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1 **Title:**
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30 **Data availability:**

31 Data and analyses on wing membrane thickness in 87 ithomiine butterflies are available in a Github
32 public repository <https://github.com/pontikaki/Thickness.git>
33

34 **Abstract :**

35 Transparent animals are often thin, which raises the question of their fragility. In Ithomiine
36 butterflies, a tribe with closely-related opaque and transparent toxic species, we explore the
37 variations of wing membrane thickness and interpret these variations as potentially selected by
38 optical and mechanical constraints. We find that species with partially transparent wings have a
39 thinner membrane in the transparent zone than in the opaque one, which likely helps light getting
40 through. Yet, we find that more transparent species have a thicker membrane in their transparent
41 zone, and that species with a higher wing proportion occupied by transparency have a thicker
42 membrane in their opaque zone, and a greater difference in thickness between opaque and
43 transparent zones. These results agree with predominant mechanical constraints selecting for the
44 maintenance of overall wing resistance, especially in species with a large wing proportion occupied
45 by transparency. Although optical constraints are likely at play, the influence of membrane thickness
46 on light transmission is likely limited, and the thicker membrane in more transparent species is likely
47 offset by the antireflective effect of nanostructures which are present in all of these species. Despite
48 their transparency, the fragility of the wing membrane in these species is likely limited.

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52 **Keywords:**

53 Transparency, trade-off, mechanical resistance, optical constraints, butterflies

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57 **Introduction**

58 Transparency in live beings is common in water, where it enables prey to be concealed from
59 predators (Johnsen 2001). Bouguer-Beer-Lambert law states that the loss of the intensity of light
60 transmitted through a very thin layer of a homogeneous medium is proportional to the absorption
61 characteristics of the medium (concentration in absorbers and their efficiency at absorbing light) and
62 to the layer thickness (Mayerhöfer et al. 2020). Rather intuitively, thinner objects pass more light.
63 Selection for camouflage favours transparent organisms with thinner membranes and tissues and
64 water buoyancy helps reducing tissue thickness. Transparent planktonic animals are often gelatinous,
65 delicate and fragile, especially when having a few layers of tissue (Johnsen 2000) and this fragility of
66 thin transparent bodies, which often hinders even scientific examination (Johnsen 2000; Miller 2009),
67 may entail significant costs in terms of mechanical resistance.

68 On land, transparency is rare and almost confined to insect wings, but it has also been shown
69 to reduce detection by predators, particularly in Lepidoptera (Arias et al. 2019; Arias et al. 2020; Arias
70 et al. 2021; Gomez et al. 2021). Whether transparency in terrestrial animals means thin and fragile
71 tissues remains an open question. While optical constraints likely select for thinner wings, the low air
72 buoyancy imposes self-supporting structures, hence thicker membranes and tissues (Johnsen 2001).
73 Wings are selected to offer a certain mechanical resistance ensuring they can be flapped for millions
74 of cycles without failure. Hence, transparent wings have likely evolved as a compromise between
75 antagonistic optical and mechanical selective pressures.

76 Lepidoptera are an outstanding model group to explore this question because transparent
77 species are relatively uncommon in this group, and have closely-related opaque sister species,
78 enabling a comparison of species otherwise very similar in terms of size, behaviour, history, and
79 ecology, and even wing morphology and venation, which are important to determine stiffness
80 (Combes and Daniel 2003). Do transparent butterfly species have thinner wing membranes than
81 opaque butterfly species? We studied Ithomiini, a speciose tribe of Neotropical rainforest toxic
82 butterfly species, whose wings range from fully opaque (in 20% of species) to nearly fully
83 transparent. In this group, the wing membrane is made of chitin and unpigmented, while coloration
84 is borne by scales. Scales are classic flat lamellar scales in opaque patches while they are typically
85 reduced to erect piliform scales in the transparent patches of most species in this group, with only
86 basal lineages like *Methona* butterflies having erect lamellar scales in the transparent patches (Pinna
87 et al. 2021).

