

1 **Micropyle number is associated with elevated female promiscuity in**

2 **Lepidoptera**

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7

8 **Abstract**

9 In the majority of insects, sperm fertilise the egg via a narrow canal through the outer chorion called  
10 the micropyle. Despite having this one primary function, there is considerable unexplained variation  
11 in the location, arrangement and number of micropyles within and between species. Here, we  
12 examined the relationship between micropyle number and female mating pattern through a  
13 comparative analysis across Lepidoptera. Three functional hypotheses could explain profound  
14 micropylar variation: (i) increasing micropyle number reduces the risk of infertility through sperm  
15 limitation in species that mate infrequently; (ii) decreasing micropyle number reduces the risk of  
16 pathological polyspermy in species that mate more frequently; (iii) increasing micropyle number  
17 allows females to exert greater control over fertilisation within the context of post-copulatory sexual  
18 selection, which will be more intense in promiscuous species. Micropyle number was positively  
19 related to the degree of female promiscuity as measured by spermatophore count, regardless of  
20 phylogenetic signal, supporting the hypothesis that micropyle number is shaped by post-copulatory  
21 sexual selection. We discuss this finding in the context of cryptic female choice, sperm limitation and  
22 physiological polyspermy.

23 **Keywords**

24 Cryptic female choice, Lepidoptera, micropyle, polyspermy, sperm competition, spermatophore  
25 count

26

27 **1. Introduction**

28 Micropyles (from the Greek *mikros*, small, *pulē*, gate) are small openings that allow male gametes to  
29 enter and fertilise the ovum in a wide diversity of taxa including insects, fishes, cephalopods and  
30 plants [1]. Amongst insect orders, micropyles exhibit considerable variation in position, arrangement  
31 and number. For example in some species micropyles protrude from the egg chorion on ‘stalks’  
32 (micropylar processes) such as *Drosophila spp.* [2] whereas others are located in micropylar pits as in  
33 some Lepidoptera [e.g. 3], whilst others are superficial [1,2]. Within the Heteroptera variation in  
34 micropyle number is extensive: 0 – 70 [4], whilst in the Lepidoptera there are between 1-20 [1], with  
35 some evidence of intraspecific variation [5,6]. Despite such large and obvious differences between  
36 species, few authors have attempted to seek functional explanations for this variation.

37         Here we use a comparative approach to investigate variation in micropyle number, testing  
38 between three hypotheses associated with female mating pattern. If micropyles only act to facilitate  
39 fertilisation success, we predict more micropyles in those species at greater risk of fertilisation  
40 failure due to sperm limitation. This could occur in populations that have a strongly female-biased  
41 operational sex ratio [7] and/or in populations in which females mate infrequently. For example,  
42 female *Drosophila pseudoobscura* that copulate only once appear to have insufficient viable sperm  
43 stores to maintain fertility [8]. Hence, if greater micropyle number increases fertilisation success we  
44 predict a negative association with the likelihood of female promiscuity. Similarly, if micropyle  
45 number functions to mitigate against pathological polyspermy (embryonic failure due to more than  
46 one sperm entering the oocyte cytoplasm [9]), then we predict a negative association between  
47 micropyle number and female mating frequency, such that promiscuous species at greater risk of  
48 pathological polyspermy have fewer micropyles. (It should be noted that our approach cannot  
49 distinguish between these two hypotheses.) By contrast, if micropyles are shaped by post-copulatory

50 sexual selection, then greater micropyle number is predicted to be positively associated with  
51 promiscuous mating patterns. In species where ejaculates from more than one male compete to  
52 fertilise a female's set of eggs cryptic female choice can operate to bias fertilisation success in favour  
53 of particular male traits [10]. In species with internal fertilisation, this can manifest itself as a number  
54 of male-female interactions [11] including those at the sperm-egg interface [12]. Thus it is possible  
55 that variation in micropyle number could be driven by post-copulatory sexual selection if females are  
56 able to use these structures to exert control over fertilisations. However, to our knowledge, no  
57 studies have yet examined this novel hypothesis.

58 We therefore compare micropyle number against the extent of female promiscuity, using  
59 lepidopteran species that vary greatly in both female mating pattern and egg micropyle number. The  
60 Lepidoptera are especially suitable for this study because mating pattern can be quantified from  
61 spermatophore counts which persist within the female bursa copulatrix [13]. We hypothesise that  
62 variation in micropyle number functions to: (i) reduce the risk of sperm limitation and egg infertility,  
63 (ii) reduce the risk of pathological polyspermy, or (iii) allow greater control over paternity.

64

## 65 **2. Materials and methods**

### 66 a) Data collation

67 Species-specific average micropyle number and spermatophore count (number of spermatophores  
68 recovered from the bursa copulatrix) were collated from the literature alongside egg size (a potential  
69 co-variate; [14]) for 56 species of Lepidoptera from 15 families (25 butterflies and 31 moths).

