Scramble competition for molting females as a driving force for extreme male dwarfism in spiders

Scramble competition for moulting females as a driving force for extreme male dwarfism in spiders

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Extreme sexual size dimorphism in spiders is generally assumed to result from female gigantism, male dwarfism or both. However, field evidence demonstrating the existence and nature of these driving mechanisms is still lacking. In this study we performed field monitoring and laboratory manipulations on the giant wood spider, *Nephila pilipes*, to assess pre- and postcopulatory sexual selection pressures on males. Results of field monitoring showed that females frequently changed web sites and travelled for long distances and consequently the advantage of large males in occupying favourable positions on webs did not guarantee them higher mating opportunities. Most of the mating events occurred during female moulting but there was typically only one male present on a female's web, indicating a low level of precopulatory male–male physical competition. Results of staged double-mating experiments using the sterile-male technique showed that sperm competition in *N. pilipes* followed a mixed-priority pattern. So males able to find a moulting female earlier than their rivals, to monopolize it to transfer more sperm, are favoured. Therefore, scramble competition for suitable but ephemeral penultimate-stage females is one major force driving protandry and maintaining small body size in spiders with extreme sexual dimorphism.

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Sexual size dimorphism (SSD), a phenomenon where body size differs between males and females, is common in animal taxa. Generally, SSD evolves because males and females have different selection pressures during maturation (Higgins 2002). The relative sizes of males and females vary among taxa (Blanckenhorn 2005). SSD in birds and mammals is usually male-biased, which is generally attributed to strong sexual selection pressures favouring larger males via male–male competition or female choice (Andersson 1994). SSD in invertebrates, however, shows an opposite trend and females are usually larger than males (Fairbairn 1997). Legrand & Morse (2000) argued that female-biased SSD actually occurs more often in nature. Large female body size is regarded as advantageous since it is typically related to an increase in fecundity (Shine 1988; Blanckenhorn 2005).

In most species of spiders the body size of females is larger than that of males (Head 1995; Hormiga et al. 2000). Certain orb-weaving spiders such as golden orb-weaving spider (genus *Nephila*) are well known for their extreme SSD (Kuntner & Coddington 2009). In some

Nephila species females are more than 12 times larger than males (Robinson & Robinson 1974) and may weigh over 100 times more than males (Vollrath & Parker 1992; Coddington et al. 1997). Therefore, spiders have long attracted much attention in the study of SSD. Different hypotheses have been proposed to provide a general explanation for SSD in spiders and among them are two major ones arguing for opposing mechanisms. One hypothesis proposed female gigantism as the cause and argued that SSD in spiders was generated by fecundity selection favouring larger females (Coddington et al. 1997). These researchers used a phylogenetic approach that supported a female gigantism mechanism for many species (Hormiga et al. 2000; Kuntner et al. 2009). The other hypothesis proposed male dwarfism because the males of orb-weaving spiders suffer high mortality rates during mate searching, which consequently reduces male–male competition and favours protandry by early maturation (Vollrath & Parker 1992). However, other studies have proposed that in some orb-weaving spiders larger males might be favoured through male–male competition (Elgar 1998; Foellmer & Fairbairn 2005a; Kasumovic et al. 2008). Empirical evidence showed that large males usually won fights against smaller rivals and obtained a favourable position on the web near the hub (Christenson & Cohn 1988; Christenson 1989; Miyashita 1993; Elgar et al. 2003a, b). In *Nephila clavipes* larger males also have more sperm, potentially

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giving them an advantage in sperm competition (Danielson-François 2002). In the absence of opposing selection forces, such evolutionary pressures should lead to an increase in male size (Huber 2005). Currently, what keeps orb-weaving spider males small is still unclear and the underlying mechanisms await further investigation.

Several hypotheses have been proposed to argue for small male advantage in spiders. In *Nephila edulis*, smaller males copulated for longer than larger ones and fertilized more eggs (Schneider et al. 2000). During mate searching, smaller males might have a mobility advantage because they have higher locomotion speed, greater climbing performance, better bridging ability and consequently might find females faster or escape predators more easily (Moya-Laraño et al. 2002, 2009; Linn et al. 2007; Corcobado et al. 2010). In *N. plumipes*, males suffered high mortality during mate searching (Kasumovic et al. 2007) and this cost might have severely weakened the intensity of male–male competition and relaxed the selection pressure for larger males. The aforementioned reasons may explain why smaller males are favoured compared with larger ones. However, empirical evidence of small male advantage from direct observations of male and female sexual interactions in the field is lacking (Kasumovic et al. 2008).

Multiple matings by females allow sexual selection to persist even after mating has taken place. When sperm from rival males coexist within the female's reproductive tract, sperm competition may occur (Parker 1970). Currently, studies connecting sperm competition and male body size in spiders are few (Simmons & Siva-Jothy 1998). Schneider et al. (2000) showed that smaller male *N. edulis* exhibited a reproductive advantage by copulating for longer and fertilizing more eggs than larger ones. Huber (2005) proposed that if there was relaxed precopulatory male–male competition and at the same time postcopulatory selection pressure favoured males that arrived first (e.g. by first-male sperm priority), these conditions would generate selection pressures favouring protandry and early maturation. Under such circumstances the optimal size of males would be small (Kasumovic et al. 2008). Currently, there is still no evidence generated from field studies to support this hypothesis. In this study, we investigated factors maintaining small male body size in orb-weaving spiders by using the giant wood spider, *Nephila pilipes*, a species with extreme SSD. By conducting long-term field monitoring and staged double-mating experiments, we assessed how pre- and postcopulatory sexual selection pressures in the form of male–male physical interaction and sperm competition influenced male body size in orb-weaving spiders.