88 Considering wing membrane as the result of optical and mechanical constraints, we can
89 formulate several predictions concerning wing membrane thickness in Lepidoptera. On the one hand,
90 in agreement with Bouguer-Beer-Lambert law, optical constraints should select for a thinner wing
91 membrane in the transparent zone than in the opaque zone as it transmits more light; in addition,
92 more transparent species should have thinner membrane as it would increase transparency. Yet, the
93 optical benefit of a thinner membrane may be limited: the optical gain can come more from the
94 presence of a nanorelief covering the wing membrane, which efficiently reduce parasitic reflections
95 and increase wing transparency (Siddique et al. 2015; Pinna et al. 2021; Pomerantz et al. 2021).
96 Optical constraints on transparency should not operate in the opaque zone, where light is stopped by
97 coloured scales and the wing membrane is not accessible to light.

98 On the other hand, mechanical constraints should select for a thicker membrane. A thicker
99 membrane can increase wing resistance to predator attacks. It has been shown that toxic butterfly
100 species have thicker wing membrane than palatable species, likely in response to resistance to
101 predator attacks and handling (DeVries 2002). This is especially important considering that
102 unpalatable butterflies have a longer life span than palatable butterflies (Beck and Fiedler 2008). A
103 thicker membrane can also increase resistance to deformation in flight, and may better sustain
104 scales, especially in opaque patches where scales are densely packed. Species with larger transparent
105 patches may compensate the greater loss of mechanical resistance (coming from transparency
106 occupying a greater wing surface) by a thicker membrane in the opaque zone, thus maintaining wing

107 overall resistance. In other words, opaque patches may act as zones of increased resistance between
108 which transparent patches are zones of poorer resistance. To our knowledge, no study has hitherto
109 assessed the potential role of scales in the mechanical properties of butterfly wings but veins seem
110 to help strengthening wings like the tubular structure in a kite (Wootton 1992; Wootton 1993;
111 Combes and Daniel 2003).

112 Here we investigate how wing membrane thickness relates to transparency/opacity and to
113 the proportion of transparent surface on the wing in ithomiine butterflies to explore the balance
114 between optical and mechanical constraints.

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116

117 **Methods:**

118 In 87 ithomiine species with partially transparent wings, issued from various lineages, we took
119 specimen photographs and measured on the forewing the length from insertion to apex, the total
120 wing area and the proportion of transparent area (proportion of the total wing area covered by
121 transparent patches). While a lower resistance can be advantageous as a predation escape strategy
122 especially in hindwings (marginal hindwing patches in Hill and Vaca 2004; hindwing tail in Chotard et
123 al. 2022) attacks on forewing have more detrimental consequences for flight and forewings are less
124 damaged than hindwings (Korkmaz et al. 2023). Compared to hindwings, forewings are likely more
125 strongly selected to be resistant to damage. This is the reason why we focused on forewing in this
126 study.

127 We quantified transmittance (percentage of light transmitted through the wing) in the
128 transparent zone of the forewing, using a custom-built spectrometric set-up composed of a 300 W
129 Xenon lamp (200–1160 nm emission range), a collimated emitting optic fibre (UV-VIS-NIR multimod
130 fibre with a core diameter of 50 μm) illuminating the wing sample with a 1 mm diameter spot and a
131 collimated collecting optic fibre (Avantes UV-VIS-NIR multimod fibre with a core diameter of 200 μm ,
132 FCUVIR200–1) connected to the spectrometer (SensLine AvaSpec-ULS2048XL-EVO, Avantes, 0.5 nm
133 resolution). Fibres were aligned and 22 cm apart. The wing was placed perpendicular to the fibres at
134 equal distance with the ventral side facing the illuminating fibre. Transmittance was measured
135 relative to a dark (light patch blocked at the end of the illuminating fibre) and to a white reference
136 (no sample between the fibres). Based on the spectra, we quantified transparency by computing the
137 mean percentage of light transmission over 300-700 nm.

138 We cut from the specimen forewing a section including a transparent zone and an opaque
139 zone. We gold-coated it (10 nm thick layer) before performing SEM imaging using a Zeiss Auriga 40.
140 We computed wing membrane thickness in the opaque zone (MTO) and in the transparent zone
141 (MTT) using ImageJ 1.52a (Schindelin et al. 2012) and the difference in thickness between opaque
142 and transparent zone ($\text{DMT} = \text{MTO} - \text{MTT}$). We excluded the thickness of the nano-sculpturing from the
143 calculation of the wing membrane thickness.

