Protein restriction affects sperm number but not sperm viability in male ants

Francisco Dávila*, Serge Aron

Evolutionary Biology and Ecology, Université Libre de Bruxelles, Avenue F.D. Roosevelt 50, CP160/12, 1050 Brussels, Belgium

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ABSTRACT

Sperm cells are costly to produce; diet should therefore affect sperm number and/or viability. In non-social insects and vertebrates, there is compelling evidence that diet influences sperm production. Less is known about this relationship in eusocial hymenopterans (all ants and some bees and wasps), whose mating systems impose unique selective pressures on sperm production. Males face physiological constraints: they acquire all of the resources they will use in future reproductive efforts as larvae and emerge from the pupal stage with a fixed, non-renewable amount of sperm. Furthermore, males die shortly after copulation, but their genetic material persists for years since their spermatozoa are stored in their mates' spermathecae. We examined the effects of protein restriction during larval development on sperm number and viability in the Argentine ant *Linepithema humile*. We also looked at its impact on male development, adult mass, and adult fluctuating asymmetry. We found that protein restriction during larval development significantly reduced sperm production, but not sperm viability. It did not affect the number of males reared, male mass, or male asymmetry. However, males from protein-restricted nests developed much more slowly than males from protein-supplemented nests. These results suggest investing in sperm quality and in somatic growth, which enhances a male's ability to disperse and find a mate, are critical to successful male reproduction.

1. Introduction

The production of sperm cells is energetically costly (Dewsbury, 1982; Reznick, 1985; Simmons, 2001; Parker and Pizzarri, 2010). In several species, there is an energetic trade-off between sperm production and other physiological functions, namely growth (Olsson et al., 1997; Sella and Lorenzi, 2003), reproduction (Simmons et al., 2010), immunity (Simmons and Roberts, 2005; Skau and Folstad, 2005; Simmons, 2011; Radhakrishnan and Fedorka, 2012; Stürup et al., 2014; Schwenke et al., 2016), and survival (Van Voorhies, 1992). A number of studies have also found evidence of trade-offs among sperm traits, including between sperm size and number (Parker, 1970; Pitnick, 1996; Stockley et al., 1997; Oppliger et al., 1998; Tourmente et al., 2009; Immler et al., 2011) and between sperm size and viability (Stockley et al., 1997; Levitan, 2000; Bakker et al., 2014). Such trade-offs exist even though sperm competition can result in selection that works to simultaneously maximize sperm size, number, and morphology (Fitzpatrick et al., 2009; Gómez Montoto et al., 2011; Rowe and Pruett-Jones, 2009; Decanini et al., 2013; Tourmente et al., 2015).

Given that males have a finite amount of energy and nutrients to dedicate to sperm production, dietary quality should affect sperm number and/or viability. When males experience dietary restrictions, sperm number declines; this pattern has been documented in both invertebrates (Gage and Cook, 1994; Fedina and Lewis, 2006; McGraw et al., 2007; Bunning et al., 2015) and vertebrates (Louis et al., 1994; Coulter et al., 1997; O'Dea et al., 2014; Kahrl and Cox, 2015), including humans (Olsen and Ramlau-Hansen, 2012).

Eusocial hymenopterans provide an interesting model system with which to study the effects of diet on sperm traits. First, in this group, males have usually completed spermatogenesis by the time they reach adulthood (Hölldobler and Wilson, 1990). They acquire all the resources they will use in future reproductive efforts as larvae and emerge from the pupal stage with a fixed, non-renewable amount of sperm. They cannot later increase their supplies because their testes degenerate before or shortly after eclosion. Thus, the energy available for sperm production is determined by a larva's diet, and the number of sperm stored in the accessory testes at eclosion reflects the total investment in sperm production. Second, the reproductive systems of ants, bees, and wasps impose unique selective pressures on sperm production. Both sexes typically mate during a short window of time at the beginning of their adult lives. Queens store a lifetime's supply of sperm in their spermathecae and use it to fertilize their eggs, sometimes for decades. Fertilization therefore goes on long after insemination has occurred. Consequently, although males die shortly after copulation, their genetic material persists *post mortem* for years. Therefore, males are under strong selective pressure to maximize sperm quantity and

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quality, since it directly influences the lifetime reproductive success of the partners (Baer, 2003, 2011; Boomsma et al., 2005).