METHODS

Study Organisms

Nephila pilipes is widely distributed in the Asian–Australian region ranging from southern Japan to northern Australia (Su et al. 2007, 2011). It is a spider species with extreme SSD (Fig. 1). Adult females can be 12 times longer than adult males and weigh over 100 times more (Robinson & Robinson 1974; Head 1995). To reach maturity, juvenile females have to undergo at least 11 moults while males need just seven (Robinson & Robinson 1974). Once mature, males leave their own webs to search for females and may sometimes act as kleptoparasites and steal leftover prey from a female's web (Robinson & Robinson 1974).

Quantifying Precopulatory Selection on Males

Study site

In 2 consecutive years we performed field monitoring in two sites located in secondary forests. The first field study was performed between 15 August and 5 September 2008 in Ape Hill

(22°38′19″N, 120°15′54″E) near National Sun Yat-Sen University, Kaohsiung City, Taiwan. We searched for and marked all *N. pilipes* on both sides of a 200 m long trail. The second field study was performed between 28 June and 20 July 2009 in Huoyan Mountain (24°06′42.2″N, 121°11′51.1″E), Sanyi Township, Miaoli County, Taiwan. Along a 100 m hiking trail, we searched for and marked all *N. pilipes* individuals inside the forest.

Field monitoring

During both study periods, no juvenile males were found and adult males were mostly found on females' webs. Male and female spiders were anaesthetized with carbon dioxide for about 30 s in a sealed vial following the method of Fromhage et al. (2007). After the spiders were anaesthetized nail polish was applied to the first leg of the female and to the abdomen of male spiders for individual identification. The following body characteristics were measured during this time: body length (in mm), cephalothorax width (in mm), number of legs and the status of the copulatory organ (i.e. whether the embolus was broken off or not). The female epigynum was examined to distinguish adults from juveniles by using a hand-held field magnifying lens. For mature males, their emboli were examined to identify mating history because only mated males break off the tip of the embolus. When all the procedures were completed and spiders returned to normal activity they were released back to their webs.

We surveyed each web once each hour each day continuously for 24 h in 2008 and once each hour from 0800 to 1800 hours each day in 2009. While surveying the webs we recorded the following

Figure 1. Female giant wood spider *Nephila pilipes* with a dwarf male (indicated by arrow) on her web. Scale bar = 1 cm.

male–male and male–female interactions: number of males and male's position on a female's web, occurrence of male–male competition, mating events, moulting events, web relocation and travel distance. Sometimes when a female used the same frame silk the distance between an old web and a new one could be up to 1.5 m (C. Hou, unpublished data). Therefore, when a female moved more than 2 m from the original site to build a new web, we regarded that as a web relocation event.

Statistical analyses

Analyses were performed with SYSTAT 9.0 (Wilkinson et al. 1992) and SAS software (SAS Institute Inc. 2001). All variables were first inspected for normal distribution by using Kolmogorov–Smirnov one-sample tests. Previous studies had demonstrated that phenotypic characteristics of male spiders might vary significantly between different demographic regions (Kasumovic et al. 2008, 2009). Therefore, two-way ANOVA tests were used to analyse the size difference between mated and unmated males using study site as another factor. The size difference between males on hub and nonhub positions on a female's web was analysed by a Student's *t* test because such data were only available from Huoyan Mountain study site. Logistic multiple regressions were used to test which measured male characteristic correlated significantly with their mating success. The variables examined included study site, body length, cephalothorax width, leg number, the number of days for which the marked individual could be traced, the number of days spent with a particular female, the number of females visited and whether or not the mating occurred while the female moulted.

Quantifying Postcopulatory Selection on Males

Collection and maintenance of spiders

Subadult female and adult male *N. pilipes* were collected in June and July 2008 from secondary forests at Dawu Mountain, Pingtung county in southern Taiwan (22°24′59″N, 120°54′40″E) and Huoyan Mountain. Males were collected as adults from the webs of females. Similar to other *Nephila* species (Robinson & Robinson 1974; Higgins 2002), males appear to mature earlier than females in the field: adult males were found while most females were immature. All spiders were mated within a month of being collected. All females were adults and aged several days before being used in the staged mating trials; no newly moulted females were used in order to control for this variable, as it was not possible to put males on newly moulted females in the short time frame of our study. We also noted field observations of females completing moulting without a male nearby, so we feel this choice was justifiable.

Sperm transfer and uptake appear to vary with female age in the genus *Nephila*, that is, the first male to mate with a newly moulted *N. clavipes* fertilizes most of her offspring, while the second male does not gain much paternity (about 15–21%) as noted by Christenson & Cohn (1988). However, further studies have shown that female age does not influence sperm uptake, whereas repeated matings by the first male do. When these extra matings are not allowed, older *Nephila* females take up sperm from later males freely, as much as newly moulted females (Danielson-François 2002). It was important for our study to determine whether males can transfer sperm to older females. Our control matings with older females revealed that fertile (N) males are able to transfer enough sperm to fertilize on average about 97% of an eggsac (see Results).

