include at least 4 and up to 10 keywords.

Main text

The organization of the main text should be chosen to fit the work reported. All papers should begin with an introduction that describes why the work is important and end with a discussion of the significance of the results and their relation to other work. The following format is recommended for empirical studies:

- (1) Introduction. The introduction should summarise briefly the background and aims, and end with a very brief statement of what has been achieved by the work.
- (2) Materials and methods. This section should contain sufficient detail so that all procedures can be repeated (in conjunction with cited references).
- (3) Results. The Results section should present the experiments that support the conclusions to be drawn later in the Discussion. The Results Section should conform to

a high standard of rigour. Extended lines of inference, arguments or speculations should not be placed in the Results.

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