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

2 **Lepidoptera**

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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

1. Introduction

27

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

2

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 ((½ egg length x ½ egg width^2 x π)

72   4/3) for butterfly eggs and half-oblate spheroids for the moth eggs (((½ egg length x ½ egg width^2 x

73   π) 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 λ [15; 17; 18] which measures the degree to

81   which the matrix follows a Brownian model; λ 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 λ = 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 ± 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]

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137 **Authors' contributions**

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219   (http://dx.doi.org/10.5061/dryad.2c5m1)

220

221   Table 1 Average micropyle number across lepidopteran family

222

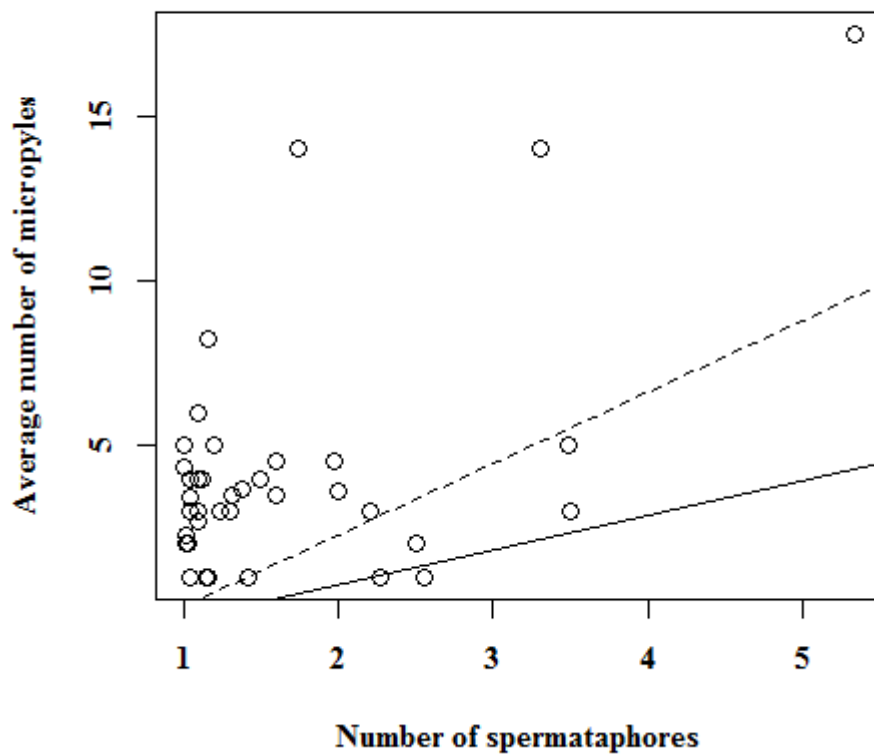| Family	Number of species	Average micropyle number
Arctiidae	1	4 (4-6)
Erebidae	1	2
Gelechiidae	1	3
Heliothinae	1	3 (3,4)
Lycaenidae	2	3.5
Noctuidae	14	5.2
Notodontidae	2	10.5
Nymphalidae	18	3.6
Papillonidae	1	7
Pieridae	2	1.5
Pyralidae	4	2
Saturnidae	1	7
Sphingidae	1	1
Tortricidae	2	1

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224

225 Table 2 PGLM model results for the relationship between micropyle number and spermatophore count. For each model the β ± SE, t and p values are

226 presented. In addition, the estimate of Pagel's λ (Pagel 1999) is calculated.

227

Model	Parameter	N	Non-phylogenetically corrected			Phylogenetically-corrected			
			B±SE	t	p	B±SE	λ	t	p
	Intercept	39	-0.08±1.19	-0.07	0.946	1.09±1.54	1.00	0.70	0.485
	Egg volume		1.86±1.60	1.16	0.252	1.14±1.49		1.76	0.449
	Spermataphore count		2.30±0.55	4.20	<0.001	1.09±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.

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