In the laboratory, females were individually housed in cages made of frames and mesh (30 × 30 × 30 cm) and were watered and fed one mealworm, *Zophobas morio*, or one field cricket, *Gryllus* sp., daily until mature. Males were maintained in plastic cups (500 ml)

and were watered and fed several *Drosophila melanogaster* daily. The total body length and cephalothorax width of every individual were measured and males were carefully inspected for pedipalp status and length of the embolus. In *N. pilipes*, determining the length of the embolus can be assessed relatively easily with either live or dead males.

Staged double-mating trials

Patterns of paternity were determined using standard double-mating trials (Parker 1970). Mature males were randomly assigned to either normal or irradiated treatments. The irradiation treatment used was an X-ray radiation dose of 2000 rads, conducted at the Taichung Veterans Hospital in Taichung City, Taiwan. Adult females were randomly assigned to one of four treatments: (1) NI, a normal male followed by an irradiated male ($N = 12$); (2) IN, an irradiated male followed by a normal male ($N = 11$); (3) I, an irradiated male ($N = 2$) as a control for sterilization success; and (4) N, a normal male ($N = 4$) as a control for number of unfertilized eggs in a normal eggsac. One female (I treatment) was also killed before egg laying as a control to determine irradiated sperm motility. Matings were staged by placing a male on the periphery of a female's web. Before mating, the females were fed one mealworm to prevent cannibalism from happening during mating. Shortly after the female started to eat, the male would move onto her body, and begin courting her by running over her abdomen and then began inserting his pedipalps into her insemination ducts. The first male was given at least 1 h in the cage to mate, but once mated was removed and a second male was placed in the cage. We found that a male is able to transfer enough sperm in 1 h to fertilize about 97% of an eggsac. To prevent males breaking off the embolus on each palp (to plug the female), males were removed after finishing the insertion of both pedipalps and were frozen at −20 °C for later sperm counting. Sperm remaining in the pedipalps of mated males were counted to determine the amount of sperm released to a female by each male. This procedure was necessary to control for differences in the number of sperm released by two males to the same female, which might bias the paternity pattern. When egg-sacs were laid (after about 1–3 months) they were placed in an environmental chamber kept at room temperature (25 °C) until they hatched (after about 4 months). After the eggs hatched, hatchlings and unfertilized eggs were preserved in 70% alcohol and counted to calculate the observed P_2 value, the percentage of eggs fertilized by the second male.

Observed paternity, P_2

Based on the criteria of Christenson & Cohn (1988), we categorized paternity patterns into first-male priority (when $P_2 < 0.33$), mixed priority ($0.33 < P_2 < 0.66$) and last-male priority (when $P_2 > 0.66$).

Paternity patterns for IN and NI treatments were compared with a nonparametric Wilcoxon two-sample test. These observed paternity values were later compared with the amount of sperm released to each female to determine whether paternity and sperm release were correlated in this species (see below 'Testing whether sperm transfer is correlated with paternity').

Sperm quantification

Sperm quantification was based on a revised sperm-counting method from Bukowski & Christenson (1997). Right and left palps were removed and individually placed into separate 1.5 ml poly-propylene centrifuge tubes. To each tube was added sperm-counting solution composed of 1 ml of 0.9% saline and 10 μl of 10% Triton-X100 detergent drawn from a common stock. Appropriate treatments were necessary to avoid sperm aggregation and to facilitate homogeneous distribution of sperm within a sample.

Each palp was crushed with forceps and was ultrasonicated using a Microson XL-2000 sonicator (Misonix Inc., Farmingdale, New York, U.S.A.) at low level for about 20 s. A sample was then immediately drawn and placed on a haemocytometer, and set aside for about 1 min until all the sperm settled. Sperm were counted under a light microscope at $400\times$ magnification. All of the values reported are estimates of total numbers of sperm.

Measures of sperm count in unmated and mated males

We examined unmated males ($N = 20$) and determined the amount of sperm in their palps using the procedure given above. We then examined mated males and determined the amount of sperm remaining in their palps after mating ($N = 46$). We compared the amount of sperm in unmated and mated males and the amount of sperm remaining in the palps of the first and second males to mate.

Testing whether sperm transfer is correlated with paternity

Paternity can be influenced by many factors. One factor that is not often examined is sperm release because it is currently impossible to estimate. Whether the amount of sperm transferred is correlated with paternity is unknown in many spider species. We wished to determine whether paternity was correlated with sperm release and whether variation in one corresponded to variation in the other. To estimate sperm release, we subtracted the amount of sperm remaining in the palps of mated males with the average amount contained in the palps of unmated males, following the methods of Danielson-François & Bukowski (2005). Although this is not a perfect method to determine sperm release, it is currently the best method available for estimating sperm transfer, as the time spent mating is often not correlated with sperm transfer in spiders, as noted in Danielson-François & Bukowski (2005). We estimated the relative numbers of sperm released by the first and second male mated to a particular female and determined the expected SR_2 the amount of paternity predicted by sperm release for the second male to mate. This prediction was compared to the actual observed paternity, P_2, as measured from each eggsac using the sterile-male technique. If the SR_2 prediction based upon sperm transfer number matched the observed paternity for each eggsac, this would suggest that the sperm numbers transferred are correlated with paternity in this species. Currently, whether paternity and sperm release are correlated in this species is unknown.

