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Dormancy induction in female meadow brown butterflies
Maniola nurag and *M. jurtina*

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Introduction

Day length plays a major role in regulating the seasonal life cycles of insects. It has a strong influence on different aspects of an insect's life, like behaviour, growth and form, reproduction, dormancy and geographical distribution (Beck, 1980). According to Košťál (2006) diapause is a specific sub-type of dormancy. While the generic term dormancy covers any state of suppressed development, diapause is a more profound, endogenously and centrally mediated interruption, usually starting prospectively before the onset of adverse conditions. This study focuses on imaginal dormancy commonly called “reproductive diapause” in butterflies; during this phase adults often still remain active and the key arrest takes place in the maturation of their ovaries (Danks 1987). When referencing other studies the term given in the cited literature is utilized, since it is not always clear what physiological type of dormancy was dealt with.

Dormancy is often regulated by the photoperiod an organism experiences (Tauber & Tauber 1976) because photoperiod is reliable and predictable and therefore a perfect parameter to time essential ecological decisions. Particularly in seasonal environments, the right timing of dormancy is necessary to survive unfavourable conditions and to make sure that the next generation can develop successfully in suitable conditions (Tauber & Tauber 1976, Danks 1987, Müller 1992). In temperate zones most insects develop and reproduce during the summer and become dormant in winter (Masaki 1980): for example adults of *Drosophila melanogaster* undergo a reproductive diapause under short-day conditions (Saunders et al. 1989). In areas where the summers are hot and dry and winters can still be cold, dormancy may occur in both seasons (Masaki 1980). Therefore some insects avoid summer as well as winter, like the cabbage beetle *Colaphellus bowringi*, which aestivates and hibernates as an adult in the soil. Wang et al. (2004) showed that day lengths over twelve hours during 24-h light-dark cycles induced summer diapause in *C. bowringi*.

In butterflies dormancies induced by changes in photoperiod are frequent and can occur in different life stages: e.g. larval summer and winter diapause in the burnet moth *Zygaena trifolii* (Wipking 1995) or pupal diapause in the large white butterfly *Pieris brassicae* (Spieth et al. 2011). Also adult dormancies are reported, though far less frequently. One example is the monarch butterfly *Danaus plexippus*: adults overwinter and it has been found that decreasing day lengths lead to a reproductive diapause in females and similarly a smaller ejaculatory duct mass in males (e.g. Herman 1981, Goehring et al. 2002).

In the family Nymphalidae aestivation behaviour of adult females was described in the Australian genus *Heteronympha*, for example in *H. mirifica* and *H. merope* (Braby 2000,

Edwards 1973). In Europe, particularly in Mediterranean populations of *Hipparchia semele* (García-Barros 1988), and of the genus *Maniola* (Scali 1971, Grill et al. 2006) a delayed oviposition towards the end of summer was reported. For these species it was proven that dormancy is indeed related to a delayed ovarian maturation, but how this is triggered is as yet unknown. In particular, it is not clear which factors determine if females enter aestivation or not and if this can be manipulated by changing environmental parameters. I used females of the genus *Maniola* to investigate the reproductive dormancy in adult butterflies. All *Maniola* species are strictly univoltine and occur exclusively in the Western Palaearctic region. The Meadow Brown *Maniola jurtina* is one of the most common and widespread European butterflies (van Swaay & Warren 1999, Schmitt et al. 2005) but detailed comparative studies about the life history traits of populations from different geographic areas are rare. In contrast to *M. jurtina*, *M. nurag* is endemic on Sardinia where it occurs in close proximity to *M. jurtina* (Grill 2003). Hence it is not only possible to analyse differences between *M. jurtina* populations (Mediterranean and Central European population) but also between the two species from the same origin.

In this study I pose three main questions: (1) Is summer dormancy in female *Maniola* butterflies controlled by day length? Day length was described to influence adult dormancy in different species and in *D. plexippus* hibernation could be induced by decreasing day lengths (Goehring et al. 2002). Therefore I expect that the two *Maniola* species will be affected by different photoperiods, with long-day, that mimics summer conditions, inducing dormancy (aestivation). (2) Can the availability of fresh grass as oviposition substrate and as a food resource for the offspring influence the female's egg-laying strategy? Female butterflies recognise potential host plants by plant-derived volatile chemical cues (e.g. Thompson & Pellmyr 1991, Bruce et al. 2005). Scali (1971) found that in Mediterranean regions *Maniola* butterflies begin egg deposition in autumn, when grasses start to resprout after the first rainfalls following a long summer drought. Thus, I hypothesize that females supplied with fresh grass will start ovipositing earlier, as the chemical cues might stimulate them, while individuals without grass will wait for better conditions. (3) Is total lifetime fecundity influenced by environmentally induced changes in egg-laying strategy or is it determined by species membership or size? Egg numbers often differ between species and a correlation between body size and fecundity has been reported for many insects, including different Lepidoptera (Honěk 1993). As wing size is a widely accepted index of adult body size (e.g. Miller 1977, Van Hook et al. 2012 and references therein), it is used in this study to measure size differences between individuals. Beside these fixed factors, also changes in egg

deposition, like the length of dormancy or of the reproductive period, could have an effect on total egg numbers. Dormant individuals can have disadvantages depending on the duration of dormancy, and trade-offs, for example between energy needed to survive and post-diapause fitness, might be unavoidable (Danks 2006). I therefore expect that lifetime fecundity primarily depends on species membership and size, but that it will be negatively affected by a prolonged dormancy.

To test these hypotheses *M. nurag* and *M. jurtina* females from Sardinia and *M. jurtina* from Austria were captured in the field and subsequently tested under different light and grass conditions in the lab. Eventually, I collected the following data for each individual: (a) duration of dormancy (= number of days from capture to first egg laid, definition of “first egg laid” see Material and Methods), (b) length of reproductive period (= time span between first and last egg laid), (c) lifespan (= number of days from capture to death), (d) fecundity (= total number of eggs laid, not accounting for mature oocytes that may have been left in the ovaries upon a female's death), and (e) wing size (= length of the discoidal cell of the hind wing).