Surprisingly, the effect of resource availability on sperm number and viability remains poorly studied in social Hymenoptera, and the available data are ambiguous. In the stingless bee *Melipona beecheii*, males reared in pollen-deficient colonies were significantly smaller and produced fewer spermatozoa than did males reared in pollen-supplemented colonies (Pech-May et al., 2012). In the honeybee *Apis mellifera*, in contrast, pollen restriction during development or adulthood did not have a significant effect on sperm number or viability (Stürup et al., 2013; Czekonska et al., 2015). Similarly, dietary restrictions at adulthood did not affect sperm number in the neotropical ant *Ectatomma ruidum* (Shik et al., 2012).

Here, we investigated the effects of protein restriction during larval development on sperm number and viability in the Argentine ant *Linepithema humile*. In this species, males are the dispersing sex: they fly away from the natal nest within a few days after eclosion and enter neighboring colonies to mate with virgin females (Markin, 1970; Keller and Passera, 1992, 1993; Passera and Keller, 1992). Males and queens mate only once (Krieger and Keller, 2000), which relaxes the potential selective pressure exerted by sperm competition on sperm traits. Consequently, males should be expected to allocate resources towards traits that maximize the chances of insemination, namely finding a mate and fertilizing all her eggs. We compared the number and viability of spermatozoa stored in the accessory testes at the time of eclosion for males reared in protein-restricted *versus* protein-supplemented nests. Given that sperm number (and possibly viability) might be correlated with male size, we examined how protein availability affected male mass, number, and development time within nests. Finally, because environmental stress during growth can result in developmental instability (Palmer and Strobeck, 1986; Parsons, 1994), we assessed the effect of protein availability on fluctuating asymmetry (FA), which is the difference in size between the right and left sides of bilaterally symmetrical organisms (Van Valen, 1962).

2. Material and methods

2.1. Field sampling and laboratory rearing

Large numbers of *L. humile* were collected in Giens (southern France) in early March 2015. In French populations of *L. humile*, queens stop laying eggs in the winter. Males develop from overwintering brood (i.e., eggs laid by queens in late fall) and the very first eggs produced by queens after hibernation (Benois, 1973; Passera et al., 1988). Colonies were collected before queens had resumed egg laying. From these, we established 50 experimental nests, each consisting of 30 queens, 3000 workers, and no brood. They were maintained under standard laboratory conditions (25 ± 2 °C, 12:12 light:dark cycle, ~40% humidity) and given sugar water and mealworms *ad libitum*. After three weeks, queens were removed from the experimental nests to trigger the rearing of reproductives from eggs laid by the queens (Keller et al., 1989). Therefore, at this point in time, the brood consisted exclusively of eggs. The nests were then randomly assigned to one of two dietary treatments. Twenty-five nests were in the protein-restriction treatment and were given only sugar water (1 M sucrose). The other 25 nests were in the protein-supplementation treatment and were given sugar water plus 0.5 g of protein (ground mealworms mixed with sunflower oil) five days/week. The nests were monitored each day for six weeks, and the numbers of males produced were counted. Following eclosion, males were weighed and set aside (with a group of workers) to ensure they reached full sexual maturity without copulating. In *L. humile*, spermatogenesis is complete one to three days after eclosion, and males are able to mate quite quickly (Passera and Keller, 1992). The males were dissected to determine sperm number and viability; their heads and thoraces were stored in ethanol (90%) for quantification of the FA (see below).

2.2. Sperm number and viability

The number of spermatozoa stored in the accessory testes as well as sperm viability (i.e., the percentage of total sperm cells that were alive) were determined using flow cytometry (FCM), in accordance with the method described in Paynter et al. (2014). FCM is rapid and accurate; it saves time and effort and, more importantly, reduces the potential photobleaching that occurs with conventional microscopy (Paynter et al., 2014).

