1 **Male wing color properties predict the size of nuptial gifts given during mating in the**

2 **Pipevine Swallowtail butterfly (*Battus philenor*)**

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6 **Abstract**   In many animals, males bear bright ornamental color patches that may signal both the

7 direct and indirect benefits that a female might accrue from mating with him. Here we test

8 whether male coloration in the Pipevine Swallowtail butterfly, *Battus philenor,* predicts two

9 potential direct benefits for females, copulation duration and the quantity of materials the male

10 passes to the female during mating. In this species, males have a bright iridescent blue field on

11 the dorsal hindwing surface while females have little or no dorsal iridescence. Females

12 preferentially mate with males who display a bright and highly chromatic blue on their dorsal

13 hindwing. In this study, we show that the chroma of the blue on the male dorsal hindwing and

14 male body size (forewing length) significantly predict the mass of material or spermatophore that

15 a male forms within the female's copulatory sac during mating. We also found that

16 spermatophore mass correlated negatively with copulation duration, but that color variables did

17 not significantly predict this potential direct benefit. These results suggest that females may

18 enhance the material benefits they receive during mating by mating with males based on the

19 coloration of their dorsal hindwing.

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32   **Introduction**

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34   In many animals, males display elaborate secondary sexual characteristics that females do not

35   have. Many of these ornaments are an evolutionary product of sexual selection in the context of

36   either female mate choice or male-male competition for mates (Andersson 1994). When females

37   display a preference for ornamented males, the honest-signaling or indicator hypothesis suggests

38   that there are two broad classes of benefits for females that may drive the evolution of the

39   preference (Andersson 1994). If the development of the ornament is correlated with male genetic

40   quality, females may gain indirect benefits in the form of high quality genes for their offspring.

41   Alternatively, male ornaments may indicate direct benefits that females and their offspring may

42   receive, such as superior parental care, disease avoidance, or other material aid the male may

43   offer.

44   Butterflies offer practical and interesting opportunities to examine these potential

45   relationships between male ornament characteristics and the benefits females may gain by mating

46   with highly ornamented males. In many butterflies, males display color patterns that are brighter,

47   more chromatic, and more boldly patterned than those of females. Recent studies suggest that

48   females preferentially mate with males with such color features (for review, Kemp and Rutowski

49   2011), as Darwin (1871) suggested, and that, because in some species male coloration is

50   heritable, there may be indirect benefits for females in choosing colorful males (Kemp and

51   Rutowski 2007; Kemp 2008).

52   The evidence that male color ornaments signal potential direct benefits in butterflies is

53 more equivocal. During mating in butterflies, a male forms a package of sperm and nutrients in

54 the female's reproductive tract called a spermatophore. The contents of a spermatophore may

55 provide females with direct benefits in the form of nutrients that she can use in egg production

56 and in her own somatic maintenance to enhance her reproductive output (Boggs and Gilbert 1979;

57 Rutowski et al. 1987; Watanabe and Sato 1993; for a recent review, see South and Lewis 2011).

58 This suggests that females might receive enhanced material benefits from mating with males with

59 features that suggest they will produce a large spermatophore during mating.   Such features

60 could include a male's body size which in the Lepidoptera is often correlated with the size of the

61 spermatophore he is likely to produce (Bissondath and Wiklund 1995; Hughes et al. 2000; Lewis

62 and Wedell 2007). A male's coloration is another potential indicator trait. However, Kemp et al.

63 (2008) found that in the Orange Sulphur butterfly, male UV coloration, which is used by females

64 in mate choice, is not correlated with the quality of the spermatophore she is likely to receive

65 from the male during mating.

66   Nonetheless, in other species, color might be an indicator of the material benefits a male

67 can give a female during mating. For example, coloration degrades with age and wing wear

68 (Kemp 2006), so bright coloration may indicate a low probability of previous mating and the

69 large size or high quality of a spermatophore a young male is likely to produce during copulation.

70 Empirical studies show that age and prior mating history both may negatively influence the size

71 of the spermatophore a male produces during mating (Svard and Wiklund 1986; Rutowski et al.

72 1987; Oberhauser 1988). Also, females that mate with recently mated males not only receive

73 smaller spermatophores but also endure longer copulation durations and decreased reproductive

74 output (Rutowski et al. 1987; Hughes et al. 2000).

