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Elusive *Parnassius mnemosyne* (Linnaeus, 1758) larvae: habitat selection, sex determination and sex ratio (Lepidoptera: Papilionidae)

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Abstract

The charismatic and regionally declining *Parnassius mnemosyne* (Linnaeus, 1758) is notable for surpluses of males in mark-recapture studies, as well as for poor detectability of its larvae, which develop on spring ephemeral plants, *Corydalis* spp. In order to study whether the bias towards male exists already in larval stage, we searched for the larvae at three localities in the Czech Republic and attempted to sex the larvae using two alternative methods: (i) by identification of the female specific sex chromatin formed by multiple copies of the W chromosome in polyploid somatic nuclei of the Malpighian tubule cells and (ii) by dissection of ovaries and testes. Finding the larvae in the field was extremely difficult, and 38 person-days of fieldwork yielded only 78 larvae. Consistent with the literature, they dwelled under sparse canopy of oak dominated mature woodlands or on woodland margins. Sexing by genitalia dissection worked well (N = 22) and revealed a prevalence of females (16 vs. 6). In contrast, the identification of sex chromatin was not possible as sex chromatin was absent (N = 56). Further cytogenetic analysis confirmed the haploid number of chromosomes n = 29, which is by one smaller than in the congeneric *Parnassius apollo* (Linnaeus, 1758). This reduction of chromosome number is probably the result of a fusion of sex chromosomes with a pair of autosomes, a situation not uncommon in Lepidoptera. The female-biased larval sex ratio, contrasting with male biases found so often in adults, is based on just 22 genitally dissected larvae, and may be biased by faster growth rate of males.

KEY WORDS: Lepidoptera, Papilionidae, habitat, sex determination, sex ratio, Czech Republic.

Dificultad de las larvas de *Parnassius mnemosyne* (Linnaeus, 1758), selección de hábitat, determinación y ratio sexual (Lepidoptera: Papilionidae)

Resumen

Parnassius mnemosyne (Linnaeus, 1758) es una carismática especie cuyos números están en declive regional. Es notable por su sobrerrepresentación de machos en estudios de marca y recaptura, al igual que la dificultad en detectar sus larvas, quienes se desarrollan en plantas efímeras primaverales (especies de *Corydalis*). Para estudiar si el sesgo hacia la sobrerrepresentación de machos es evidente en la etapa larval, buscamos larvas en tres localidades en la República Checa, e intentamos determinar el sexo de las larvas usando dos métodos distintos: identificación de la cromatina sexual específica de las hembras, formada por copias múltiples del cromosoma W en el núcleo somático de células poliploides de los túbulos de Malpighian; y disecciones de ovarios y testículos. Encontrar larvas en el campo fue extremadamente difícil; 38 días-hombre de trabajo de campo cedieron a penas 78 larvas. De acuerdo con la literatura, las larvas habitan bajo pabellones escasos de encinales maduros o al margen del bosque. Determinación sexual por disección fue eficiente (N = 22) y demostró alta prevalencia de hembras (16 vs. 6). Nos resultó imposible la identificación de la cromatina sexual (N = 56). Análisis citogenético confirmo el número haploide de cromosomas n = 29, uno menos que el congénere *Parnassius apollo* (Linnaeus, 1758). Esta reducción de cromosomas se debe a una fusión de los cromosomas sexuales con un par de autosomas, una situación bastante inusual en Lepidoptera. La sobrerrepresentación femenina de las larvas, se basa en 22 muestras, y contrasta con la sobrerrepresentación reportada en adultos, esta puede ser resultado de una tasa de desarrollo más rápida en los machos.

PALABRAS CLAVE: Lepidoptera, Papilionidae, habitat, determinación sexo, ratio sexual, República Checa.

