

Nutrients intake determines the post-maturity molting in the golden orb-web spider *Nephila pilipes* (Araneae: Nephilidae)

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ABSTRACT

While molting occurs in the development of many animals especially in arthropods, post-maturity molting (PMM, organisms continue to molt after sexual maturity) has received little attention. Mechanism of molting has been studied intensively, however, the mechanism of PMM remains unknown although it is suggested to be crucial for the development of body size. In this study, we investigated factors that potentially induce PMM in the golden orb-web spider *Nephila pilipes*, which has the greatest degree of sexual dimorphism among terrestrial animals. We manipulated the mating history and the nutrient consumption of the females to examine whether they can affect PMM. The results showed that female spiders under low nutrition were more likely to molt as adults, and mating had no significant influence to the occurrence rate of PMM. Moreover, spiders that experienced PMM lived longer than those without and their body sizes were significantly increased. Therefore, we concluded that it is the nutritional condition rather than the mating history that has affected PMM.

KEY WORDS: Arthropod development, *Nephila pilipes*, Nutrients intake, Orb-web spider, Polyandry, Post-maturity molting

INTRODUCTION

Molting is a common physiological phenomenon that occurs during the development of many animals. It involves the shedding of fur, feathers or skin in vertebrates, and the exoskeleton or cuticle in invertebrates (Ewer, 2005b; Nation, 2015). Through molting animals can respond to environmental change, eliminate parasites, or break constraints of body size (Raven and Johnson, 2008). In arthropods, the exoskeleton grows in discrete steps through molting as body mass and size change continuously (Nijhout, 2013). Though the growth rate and developmental trajectories (e.g. inter-molt duration and instar number) may vary in response to variation in environmental conditions such as temperature and food supply (Flenner et al., 2010; Kleinteich and Schneider, 2011), molting normally stops once sexual maturity is achieved.

Interestingly, there are several animal species that continue to molt after sexual maturity (Foelix, 2011). This post-maturity molting (PMM) behavior has been observed in insects (e.g. Thysanura and Collembola) (Christiansen, 1964; Joosse and Veltkamp, 1969) and many species of lobster, crab and shrimp (Nijhout, 2013). In spiders, PMM was predominantly observed in more ancestral lineages of long-lived sexually monomorphic species, such as liphistiids (i.e. tube-dwelling spiders) (Foelix, 2011; Haupt, 2003) and mygalomorphs (e.g. tarantulas) (Baerg, 1958; Miyashita, 1992). The occurrence of PMM has also been reported in other, more derived species, such as the black widow spider *Latrodectus mactans* (Theridiidae) (Kaston, 1968), *L. hesperus* (Theridiidae) (Kaston, 1968), and the brown huntsman spider *Heteropoda venatoria* (Sparassidae) (Kayashima, 1981). *Nephila pilipes* (Nephilidae) is the only orb-web spider that has been reported to have PMM so far. In addition, this species displays the greatest degree of sexual size dimorphism among all the terrestrial animals (Kuntner et al., 2012b). PMM is considered to be one of the driving forces

behind the extreme sexual dimorphism in this species (Kuntner et al., 2012b), which gives rise to *N. pilipes* female carapaces being, on average, 4.27 times longer than the males', and female mass averaging 125 times that of the male (Head, 1995; Kuntner et al., 2012b; Robinson and Robinson, 1976).