75   Here we report on the relationship between a male's coloration and two measures of the

76 potential direct benefits of mate choice, spermatophore size and copulation duration, in the

77 Pipevine Swallowtail, *Battus philenor*. Males of this butterfly species display blue iridescent

78   patches on the ventral and dorsal surfaces (Fig. 1) that serve various signal functions (Rutowski et

79   al. 2010). The ventral iridescent areas are recognized by predators as a warning signal (Brower

80   1958; Codella and Lederhouse 1990; Pegram et al. 2013) while the dorsal iridescent coloration is

81   an intersexual signal. Dorsal iridescent patches are brighter and more chromatic in males than in

82   females, and females prefer to mate with males with a more chromatic dorsal iridescence

83   (Rutowski and Rajyaguru 2013). Here we test the prediction that male iridescent dorsal

84   coloration generally, and specifically the chroma of that iridescence, is positively correlated with

85   the size of the spermatophore he is likely to produce during mating and negatively correlated with

86   the duration of the copulation.

87

88   **Materials and methods**

89

90   Source of animals

91

92   Eggs and larvae of *B. philenor* were collected from the area surrounding the confluence of

93   Mesquite Wash and Sycamore Creek (33.2º N, 111.7º W) in the Mazatzal Mountains of central

94   Arizona during the summer months of 2008 and 2009. Larvae and pupae were reared in an

95   environmental chamber (for details see Rutowski et al. 2010). All larvae were fed *ad libitum* on

96   cuttings from their local host plant, *Aristolochia watsonii*. Upon eclosion, animals were stored in

97   a refrigerator at 4ºC until use.

98

99   Matings

100

101   Virgin females that had been refrigerated for no more than 4 days (average = 0.78 days) were

102   taken to the field site described above. Each female was tethered by tying one end of a 0.5m long

103   piece of thread around the base of her abdomen where it narrowly joins the thorax. The other end

104 of the tether was tied to the end of a 1 m long stick. Each tethered female was presented to free-

105 flying males until one of the males courted and mated with the female. After copulation began,

106 we placed the mating pair in a 500 ml covered cup and checked them regularly until they

107 separated, at which time we recorded the duration of the copulation and placed both animals in a

108 freezer to euthanize and store them until the measurements described below were made.

109

110 Body size, male age, and male color assessments

111

112 We used forewing length and body mass as indicators of body size for males and females.

113 Forewing length was measured with digital calipers to the nearest 0.1 mm from where the costal

114 vein inserts into the thorax to the tip of the wing. Body mass was measured to the nearest 0.1 mg

115 on an analytical balance after the butterflies were dried under vacuum for 24 hr. The abdomen of

116 each female was dissected under Ringer's solution and her bursa copulatrix removed. We

117 measured the dry mass of each bursa and its contents after 24 hrs of drying under vacuum to

118 remove water. Previous work indicated that immediately after mating in butterflies the mass of a

119 bursa is negligible relative to the mass of the material in it received from the male (Marshall

120 1980).

121   After making these measurements we removed the hindwings from males and females

122 and mounted them on black cardboard using 3M photo mount adhesive. The left hindwing was

123 mounted ventral side up and the right hindwing was mounted dorsal side up. For males, we

124 assessed wing wear as a surrogate for age. Wear was scored on a scale of 1 to 5 using the

125 following criteria: 1, no evident wing area or scale loss or tattering of wing edges; 3, moderate

126 wing area and scale loss and tattering of wing edges; 5, extensive loss of wing area and scale, and

127 tattering of wing edges.

128   We collected reflectance spectra from both the dorsal and ventral iridescent patches of

129 the male hindwings using techniques described in detail in Rutowski et al. (2010).   Reflectance

130   relative to a magnesium oxide white standard was measured between 300 and 700 nm from the

131   regions on the wings shown in Fig. 1. Using the software, CLR (Montgomerie 2008) the

132   following color parameters were extracted from each spectrum after binning the data into 1nm

133   bins.