Males were placed in semen diluent (50 nM Tris (hydroxymethyl) aminomethane, 188.3 mM NaCl, and 5.6 mM glucose; pH 8.7) and dissected under a Leica MZ6 stereomicroscope (Leica Microsystems, Wetzlar, Germany). All the males sampled had degenerated testes, and their accessory testes were filled with spermatozoa ready to be ejaculated, an indication that the ants were fully mature. For each male, both accessory testes were transferred to a drop of semen diluent. The testes were then punctured and carefully squeezed; the released semen was transferred to a vial, and semen diluent was added to bring the final volume of the suspension to 800 μl. Sperm cells were stained using the Live/Dead Sperm Viability Kit (Life Technologies, California, USA); SYBR 14 labels live sperm with green fluorescence, while propidium iodide (PI) dyes membrane-compromised or dead sperm with red fluorescence. We proceeded as follows: first, 100 μl of 1 μM SYBR 14 dye was added to the semen suspension, and the resulting mixture was incubated in the dark at room temperature for 15 min; second, 100 μl of 120 μM PI was also added, and the modified mixture was incubated for another 15 min under the same conditions. The final suspension was gently mixed, filtered through a nylon filter with a 30 μm mesh diameter, which allowed sperm to pass through while removing any remaining tissue of larger size, and transferred to a 3.5 ml Sysmex Partec FCM tube. The concentration of sperm cells and their viability were estimated using a CyFlow Counter cytometer (Sysmex Partec, Germany), employing the same parameter values as Paynter et al. (2014). Briefly, events were triggered by forward scatter and side scatter. Both the SYBR 14 and PI in the solution were excited by a 488 nm blue solid state 50 mW laser. SYBR 14 fluorescent emissions were collected at 530 nm (bandpass FITC filter), whereas PI fluorescent emissions were collected at 630 nm (PE-TR-Tandem filter); there was no compensation for spectral overlap. We recorded the height of the resulting spectra. Data were analyzed with using *FloMax Software©* (v. 2.9; Quantum Analysis GmbH).

2.3. Fluctuating asymmetry

To quantify the effects of protein restriction during larval development on FA, we measured 17 morphological characters for each male (n = 30): scape length, antenna length, coxa + trochanter length, femur length, tibia length, basitarsus length, and tarsus length for the forelegs, midlegs, and hind legs. The antennae and legs were mounted on slides using Euparal. They were photographed using light microscopy at 50x magnification (MZ6 stereomicroscope; Leica Microsystems, Wetzlar, Germany), and the length of each character was measured to the nearest 0.01 mm using IMAGEJ software (v. 1.8; NIH, Bethesda, MD, USA; http://imagej.nih.gov/ij/). Each character was measured twice, and at least 24 h was allowed to elapse between the two measurements (Palmer, 1994). There was a strong correlation between the first and second measurements (Spearman correlation test: $r_s = 0.995$, $P < 2.2 \times 10^{-16}$), indicating that measurement error was very low and could be ignored. For each character, we measured the difference in length between the right and left sides (R_i-L_i) and compared this difference for males from protein-restricted *versus* protein-supplemented nests (Palmer and Strobeck, 1986; Keller and Passera, 1993). There was no association between a character's mean value (($R_i + L_i$)/2) and its estimated asymmetry ($|R_i$-$L_i|$) (Spearman correlation test: $P > 0.05$ for all characters).

2.4. Statistics

To test for treatment effects on sperm number and viability, we used nested ANOVAs where sperm number or viability was the dependent variable, colony was a random effect and dietary treatment was a fixed effect. Males were considered to be replicates within colonies, and the different colonies were replicates within treatments. When appropriate, Box-Cox transformations were applied to achieve normality. Treatment-based differences in the number of males reared and male body mass were tested for significance using a Wilcoxon-Mann-Whitney test and a Student's *t*-test, respectively, after checking the assumption of normality using a Shapiro–Wilk test. A Cox proportional hazards regression analysis was used to compare the number of males produced over time between treatments. All statistical analyses were conducted using R (v. 3.2.1; R Development Core Team 2013). Means $\pm$ SD are given.

3. Results

Forty-three of the experimental nests produced adult males. The treatments affected neither the number of males reared (Wilcoxon-Mann-Whitney test: $W = 673.5$, $P = 0.698$) nor male mass (Student *t*-test: $t = -0.069$, df $= 41$, $P = 0.945$) (Table 1). However, males from protein-restricted nests grew more slowly and reached adulthood later than those from protein-supplemented nests (Cox proportional regression: Wald statistics: 65.08; 95% CI: 3.84–9.10, $P = 6.7^{-16}$; Fig. 1): it took 46 days for all males to reach maturity in the former, but 34 days to do so in the latter.

Protein availability during development significantly influenced sperm number (nested ANOVA: $F = 34.618$, $P = 5.43 \times 10^{-7}$; treatment $\times$ colony interaction: $F = 0.638$, $P = 0.429$) (Fig. 2a). On average, males from protein-restricted nests produced 1.7-fold fewer sperm than males from protein-supplemented nests (Table 1). In contrast, sperm viability did not differ between treatments (nested ANOVA: $F = 1.407$, $P = 0.246$; treatment x colony interaction: $F = 0.038$, $P = 0.847$) (Fig. 2b).