The neuroendocrine mechanisms of molting or ecdysis have been extensively studied (Ewer, 2005a; Žitňan et al., 2007). For example, molting in some bird species was reported to be induced by thyroid-excreted substances (Elterich, 1936; Takewaki and Mori, 1944). In arthropods, ecdysis is driven by complex interactions among multiple neuropeptide signaling systems (Arakane et al., 2008). Initiation is caused by the secretion of a brain neuropeptide called prothoracicotropic hormone (steroid 20-hydroxyecdysone, 20E) in response to certain signals that integrate the arthropods' size, weight, nutritional status, as well as photoperiodic information (Ewer and Reynolds, 2002; Schneiderman and Gilbert, 1964; Truman, 2005; Žitňan et al., 2007; Žitňan et al., 1996). In spiders, molting is also believed to be triggered and controlled by hormones, as increased hormone levels were found in the hemolymph of spiders a few days before ecdysis (Bonaric and De Reggi, 1977; Eckert, 1966). The ecdysone, which controls molting, is probably produced by the endocrine tissues in the prosoma and in the central nervous system or Schneider's organs of the spiders (Legendre, 1958).

Although arthropod molting is regarded as mainly triggered and controlled by hormones, environmental factors may also affect ecdysis. For example, heavy metal loads in the exoskeleton of some arthropods may trigger the molting of terrestrial as well as marine arthropods, such as woodlice (Raessler et al., 2005) and fiddler crabs (Bergey and Weis, 2007). In addition, stimulation generated by body stretching can also induce molting in some insects like the milkweed bug *Oncopeltus fasciatus* (Nijhout, 1979).

Comparatively, however, the mechanisms of PMM have rarely been experimentally explored and we have little information about the endocrine and exocrine systems as well as environmental factors that might affect PMM. Kuntner et al. (2012a) proposed that in *N. pilipes* PMM may be stimulated by the quantity of sperm that the female possessed, as PMM is more likely to occur in females exposed to lower levels of polyandry had not produced an egg-sac (Kuntner et al., 2012b). In addition, the PMM exuvia resulting from PMM in *N. pilipes* does not contain any epigynal structures (the epigynum is the external genital structure of female spiders located in front of the genital opening) (Kuntner et al., 2012a), suggesting that sperm collected in the spermathecae of molting females can be retained. This suggests that PMM may be affected by the female's mating history because it may extend her life span and thus increase the potential to obtain more sperms.

Molting, including PMM, may also be affected by other factors, such as the body size and nutrient intake. For example, molting in crustaceans is closely correlated to nutrition, and a low nutrient intake inhibits the molting of larvae (McConaughy, 1985). Arthropods often initiate a molting cycle when they reach a critical body weight after consuming sufficient nutrient (Nijhout, 1979). In *N. pilipes*, females may increase body size via PMM to obtain reproductive advantages, especially when food is abundant.

We hypothesize that mating history and nutrition play an important role in determining PMM. To test this hypothesis, we manipulated the mating history and nutrient intake of *N. pilipes* females to see how the occurrence rate of the PMM is affected by these two factors. We predict that a lower level of polyandry and a higher level of nutrition will stimulate the females to molt again after maturity.

MATERIALS AND METHODS

Study species and maintenance

Nephila pilipes (Araneae: Nephilidae) is a brightly colored spider, which is widely distributed in an area spanning East Asia, India, Southeast Asia, West Pacific islands and North Australia (Su et al., 2007). It constructs large orb webs in the understory of tropical/subtropical forests (Murphy and Murphy, 2000). The spiders used in this experiment were collected from subtropical forests of Taiwan. We collected sub-adult females and reared them until adulthood in the laboratory. We also collected adult males from sub-adult females' webs and carefully inspected male pedipalp status and length of the embolus before use in experiments. Pedipalps are situated on second pair of appendages of the spider prosoma, which act as copulatory devices in mature males. The embolus is a narrow whip-like extension of the palpal bulb, which is inserted into the female genital opening during sperm transfer. Only those males with intact pedipalps (i.e. without any damage) were used in the experiments. During the mating season, there are up to eight males waiting in a sub-adult female's web, and mating usually results in male pedipalp damage in this species (Kuntner et al., 2012b; Kuntner et al., 2009). Therefore, we assumed that the males with intact pedipalps we used were unmated. We kept females individually in plastic frames (40×30 cm and 10 cm high) with translucent screens, and kept males individually in plastic cups (500 ml). All spiders were kept in the laboratory under controlled environmental conditions (temperature: $25 \pm 1^\circ\text{C}$; relative humidity: $80 \pm 10\%$; photoperiod: 12:12 h light: dark with lights on between 0800 and 2000 hours). We watered all spiders daily and fed males with *Drosophila* flies, and fed females with mealworms *Zophobas morio*. The body length and cephalothorax width of the females were measured one day after they had molted into adults.