70 Lepidopteran eggs fall broadly into two shapes: fusiform (butterflies) and flat/round (moths). Thus  
71 volume was approximated using the formula for a prolate ellipsoid ( $(\frac{1}{2} \text{ egg length} \times \frac{1}{2} \text{ egg width}^2 \times \pi)$ )

72  $4/3$ ) for butterfly eggs and half-oblate spheroids for the moth eggs ( $(\frac{1}{2} \text{ egg length} \times \frac{1}{2} \text{ egg width}^2 \times$   
73  $\pi) 4/3/2$ ). In particular estimates of species-level promiscuity were gained primarily from field  
74 studies (as opposed to lab-based studies) which reported spermatophore count (for a discussion on  
75 using this method see [13]).

76

### 77 Statistical analyses

78 We used a phylogenetic generalized least squares regression (PGLM) [15, 16] between mean  
79 micropyle number and spermatophore count. The `pglmEstLambda` function of the 'CAIC' package  
80 was used to identify the maximum likelihood value of  $\lambda$  [15; 17; 18] which measures the degree to  
81 which the matrix follows a Brownian model;  $\lambda$  can vary between 0 (no phylogenetic autocorrelation)  
82 to 1 (complete phylogenetic autocorrelation). We present results from the PGLM along with the  
83 ordinary least squares (OLS) for comparison [19]: where  $\lambda = 0$ , the resulting model is equivalent to a  
84 standard linear model. Analysis was carried out using R code kindly provided by R.P. Freckleton  
85 (University of Sheffield). We used butterfly phylogenies available on the Tree of Life Web Project  
86 [20] with branch lengths set to one. All analyses were run in R version 2.15.2 [21].

87

### 88 **3. Results and Discussion**

89 Species-specific micropyle number varied from 1 to 15 (mean  $4.06 \pm \text{S.E.M. } 0.43$ ) across the  
90 Lepidoptera sampled (Table 1). We found no evidence that micropyle number was associated with  
91 risk of sperm limitation and infertility, or that fewer micropyles were associated with a likely  
92 increased risk of polyspermy. Rather, micropyle number was positively correlated with our estimate

93 of female promiscuity. (Table 2). Micropyle number was positively related to spermatophore count  
94 in corrected and non-corrected PGLS (Table 2, Figure 1).

95           At a functional level more micropyles would suggest greater potential for multiple sperm  
96 entry into the egg. This raises two questions: i) why allow multiple sperm to enter the egg? And ii)  
97 why make this easier in species with greater female promiscuity? Physiological polyspermy is  
98 widespread in nature, being the norm in Urodeles and birds but also reported in other taxa [9, 22]  
99 including Lepidoptera [23]. In physiological polyspermy, several sperm enter the egg but only one  
100 fuses with the female pronucleus. The remaining supernumerary sperm nuclei degenerate [2]. Why  
101 physiological polyspermy occurs only in some taxa is unclear, although a recent study by Hemmings  
102 & Birkhead [24] indicates that polyspermy is essential for early embryonic development in both the  
103 domestic fowl and the zebra finch (*Taeniopygia guttata*). Physiological polyspermy enables the  
104 intriguing possibility of mate choice within an egg cell [25]. In the polyspermic ctenophore (*Beroe*  
105 *ovata*) the female pronucleus migrates among male pronuclei within the egg before fusing with one  
106 [26]. Thus, the presence of multiple micropyles could increase the opportunity for post-copulatory  
107 female choice within the egg environment. Such mechanisms are likely to be most relevant for  
108 polyandrous species where selection has acted on mating pattern to increase the opportunity for  
109 sperm choice.

110           Alternatively, more micropyles may represent a bet-hedging strategy for the female where  
111 sperm numbers are limited. Although sperm are cheaper to produce than eggs, they still involve a  
112 reproductive cost. When sperm competition is high, males can allocate their ejaculates prudently  
113 resulting in sperm limitation for females [27]. Thus the presence of a greater number of micropyles  
114 may represent an evolved mechanism to counter male traits which incidentally lower female fitness.

115           Lastly, the micropyle opening is only one component of micropyles. The micropylar  
116 openings lead to canaliculi, minute ducts through the chorion. In some species these canals show  
117 complex structuring; for example, *Bombyx mori* have a single external micropyle which branches to  
118 3-5 canaliculi which lead to the chorion [28]. Given that the number of sperm entering eggs in *B.*  
119 *mori* varies from 1 to 11, Kawaguchi et al. [6] proposed that the number of canals is related to the  
120 degree of polyspermy. Such diversity in internal structuring of the micropyles suggests a greater  
121 degree of complexity to their function than has been considered previously and a possible role in  
122 polyspermy in insects.