Material and Methods

Study organisms

Maniola jurtina (Linnaeus 1758) and *M. nurag* (Ghiliani 1852) are two of the seven European *Maniola* species. Both species can be found in Sardinia, but while *M. nurag* is endemic to the island, *M. jurtina* is widespread over Europe. They are univoltine and protandric, i.e. the males hatch at least one week earlier than the females (Ebert & Rennwald 1991, Hesselbarth et al. 1995, Grill 2003).

On Sardinia the two species are to a certain degree spatially separated by the elevation of their preferred habitats and temporally by different emergence times of adult butterflies, but they are often found in mixed, occasionally hybridizing populations. *M. nurag* butterflies occur from May to September and *M. jurtina*, from late April to September (Grill 2003, Grill et al. 2006). Depending on altitude, *M. jurtina* flies commonly 1-2 months later in more northern regions of Europe, with a peak in July and August (Ebert & Rennwald 1991). In warm localities of Austria the first males can be found from late May (Grill et al. 2013).

In Sardinia the females of both species perform an imaginal dormancy during summer (aestivation: Scali 1971, Grill et al. 2006). This behaviour has also been described for other *Maniola* species that live in hot Mediterranean habitats, like *M. megala*, *M. telmessia*, *M. halicarnassus* and *M. chia* (van Oorschot & van den Brink 1992, Hesselbarth et al. 1995).

Caused by the fact that the males hatch earlier, mating occurs soon after eclosion of the females (Brakefield 1982a+b) in early summer, hence in Mediterranean populations before aestivation, even though the maturation of the female's ovaries is not yet completed (Scali 1971): while males are sexually mature right upon eclosion, females have unripe ovarioles, but the normal development of other parts of the genital apparatus allows mating and storage of a spermatophore after hatching. By performing the dormancy Mediterranean *Maniola* females avoid that their offspring has to face problems caused by heat and drought in summer, like the lack of larval food resources. Females survive the hottest part of the summer by reducing their activity: they fly less and actively seek shady places. At the end of summer their ovarioles mature and the eggs are fertilized at egg-laying with the sperm stored weeks or months earlier (Scali 1971, García-Barros 1987). In Mediterranean populations egg deposition starts in September on grasses and females can be found until then, while males normally do not occur anymore. They disappear soon after successful mating, but in some continental populations of *M. jurtina* a few males can be found until September (Ebert & Rennwald 1991). In more northern and therefore cooler and more humid regions where food for larvae is abundant in summer *M. jurtina* commonly do not perform an aestivation. In Central Europe a delayed egg deposition could further be problematic as larvae might not grow enough before hibernation (Goulson 1993). A few exceptions have been observed in Germany by H. Steffny and there are probably some more, but these are in the minority (Ebert & Rennwald 1991).

Experimental design

During two weeks of field work in Sardinia (29.5. – 12.6.2012) females of *M. nurag* (abbreviated “N” hereafter, 57 individuals) and *M. jurtina* (“J”, 56 individuals) were collected with a hand-held net at different sampling sites in the mountains (between 500 and 950m a.s.l.). *Maniola* species are protandric and males are actively searching for freshly eclosed females, with which they mate within a few hours (A. Grill, unpublished data). Therefore, I assumed that the collected butterflies were most probably all mated. To investigate the influence of geographic provenance within a species, *M. jurtina* females were captured in Austria (National Park Donauauen, 20.6. – 3.7.2012) to represent a Central European population (“JOE”, 36 individuals).

The butterflies from the three populations (N, J and JOE) were further separated into three treatment groups (Table 1). Butterflies were kept under controlled conditions in two climate chambers (Binder, KBW 400, E5.1) under 24-h light-dark cycles. Females were kept individually in transparent plastic containers (1 litre volume), which were closed with a gauze

lid and had humid paper towels at the bottom. Sugar water (5% fructose solution) as nutrition was available ad libitum. Whilst temperature was the same in both settings (24°C during light, 16°C during dark), the photoperiod was set differently to examine the effect of day length on aestivation behaviour: long-day with L16:D8 and short-day with L11:D13. The long-day treatment mimicked natural conditions during summer, while the short-day treatment mimicked autumn conditions, when Mediterranean females in the wild start egg deposition.

To investigate the influence of the availability of a food resource for the offspring two groups were offered fresh grass (*Lolium perenne*) daily. There were three treatments per population, so I analysed nine groups in total (Table 1): long-day + fresh grass (T1: N1, J1 and JOE1), long-day without grass (T2: N2, J2 and JOE2), and short-day + fresh grass (T3: N3, J3 and JOE3). Females that died a few days after capture or during transport, probably due to stress, were excluded from all analyses: 7 *M. nurag*, 2 *M. jurtina* from Sardinia and 2 *M. jurtina* from Austria.

Table 1: Number of analysed individuals per group

Origin	Species	Group	Treatment	Individuals
Sardinia	<i>Maniola nurag</i>	N1	long-day + fresh grass	19
		N2	long-day, no grass	16
		N3	short-day + fresh grass	15
Sardinia	<i>Maniola jurtina</i>	J1	long-day + fresh grass	19
		J2	long-day, no grass	19
		J3	short-day + fresh grass	16
Austria	<i>Maniola jurtina</i>	JOE1	long-day + fresh grass	12
		JOE2	long-day, no grass	10
		JOE3	short-day + fresh grass	12

I checked the containers daily for eggs, removed and counted them. Eggs were reared until larvae hatched, to control if the females really had been mated and if they showed a different egg-laying behaviour when reared unmated. In all, from 117 of 130 females (=90%) larvae hatched and no unusual behaviour could be observed when this was not the case. It can largely be excluded that females had laid eggs before they were caught, because they were captured soon after eclosion, right at the beginning of the flight season. None of the butterflies started to lay eggs immediately after capture, confirming this assumption.