Mating trials

To examine how nutrition and mating history may affect female PMM, we performed mating trials in the laboratory in female webs. Females were randomly subjected to two mating (mating vs. without mating) and two feeding regimes (high nutrition vs. low nutrition, we assumed high diet as high nutrition in this study). Four groups were set up in this experiment: high nutrition and no mating (HN, N = 40); high nutrition and mating (HM, N = 20); low nutrition and no mating (LN, N = 40); low nutrition and mating (LM, N = 20). In the mating groups, female mated three times with three different males consecutively, and each mating event was allowed to occur for 15 minutes. Kuntner et al. (2012b) and Danielson-François et al. (2012) reported that the mating duration of a male can last up to 1 hour. In this highly polyandrous species, sperm from different males may compete with each other inside the female spermatheca. It is unknown how much sperm a female *N. pilipes* will accept before she refuses further mating attempts. Therefore, we assumed that the longer the pedipalp insertion, the more sperm a female can receive, and considered 45 minutes long enough for a female to obtain enough sperm in order to produce egg sacs. We started each mating trial by gently introducing a male to a female's web about 15 cm away from the female and recorded all occurrences of pedipalp insertions. We interrupted the mating of each male when mating duration reached 15 minutes. We introduced another male to the female web 10 minutes after mating with the previous male was interrupted. We discarded the data if the total insertion time of a male did not reach 15 minutes. All females used in the experiment were aged two to three weeks after maturity. Females were fed one mealworm before mating trials to decrease the cannibalistic tendency of the female. After mating, we kept the females individually and reared them until they died. We discarded the data if females escaped or died accidentally. Each male was used for mating only once. In the high nutrition group, we fed

females 12 mealworms per week, while in the low nutrition group we fed females 3 mealworms per week.

Data analysis and statistics

We used a linear mixed model to compare the body length of females before and after PMM, where spider individual was treated as a random factor. Some spiders escaped or died accidentally during the experiment, so some groups have a smaller sample size. To fit data with the smaller sample sizes, we performed an exact logistic regression (Zamar et al., 2007) to test the relationship between the carapace width and mating history of adult female with the probability of PMM. We used a Firth's bias-reduced penalized-likelihood logistic regression to determine whether nutrition (high and low) and female carapace width affect the probability of PMM. A Cox proportional hazards regression model based on the Kaplan-Meier method was used to fit the duration of PMM among nutrition treatments given the same carapace width. The Kaplan-Meier method was used to estimate the survival rate of the individual, which was then examined by a Cox proportional hazards regression model to check whether nutrient intake, PMM and carapace width had any impact to the survival rate.

RESULTS

In total, we obtained 36 mating trials in the high nutrition and no mating (HN) group, 9 mating trials in the high nutrition and mating (HM) group, 35 mating trials in the low nutrition and no mating (LN) group, and 8 mating trials in the low nutrition and mating (LM) group. Among these groups, we observed 5, 0, 16, and 4 cases of PMM, respectively. The carapace width of the female was significantly increased after PMM in both high nutrition and low nutrition groups ($P < 0.0001$) (Fig. 1, Table 1), suggesting that body size of the spider can increase through PMM. In addition, females receiving high nutrition had significantly larger body sizes than those receiving low nutrition, either before or after PMM

($P = 0.018$) (Fig. 1, Table 1). However, we did not find significant variation in average growth ratio (ratio of carapace width of spiders when finishing PMM to that of spiders just achieving maturity) between spiders from the two different food treatments (the interaction between nutrition and pre-/post- measurement; $P = 0.843$) (Fig. 1, Table 1). Females in low nutrition groups were significantly more likely to molt after maturity than those in high nutrition groups (odds ratio = 0.141, exact logistic regression, $P < 0.001$). Mating did not significantly affect the frequency of PMM (exact logistic regression, $P = 0.329$) (Fig. 2, Table 2).