134   1) Hue: the wavelength (nm) at which percent reflectance relative to the white standard was

135   greatest

136   2) Chroma: the percent of the total reflectance from 300 – 700 nm found within a

137   wavelength segment spanning 50 nm on either side of the wavelength of peak reflectance

138   3) Brightness: the average percent reflectance in wavelengths from 300-700 nm

139

140   Statistical Analysis

141

142   To determine which phenotypic variables best predicted spermatophore mass and copulation

143   duration, we ran two stepwise linear regressions with spermatophore mass and copulation

144   duration as the dependent variables, respectively. Some of our predictor variables were

145   correlated and so to reduce the effects of multicollinearity, we first evaluated the strength of these

146   correlations using a Pearson correlation analysis with a two-tailed test.   For any pair of

147   significantly correlated variables, we removed one of the variables in the two ensuing regression

148   analyses (see list of variables included in each model and explanation in the Results). This was

149   done in lieu of a principal components analysis to facilitate interpretation of the results. We used

150   SPSS v. 21 (IBM, Armonk, NY) and a 0.05 level of significance for all statistical analyses.

151

152   **Results**

153

154   The data collected from 75 mated pairs are summarized in Table 1. There was substantial

155   variation for our dependent variables, spermatophore mass and copulation duration, both ranging

156   over almost an order of magnitude.

157   The correlation analysis on our independent variables revealed several significant

158   correlations that were taken into consideration in our decisions about which variables to include

159   in the regression analysis. First, there were strong correlations between forewing length and body

160   mass (males: $r = 0.84$, $p < 0.001$; females: $r = 0.771$, $p < 0.001$). We used only forewing length in

161   subsequent analyses because this measure does not change with an individual's age and adult

162   history whereas mass is expected to decrease with age as indicated by wing wear, as it did at least

163   for males ($r = -0.25$, $p = 0.03$).

164   All ventral hindwing color parameters as well as dorsal hindwing brightness were also

165   omitted from the independent variables for several reasons. First, experimental studies suggested

166   that females attend to male dorsal and not ventral hindwing coloration and so correlations

167   between ventral coloration and our dependent variables were not of interest (Rutowski and

168   Rajyaguru 2013). Second, several correlations between ventral and dorsal hindwing color

169   parameters suggested this was appropriate. From prior studies (Rutowski et al. 2010) and in this

170   data set, there were significant positive correlations between dorsal and ventral hindwing hue ($r =$

171   0.63, $p < 0.001$) hence we included only dorsal hindwing hue in the regression analysis. Also, we

172   did not include any measure of hindwing brightness in the analysis for two reasons. First,

173   previous experiments indicated that it was not correlated with male mating success (Rutowski and

174   Rajyaguru 2013) and dorsal brightness was negatively correlated with dorsal chroma ($r = -0.506$,

175    $p < 0.001$) on the hindwing. Finally, wing wear was included in the analysis but ventral hindwing

176   chroma was not because it was negatively correlated with wing wear ($r = -0.432$, $p < 0.001$).

177   Again, these exclusions were done to control confounding covariation among

178   independent variables and facilitate interpretation of the results. The final multiple regression

179   model included these independent variables: male forewing length, female forewing length, male

180   wing wear, male dorsal hindwing chroma, and male dorsal hindwing hue. In a stepwise multiple

181   regression this suite of variables significantly predicted spermatophore mass (ANOVA, $p = $

182   0.004) but did not predict copulation duration (ANOVA, $p = 0.453$). Moreover, the only

183   variables that contributed significantly to the prediction of spermatophore mass were (1) male

184   forewing length ($p = 0.014$), i.e., larger males produced larger spermatophores, and (2) dorsal

185   hindwing chroma ($p = 0.045$), i.e., more chromatic males produced larger spermatophores (Fig.

186   2).

187   Although no independent variable included in our regression model predicted copulation

188   duration we did find that the mass of the spermatophore produced in copulation was negatively

189   correlated with the duration of copulation (Fig. 3).

190

191   **Discussion**

192

193   The results help identify those features of the participants in a mating that may influence the size

194   of the spermatophore the female receives. Larger males produce larger spermatophores which is

195   consistent with prior reports for *B. philenor* (Rutowski et al. 1989) and other butterfly species

196   (Rutowski 1984; Rutowski and Gilchrist 1986, 1987; Svärd and Wiklund 1986; Bissoondath and

197   Wiklund 1996; Wedell and Cook 1999; Hughes et al. 2000) and may reflect that larger males

198   have more resources to commit to the production of a spermatophore and its contents. We found

199   not relationship between female body size and the size of the spermatophore she receives Which

200   agrees with results from other butterfly species (Bissoondath and Wiklund 1996; Hughes et al.