As an index for wing size the length of the discoidal cell on the ventral side of the hind wing was measured after the butterfly had died. It is more common to use the length of the forewing, but this was not possible, because the forewings of most individuals were too

damaged for a proper measurement. In contrast, the discoidal cell was well preserved and could easily be measured through a stereo-microscope.

Data analysis

I defined the date of the “first egg laid” to the first day of a five day period, during which an individual laid at least four eggs. In doing so, a few cases of single eggs that were laid long before all other eggs were excluded, as these singleton eggs were apparently not the start of the true reproductive period. For the total lifetime egg number, however, these occasional outlier eggs were taken into account. Individuals that laid fewer than 20 eggs in total were considered for analyses of the duration of dormancy and lifespan, but were excluded from all egg-related analyses (reproductive period, total number of eggs, cumulative number of eggs; 9 ind.). Females that did not lay any eggs were only considered for lifespan analysis (8 ind.).

To test which factors influence lifespan and egg-laying strategy (dormancy, reproductive period, total number of eggs) I performed GLMs. Data were split into two datasets, which I analysed separately: one for Sardinian females (N and J), and one for *M. jurtina* females (J and JOE). As there was only a “no grass”-treatment at long-day, the ANOVAs were performed pairwise between long-day + grass (T1) and short-day + grass (T3), to test the effect of day length, and between long-day + grass (T1) and long-day no grass (T2), to test the grass-effect respectively.

On the basis of mean egg numbers laid each day since capture, a curve of the relative cumulative number of eggs was calculated for each experimental group, showing the increasing percentage of laid eggs. In this way it is possible to get a better insight into the different oviposition strategies with regard to their timing.

Data were not (reproductive period, wing size), square-root (total number of eggs) or log (duration of dormancy, lifespan) transformed to ensure normal distribution. The statistical analyses were run in Microsoft Excel (2003) and RStudio (v. 0.97.173, R Core Team 2013).

Results

Dormancy and lifespan

As expected day length had a pronounced effect on lifespan and timing of oviposition of Sardinian females. Long-day treatment led to delayed egg-laying (viz. longer reproductive dormancy) and thereby an extremely prolonged life (Figure 1, A+B). Mean dormancy in long-day treatments was two to three times longer (2-3 months) than short-day dormancy (3-4 weeks). The same was true for lifespan (4 months as opposed to 2 months).

Austrian *M. jurtina* did not perform a reproductive dormancy under any of the treatments and behaved similarly to short-day females from Sardinia (Figure 1, A+B). Whilst mean lifespan was quite the same for all Austrian groups and resembled the Sardinian short-day treatments (on average about 8 weeks), they started slightly earlier with oviposition (after about 2-3 weeks). Many Sardinian long-day females exceeded all expectations with regard to the length of the dormancy period and their total lifespan. One *M. nurag* female, N29 (T1, long-day + grass), started laying eggs after 185 days and lived for 246 days, which means that it lived at summer-like temperatures for more than eight months.