When comparing the PMM rate in high nutrition and low nutrition group by logistic regression, we found that PMM rate was influenced by the disordinal interaction between nutrition and carapace width ($P = 0.045$). Results showed that in high nutrition groups, females with larger carapace width were more likely to undergo PMM comparing with those with smaller carapace width. In contrast, in low nutrition groups, females with smaller carapace width were more likely to molt again after maturity (Fig. 3, Table 3).

We also compared the number of days from reaching adulthood to post-maturity molting in groups that did not mate. The results showed that this duration was significantly affected by nutrition ($P = 0.012$). Given the same length of time, females in high nutrition groups had much higher probability of performing PMM (~ 5.54 times higher) than those in low nutrition groups (Fig. 4, Table 4). However, the carapace width did not have a significant effect on number of days it took to reach PMM after maturity (Fig. 4, Table 4). The results suggested that spiders in low nutrition groups might need more time to accumulate enough energetic resources to perform PMM. Spiders in high nutrition groups had wider carapace than those in low nutrition groups, which were also more likely to molt again after maturity. In addition,

we found that survival rate of adult spiders undergoing PMM was three times greater than those that did not ($P = 0.0006$) (Fig. 5, Table 5).

DISCUSSION

We studied factors that potentially induce post-maturity molting in the golden orb-web spider *N. pilipes*, a topic that has rarely been examined in arthropods. We predicted that spiders would be more likely to molt as adults under higher levels of nutrition and a lower levels of polyandry. However, our results showed that PMM is more closely associated with nutrient consumption as mating history is comparatively less relevant. Females in low nutrition groups were more likely to undergo PMM, no matter if they had mated or not. Though the sample size of the data was small in the two mating groups, the results clearly showed that nutrition is more likely to influence PMM rate than mating history (Fig. 2, Table 2).

In arthropods, female body size is positively correlated with female fecundity (Head, 1995; Higgins, 1992). In spiders, sexual size dimorphism is believed to be generated and maintained by fecundity selection on female body size (Head, 1995; Kuntner and Elgar, 2014). In theory, an increase in female body size via PMM may enhance reproductive performance, especially when food is abundant. However, our results showed that with similar size females that consumed low nutrition had higher rate of PMM than those that consumed higher nutrition (Fig. 3, Table 3). These results imply that PMM might not be selected for through a fecundity advantage, even though PMM can considerably increase body size (Fig. 1, Table 1). However, body size does have some impact on PMM (Fig. 4). As sit-and-wait predators, orb-web spiders have limited options in adjusting their prey availabilities. Available means to enhance foraging include relocating the web site/web position, and modifying web/silk properties (Blackledge et al., 2011; Heiling and Herberstein, 1999; Herberstein and Tso, 2011). Therefore, spiders with certain growth and developmental

plasticity can respond to variations in foraging opportunities. Studies on *N. clavipes* showed that the number of molts and inter-molt duration were plastic due to variation in food resources (Higgins and Rankin, 1996). Results of our study showed that adult female *N. pilipes* also adjusts molting to variations in foraging success, possibly maximising its reproductive potential. *N. pilipes* inhabits both tropical and subtropical areas ranging from southern Japan to northern Australasia (Su et al. 2007). PMM, an uncommon phenomenon in modern spider clades, may have evolved as an adaptation in this widely distributed species early in its geographic expansion. This adaptation may have enabled spiders to cope with a diverse array of habitats and associated spatial and temporal heterogeneities in foraging conditions.