None of the 17 characters studied showed a significant treatment-related difference in FA (Wilcoxon-Mann-Whitney test, $65.5 \leq W \leq 144$, $n = 30$, all $P > 0.05$), indicating that protein restriction had a weak effect on asymmetry of bilateral traits, if any at all.

4. Discussion

Our study shows that protein restriction during larval development significantly reduces sperm number, but not sperm viability, in the Argentine ant *Linepithema humile*. This suggests that, in this species, sperm viability may play a more important role than sperm number in male fertility. These results contrast with those reported for the honeybee (*Apis mellifera*), where pollen restriction during development

Table 1
Total number of males reared, and mean $\pm$ SD of the number of males produced, male mass, fluctuating asymmetry, sperm number and sperm viability, for protein-restricted *versus* protein-supplemented nests of the Argentine ant *Linepithema humile*.

	Protein-restricted nests (n = 20)	Protein-supplemented nests (n = 23)
Total number of males reared	76	81
Number of males produced per nest	3.8 $\pm$ 1.7	3.5 $\pm$ 1.5
Male mass (mg)	1.3 $\pm$ 0.2	1.3 $\pm$ 0.1
Fluctuating asymmetry (mm)	0.022 $\pm$ 0.025	0.028 $\pm$ 0.029
Sperm number	381 328 $\pm$ 157 388.9	674 675 $\pm$ 179 727.4
Sperm viability (%)	90.1 $\pm$ 5.4	88.7 $\pm$ 4.1

has no effect on sperm number or viability in adult males (Czekonska et al., 2015). Such a discrepancy might stem from phylogenetic differences, or from disparities in the intensity of post-copulatory sexual selection. Honeybee queens mate with 12 males on average, although this number can exceed 40 (Tarpy and Nielsen, 2002). Consequently, ejaculates from multiple males may co-occur in the female's reproductive tract and sperm may compete to reach the queen's spermatheca. Recent work has indeed shown that proteins in the seminal fluid, which are produced by the males' accessory glands, reduce the survival of rival male sperm in the female genital tract (den Boer et al., 2010, 2015). Sperm competition is expected to generate intense directional selection on sperm traits that enhance fertilization success, such as sperm number and viability (Simmons, 2001; Birkhead et al., 2009). However, to date, only a few studies have explored the relationship between sperm number and viability. They show that the two can be uncorrelated (cockroaches: Moore et al., 2004) or positively associated (*Drosophila*: Holman and Snook, 2008; Decanini et al., 2013; rodents: Gómez Montoto et al., 2011), suggesting that post-copulatory sexual selection does not underlie trade-offs between sperm number and quality in polyandrous species. In contrast, in monandrous species, selection is relaxed on competition-related sperm traits, such as sperm number (Simmons, 2001; Parker and Pizzarri, 2010). Indeed, in ants, there is evidence that the level of sperm production covaries with the level of sperm competition (Baer and Boomsma, 2004; Aron et al., 2016). In our study species, the ant *L. humile*, males and females mate only once and the number of sperm cells transferred is largely sufficient to ensure the fertilization of all the eggs laid during a queen's lifetime (see Table 1) (Keller and Passera, 1992; Cournault and Aron, 2008). In support of this argument, queens of the Argentine ant store as many sperm cells as present in one male's entire ejaculate (Keller and Passera, 1992), they are short lived (lifetime expectancy of about 1 year; Keller et al., 1989), and die fully fertile while still harbouring large amounts of sperm in their spermatheca (Keller et al., 1989; L. Passera, pers. comm.). Therefore, sperm limitation does not constrain lifetime reproductive success in this species. It would appear that the percentage of live, fertilization-competent sperm transferred to queens is a key element of fitness for both sexual partners. There is thus strong selective pressure on males to produce a high percentage of viable sperm.