144 We explored the link between the thickness of the wing membrane and its transparency
145 using PGLS models from caper package (Orme et al. 2023) on MTT, MTO or DMT with percentage of
146 light transmission (Transmittance), proportion of transparent area (PropTransp) and forewing length
147 (FWLength) as explaining factors, letting the program find the appropriate correction for Pagel's
148 lambda.

149

150 **Results and discussion:**

151 We found that while correcting for wing length, species transmitting more light (higher transmittance
152 values) had a higher MTT, i. e. thicker wings in their transparent zone (Fig 1A, Table 1). Species where
153 transparency occupied a higher wing proportion had a higher MTO, i. e. a thicker membrane in their
154 opaque zone (Fig 1B, Table 1) but they had not a thinner or a thicker membrane in their transparent
155 zone (Table 1). The difference in thickness between the opaque and the transparent zone DMT was
156 negative for 17% (15/87) species while it was positive for the vast majority of species (83%, i. e.
157 72/87 species). For instance, the difference was negative in both *Methona* species which make

158 transparency with erect coloured lamellar scales. It was also negative in many *Oleria* species, in two
159 *Hyaliris* species.

160 DMT - the difference in thickness between the opaque and the transparent zone - increased in
161 species where transparency occupied a higher wing proportion (Fig 1C, Table 1).

162 Optical constraints likely operate: 83% of the ithomiine species studied here have a positive
163 DMT, i. e. a thinner membrane in the transparent zone compared to the opaque zone. This result is
164 consistent with the selection of a thinner membrane for a higher transmittance. Yet and surprisingly,
165 more transparent species have a thicker membrane while Bouguer-Beer-Lambert law states that a
166 thicker layer transmits less light. Ithomiine species present nanostructures on their wing membrane
167 which have been repeatedly shown to be efficient antireflective devices (Siddique et al. 2015;
168 Pomerantz et al. 2021). Hence, the optical gain ensured by nanostructures seems to largely outweigh
169 the potential optical loss, if any, of a thicker wing membrane in the transparent zone in more
170 transparent species.

171 Our results support the predominance of mechanical constraints in the evolution of the
172 thickness of the wing membrane. In Ithomiini, the vast majority of species present erect piliform
173 scales in their transparent zone and lamellar scales in their opaque zone (Pinna et al. 2021). Besides
174 being due to optical constraints, a thinner membrane in the transparent than in the opaque zone
175 could arise from a lower need for scale sustainability in the transparent zone where scales are
176 reduced to piliform scales. The thicker membrane in the transparent zone in the genus *Methona* is
177 consistent with the great need of scale sustainability in the transparent zone where transparency is
178 made with erect coloured scales (Gomez et al. 2021; Pinna et al. 2021). Yet, we have shown that
179 transparency increases when scales are in lower density (Gomez et al. 2021; Pinna et al. 2021). It is
180 reasonable to think that in Ithomiini, more transparent species have scales in lower densities. Hence,
181 if wing thickness unique role were to support scales, it would be thinner in more transparent species,
182 which is not the case.

183 If scales contribute to wing mechanical resistance, an assumption that needs further testing
184 and exploration, the increase in membrane thickness in more transparent species can be easily
185 explained if we think that more transparent species have less densely-packed scales in the
186 transparent zone, as shown in previous studies (Gomez et al. 2021; Pinna et al. 2021). The thicker
187 membrane in the opaque zone can offer a valuable compensation, especially in species where
188 transparency occupies a higher wing proportion. Finally, the higher difference in membrane
189 thickness between the opaque and the transparent zone in species where transparency occupies a
190 higher wing proportion could be of special importance as it could compensate for the higher fragility
191 conferred by the larger transparent zone.