Female spiders with larger body sizes have higher reproductive potential (Schneider and Andrade, 2011). Our findings indicated that when food is abundant, the larger-bodied females might invest energy into PMM to increase body size and thereby maximising reproductive potential. Smaller-bodied females may transfer energy into reproductive potential rather than investing in increasing body size. However, when food resources are scarce, larger-bodied females may save energy and use it in egg production rather than in PMM. On the other hand, smaller-bodied females may dedicate to a final effort to attract males by releasing sexual pheromones via PMM, as molting fluid may contain volatile sexual pheromones (Miyashita and Hayashi, 1996).

Protandry, a phenomenon where males become sexually mature earlier than the females, is common in spiders (Gunnarsson and Johnsson, 1990; Maklakov et al., 2004). *N. pilipes* is a protandrous species (Danielson-François et al., 2012), and males mature on average one month before the females (Zhang's observation). In many spiders, including *N. pilipes*, the population of males would drop drastically after the peak of adult abundance (Schneider and Andrade, 2011). Therefore, maturing during the abundance peak is important for the polyandrous females, as they would encounter more males to carry on mate selection. We observed in the field that towards the end of the mating season late maturing females are generally smaller than females that matured and were active earlier in the mating season. We assumed that after the adult male abundance peak females would speed up the maturity process and molt to adulthood, even when body size is still relatively small, in order to catch up with diminishing reproductive opportunities. Future studies may investigate whether PMM is correlated to the developmental history of the spiders.

In summary, the presented study is the first demonstration that nutrition can determine the occurrence rate of PMM in invertebrates. Our results showed that nutrient intake is more important than mating history in triggering PMM in *N. pilipes*. Therefore, we speculate that in this species PMM may have evolved as an adaptation to diverse foraging conditions, especially food scarcity during the initial expansion from tropical to subtropical zones.

Competing interests

The authors declare no competing or financial interests.

Author contributions

R. C., S. Z. and I. T. designed the study. S. Z., Y. C., C. L., and H. Y. performed the experiment. R. C., S. Z. and C. L. did the statistical analysis. S. Z. D.P and I. T. wrote the paper.

Funding

The study was funded by the National Science Council, Taiwan (NSC-99-2632-B-029-001-MY3; NSC-102-2311-B-029-001-MY3; MOST 103-2621-B-029-001-MY3) grants to I.M.T and the Ministry of Science and Technology, Taiwan (MOST 103-2811-B-029-001; 103-2811-B-029-003) grants to S.Z.

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Figure legends:

Figures

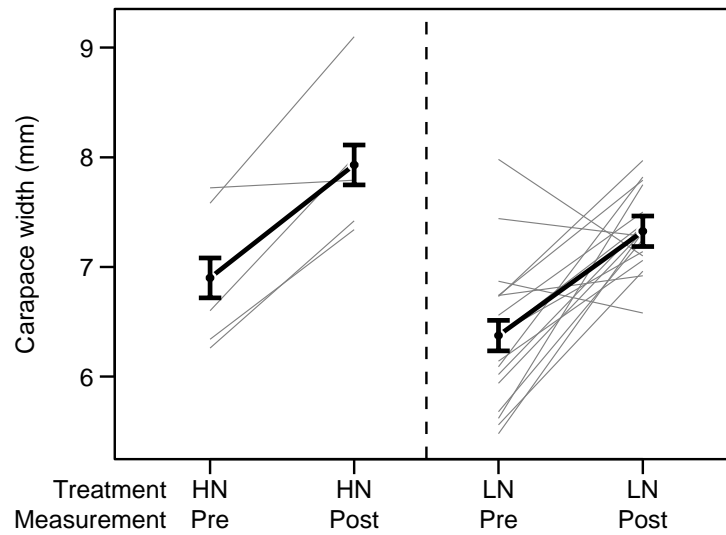


Fig. 1. Mean (+SE) carapace width of female *Nephila pilipes* that did not mate before and after maturity molting in high nutrition and low nutrition manipulations. Dotted lines represent data of individual spiders. (HN: high nutrition treatment, $N = 36$; LN: low nutrition treatment, $N = 9$; Pre: before maturity molting; Post: after maturity molting).