123

## 124 **Conclusions**

125 This is the first study to show that micropylar variation is in part driven by the degree of female  
126 promiscuity. Micropyles allow sperm entry into the egg, hence more micropyles should aid sperm  
127 entry in to the egg, reducing the likelihood of infertility, whilst at the same time increasing the  
128 likelihood of physiological polyspermy. Whether physiological polyspermy benefits early  
129 embryogenesis in insects as it appears to do in birds [24] and/or offers an alternative site for cryptic  
130 female choice [25] requires further study.

## 131 **Ethics**

132 **Ethical approval was granted from University of Lincoln's College of Science Ethics Committee**  
133 **(COSREC37).**

## 134 **Data accessibility**

135 Data are available on the Dryad Digital Repository doi:10.5061/dryad.2c5m1[29]

136

137 **Authors' contributions**

138 G.I. and P.E. designed the study, G.I. collated and analysed the data, G.I. and P.E. drafted the  
139 manuscript. M.J.G.G. shared his butterfly sperm dataset, contributed to the study design and helped  
140 draft the manuscript. All authors gave final approval for publication and agreed to be accountable  
141 for all aspects of the content therein.

142

143 **Competing interests**

144 We have no competing interests.

145

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220

221 Table 1 Average micropyle number across lepidopteran family

222

Family	Number of species	Average micropyle number
Arctiidae	1	4 (4-6)
Erebidae	1	2
Gelechiidae	1	3
Heliiothinae	1	3 (3,4)
Lycaenidae	2	3.5
Noctuidae	14	5.2
Notodontidae	2	10.5
Nymphalidae	18	3.6
Papilionidae	1	7
Pieridae	2	1.5
Pyralidae	4	2
Saturnidae	1	7
Sphingidae	1	1
Tortricidae	2	1

223

224

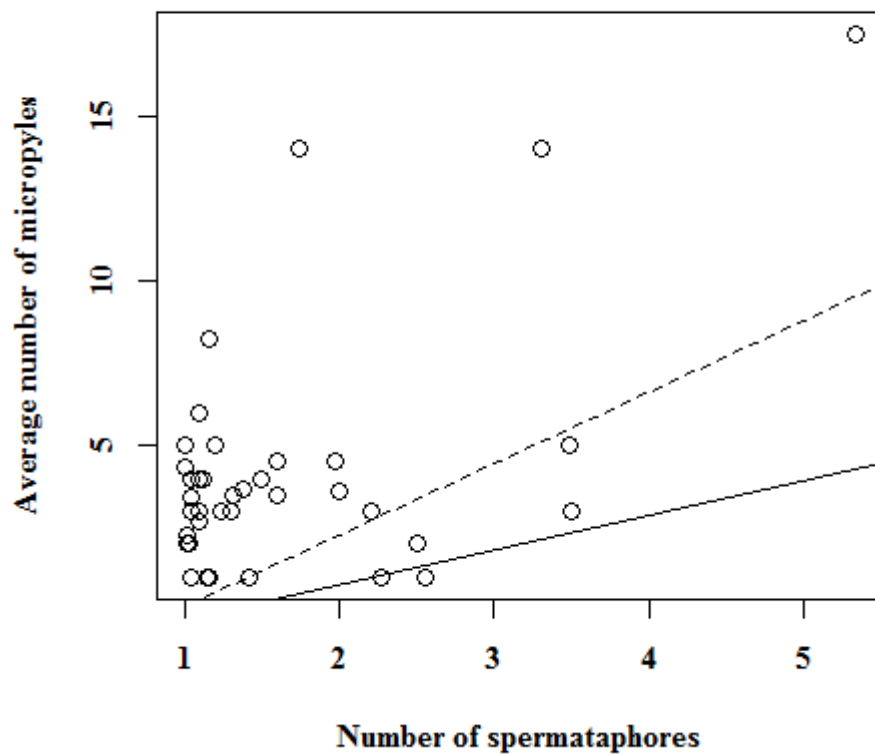
225 Table 2 PGLM model results for the relationship between micropyle number and spermatophore count. For each model the  $\beta \pm SE$ , t and p values are  
226 presented. In addition, the estimate of Pagel's  $\lambda$  (Pagel 1999) is calculated.

227

Model	Parameter	N	Non-phylogenetically corrected			Phylogenetically-corrected			
			B $\pm$ SE	t	p	B $\pm$ SE	$\lambda$	t	p
	Intercept	39	-0.08 $\pm$ 1.19	-0.07	0.946	1.09 $\pm$ 1.54	1.00	0.70	0.485
	Egg volume		1.86 $\pm$ 1.60	1.16	0.252	1.14 $\pm$ 1.49		1.76	0.449
	Spermatophore count		2.30 $\pm$ 0.55	4.20	<0.001	1.09 $\pm$ 0.45		2.40	0.022

228 Figure 1 The relationship between micropyle number and spermatophore count in corrected (solid  
229 line) and non-corrected (dotted line) PGLS.

230



231