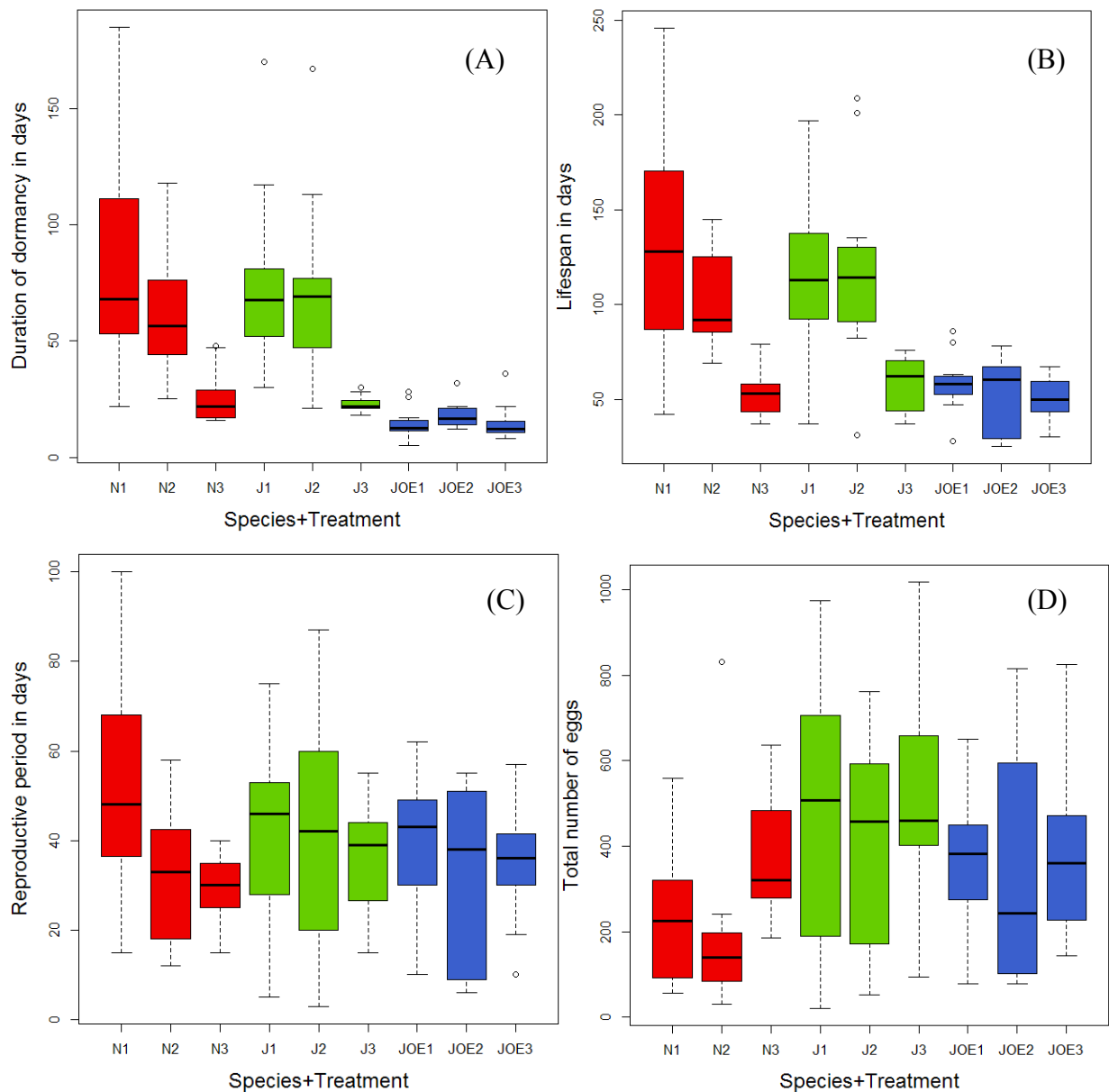


Figure 1: Boxplots of duration of dormancy (A), lifespan (B), reproductive period (C), and total number of eggs (D) across species and treatments; red = *M. nurag* (N), green = *M. jurtina* from Sardinia (J), blue = *M. jurtina* from Austria (JOE); boxplot: band = median; box = interquartile range (IQR); whiskers = lowest/highest data points within 1.5 IQR; spots = outliers.

GLMs proved the significant influence of day length and geographic provenance on duration of dormancy and lifespan (Table 2). *M. nurag* and *M. jurtina* from Sardinia showed the same reaction to the photoperiod treatment (dormancy: day length×species interaction, $F = 0.007$, $p = 0.932$), while there was a significant difference between the Mediterranean and the Central European *M. jurtina* populations (day length×population $F = 26.764$, $p < 0.001$). The availability of fresh grass had no effect on dormancy or lifespan in any of the groups. *M. nurag* individuals were with 8.6mm (mean, SD = 0.49, N = 50) length of the discoidal cell significantly smaller than *M. jurtina* females (mean = 9.9mm, SD = 0.62, N = 88), but size did never significantly influence dormancy or lifespan.

Table 2: Results of GLMs showing the effect of grass availability, day length, body size and species/population on duration of dormancy and lifespan; all values marked in bold remained significant at $p < 0.05$ after application of a table-wide false-discovery rate correction.

Analysed group			Dormancy			Lifespan		
			df	<i>F</i>	<i>p</i>	df	<i>F</i>	<i>p</i>
Sardinia	long day	grass	1	1.963	0.166	1	1.174	0.282
		species	1	0.055	0.816	1	0.033	0.856
		size	1	0.005	0.945	1	2.076	0.154
		grass×species	1	0.451	0.504	1	0.403	0.528
Sardinia	+ grass	day length	1	107.97	< 0.001	1	79.41	< 0.001
		species	1	0.264	0.610	1	0.018	0.895
		size	1	1.092	0.300	1	2.935	0.092
		day length×species	1	0.007	0.932	1	1.197	0.278
<i>M. jurtina</i>	long day	grass	1	0.993	0.323	1	0.165	0.686
		population	1	141.98	< 0.001	1	51.98	< 0.001
		size	1	0.085	0.772	1	0.667	0.418
		grass×population	1	2.247	0.140	1	0.358	0.552
<i>M. jurtina</i>	+ grass	day length	1	44.74	< 0.001	1	37.10	< 0.001
		population	1	115.02	< 0.001	1	27.96	< 0.001
		size	1	0.544	0.464	1	1.06	0.308
		day length×population	1	26.76	< 0.001	1	10.97	0.002

Egg numbers and dynamics of egg-laying

The duration of the reproductive period (Figure 1, C) was the same in both species and was neither significantly influenced by day length nor by the availability of grass: one might only

guess a trend, with females of T1 (long-day + fresh grass) having the longest reproductive period, ahead of T2 (long-day no grass) and T3 (short-day + fresh grass): $T1 > T2 > T3$. However, this could not be confirmed through statistical analyses.

Total egg numbers (Figure 1, D) differed between the two species: *M. nurag* laid on average substantially fewer eggs (mean = 273, SD = 187, N = 38) than *M. jurtina* (mean = 425, SD = 247, N = 83). When comparing Mediterranean and Central European *M. jurtina* populations it turned out that Sardinian females laid more eggs: J mean = 467 (SD = 264, N = 50) > JOE mean = 361 (SD = 204, N = 33). The maximum number of eggs was deposited by a Sardinian *M. jurtina* with 1018 eggs in total. In Figure 1 (D) there is an outlier at N2 (832 eggs), which in reality was probably a misidentified *M. jurtina* female, and 636 was the confirmed maximum number of eggs laid by *M. nurag*.

Total egg numbers of the species were influenced in a different way by the tested factors (Table 3). Body size played a minor role and did not modulate egg numbers significantly. For *M. nurag* the length of dormancy had the biggest impact on egg numbers, followed by the duration of the reproductive period, whereas it is vice versa in Sardinian *M. jurtina* populations. In both species the length dormancy had a negative and the duration of the reproductive period a positive effect. The variation of intensity of the responses can be seen in Figure 1 (D): egg numbers of *M. nurag* decrease significantly under long-day conditions. Austrian females were only affected by the duration of the reproductive period.

Table 3: Results of GLMs showing the effects of body size, duration of dormancy and length of reproductive period on number of eggs laid by *M. nurag* (N=38) and *M. jurtina* (Austria: N=33, Sardinia: N=50); all values marked in bold remained significant at $p < 0.05$ after application of a table-wide false-discovery rate correction.