Introduction

The Clouded Apollo, *Parnassius mnemosyne* (Linnaeus, 1758) is a charismatic West Palaearctic butterfly associated with sparse woodlands, clearings, wooded meadows and forest-steppes of temperate vegetation belt. It is declining in many parts of its range (VAN SWAAY & WARREN *et al*., 1999; BENEŠ *et al*., 2002) and became a target of conservation actions in several countries (e.g., KONVIČKA & KURAS, 1999; GÄRDENFORS, 2015). In censuses of its populations, remarkably male-biased adult sex ratios were repeatedly reported (KUDRNA & SEUFERT, 1991; KONVIČKA & KURAS, 1999; MEGLÉCZ *et al*., 1999; KONVIČKA *et al*., 2001; VÄISÄNEN & SOMERMA, 1985; VLAŠÁNEK *et al*., 2009) and the same holds true for other *Parnassius* species (SCOTT, 1973; MATSUMOTO, 1985; BROMMER & FRED, 1999; ROLAND *et al*., 2000, ADAMSKI, 2004; AUCKLAND *et al*., 2004). Surpluses of males in populations decrease the effective population size, which can be serious problem in conservation management due to genetic consequences (KUUSSAARI *et al*., 1998, 2015). Hence, it is worth asking whether the census male surpluses in *Parnassius*, and related butterflies (e.g., SLANCAROVA *et al*., 2015), are real or artefactual.

Butterfly mark-recapture censuses may suffer biases related to different activity of sexes. Males often defend territories or actively patrol over a habitat to locate females. Females, contrarily, may not need repeated mating (but see VLAŠÁNEK & KONVIČKA 2009) and mainly spend time laying eggs or feeding to boost eggs development. As a result, males are more visible to researchers than females (KONVIČKA & KURAS, 1999; STOKS, 2001; ADAMSKI, 2004). Captive rearing reports, in which *Parnassius* sex ratio did not deviate from 1:1 (ADAMSKI, 2004), support the existence of biases due to differences in male and female activity.

EHRLICH *et al*. (1984) proposed that surpluses of adult butterfly males may be caused by a higher pre-adult female mortality, a higher rate of female emigration, or a higher adult female mortality. However, a particularly detailed mark-recapture study of *P. mnemosyne* adults that controlled for biases due to female emigration of mortality revealed male biased sex ratio as well (VLAŠÁNEK *et al*., 2009).

It follows that answer to the *Parnassius* sex ratio riddle should be sought by studying preadult stages. There are several methods how to sex butterfly larvae, varying in laboratory skills requirement; they include ovaries and testes dissection (YOSHIDO *et al*., 2014), identification of the female specific sex chromatin formed by multiple copies of the W chromosome in polyploid somatic nuclei (TRAUT & MAREC, 1996; FUKOVÁ *et al*., 2009), PCR amplification of a molecular marker derived from the female-sex-determining W chromosome (FUKOVÁ *et al*., 2009), and determination of a Z-linked gene dose in genomic DNA by quantitative real-time PCR (qPCR) (NGUYEN *et al*., 2013). Working with *P. mnemosyne* larvae, however, is further complicated by poor detectability of the larvae in the field. They live solitarily in early spring, when their *Corydalis* spp. host plants are not yet fully developed, and tend to avoid detection by falling into abundant leaf litter immediately after being disturbed.

In this contribution, we describe sex identification of field-collected *P. mnemosyne* larvae. We first describe the conditions under which the larvae occur in field, and investigate the sex ratio and sex determining system in this charismatic species.

Material and methods

The single annual brood of *Parnassius mnemosyne* is on wings from early May to early July, depending on weather and altitude. The singly laid egg is the overwintering stage. The larvae hatch in early spring, when their host plants, the spring ephemerals *Corydalis* spp., sprout in deciduous woodlands understory. Larval development takes a few weeks, pupal stage about three weeks and the adults emerge when the host plants are already decayed (KONVIČKA & KURAS, 1999). The notably protandrous males monopolize mated females by production of sphragis - a waxy structure reducing further mating efforts (VLAŠÁNEK & KONVIČKA, 2009).

We surveyed *P. mnemosyne* larvae at two localities with known high adult densities, where we observed egg laying females in the preceding seasons: in Milovický Wood (48.82N, 16.72E), Boří Wood (48.76N, 16.84E) and near Vranov Reservoir (48.93N, 15.75E), all South Moravia, SE Czech Republic. The surveys were carried out for 2-3 days every April week, 2010, and for 2-3 days during the first and second April weeks, 2011, and took 38 person/days in the field in total. We searched for feeding damages on *Corydalis* plants and surveyed close vicinity of the plants. All located larvae were taken to laboratory and the conditions of all larval records were noted down: *Corydalis* species, behaviour of larva, shading of locality. Proportion of ground coverage components (grass, herbs, leaf litter, exposed ground, *Corydalis* plants, shrubs and trees; components could lay over each other and so the final proportion was higher than 100%) was estimated at microsite (0.5 m radius) and at macrosite (10 m radius).