Statistical Analysis

Student's two-tailed t tests were used to compare differences in the amount of palp sperm between unmated and mated males as well as between first and second males to mate. Wilcoxon two-sample tests were also used to compare the paternity of females receiving NI and IN treatments. The measures of paternity obtained from P_2 were compared to the predictions from sperm release, SR_2, using the Wilcoxon two-sample test.

RESULTS

Precopulatory Selection on Males

Population characteristics

In the Ape Hill study site, females ($N = 76$) could be traced for 4.4 ± 0.4 days and males could be traced for 1.6 ± 0.2 days. On average, males ($N = 45$) spent 1.5 ± 0.2 days on a female's web. The abundance of males remained lower than those of immature and adult females throughout the whole study period and the operational sex ratio was 0.94 (adult male/adult female = 45/48). In the Huoyan Mountain study site, females ($N = 88$) could be tracked for

10.7 ± 0.8 days and males ($N = 102$) could be tracked for 4.6 ± 0.4 days. On average, males spent 3.7 ± 0.4 days on a female's web. Marked females might travel 2–30 m (mean $\pm$ SE = 4.7 ± 2.0, $N = 13$) and the longest travel distance recorded for marked males was 15 m. The temporal abundance pattern of males seemed to coincide with that of adult females and the operational sex ratio was 1.89 (adult male/adult female = 102/54).

Female–male interactions

At the Ape Hill study site, a female was visited by zero to five males, with a mean of 0.53 (Fig. 2a). Most females were either not visited by any male (61%) or were visited by one male (26%) and only a few females (13%) were visited by multiple males sequentially. Nine females (12%) had multiple males present on webs at the same time, with two or three coexisting males. Overall, most males (93%) visited one female and only one male (2%) visited more than one female (Fig. 3a). Eight females were observed to mate and no multiple matings were observed during this study period. At the Huoyan Mountain study site, a female was visited by zero to five males, with a mean of 1.5 (Fig. 2b). In total, around 60% of females were either not visited by any male or were visited by only one male and 44% of marked females were visited by multiple males sequentially. We observed 32 females (36%) having multiple males present on webs at the same time, with two to four coexisting males. Overall, most males (73%) visited only one female, about 25% of males visited two females and 2% of males visited more than two females (Fig. 3b). A total of 26 females were observed to mate and most of them (92%) mated with only one male. Only one female mated with two males and another mated with three males. Multiple matings by males were only observed in one of 26 mating events.

Male–male interactions

In this part of the study in most analyses the data collected from Ape Hill and Huoyan Mountain study sites were pooled. Only female webs with two or more males and with complete male
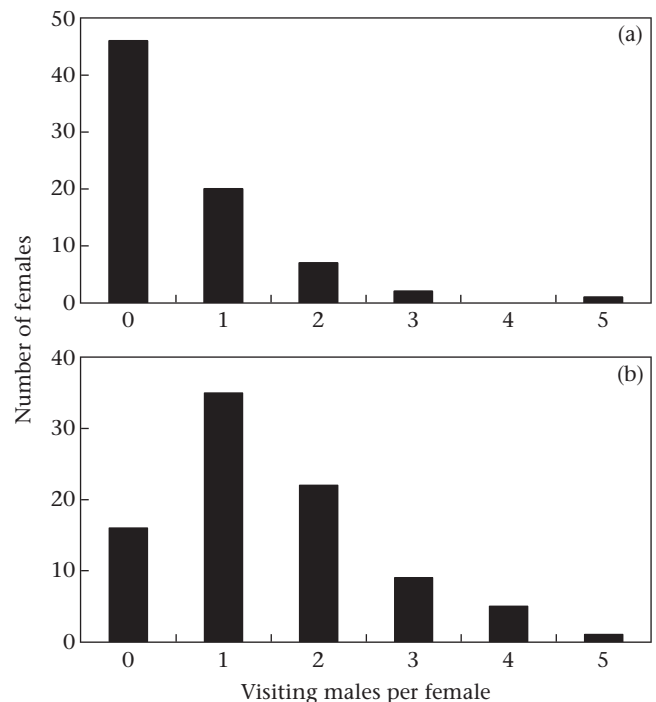
Figure 2. Total number of visiting males per female *Nephila pilipes* recorded from (a) Ape Hill and (b) Huoyan Mountain study sites.
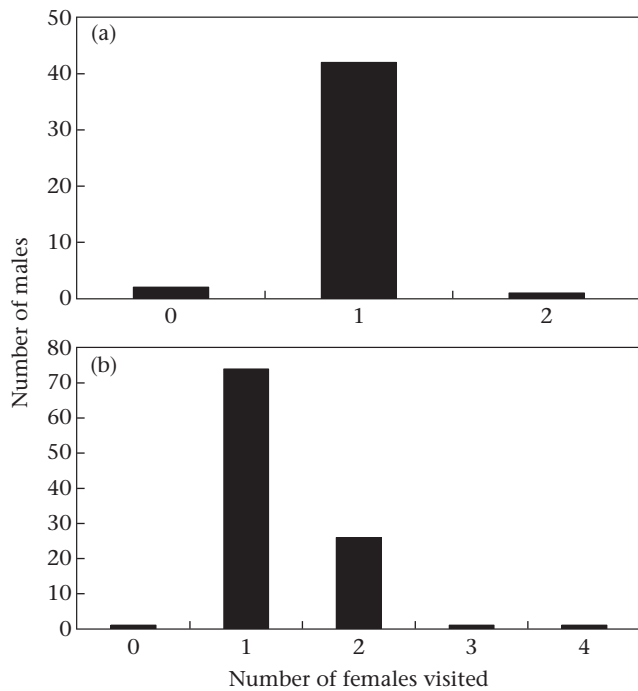
Figure 3. Frequency of male *Nephila pilipes* visiting various numbers of females recorded from (a) Ape Hill and (b) Huoyan Mountain study sites.