Origin	Species		<i>t</i>	<i>p</i>
Sardinia	<i>M. nurag</i>	size	1.912	0.064
		dormancy	- 3.879	< 0.001
		reproductive period	2.555	0.015
Sardinia	<i>M. jurtina</i>	size	1.974	0.054
		dormancy	- 2.781	0.008
		reproductive period	5.694	< 0.001
Austria	<i>M. jurtina</i>	size	1.944	0.062
		dormancy	- 0.927	0.362
		reproductive period	5.175	< 0.001

By taking a closer look at the cumulative number of eggs and therefore oviposition strategies (Figure 2), it can be clearly seen that again Austrian *M. jurtina* (JOE1, JOE2, JOE3) and the short-day treatments of Sardinian females (J3, N3) grouped together, while females in long-day treatments showed very different dynamics of egg-laying (J1, J2 and N1, N2). These females started later with oviposition, but it also took them longer until they had laid all eggs. For *M. jurtina* individuals it did not make a difference if fresh grass was available or not. Nevertheless, it did make a difference in *M. nurag*: the group with fresh grass (N1) needed longer to fully deposit their egg load than the one without access to grass (N2).

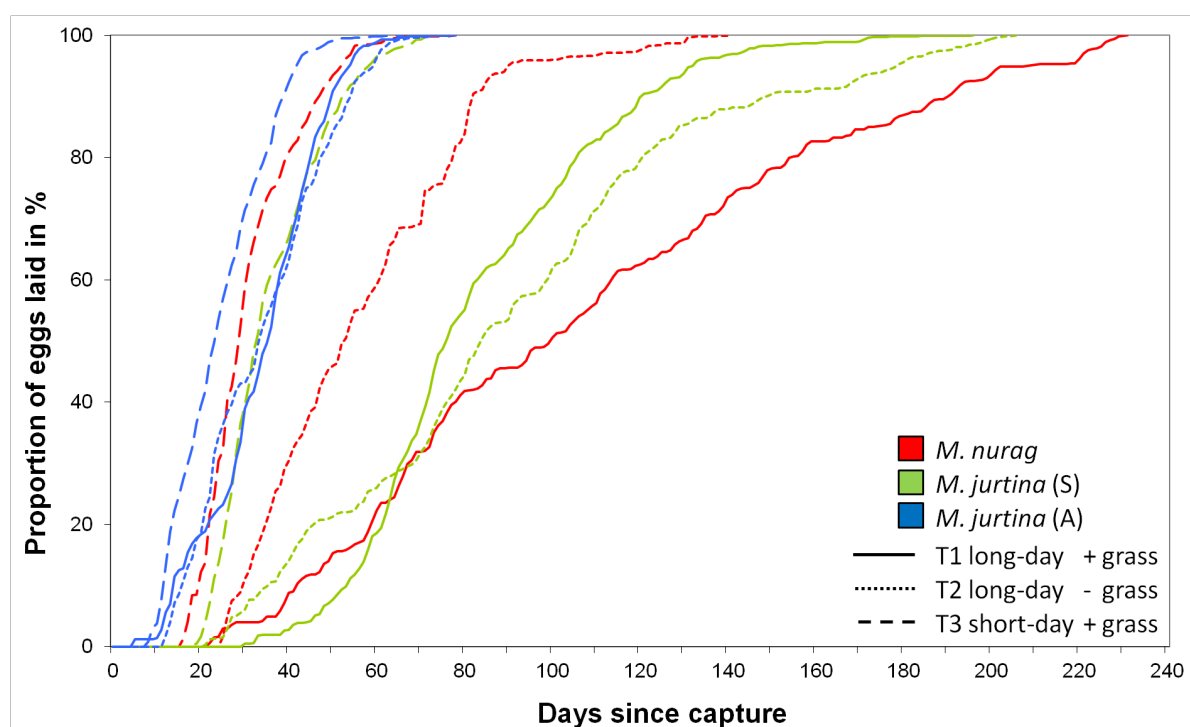


Figure 2: Cumulative number of eggs laid since capture for the nine groups; red = *M. nurag* (N), green = *M. jurtina* from Sardinia (J), blue = *M. jurtina* from Austria (JOE); continuous line = T1 (long-day + grass), dotted line = T2 (long-day no grass), dashed line = T3 (short-day + grass).

Discussion

Is summer dormancy in female Maniola butterflies controlled by day length?

As expected long-day conditions, imitating summer photoperiods, led to a prolonged aestivation and an extremely delayed onset of oviposition in Sardinian females, while individuals in the short-day treatment started laying eggs already after three weeks, thus much earlier than in natural populations under a Mediterranean climate regime (Figure 1 A). This was the case in the island endemic *M. nurag* as well as in the widespread *M. jurtina*: the two species were influenced in the same way. The similar egg-laying strategy of Sardinian *M. nurag* and *M. jurtina* females show that they are clearly well, and in the same way, adapted to

the Mediterranean habitat by undergoing a reproductive dormancy. In Austrian populations of *M. jurtina*, it was not possible to induce a reproductive dormancy through any of the treatments. These populations did not react to the photoperiodic regimes and behaved like Sardinian short-day females. Such a pronounced difference between the two *M. jurtina* populations was unexpected. This leads to the conclusion that in *Maniola* females oviposition strategy depends on geographic provenance rather than on species membership.

A comparable distribution of different diapause behaviour was reported by Spieth et al. (2011) in the large white butterfly *Pieris brassicae*. In contrast to *Maniola* species, *P. brassicae* is multivoltine and produces, under optimal conditions, up to nine generations per year. What the species have in common is that normally only some Mediterranean populations aestivate. In *P. brassicae* in general just hibernation of pupae occurs and unfavourable summer conditions are avoided by migration, but in populations from the southern Iberian Peninsula pupae may also aestivate. In Spieth et al.'s experiments, only the populations with aestivation potential responded to laboratory long-day conditions by undergoing summer diapause. By crossing *P. brassicae* individuals from aestivating with non-aestivating populations from Denmark it was discovered that in the F1 generation aestivation was not or scarcely present. When crossing with non-aestivating individuals from Southern France the photoperiodic response of F1 hybrids was intermediate between that of their parents, concluding that the genetic precondition for aestivation decreases from south to north. With two inbred lines this study also showed that the heritability of aestivation and of non-aestivation is equal. Following these results of Spieth et al. (2011) one could test if the ability to aestivate is heritable in *Maniola* butterflies, by crossing Austrian and Sardinian *M. jurtina* individuals.