The larvae collected in 2010 were fixed in a mixture of ethanol, chloroform and acetic acid (6:3:1, Carnoy´s solution) and their Malpighian tubules were dissected, stained in 1.5% lactic acetic orcein and inspected in a light microscope for the presence of sex chromatin. Lepidoptera employ the *Abraxas* type of sex determination (i.e., a WZ/ZZ sex chromosome system), in which females are the heterogametic sex (TRAUT *et al*., 2007). Larvae of most butterflies can be sexed by observing a conspicuous spherical body (or bodies), the so-called sex chromatin, composed of multiple copies of the W sex chromosome in polyploid interphase nuclei of some somatic tissues of females (TRAUT & MAREC, 1996). In male larvae, the W sex chromosome is absent and sex chromatin is not formed. In 2011, the larvae were not immediately fixed but were sexed by dissection of their ovaries/testes in a physiological solution. Ovaries were then fixed in Carnoy´s solution and used for detailed analysis of chromosomes in the pachytene stage of meiotic oocytes as described in MEDIOUNI *et al*. (2004). Preparations from both the ovaries and testes were used for counting of metaphase chromosomes in the mitotically dividing oogonia and spermatogonia, respectively.

Results

We found 56 larvae in 2010 and 22 larvae in 2011. They were all from second to fourth instars; Figure 1 shows sizes of head capsules.

All larvae were found underneath very sparse canopy of oak dominated mature woodlands or on sunny woodland margins or clearings. Since in Central Europe the canopy trees are not yet foliaged in April, the larval habitats were fully exposed to sun. In microsites, dry leaf litter from last year dominated (63%) over grasses (17%) and herbs (13%). In macrosites, the coverage was similar with dominating leaf litter (54%), grasses (29%) as well as herbs (15%) (Figure 2). The larvae were feeding on *Corydalis* plants (18%), basking on upper litter layer (33%) or hiding in lower litter layers (49%). All the findings were during sunny days. The host plant was always *Corydalis pumila* which is in the Czech Republic limited to thermophyticum of Central Bohemia and South Moravia (HEJNÝ & SLAVÍK, 1997).

No sex-chromatin was found in highly polyploid nuclei of the Malpighian tubule cells in the fixed larvae collected in 2010, indicating that sex discrimination by this trait is impossible (Figure 3a).

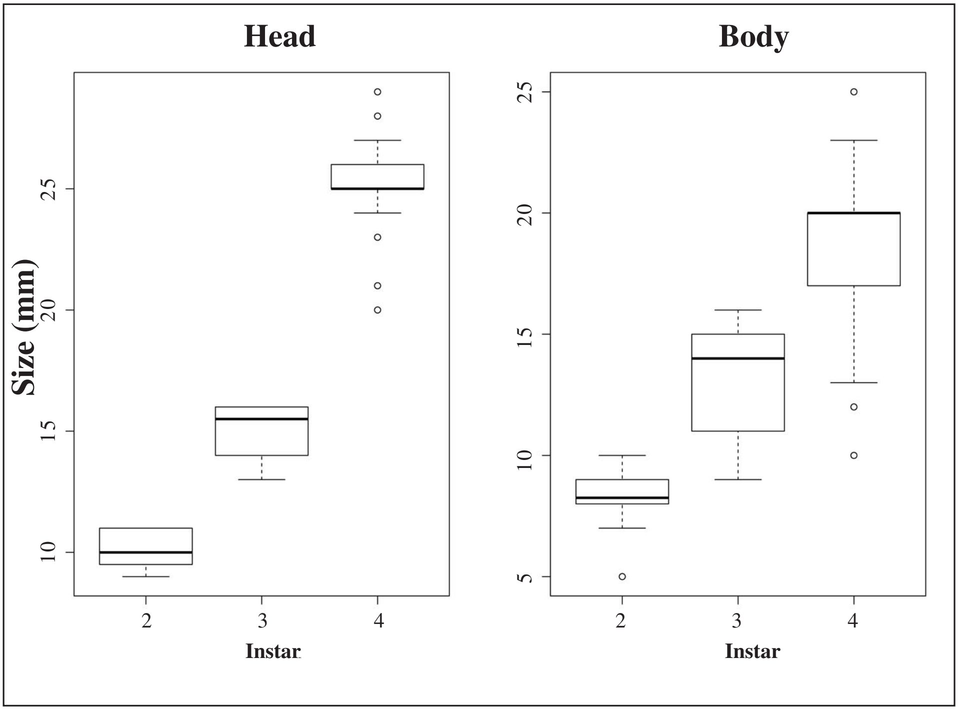

Figure 1.– Size of head and body of larvae in second, third and fourth instars of *Parnassius mnemosyne* larvae sampled in field.