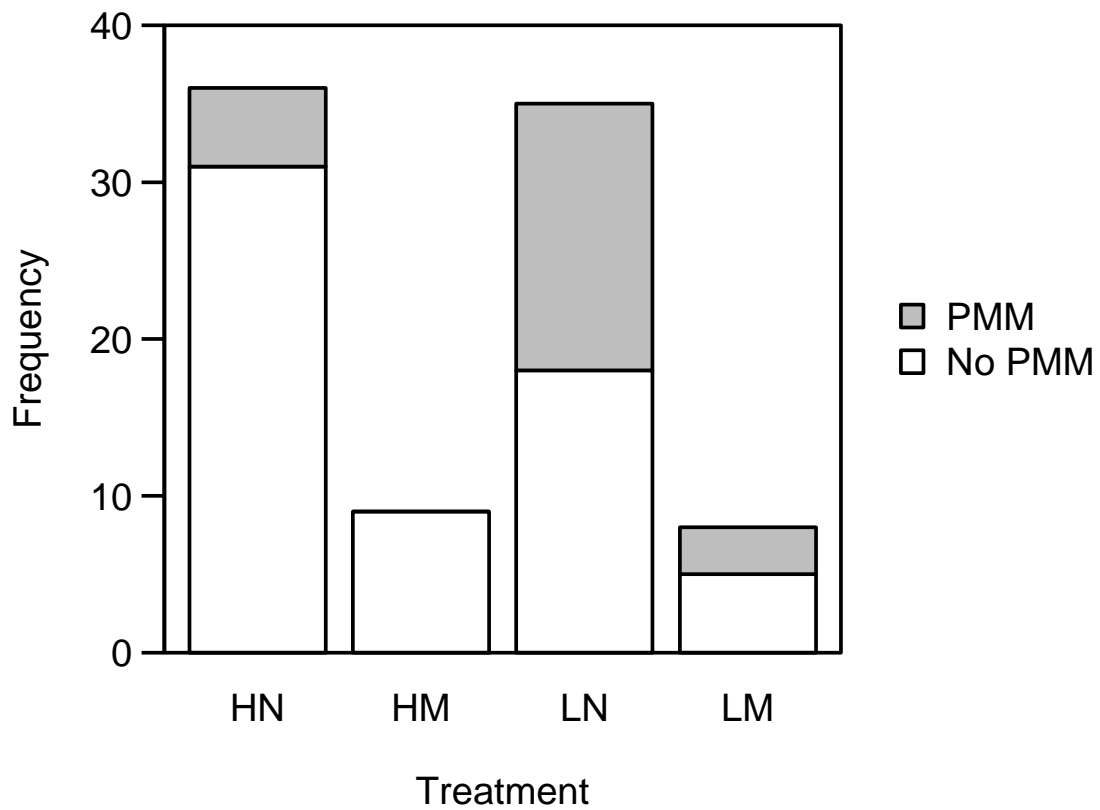


Fig. 2. Frequency of female *N. pilipes* performing post-maturity molting (PMM) in treatment groups receiving different nutrition and mating/no mating. (HN: High nutrition and no mating, $N = 36$; HM: High nutrition and mating, $N = 9$; LN: Low nutrition and no mating, $N = 35$; LM: Low nutrition and mating, $N = 8$).