Sardinian short-day and Austrian females behaved similarly, but the Mediterranean individuals started on average a little later with egg deposition. Scali (1971) described that the dormancy in *M. jurtina* is connected to a delayed maturation of the female's ovaries. This explains why Sardinian females could never oviposit directly after capture: while most of the Austrian females were ready for oviposition after two weeks, individuals from the Sardinian population had a deficit, probably due to their unripe ovaries. I assume that the development of the ovaries was accelerated through short-day, mimicking autumn conditions. Although Austrian females did not perform a reproductive dormancy, they also showed a delayed onset of egg deposition (two weeks). Timing of oviposition of the Austrian females in this experiment resembled that of Pannonian *M. jurtina* populations described by Grill et al. (2013): they started a little later than females from Alpine habitats, but there was no

significant difference. By repeating my experiments with *M. jurtina* populations along a latitudinal cline, it would be possible to find out where and how their oviposition strategy changes.

The facts that *Maniola* females from Sardinia showed the same aestivation response, while there was a pronounced difference between the two *M. jurtina* populations and that Austrian and Sardinian females could not be synchronised under the same conditions lead to the assumption that there are genetic differences in the reaction norms in *Maniola*. The phenotypic expression of a genotype can differ under a range of climatic conditions and therefore it is possible that the intraspecific difference between geographically isolated populations is bigger than between two species from the same origin.

As day length stayed fixed for the whole experiment, the long-day females terminated aestivation spontaneously after some time. This is compatible with findings in *D. melanogaster*: under diapause inducing short-day conditions all flies terminated diapause after six weeks within one week (Saunders et al. 1989). A critical, in the present study shorter, photoperiod as a cue to end was not necessary to terminate dormancy, but my results show that the duration of dormancy among long-day females was highly variable. Considering further that increased body mass normally correlates with greater reserves (Hahn & Denlinger 2007), one can assume that bigger individuals might be able to aestivate for longer periods. Nevertheless, size could not be confirmed as an explanation for the high variation: wing size had no effect on duration of dormancy. Variation in dormancy duration was less in short-day females, leading to conclude that with decreasing day lengths or a transfer to short-day conditions the beginning of egg deposition would probably become more synchronised.

The delayed oviposition led to an extremely prolonged life in some of the long-day females. The majority of Lepidoptera are short-lived and as adults they typically do not live longer than a few weeks (Carey 2001). But there are some exceptions: a study from Mollemann et al. (2007) on fruit-feeding butterflies in a tropical forest (Uganda) included the longest active lifespans of butterflies that have been published, with up to 293 days in *Euphaedra medon*. Of 62 observed species twelve lived longer than five months and five species lived longer than seven months. Long-day females of *M. jurtina* and *M. nurag* reached on average an adult life span of 116 days, i.e. nearly four months. But some females lived longer and one survived for eight months (246 days). Beck and Fiedler (2009) found that fruit-feeding butterflies live longer than nectar feeders, hence it is remarkable that in this experiment the lifespans of Sardinian long-day females are close to those of the tropical species. As sugar water was

available, females were not dependent on their reserves and therefore it was possible for them to reach this age.

Can egg deposition behaviour be influenced by the availability of fresh grass as a food resource for the offspring?

Earlier studies described that *M. jurtina* started with egg deposition after the first rainfalls in autumn, when grasses start to sprout again after summer drought (Scali 1971). For butterflies in the seasonal tropics it was suggested to be advantageous to spend the dry season as an adult, because adult food remains available, while larval resources disappear (Jones 1987). Hence it appeared quite reasonable that in Mediterranean females the availability of fresh grass could stimulate them to start earlier with oviposition. The results of this study do not support this: neither in Sardinian nor Austrian females had the availability of fresh grass a detectable effect on the duration of dormancy (Table 2, Figure 1, A). Rather, the opposite trend might be seen at the length of the reproductive period and the egg-laying curves (Figure 1 C; Figure 2): especially in *M. nurag* females in the long-day treatment with grass the oviposition period took longer than in the ones without grass. But this non-significant result could be an artefact, as *M. jurtina* did not show a similar behaviour.

A few studies exist about the influence of food quality on entering larval diapause. For example in the moth *Choristoneura rosaceana* low-quality diet can favour diapause induction (Hunter & McNeil 1997). Only some studies where the abundance of larval food resources perhaps play a role can be found. The afrotropical butterfly *Bicyclus anynana* shows a seasonal polyphenism with a wet and a dry season form. During the warmer wet season food is abundant and reproduction and larval growth are favoured. In the cooler dry season, when food is scarce, adults survive for months and reproduce with the onset of rains (Roskam & Brakefield 1999, Brakefield et al. 2007). It was shown that temperature is the major cue modulating the pathway of development, but that also rainfall, which might be a predictor for larval food resources, can be a determinant (Roskam & Brakefield 1999). Braby (1995) further suggested, that it might be advantageous for Australian satyrines to spend the late dry season as an adult (diapausing or with mature eggs) as new grown grasses at the beginning of the wet season can be utilized faster. However, experiments on how the availability of food resources for offspring can change dormancy decisions in the adult stage do not exist.

What could have been a problem is that the individuals of the treatments with and the one without grass were kept in the same climate chamber, because only two such chambers were available for this study. I tried to separate them by putting them on different levels, so that the

females without grass at least could not perceive visual cues. Nevertheless, volatiles from the grass, which could have had an influence on *Maniola* females, were probably present in the whole climate chamber, therefore reducing a potential differential effect. For more detailed studies a complete separation would be recommended. I observed that females in the grass treatments did not only lay eggs on the grass, but also randomly on the walls of the plastic container and the net, so maybe in the end they are not affected at all by the presence of grass. As females laid eggs whether grass was available or not, egg-laying in *Maniola* butterflies is not dependent on the presence of larval food resources.

Is fecundity influenced by changes in egg-laying strategy or is it determined by species membership or body size?