201   2000; Rutowski 1984).

202

203   Direct benefits and male coloration

204

205   The chroma of a dorsal male's hindwing coloration was the only color variable that regression

206   analysis revealed to be a significant predictor of the quantity of material he placed in the female

207   during mating. This relationship was positive, i.e., more chromatic males produced on average

208   larger spermatophores, as we expected given the results of our mate choice experiments in which

209   the males with the highest mating success were those that were most chromatic (Rutowski and

210   Rajyaguru 2013). This supports the hypothesis that females are choosing among males on the

211   basis of the properties of an ornament in a way that will maximize the direct benefits they accrue

212   from the mating. This is one of only a few studies showing that a known sexual signal that is

213   important in mate choice may reliably predict the nuptial gift a female is likely to receive from a

214   male in species in which the quality of the nuptial gift cannot be directly evaluated (Dussourd et

215   al. 1991; Lewis and Cratsley 2008).

216   Brief copulation duration is another potential direct benefit. In butterflies, males control

217   the duration of copulation (Wickman 1985) and mating pairs are perhaps exposed to greater risk

218   of predation, and so the briefer the copulation better. However, in this study no color parameter

219   included in the analysis was a significant predictor of copulation duration, including dorsal

220   hindwing chroma.

221   We emphasize that male dorsal coloration is most likely an indicator of his overall

222   potential to produce material benefits and not his recent mating history. A male's previous

223   mating history affects the mass of the spermatophore he can produce at a given time in his life

224   (Bissoondath and Wiklund 1996; Hughes et al. 2000) which can have fitness consequences for

225   females (Rutowski et al. 1987, Svard and Wiklund 1991). Replenishment of the materials

226   available to put into spermatophores can take several days in *B. philenor* (Rutowski et al. 1989).

227   However, recent mating history cannot in butterflies lead to changes in a male's color signal

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228   which is set at eclosion and in *B. philenor* surprisingly changes little, if at all, with age (Rutowski

229   et al. 2010).

230

231   Iridescent reflections as signals

232

233   The iridescent properties of the male's blue coloration mean that because of the changes in the

234   relative positions of the female and the male's wings during courtship, the perceived color of the

235   male's dorsal wing surface may change dramatically over a range of wavelengths (Rutowski et al.

236   2010).   For a given position of receiver and light source above a wing surface, both the

237   brightness and the chroma of the reflection seen by the viewer will change as the wing moves

238   during a wing beat cycle. Hue might also vary if the light source that contributes to a visible

239   reflection can come from multiple directions above the wing surface, i.e. from different points in

240   the blue sky and so arrive at different angles of incidence. These effects are expected to make it

241   difficult for a receiver to assess reliably the relative chromaticity or brightness, and hue of an

242   individual male's reflection relative to some threshold or internalized standard. This problem

243   could be reduced if the relative positions of male and female are somehow "standardized" during

244   courtship. For some birds that use iridescent color signals, signaler behavior does appear to be

245   structured to maximize the transmission of their iridescent signal to the intended receiver

246   (Hamilton 1965; Loyau et al. 2007).   In sulphur butterflies, males position themselves relative to

247   conspecifics in ways that enhance their ability to assess whether or not an approached conspecific

248   has an iridescent UV reflection (Rutowski et al 2007). Those that do not, namely, females, are

249   courted. High speed video recordings of the behavior of *B. philenor* males and females in

250   courtship that are currently being analyzed in our lab suggest this is the case (unpubl. data).

251

252   Is male dorsal hindwing iridescence costly?

253

254 Our results with *B. philenor* provide evidence that female color preference may be adaptive in

255 that it maximizes the size of the nuptial gift she receives during mating. However, for such an

256 indicator signal to evolve, it requires that there be costs associated with its production that prevent

257 males from cheating, that is, developing a colorful ornament even when they are not able to

258 produce the costly contents of a spermatophore. During development, the photonic structures that

259 produce iridescent colors in animals may be especially costly to build because of the precision

260 required in nanoscale construction to produce a bright and chromatic iridescent reflection

261 (McGraw et al. 2002, Kemp 2006, Kemp and Macedonia 2006, Kemp and Rutowski 2007) and

262 therefore exhibit condition dependence (e.g. Doucet et al. 2006. Kemp 2006, Kemp and Rutowski

263 2007). Pegram et al. (in press) have examined which features of the coloration of *B. philenor* are

264 affected by food deprivation. Food deprivation had negative effects on body size, a measure of

265 condition, in that study and so offered an indicator of whether color features, especially dorsal

266 hindwing chroma are costly to produce (Cotton et al. 2004). Contrary to expectation, chroma was

267 not affected by food deprivation which leaves open the question of whether chroma could evolve

268 as a reliable indicator trait.