body size data were included in the analysis comparing size difference of males occupying different positions on a female's web ($N = 25$). Such data were only available from the Huoyan Mountain study site so a Student's t test was used. Males that were positioned at the hub had a larger body length and cephalothorax width than those occupying other positions (body length: Student's t test: $t_{0.05, 48} = 2.253$, $P = 0.029$; cephalothorax width: $t_{0.05, 48} = 2.67$, $P = 0.01$; Fig. 4a, b). For most of the time males could stay on the same web peacefully and only when smaller males tried to approach the females would larger males actively defend their position. Larger males usually vibrated the web to chase invading males away but sometimes direct competition occurred. We observed one case in which a smaller male was eaten by a larger one (Supplementary Fig. S1) and another in which two males with similar body size competed fiercely to mate with a moulting female (Supplementary video). In a total of 34 mating events recorded from two study sites, 22 (65%) had only one male and 12 (35%) had multiple males visiting the female webs. In eight of the 12 aforementioned cases the larger males mated with the female first whereas in the other four cases the smaller males mated with the female first ($\chi^2_1 = 0.4$, $P = 0.79$). This nonsignificant statistical comparison result showed that males occupying the hub might not be guaranteed the opportunity of being the first or only male to mate with a female. To test whether mated males ($N = 31$) had a larger body size than unmated males ($N = 91$) we pooled the data sets from both field sites and found that these males did not differ in body length or cephalothorax width (Table 1, Fig. 4c, d).

Factors determining male mating success

Results of multiple logistic regressions ($N = 31$ for mated and 91 for unmated males) showed that females' moulting events were the most significant variable determining males' mating success (Table 2). We observed a total of 34 mating events at the Ape Hill and Huoyan Mountain study sites. In 24 of them mating occurred while females were moulting and only 10 occurred while females were not moulting (chi-square test: $\chi^2_1 = 5.76$, $P = 0.025$). Another significant variable is

the number of days for which a male could be tracked (Table 2). Mated males spent an average of 7.74 ± 0.99 days on a particular female's web, while unmated males only spent about 3.83 ± 0.42 days (two-tailed t test: $t_{98} = -3.078$, $P = 0.003$). The rest of the male attributes examined were not statistically significant (Table 2).

Postcopulatory Selection on Males

Paternity analysis

Observed P_2 values showed no significant difference between NI and IN treatments so we pooled the data (Wilcoxon two-sample test: $S = 108.5$, $Z = -1.419$, $P = 0.16$; NI: $N = 12$; IN: $N = 11$). The results showed that the mean P_2 value of the pooled data was 0.41 ± 0.07 ($N = 23$), showing an overall mixed paternity pattern in *N. pilipes*. The individual P_2 values varied from spider to spider (Fig. 5), similar to that reported previously in other spider species.

Fertility and sterility controls showed that fertile males fertilized on average 97% of an eggsac ($N = 2$, 98% and 97% hatching) and that sterile males had 0% hatching success ($N = 1$). One of the female fertility controls died before laying eggs. One female mated to a sterile male was killed to check for sperm motility, which was equivalent to the motility of a fertile control female.

Sperm number and transfer patterns

To determine whether site location influenced male size, we used an analysis of variance. Body size was measured as patella–tibia length and was transformed for normality; averages shown below are untransformed data for ease of comparison. We found no significant difference in male body size across location, males from Huoyan Mountain ($N = 31$) were on average 5.29 ± 0.14 mm and males from Dawu Mountain ($N = 15$) were on average 4.92 ± 0.21 mm (two-tailed t test: $t_{44} = 1.54$, $P = 0.13$). To determine whether body size and sperm number were correlated, a separate set of unmated males ($N = 20$) was measured and the sperm in their palps was counted. The amount of sperm contained in the palps of unmated males was $4\,398\,800 \pm 378\,600$ sperm, ranging from a minimum of $2\,144\,400$ to a maximum of $8\,009\,200$ (the most recorded in any spider species to date). Multiple regression analysis revealed that there was no statistically significant relationship between unmated male body size and sperm number based on patella–tibia length, femur length or cephalothorax width (Table 3). The patella–tibia length was transformed in order to generate a normal distribution for the multiple regression analysis.