During 2011, we further explored this unexpected phenomenon. Both females (not shown) and males (Figure 3b) showed a diploid chromosome number of 2n = 58 in the mitotic metaphase stage. Cytogenetic analysis of the *P. mnemosyne* karyotype thus confirmed the formerly described haploid chromosome number of n = 29 (FEDERLEY, 1938). This number is by one smaller than in the congeneric species *Parnassius apollo* (Linnaeus, 1758), in which sex chromatin is developed (TRAUT & MAREC, 1996). The chromosome number of n=30 was also reported for *Parnassius smintheus* Doubleday, [1847] and for many other papilionids (see ROBINSON, 1971), and it appears to be a modal chromosome number for the Papilionidae (EMMEL *et al.*, 1995). Subsequent analysis of sex chromosomes in pachytene oocytes of females revealed a pair of neo-WZ sex chromosomes originated by fusion of the ancestral sex chromosomes with a pair of autosomes, resulting in the reduced number of chromosomes (Figure 3c, d). This fusion is obviously responsible for the sex chromatin absence in female polyploid nuclei. Since it was impossible to determine the sex of larvae by this predicted trait, we sexed the 2011 larvae by dissection of ovaries or testes. We found 16 females (6 penultimate and 10 last instar) and 6 males (3 and 3) amongst these larvae. Dissection of fixed specimens made such determination impossible in 2010 larvae.

Discussion

P. mnemosyne larvae were found mostly at sunny woodland margins and clearings in sparse deciduous woodland which is the known habitat for the adults (LUOTO *et al.*, 2001; KONVIČKA *et* *al*., 2001; BENEŠ *et al*., 2002). Such woodland openings might be suboptimal for the host plant, which flourish in early spring before canopy trees flush (STOLLE 2004), and therefore prospers under close canopy, probably owing to lack of competition with later herbal aspect. Adult butterflies, flying when the foliage is already developed, avoid entering close canopy conditions (KONVIČKA *et al*., 2001) and oviposition at woodland margins suggests that ovipositing females seek for a balance between larval demands and sun for their activity (VALIMAKI & ITAMIES, 2005).

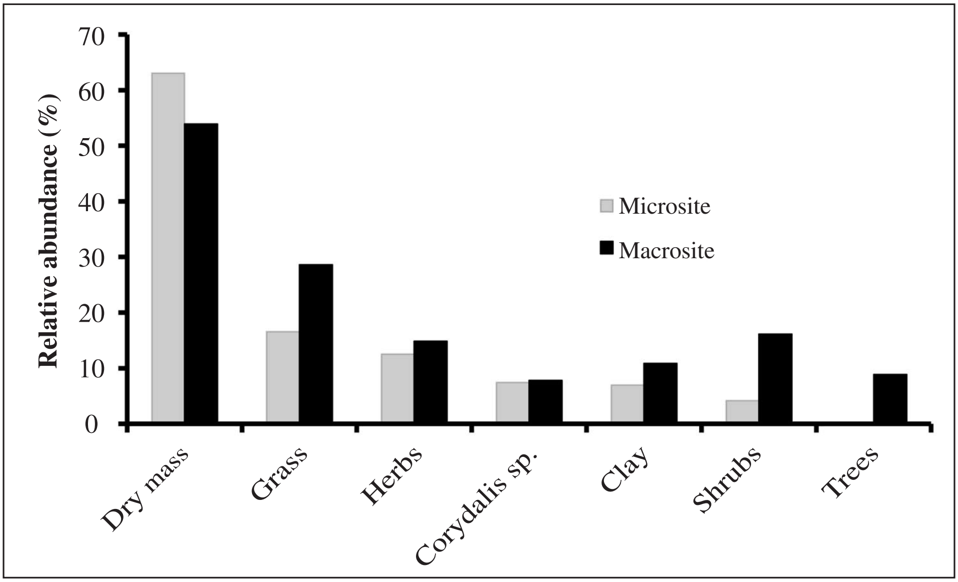

Figure 2.– Ground coverage at micro and macrosites around recorded *Parnassius mnemosyne* larvae. Note that sum of relative abundance is not 100%, estimations were done by eye.