192 Overall, maintaining membrane thickness seems crucial in clearwing Ithomiine butterflies
193 and likely reduces the potential costs of transparency in terms of loss of mechanical resistance. The
194 exact role of scales in wing mechanical properties is still unknown and only few elements are known:
195 (i) compared to Hymenoptera and Odonata, Lepidoptera showed the lowest reduction in mechanical
196 resistance with increasing air humidity, suggesting scales may play a role in that effect (Landowski et
197 al. 2020). (ii) Scales are involved in flight efficiency, and removing scales in opaque butterflies
198 decreases climbing efficiency by around a third (Slegers et al. 2017), but this property may not be
199 directly linked to wing mechanical resistance. (iii) In Odonata, wing damage heavily compromises
200 foraging success and flight efficiency, hence survival (Combes et al. 2010) suggesting wings are under
201 a strong selection for a high mechanical resistance. (iv) In Lepidoptera, species can escape predators
202 by having less resistant wing parts (marginal hindwing patches in Hill and Vaca 2004; hindwing tail in
203 Chotard et al. 2022). Yet, these elements are only present on hindwings and attacks on forewing
204 have more detrimental consequences for flight and survival, as suggested by the lower damage
205 proportion on forewing than hindwings (Korkmaz et al. 2023). (v) Flexural stiffness has been found
206 higher in bigger lepidopteran species (Combes and Daniel 2003) but the exact contribution of wing
207 membrane thickness is unknown. Future research should focus on determining the mechanical
208 properties and the structural features determining flight efficiency in opaque versus transparent

209 species and the respective role of scales (shape, density) and wing membrane thickness in building
210 these properties.
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287 Table 1. Results of analyses on membrane thickness in the transparent zone (MTT), in the opaque
 288 zone (MTO) and the difference in membrane thickness between the opaque and the transparent
 289 zone (DMT=MTO-MTT). We ran PGLS models with best lambda value, either with all factors (full) or
 290 with the factors retained in the best model (best). All variables were centred and scaled so estimates
 291 could be compared. We kept the same full model for all dependent variables to facilitate
 292 comparison. Significant effects are in bold and symbols are the following: * p<0.05, ** p<0.01, *** p
 293 <0.001. We also present λ , Pagel's lambda values. Notice that for MTO and DMT, lambda is null,
 294 which results in no need for controlling for phylogeny.
 295

Variable	Factor	PGLS models			
		Estimate \pm std error	t-value	p-value	λ
MTT full	Intercept	0.0188 \pm 0.1646	0.114	0.909	1
	Transmittance	0.0033 \pm 0.0013	2.515	0.014*	
	FWLength	0.0201 \pm 0.0035	5.821	1.07E-07***	
	PropTransp	0.0017 \pm 0.0012	1.434	0.155	
MTO full	Intercept	-0.6989 \pm 0.2231	-3.133	0.002**	0
	Transmittance	0.0051 \pm 0.0034	1.515	0.134	
	FWLength	0.0324 \pm 0.0066	4.888	4.91E-06***	
	PropTransp	0.0127 \pm 0.0032	3.963	0.0001***	
DMT full	Intercept	-0.4316 \pm 0.2137	-2.02	0.0467*	0
	Transmittance	0.0034 \pm 0.0032	1.044	0.300	
	FWLength	0.0069 \pm 0.0064	1.092	0.278	
	PropTransp	0.0092 \pm 0.0031	3.018	0.003**	
MTT best	Intercept	0.0293 \pm 0.1655	0.177	0.860	1
	Transmittance	0.0042 \pm 0.0011	3.688	0.0004***	
	FWLength	0.0212 \pm 0.0034	6.254	1.60E-08***	
MTO best	Intercept	-0.5217 \pm 0.1914	-2.725	0.008**	0
	FWLength	0.028 \pm 0.006	4.661	1.17E-05***	
	PropTransp	0.0157 \pm 0.0025	6.216	1.89E-08***	
DMT best	Intercept	-0.2131 \pm 0.1115	-1.911	0.059	0
	PropTransp	0.0116 \pm 0.0023	4.961	3.55E-06***	

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298 Figure 1: Variation of the thickness of the wing membrane in the transparent zone (A) in relation to
 299 wing transparency estimated by the mean transmittance over the range 300-700 nm, and in relation
 300 to wing size. Variation of the thickness of the wing membrane in the opaque zone (B) and the
 301 difference in thickness between the opaque and the transparent zone (C) in relation to the
 302 proportion occupied by transparency on the wing and to wing size. Solid black lines are regression
 303 lines from the best PGLS models. Point colour represents wing size. The red dashed line in C indicates
 304 when opaque and transparent zone have equal membrane thickness. Notice that in B and C, one
 305 outlier was withdrawn from the plot for clarity.