During the staged matings males transferred sperm in 43 of 46 trials. A comparison of unmated ($N = 20$) and mated males ($N = 46$) revealed that the palps of unmated males contained more sperm, on average $4\,398\,800 \pm 368\,700$ sperm and that mated males had fewer sperm remaining in their palps, on average $2\,451\,600 \pm 243\,100$ sperm (Student's two-tailed t test: $t_{64} = -4.41$, $P < 0.0001$). For mated males ($N = 46$), there were no significant differences in the amount of sperm remaining in first ($N = 23$) versus second males ($N = 23$) to mate; first males had on average $2\,189\,500 \pm 273\,700$ sperm and second males had on average $2\,713\,600 \pm 393\,900$ sperm (Student's two-tailed t test: $t_{44} = 1.09$, $P = 0.28$). Male body size and sperm count remaining in the palps after mating do not appear to be correlated ($N = 46$); the slope of this regression was -0.023, and the P value for the regression coefficient of body size was 0.98 ($t = 0.03$), suggesting that selection is not operating on male body size through differential sperm amounts. Body sizes of first and second males (5.05 ± 0.17 mm versus 5.29 ± 0.17 mm) were not significantly different (Student's two-tailed t test: $t_{44} = 0.87$, $P = 0.38$; untransformed values of patella–tibia length are shown for ease of comparison).
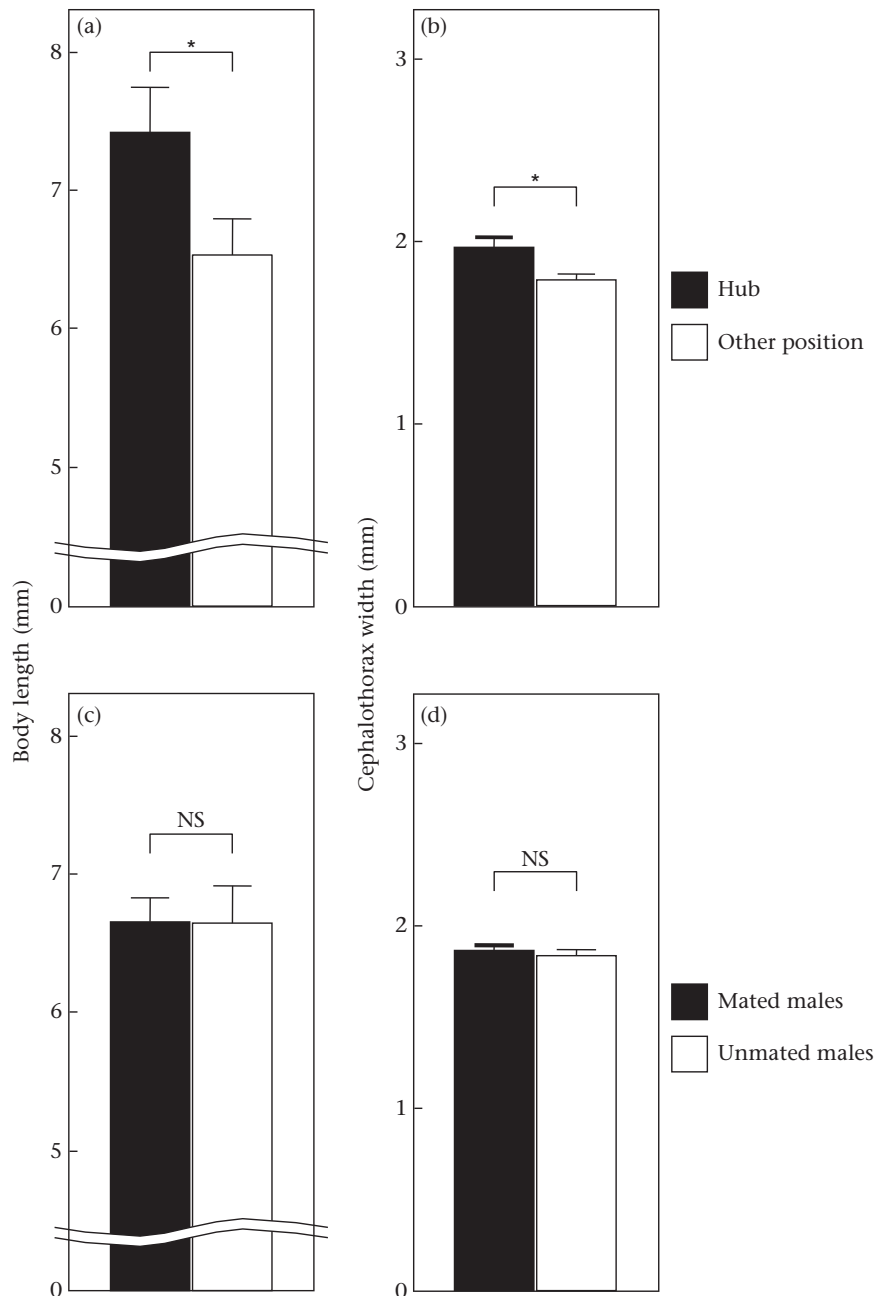
Figure 4. (a) Body length and (b) cephalothorax width of coexisting *Nephila pilipes* males at hub ($N = 25$) and other positions ($N = 25$). (c) Body length and (d) cephalothorax width of mated ($N = 31$) and unmated ($N = 91$) males. Means $\pm$ SE are shown. Data from Ape Hill and Huoyan Mountain study sites are pooled. $^*P < 0.05$.