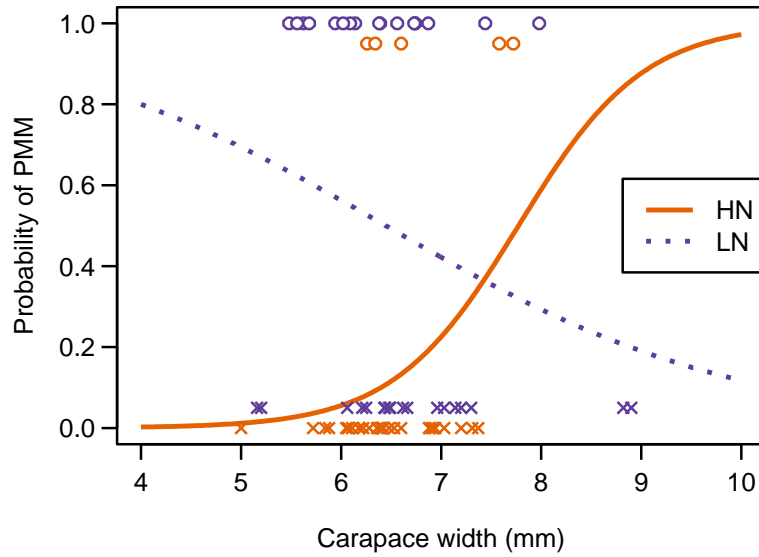


Fig. 3. Post-maturity molting probabilities of female *N. pilipes* of various carapace widths receiving high or low nutrition. (HN: high nutrition treatment, $N = 36$; LN: low nutrition treatment, $N = 9$; O: PMM; X: no PMM).

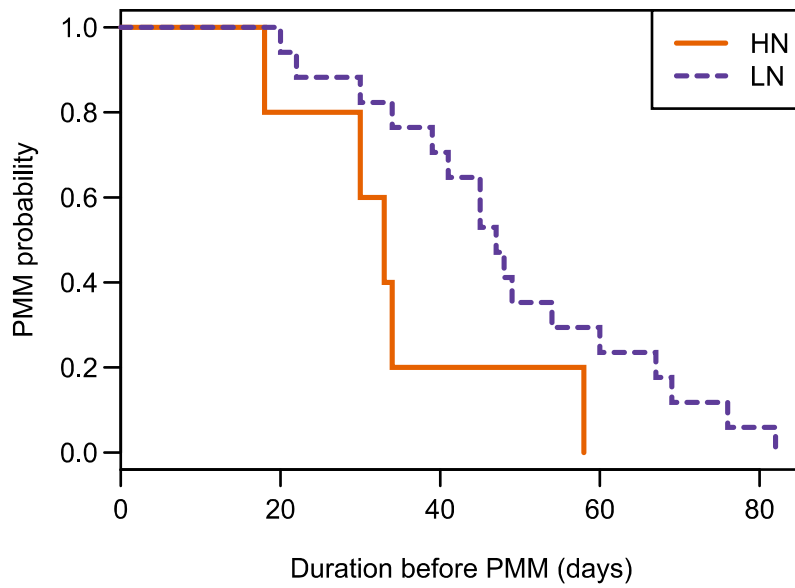


Fig. 4. Duration (number of days) before post maturity molting of female *N. pilipes* of various carapace widths receiving high and low nutrition. (HN: high nutrition treatment, $N = 36$; LN: low nutrition treatment, $N = 9$).