Our results are consistent with those of other studies showing that sperm production is positively associated with protein intake in mammals (Ng et al., 2004; Cheah and Yang, 2011), as well as in both social (Pech-May et al., 2012) and non-social insects (Gage and Cook, 1994; McGraw et al., 2007; Bunning et al., 2015; but see Cook and Wedell, 2006 or Lewis and Wedell, 2007 for evidence that diet does not affect sperm production in butterflies). Studies in mammals showed that amino acids like arginine or L-carnitine contribute to spermatogenesis, sperm maturation and motility (Ng et al., 2004; Wu et al., 2009). In a meticulous study of the effects of diet on sperm production in the speckled cockroach *Nauphoeta cinerea*, Bunning et al. (2015) demonstrated that protein and carbohydrate intake influenced sperm number, but not sperm viability. Sperm number was closely associated with the number of offspring produced by the mating partner and was maximized at a protein-to-carbohydrate ratio of 1:2. The effect of this nutritional ratio on sperm production in social Hymenoptera remains unknown. As in many ants, *L. humile* raises aphids and scale insects, whose honeydew can account for more than half of the ants' diet (Lester et al., 2003; Shik and Silverman, 2013; Shik et al., 2014). Carbohydrates are a nutritional requirement for larvae. We conducted a preliminary study in which we found that colonies fed a no-sugar diet (i.e., consisting of just water and homogenized mealworms) failed to produce any adults from their brood (Dávila, unpublished data). Similarly, larvae of the green-head ant *Rhytidoponera metallica* die within 2–3 weeks when colonies are fed a carbohydrate-poor diet (Dussutour and Simpson, 2009). More recently, it has been shown in the fire ant *Solenopsis invicta* that access to carbohydrates resulted in colonies producing higher numbers of workers and larger workers
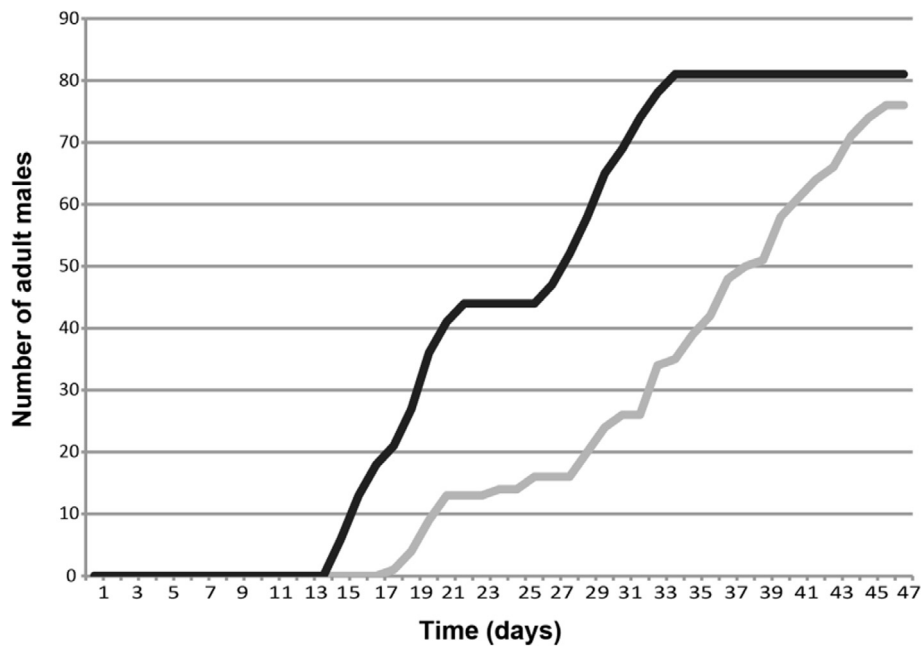
Fig. 1. Cumulated number of adult males produced across all colonies in protein-restricted (gray) and protein-supplemented (black) nests of the Argentine ant *Linepithema humile* over time.
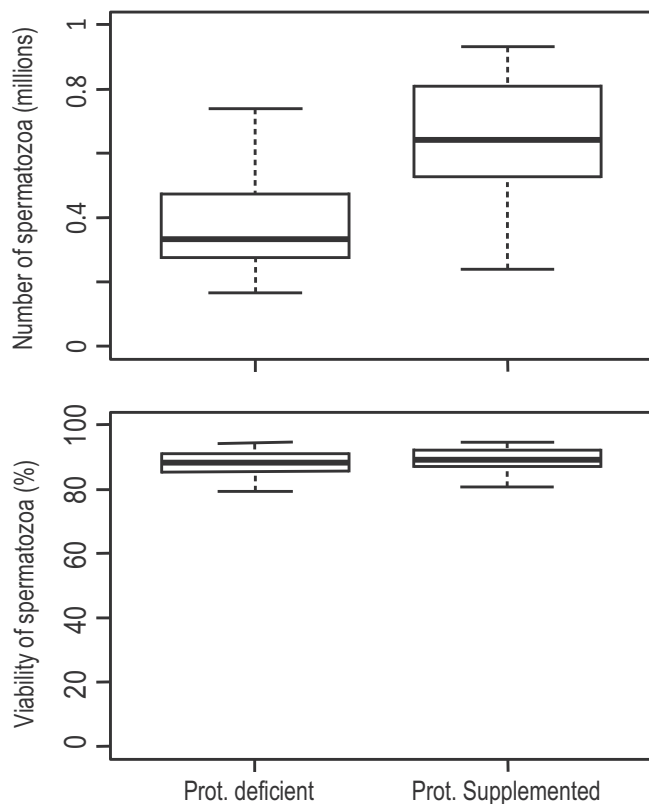