Sex of higher instars larvae of *P. mnemosyne* can be determined by dissection of gonads but not by the presence of sex chromatin, as we expected. The most likely reason for the sex chromatin absence is that *P. mnemosyne* females have a neo-WZ pair of sex chromosomes that arose by fusion of the ancestral sex chromosomes with a pair of autosomes. That the neo-W chromosome do not form sex chromatin body (or bodies) in female polyploid nuclei is probably due to the opposite tendencies of the ancestral W heterochromatin “stickiness” and the property of newly-acquired autosomal euchromatin to disperse (TRAUT *et al*., 1986; MAREC & TRAUT, 1994). It has recently been found that such sex-chromosome-autosome fusions are more common in Lepidoptera than previously thought (reviewed in NGUYEN & CARABAJAL PALADINO, 2016). However, these fusions do not usually lead to the disappearance of sex chromatin (e.g., see ŠÍCHOVÁ *et al*., 2013). Thus, the total absence of sex chromatin seems to be quite unique in moths and butterflies, and so far has been mainly reported for primitive (non-ditrysian) moths with a Z/ZZ (female/male) sex chromosome system and for a few ditrysian species which secondarily lost the W chromosome (TRAUT *et al*., 2007). In the same time, this phenomenon seems to by phylogenetically quite plastic, as it does not occur in the congeneric (although not immediately related: see MICHEL *et al*., 2008, for a *Parnassius* phylogeny) *Parnassius apollo*. A more detailed sampling of species for studies of chromosome organisations is desirable to resolve, how frequently such neo-WZ chromosomes, or other sex chromosome rearrangements that prevent sexing on the basis of sex chromatin detection, occur in butterflies and other Lepidoptera.