To determine whether sperm release patterns might be able to predict paternity patterns, we estimated the sperm release by males in the study following the protocol of Danielson-François & Bukowski (2005). On average, we estimated that the amount of sperm transferred to females during mating was $2\,252\,300 \pm 211\,200$ sperm. The amount of sperm transferred by the first male to mate was on average $2\,209\,300 \pm 273\,700$ sperm and that by the second was $2\,295\,400 \pm 327\,800$ sperm, which was not significantly different (Wilcoxon two-sample test: $S = 548$, $Z = 0.1538$, $P = 0.87$). For each mated female in the study ($N = 23$), predictions were calculated based on sperm release (SR_2), the amount of paternity predicted for the second male to mate based on the sperm release from each male. On average, the sperm release predictions estimate a mixed $SR_2 = 0.43 \pm 0.056$ paternity pattern

for *N. pilipes*, which is similar to the observed P_2 values (see above). A comparison of the observed P_2 and the SR_2 predictions revealed no significant difference between the measurement of paternity and our estimate of paternity ($N = 23$, Wilcoxon two-sample test: $S = 549.5$, $Z = 0.187$, $P = 0.85$). Both lines of evidence suggest that sperm are mixed within the sperm storage organs and randomly used in fertilization.

DISCUSSION

SSD studies that show an advantage for smaller males are rare (Blanckenhorn 2005). For smaller males to have an advantage, they must excel in enough of the following areas to offset the advantages of larger males: precopulatory search, female mate choice, sperm

Table 1
Results of two-way ANOVA analyses comparing the body length and carapace width of males inhabiting the hub and nonhub positions while considering the effect of study site

Source	Sum of squares	df	Mean square	F ratio	P
Body length					
Mating status	0.010	1	0.010	1.595	0.209
Study sites	0.000	1	<0.0001	0.079	0.780
Interaction term	0.023	1	0.023	3.678	0.058
Error	0.734	118	0.006		
Cephalothorax					
Mating status	0.000	1	<0.0001	0.323	0.571
Study sites	0.000	1	<0.0001	0.085	0.771
Interaction term	0.001	1	0.001	1.828	0.179
Error	0.070	118	0.001		

Figure 5. The distribution of P_2 values of *Nephila pilipes* used in the staged matings.
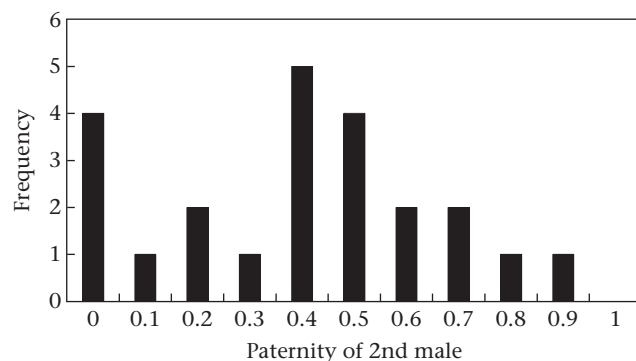
competition and protandry. Here we have demonstrated that the appropriate strategy for *N. pilipes* males seems to be maturing earlier and locating a moulting female faster to outcompete others in a scramble competition for mates. Our field data show that in male *N. pilipes* the key factor to mate successfully with a female is not being large but being able to locate a female at the right time when females are vulnerable during moulting. Although after reaching adulthood females remain receptive for a long period of time, most of the recorded mating events occurred when females were moulting to adulthood and this opportunity was relatively rare and ephemeral. In *N. pilipes*, males face a high risk of cannibalism by females (of 46 staged matings 12 ended in attacks or cannibalism, C. Hou, unpublished data) and therefore it is much riskier to mate with a female when she has completed moulting. In some Theridiidae, in which mating occurs with older females, males court the female again after inseminating one side of the reproductive tract, which allows cannibalistic females more control over paternity (Snow & Andrade 2005). In *N. pilipes*, males typically courted once and then mated with both palps. Our field data also show that subadult female *N. pilipes* change web sites frequently and travel long distances, which makes guarding of potential mates difficult for males. Opportunistic mating during female spiders' final moult has also been reported in *Argiope aurantia* (Foellmer & Fairbairn 2005a). However, in *A. aurantia* in addition to scramble competition during mate searching (Foellmer & Fairbairn 2005b) on females' webs, there was also relatively strong interference competition between males so that larger males were also favoured. In *N. clavipes*, multiple males have been observed guarding penultimate females before their moult, with the hub male excluding his rivals (Christenson & Goist 1979; Vollrath 1980). However, this intense mate guarding was not seen with the monogynous *N. fenestrata* (Fromhage et al. 2007). In *N. clavata* males similarly appear to mate once (C. Hou, unpublished data). The relaxation of direct male–male physical competition caused by the spatial and temporal pattern of female *N. pilipes* may have reduced selection pressures favouring large male body size.

Moreover, there does not seem to be a correlation between larger male body size and a higher number of sperm in *N. pilipes*, so larger males would not have an advantage in sperm competition either. Therefore, selection may have favoured protandry by early maturation, thus generating a large number of small males that enhance their mating success through the process of scramble competition. Furthermore, smaller males enjoy benefits such as ease of escaping predators, climbing upwards and bridging gaps (Moya-Laraño et al. 2002, 2009; Corcobado et al. 2010), which allows them to have more mating opportunities.