Fig. 2. (a) Sperm number and (b) sperm viability (percentage of total sperm cells that were alive) produced by males reared in protein-restricted *versus* protein-supplemented nests of the Argentine ant (number of males: n = 76 and n = 81, respectively). The median is marked by a horizontal line. The lower and upper ends of each box represent the 25% and 75% quartiles of the data, respectively, and the whiskers show the entire range of the data.

(Wills et al., 2015).

Interestingly, we found there was no difference in the number of males produced or in the male mass between protein-restricted and protein-supplemented nests. Male larvae reared in protein-restricted nests took longer to develop but reached the same mass at maturity as males reared in protein-supplemented nests. The lack of detectable male phenotypic differences between diet treatments might stem from workers having a buffering effect on the nutritional conditions. Indeed, food shortage has been shown to trigger cannibalism of siblings in ants and other social insects (Hölldobler and Wilson, 1990; Crespi, 1992). Cannibalism by workers permits tailoring of reproductive efforts of the colony to resource availability or other environmental conditions (Chapuisat et al., 1997; Wharton et al., 2008). In the Argentine ant, it has been shown that workers discriminate between sex, caste and age of the brood and selectively eliminate larvae according to social environment (Aron et al., 1994; Passera and Aron, 1996). In the protein-restriction treatment, nurse workers could have consumed developing worker or female larvae and provided the amino acids to developing male larvae via trophallaxis. Our results suggest that investment in mass (up to a certain threshold) is pivotal in maximizing fitness. Males are the dispersing sex in *L. humile*: they undergo short nuptial flights to reach neighboring nests, where they mate with virgin queens (Passera and Keller, 1992). Although dispersal distances are no more than a few meters, a male's flying abilities are key to his reproductive success. There is probably an optimal mass (or size) that maximizes dispersal capacity (Clobert et al., 2012). Lighter males might be at a disadvantage due to their underdeveloped wing muscles, whereas heavier males might face the challenge of increased wing loading and drag. Since males are short lived and die a few hours after mating, there is no need for them to allocate energy to anything but growth and reproduction. Our results show that protein restriction during larval development affects sperm number but not body mass, suggesting that dispersal and mating success take priority over investment in offspring when resources are limited. This finding is fully consistent with the optimal energy allocation model, which predicts that short-lived organisms (like male ants) should first allocate all their energy to vegetative growth and then completely shift to allocating all their energy to reproduction (Kozlowski, 1992; Roff, 1992; Stearns, 1992).

We found that male bilateral symmetry is unaffected by protein restrictions. This is consistent with other research showing that environmental conditions (in ants; Fjerdingstad and Keller, 2004) and protein availability (in honeybees; Szentgyörgyi et al., 2016) caused no or only weak differences in the fluctuating asymmetry of size and shape

in workers, males, and queens. In the same vein, a previous study showed that inbreeding had no effect on the level of fluctuating asymmetry in *L. humile* workers (Keller and Passera, 1993). These results contrast with other studies showing that nutritional stress often increases fluctuating asymmetry of bilateral traits in animals (e.g., birds: Nilsson, 1994; *Drosophila*: Imasheva et al., 1999), and lend credence to the idea that social Hymenoptera could have mechanisms maintaining developmental stability that allow them to counteract the effects of environmental stress on growth.

In conclusion, our study shows that protein restriction during larval development in *Linepithema humile* impacts sperm number but not sperm viability. Furthermore, although protein restriction increased developmental time, male mass at eclosion was unaffected. Taken together, these findings suggest that males experience selective pressure that promotes investment in sperm quality and somatic growth, which enhances their ability to disperse and find a mate. Argentine ant queens are short-lived (~1 year) and mate just once. In other social Hymenoptera, however, queens may live several years (Keller and Genoud, 1997) and/or mate with multiple males (Boomsma et al., 2005, 2009). Future studies should examine if and how investment in sperm traits depends on life-history traits, such as the queen's life expectancy and mating frequency, which could drive sperm competition.

Acknowledgments

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