269 However, there are two features of the study by Pegram et al. (in press) that leave this

270 question open. First, the effect of food deprivation was evaluated under only a single set of

271 controlled growth conditions. Perhaps there are significant effects of food deprivation on chroma

272 under other regimes of temperature and humidity in the highly variable field environment.

273 Second, only a single stressor was examined. There are other possible stressors such as disease,

274 extreme environmental variation, and foodplant quality that might affect chroma as well as a

275 male's ability to produce a spermatophore (Kemp and Rutowski 2007). These possibilities are

276 supported by the fact that the chroma of the male's dorsal hindwing of lab-reared *B. philenor* is

277 different from that of field caught-individuals, a result not fully explained by higher levels of

278 wing wear in the field-caught individuals (Rutowski et al. 2010). Wing wear has no significant

279 effect on dorsal hindwing coloration in this species.

280   There also remains the possibility that females benefit from selecting chromatic males for

281   indirect rather than direct benefits. The intrasexual variation in male coloration has a genetic basis

282   in other butterflies (Kemp and Rutowski 2007, Kemp 2008). For *B. philenor*, the details of the

283   proximate causes of naturally-occurring variation in male dorsal hindwing coloration (especially

284   chroma) and ability to produce a spermatophore, and the consequences of this variation for

285   female reproductive success, warrant further investigation and will inform our understanding of

286   the evolution of this female color preference in this and other species.

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293

294   **References**

295

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396   342

397

398 **Table 1** Descriptive statistics for the measured variables (n = 75). See text for details on

399 measurements, especially wing wear, brightness, and chroma.

400 _____

Variable	mean	SD	minimum-maximum
401 Variable	mean	SD	minimum-maximum
403 Copulation Duration (min)	147	88.1	52-507
404 Spermatophore dry mass (mg)	6.5	2.02	2.1-10.8
405 Female: Forewing length (mm)	49	3.1	39-55
406 Body mass (mg)	398	87	194-550
407 Male: Forewing length (mm)	42	3.3	32-48
408 Body mass (mg)	178	49.5	81-327
409 Wing wear	2.7	1.09	1-5
410 Dorsal iridescent patch			
411 Brightness	9.3	3.6	2.7-22.1
412 Hue (nm)	489	16.6	441-533
413 Chroma	0.501	0.057	0.392-0.717
414 Ventral iridescent patch			
415 Brightness	35.5	10.7	18.7-77
416 Hue (nm)	490	21.3	451-566
417 Chroma	0.428	0.025	0.322-0.475