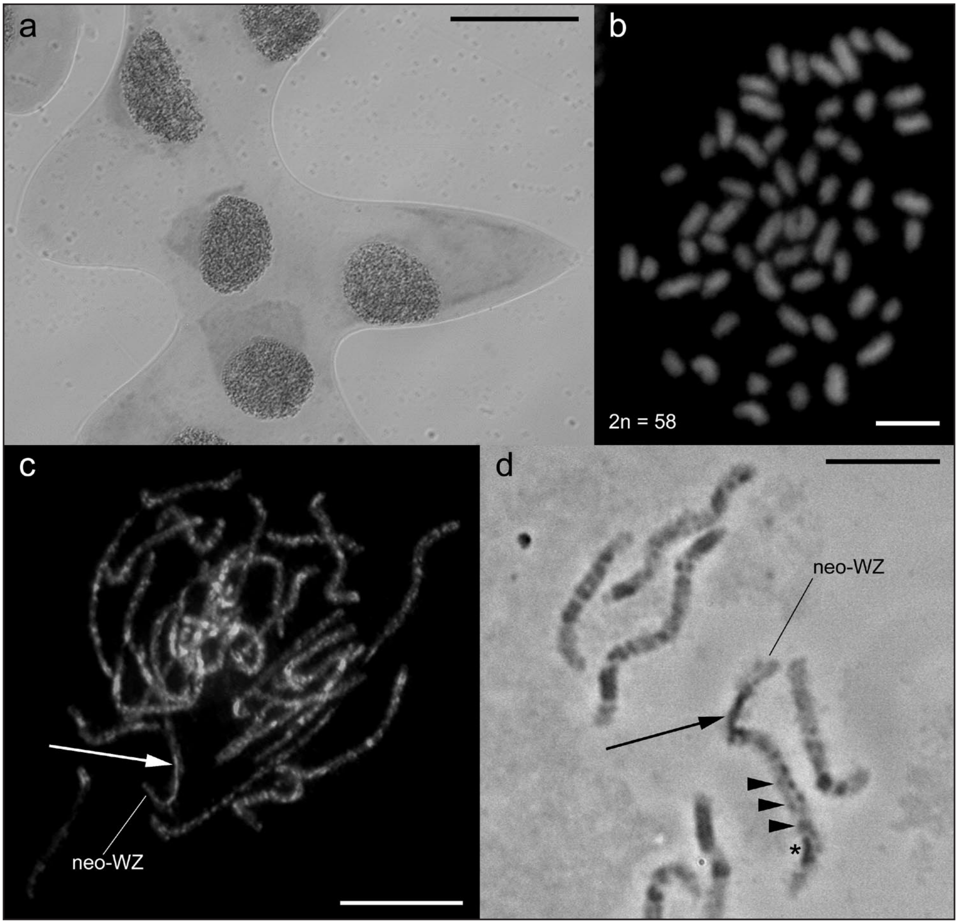

Figure 3.– Cytogenetic analysis of *Parnassius mnemosyne* female larvae. (a) Malpighian tubule showing orcein-stained highly polyploid nuclei without distinguishable sex chromatin body (or bodies). Scale bar = 50 µm. (b) DAPI-stained mitotic spermatogonial metaphase showing a diploid set of 2n = 58 of holokinetic chromosomes. Scale bar = 5 µm. (c) DAPI-stained pachytenne oocyte complement with a haploid number of chromosomal bivalents (n = 29); the neo-WZ bivalent is distinguishable by DAPI-highlighted heterochromatic segment of the ancestral part of the neo-W chromosome (arrow). Scale bar = 20 µm. (d) A part of orcein-stained pachytenne oocyte complement: apart from autosomal bivalents the neo-WZ bivalent shows a large block of heterochromatin near one end (arrow), representing the ancestral part of the neo-W chromosome. The major part of the neo-WZ bivalent is most probably of autosomal origin, where the neo-W chromosome shows several inconspicuous chromomeres (arrowheads) and additional small block of heterochromatin (asterisk) while the neo-Z chromosome has a conspicuous chromomere pattern. Scale bar = 10 µm.

In this study we found surplus of female larvae. This result was, however, based on very small number (n=22) and refers to distinctly protandrous species, in which sexual differences in growth rate are expectable. As we did not find any larvae of first or second instars, it is possible that male larvae were already pupated in time of our survey. Alternatively, the early larvae may be even more difficult to detect than the grown ones. Recall that finding these 22 larvae in 2011 required 9 persons days spent in field, a time budged hardly available, had we not worked in rather large parties, during the short time window during which *P. mnemosyne* develops from eggs to pupae. Early spring aspect is also quite variable among years, which further complicates larval counts in this species. (See SLANCAROVA *et al*., 2015, for another example of early spring larvae survey).

The riddle of the biased *Parnassius* adult sex ratios thus remains unresolved. Several hypotheses are to be posed. There is still a possibility that the primary sex ratio of laid eggs differs from 1:1. The sex of developing eggs can be ascertained either by standard PCR using a molecular marker derived from the neo-W chromosome (FUKOVÁ *et al*., 2009; YOSHIDO *et al*., 2016) or by qPCR to determine a dose, i.e. copy number of a sex-linked gene. The latter approach would require the identification of an autosomal reference gene and a gene located on the neo-Z chromosome. The neo-Z-linked gene to autosomal reference gene dose ratio is expected to be 1:1 in males, which have two copies of the neo-Z chromosome but 1:2 in females with only one copy of the neo-Z (NGUYEN *et al*., 2013). However, reports from captive rearing of other *Parnassius* species suggest that biased sex ratio is not the case here (ADAMSKI, 2004). Alternatively, the biases may arise during larval stage, due to longer time required for the larger-sized females to develop, and resulting higher risks from predation, parasitoids or weather changes. In larvae as elusive as those of *P. mnemosyne*, this hypothesis is difficult to test by field observation. ADAMSKI (2004) suggested a complementary explanation that the risks concentrate to moments after adult hatch from the pupae, as the larger females may require larger time to fully stretch and dry their wings. Finally, there still may be an aspect of less conspicuous female activity, which may bias mark-recapture results. In our earlier mark-recapture study (VLAŠÁNEK *et al*., 2009), when we invested maximum effort to minimise biases due to activity of sexes, we detected a male: female ratio as 1.5-1.6, which is considerable, but lower than in any other studies (VÄISÄNEN & SOMERMA, 1985; KUDRNA & SEUFERT, 1991; VALIMAKI & ITAMIES, 2005).

Resolved or not, the best way how to avoid demographic risks of sex ratio biases in this and other endangered species is to keep their populations sufficiently abundant, because any demography and genetic risks are mainly threatening small populations. For *P. mnemosyne*, this calls for appropriate management of its habitats (cf. KONVIČKA & KURAS, 1999; ŠEBEK *et al*., 2015) over the largest feasible areas.

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