Beside the differences in dormancy behaviour, I also observed variation in the total numbers of eggs being laid: *M. jurtina* from Sardinia laid the most eggs, followed by *M. jurtina* from Austria, and *M. nurag* laid the smallest number of eggs (Figure 1 D).

One *M. jurtina* female laid 1018 eggs in total, which was unexpected as egg numbers in the literature are often much lower. Only García-Barros (1987) described 900 eggs or more as potential fecundity in *M. jurtina*. Nevertheless, mean egg numbers of *M. jurtina* in this study are close to numbers given in different publications. While Brakefield (1982b) reported 179 as mean egg number for *M. jurtina* from the Isles of Scilly (Great Britain), García-Barros (1987) found on average 395 eggs in central Spain and Grill et al. (2013) 200-350 eggs in females from Krk and Austria, i.e. the same order of magnitude as I got in my experiments. Considering my results with a mean egg number of 467 in Sardinian and 361 in Austrian *M. jurtina* females, it seems like fecundity depends on latitude, with southern females laying more eggs. This disagrees with findings in another satyrine butterfly: even though *H. semele* shows a comparable delayed ovarian maturation as *Maniola* butterflies in southern populations, a comparison between Spanish and Swedish populations demonstrated that the northern females laid more, but smaller eggs (García-Barros 1988, 1992).

In contrast to the results of Calvo (2005) with *Streblote panda* (a moth incapable of acquiring nutrient resources in the adults stage, thus a capital breeder sensu Tammaru & Haukioja 1996), fecundity is not determined unchangeably in the larval stage in *Maniola*. The close correlation between pupal weight and fecundity among *S. panda* females (see also Honěk (1993) and Loewy et al. (2013) for a summary of similar studies) could not be found in the income breeding *Maniola* butterflies. *M. jurtina* (mean 9.9mm) and *M. nurag* (8.6mm) individuals clearly differed in size, but their variation in egg numbers was more related to

differences between species rather than intraspecific size variation. Within the species a size-effect on fecundity could not be verified as only a non-significant positive trend could be observed.

In females from Sardinia the duration of dormancy and the length of the reproductive period had a significant impact: the earlier the females started and the longer they laid eggs, the higher was their lifetime fecundity. While in *M. jurtina* the reproductive period was the most important factor, fecundity in *M. nurag* was more influenced by the duration of dormancy. Possible negative impacts of adult diapause and a long life on reproduction in insects were reviewed by Tatar and Yin (2001). Such trade-offs do not follow simple expectations and records could also depend on laboratory procedures (Danks 2006). Hodkova (2008) described this kind of a trade-off in the linden bug *Pyrrhocoris apterus*, where short-day promoted a diapause and concomitantly lead to a lower fecundity. Comparing the response of the widespread *M. jurtina* and the island endemic *M. nurag*, it turns out that the fecundity of *M. jurtina* did not suffer under dormancy, caused by long-day, whereas egg numbers in *M. nurag* decreased significantly (Figure 1 D). The egg numbers of non-dormant *M. nurag* females (short-day) were still lower compared to *M. jurtina* from Sardinia, but they resembled the numbers of the Austrian population. Whether the two species on Sardinia pursue different oviposition strategies could be further analysed by measuring egg sizes. Maybe *M. nurag* lays fewer but bigger eggs after passing through reproductive dormancy. Nevertheless, it seems like the endemic *M. nurag* reacts more sensitively to changes of the environmental conditions. Trade-offs between egg size and egg numbers have been reported several times, for example in different *Lycaena hippothoe* populations (Fischer and Fiedler 2001, 2002). Additionally to the size-number trade-off, the hatching success was higher in the *L. hippothoe* population laying the biggest eggs. But trade-offs are often restricted to stressful situations (Nylin and Gotthard, 1998). As the females in this experiment did not experience food or drought stress (sugar water was available at all times) possible trade-offs might not have occurred anyway.

Besides possible trade-offs, there still remain some other interesting aspects to be investigated. One is the influence of changing photoperiods. In the monarch butterfly *D. plexippus* more individuals were observed to hibernate under decreasing day lengths than under constant short-day (Goehring et al. 2002). Nylin (2013) assumed that in species with adult diapause the sensitivity to the direction of change might be more important, as the same day lengths are present before and after summer.

Another aspect is that this study only concentrated on day length experienced by the adults, as already hatched wild females were collected. Therefore larvae and pupae had lived under natural conditions which obviously differed between Sardinia and Austria. The sensitive stage and the stage when dormancy occurs need not be the same: in the tropical nymphalid butterfly *Hypolimnas bolina* short-day conditions experienced by the larvae lead to a facultative diapause in adult females, enduring the dry season (Pieloor & Seymour 2001). A similar picture was found by Fujita et al. (2009) in the nymphalid *Polygonia c-aureum*, where a summer (non diapausing) and an autumn (diapausing) morph can be distinguished. Which morph develops depends on temperature and photoperiodic regime experienced during the larval stage. But this study also described that diapausing behaviour could be changed in adults: long-day lead to an earlier production of eggs in the autumn morph, while short-day delayed ovarian maturation in the summer morph.

Further, the effect of temperature was neglected in this study, as it was the same in all treatments. However, it is known that temperature can modify the response to a certain day length in other insects. In *Pieris melete* photoperiodically controlled winter and summer diapause occur in pupae: whereas hibernation is compensated by temperature, aestivation is strongly dependent on it (Xiao et al. 2009). An effect of the combination of photoperiod and temperature cannot be excluded in *Maniola* and a potential influx can hardly be predicted.

Keeping these aspects in mind further studies could be performed testing for example if it is possible to induce reproductive dormancy in Austrian *M. jurtina* females in any way: for example by increasing day lengths experienced by the adults or when larvae or pupae are raised under different photoperiodic conditions. Also the influence of a combination of day length and temperature (during different life stages) on oviposition could be tested to obtain first insight what consequences climatic changes (climatic warming) could have for the life history of *Maniola* butterflies.