418 _____
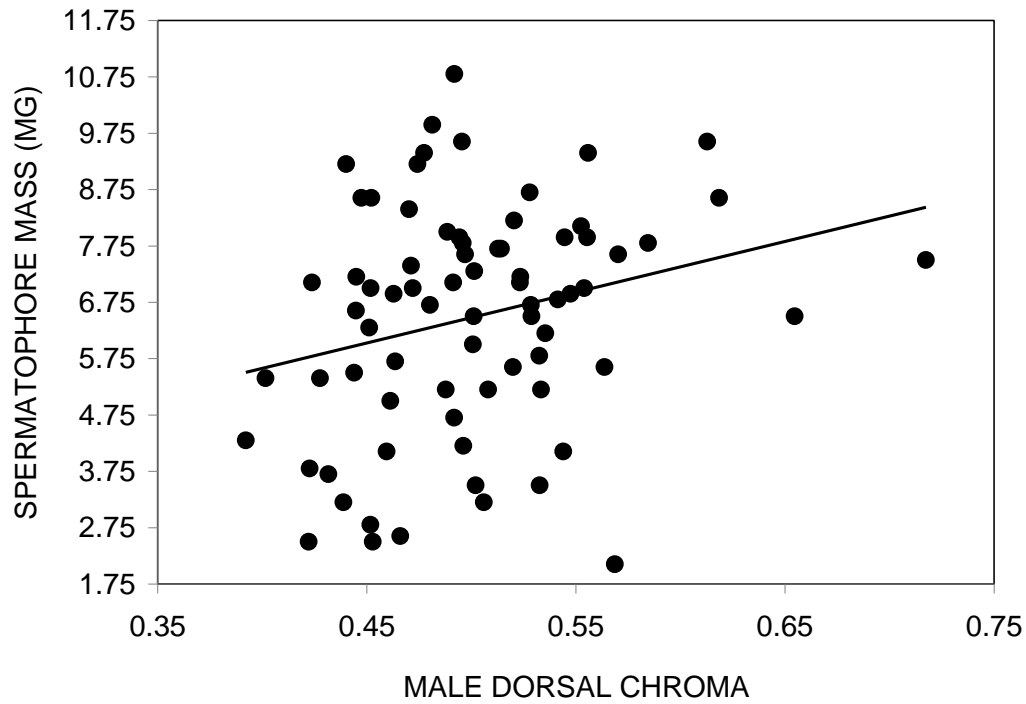
419

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Figure 1.   The dorsal (left) and ventral (right) wing surfaces of a *B. philenor* male photographed

under conditions that maximize the visibility of the iridescent blue to the camera. The red circles

show the region on each wing surface from which reflectance measurements were taken.
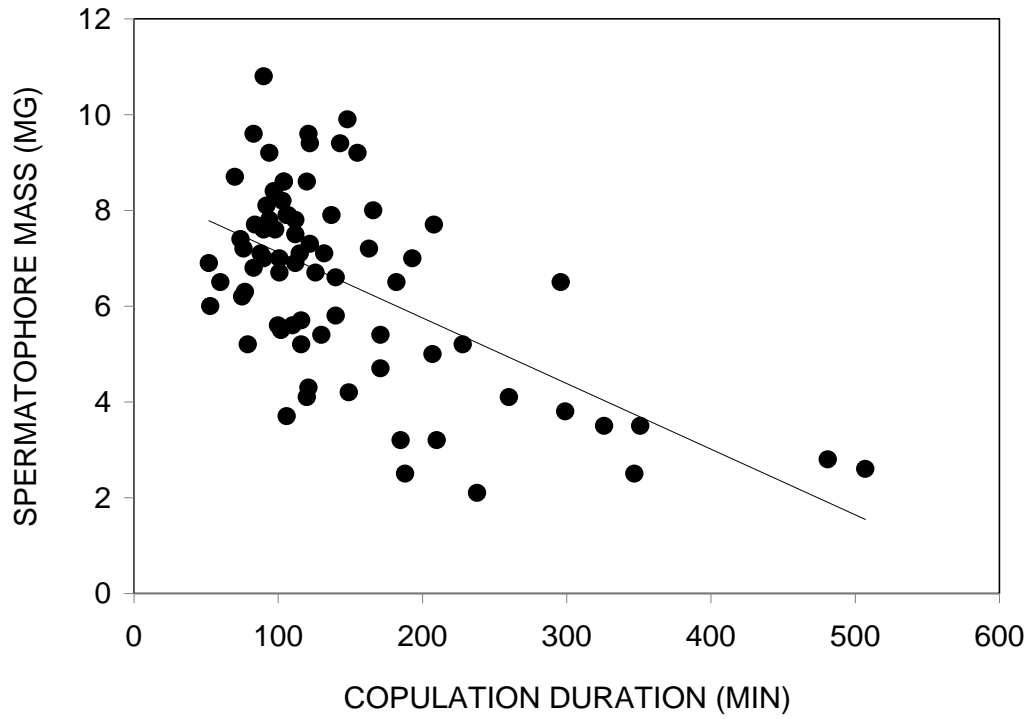
426

427   Figure 2. The relationship between the mass of the spermatophore a male produces and the

428   chroma of the iridescent area on his dorsal hindwing surface. The line is the linear best fit from a

429   simple regression ($r = 0.253$, 74 df, $p < 0.029$). The multiple regression analysis also supported

430   chroma as a predictor of spermatophore mass (see text for details).

431

432

433   Figure 3. The negative relationship between the mass of the spermatophore produced during

434   copulation and the duration of copulation ($r = -0.596$, 74 df, $p < 10^{-7}$).

435