Male *N. pilipes* could potentially gain a much higher mating success when interacting with a moulting female. The first reason is that such females cannot perform any female choice, male chasing or male cannibalism because they do not have any mobility until they start to sclerotize. Second, moulting events took at least half a day to complete (C. Hou, personal observations) and therefore a male would have enough time to transfer his sperm and fertilize more eggs. Mating with a moulting female will allow males enough time to transfer more sperm uninterrupted to combat future rivals' sperm. In *N. edulis*, if a male is allowed to have sufficient time to mate with a female twice, his paternity will be doubled (Schneider et al. 2000). If similar conditions also occur in *N. pilipes* then locating a moulting female to be able to conduct multiple copulations will greatly enhance the reproductive success of males. Moreover, several studies have demonstrated that the duration of copulation in spiders is usually longer than necessary to transfer sufficient sperm to fertilize the eggs (Eberhard 1996; Elgar 1998; Snow & Andrade 2004; Danielson-François & Bukowski 2005), so the extra time males obtain may greatly enhance their reproductive success. In *Nephila* spiders, similar to other spiders facing high male mortality (such as those with cannibalistic females), males stop sperm production after maturation and do not recharge their palps after copulation (Christenson 1989). For these so-called 'one-shot-insemination' males (Michalik et al. 2010; Schulte et al. 2010), mating with a moulting female to obtain more copulation time might be an adaptation to enhance reproductive success. Since our results show that in *N. pilipes* there is no first-male sperm priority, finding a moulting female to be able to monopolize her long enough to enhance paternity becomes vital.

Table 2
Results of a logistic multiple regression testing the effect of various male attributes on the mating success of male *Nephila pilipes*

Variables	Estimate	SE	Wald	P
Site	1.6466	1.1518	2.0438	0.153
Female moult	5.8001	1.1646	24.8034	<0.001
Days tracked	0.5741	0.2923	3.8583	0.050
Days spent with a female	−0.5401	0.3217	2.8201	0.093
Number of females visited	−1.0316	1.0200	1.0228	0.312
Body length	8.0878	7.6734	1.1109	0.292
Cephalothorax width	−18.4861	27.6809	0.4460	0.504

$N = 122$, $df = 1$.

Table 3
Results of a multiple regression testing the effect of various male attributes on the amount of sperm in the palps of unmated male *Nephila pilipes*

Variables	Estimate	SE	t	P
Femur length	−895591.8	1905227	−0.47	0.645
Carapace width	−2504758	2934799	−0.85	0.406
Leg length (patella–tibia)	217960.48	166899.9	1.31	0.210

$N = 20$, $df = 1$.

The results of the double-mating experiments suggest that *N. pilipes* exhibits a mixed rather than a first-male sperm priority pattern, indicating the presence of sperm competition in this extremely sexually dimorphic species. Congruent with this proposition are the excessively large numbers of sperm produced (the highest amount recorded for any spider, on average over 4 million with a maximum of 8 million) found in *N. pilipes*. The observed paternity pattern (P_2) matches overall the predictions made from the relative amount of sperm released (SR_2) by the second male. If the reproductive structures of female *N. pilipes* operate in a 'semi-haplogyne' rather than 'entelegyne' fashion and therefore sperm from different males are mixed (Uhl & Vollrath 1998), one would see such a mixed paternity pattern. Further morphological examinations of female genitalia could verify this explanation. Given that the average sperm release predictions ($SR_2 = 0.43$) and the average observed paternity ($P_2 = 0.41$) are fairly similar this seems likely. Although Christenson & Cohn (1988) found P_2 to be fairly low (15–21%) in *N. clavipes*, Schneider & Elgar (2005) found a nearly 0.5 P_2 value in another *Nephila* species (*N. edulis*) and mixed paternity was also observed in *N. plumipes* (Elgar et al. 2003a, b), so a mixed paternity pattern might be more common than expected in the genus *Nephila*. In most paternity studies, the value of P_2 ranges from 0 to 100% and our findings in *N. pilipes* are similar (Elgar 1998). Although typically only the average P_2 is provided, we find the range of values useful for examining the underlying variation in paternity. The forces underlying this variation in paternity are poorly understood (Simmons & Siva-Jothy 1998) but here we have provided a potential answer: variation in sperm release between males. Some of this variation in sperm release could result from males breaking off their emboli inside the female, although we were able to control for that in our study. In *N. edulis*, females were observed with an average of two or three emboli in their sperm storage organs (Fromhage et al. 2007). In *Latrodectus*, males can produce effective plugs (Andrade & Banta 2002; Snow et al. 2006) but mating plugs are not always an effective barrier to further insemination (Masumoto 1993). More research needs to be done on variation in sperm transfer and mating plugs in spiders.

Conclusion

Studies finding that smaller males have an advantage in species with SSD are not common. Our results support intense scramble competition for mates and sperm competition in *N. pilipes*, a species with extreme sexual size dimorphism. This may drive the evolution of smaller males that can move faster and further, enabling them to find suitable females faster than larger ones. Although larger males might win male–male contests, these were observed infrequently. More often a female was alone with a single male during her moult. However, after moulting some females did mate multiple times, suggesting that postcopulatory selection pressure through sperm competition is present. There was no association between sperm production and male body size, suggesting that small males do not have a disadvantage in sperm competition. The advantages exhibited by small males in precopulatory scramble competition and in postcopulatory sperm competition may constitute one major driving force of extreme SSD in orb-weaving spiders.

Acknowledgments

Supplementary Material

Supplementary material associated with this article is available, in the online version, at http://dx.doi.org/10.1016/j.anbehav.2012.07.018.

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