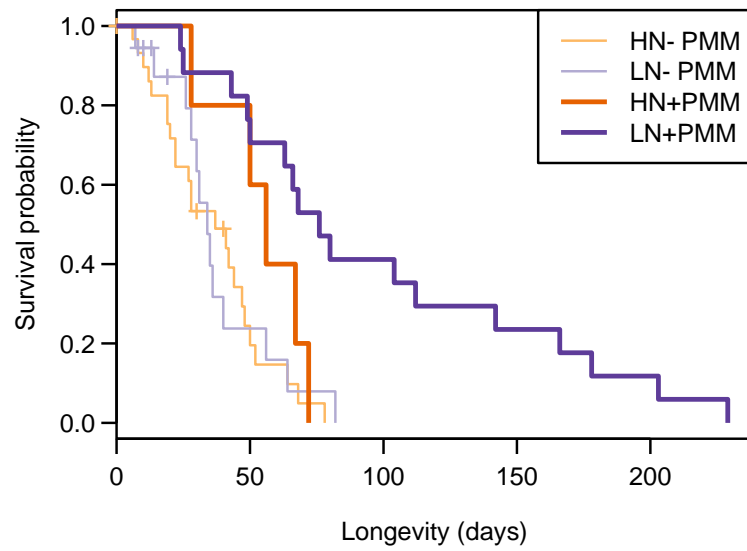


Fig. 5. Survival probability of female *N. pilipes* receiving high and low nutrition treatment with and without post maturity molting (PMM). (HN: high nutrition treatment, $N = 36$; LN: low nutrition treatment, $N = 9$).

Tables

Table 1 Results of linear mixed model assessing the change of body size of females before and after post-maturity molting.

| | Estimate | SE | T_{20} | P |
|-----------------------------------|----------|-------|----------|----------|
| Intercept (overall average) | 7.132 | 0.110 | 64.914 | < 0.0001 |
| Nutrition (high vs low) | 0.565 | 0.220 | 2.572 | 0.018 |
| Measurement (pre-PMM vs post-PMM) | 0.991 | 0.196 | 5.049 | < 0.0001 |
| Nutrition \times Measurement | 0.079 | 0.392 | 0.201 | 0.843 |

Table 2 Results of exact logistic regression assessing the effects of nutrition and mating on occurrence of post-maturity molting.

| | Estimate | Exp.(estimate) | <i>P</i> |
|-------------------------|----------|----------------|----------|
| Mating (none vs short) | 0.772 | 2.164 | 0.329 |
| Nutrition (high vs low) | -2.423 | 0.089 | < 0.001 |

Table 3 Results of Firth's bias-reduced penalized-likelihood logistic regression analyzing the relationship between nutrition treatments, carapace width and post-maturity molting rate of female *Nephila pilipes*.

| | Estimate | Exp.(estimate) | SE | Z | P |
|-------------------------------|----------|----------------|-------|--------|-------|
| Intercept (overall average) | -0.917 | 0.400 | 0.316 | -3.262 | 0.001 |
| Nutrition (high vs low) | -1.751 | 0.174 | 0.631 | -3.099 | 0.002 |
| Carapace width (centered; mm) | 0.436 | 1.589 | 0.513 | 0.941 | 0.347 |
| Nutrition × Carapace width | 1.906 | 6.723 | 1.026 | 1.991 | 0.047 |

Table 4 Results of Cox proportional hazards regression model analyzing the effects of nutrition treatments and carapace width on intervals between maturity and post-maturity molting.

| | Estimate | Exp. (estimate) | SE | Z | P |
|-------------------------------|----------|-----------------|-------|--------|-------|
| Nutrition (high vs low) | 1.713 | 5.544 | 0.682 | 2.511 | 0.012 |
| Carapace width (centered; mm) | -0.661 | 0.516 | 0.421 | -1.571 | 0.116 |
| Nutrition × carapace width | -0.960 | 0.383 | 0.824 | -1.165 | 0.224 |

Table 5 Results of Cox proportional hazards regression model assessing the effects of nutrition, post-maturity molting and carapace width on the survival of spiders.

| | Estimate | Exp. (-estimate) | SE | Z | P |
|-------------------------------|----------|---------------------|-------|--------|--------|
| Nutrition (high vs low) | -0.324 | 1.383 | 0.322 | -1.006 | 0.314 |
| PMM (PMM+ vs PMM-) | -1.232 | 3.428 | 0.361 | -3.413 | 0.0006 |
| Carapace width (centered; mm) | -0.068 | 1.070 | 0.215 | -0.315 | 0.753 |