Butterflies can respond in two different ways to climatic warming: they can prolong dormancy or they increase the number of generations. I would expect that Mediterranean *Maniola* butterflies would react with a longer reproductive dormancy, as my results show that females can survive for some months and reproduce successfully after aestivation. Further, reproductive dormancies caused by a delayed ovarian maturation were also reported for other Satyrinae from hot habitats, namely *Hipparchia semele*, *Chazara briseis* and *Kanetisa circe* (García-Barros 1988, 2002). However, in experiments following my study it was possible to induce a second generation within one year in both *Maniola* species, due to faster development under short-day/autumn conditions in the laboratory (A. Grill, unpublished

data). Even though *Maniola* butterflies are univoltine throughout their range, it was possible to induce direct development by the right cues, i.e. univoltinism in *Maniola* is not genetically fixed. In the predatory bug *Picromerus bidens* univoltinism is maintained by two periods of dormancy: a second generation is prevented through a facultative aestivation of early emerged adults, which occurs in addition to an obligatory egg winter diapause (Musolin & Saulich 2000). Altermatt (2010) reported that higher temperatures since 1980 lead to an increasing number of generations in various butterfly species. Maybe Central European *Maniola* populations would show changes in voltinism rather than a prolonged dormancy as assumed for Mediterranean populations.

Through the different dormancy behaviour *Maniola* populations are well adapted to their habitat. Moreover, Sardinian *M. nurag* and *M. jurtina* females behaved in much the same way, while there was a pronounced difference between the two *M. jurtina* populations. This proves that egg-laying strategies depend on geographic provenance and not on species membership. Nevertheless, it was possible to modulate egg-laying, hence climatic changes could affect oviposition strategies and life cycles of *Maniola* butterflies.

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Zusammenfassung

Auf Sardinien machen die Weibchen der beiden Schmetterlingsarten *Maniola nurag* und *M. jurtina* eine Sommerruhe. Sie legen erst im Herbst ihre Eier ab, da das Gras, von dem sich die Larven ernähren, während der Sommermonate vertrocknet ist. Die zwei Arten unterscheiden sich stark in ihrer Verbreitung: *M. nurag* ist endemisch auf Sardinien, *M. jurtina* kommt dagegen europaweit vor und Populationen aus kühleren Regionen durchlaufen normalerweise keine Dormanz im Sommer. Für univoltine Arten, zu denen auch *M. nurag* und *M. jurtina* gehören, ist es überlebenswichtig, den richtigen Zeitpunkt für die Eiablage zu finden. In dieser Studie wurde der Einfluss der Tageslänge und der Verfügbarkeit von frischem Gras als Futterquelle für die Raupen auf die Dormanz und das Eiablageverhalten untersucht. Ich erwartete, dass Langtag und das Fehlen von Gras (= Sommerbedingungen) eine Sommerruhe fördern und die Eiablage verzögern. Für das Experiment wurden 150 Weibchen aus Sardinien (*M. nurag* + *M. jurtina*) und Österreich (*M. jurtina*) gesammelt und in Klimaschränken unter drei verschiedenen Bedingungen gehalten: Langtag (LD = L16:D8) mit Gras, Langtag ohne Gras sowie Kurztag (SD = L11:D13) mit Gras. Langtag-Bedingungen hatten einen verlängernden Effekt auf die Dormanz der sardischen Weibchen: 72 Tage (LD) im Vergleich zu 24 (SD). Auch lebten sie länger: 117 Tage (LD) gegen 56 (SD), ein Weibchen lebte sogar acht Monate lang. Frisches Gras hatte keinen Effekt auf die Dormanz. Die österreichischen Schmetterlinge (*M. jurtina*) reagierten nicht auf die veränderten Bedingungen (Lebensdauer: 54 Tage, Dormanz: 16 Tage): sie verhielten sich wie sardische Kurztag-Weibchen. Ich folgere daher, dass die Sommerruhe der *Maniola*-Weibchen aus dem Mittelmeergebiet durch die Tageslänge induziert wird, während die Photoperiode keinen Effekt auf das Eiablageverhalten mitteleuropäischer Individuen hat.

Abstract

In Sardinia, adult females of the two butterfly species *Maniola nurag* and *M. jurtina* perform a reproductive summer dormancy, i.e. they do not oviposit before autumn. With this strategy they survive summer-drought during which grass, the caterpillar's food resource, is scarce. While *M. nurag* is endemic on the island, *M. jurtina* is widespread over Europe and populations of climatically cooler regions usually do not perform an aestivation. In this study I investigate the influence of day length and the availability of fresh grass as a food resource for the offspring on dormancy induction and egg-laying strategy. I expected that long daylength and the lack of grass (=summer conditions) would promote aestivation and therefore delay egg deposition. For the experiment, 150 females were collected from Sardinia (*M. nurag* + *M. jurtina*) and Austria (*M. jurtina*) and kept under three controlled conditions: long-day (LD = L16:D8) with fresh grass, long-day without grass, and short-day (SD = L11:D13) with fresh grass. The long-day treatment prolonged dormancy of Sardinian females from a mean of 24d (SD) to 72d (LD). Concomitantly, average adult lifespan was extended from 56d (SD) to 117d (LD); one female butterfly actually lived for eight months. Fresh grass had no effect on reproductive dormancy. *M. jurtina* from Austria did not show a reaction to the different treatments (average lifespan: 54d, days until oviposition: 16d), they behaved similar to short-day females from Sardinia. I conclude that reproductive summer dormancy in Mediterranean *Maniola* butterflies is induced by long-day, while day length has no effect on the timing of egg-laying of Central European individuals.

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Blagoevgrad, Bulgaria

Oral presentation: "Sleeping beauty - Dormancy induction in female meadow brown butterflies *Maniola nurag* and *Maniola jurtina*" (2nd place in the student's competition)

11/02 – 15/02/2013 Seminar "Epigenetics and evolution"
ENFA, Toulouse, France

27/08 – 31/08/2012 Summer School on Integrative Biology and Ecology
LabEx TULIP, Toulouse, France