Sexual selection on male development time in the parasitoid wasp *Nasonia vitripennis*

A. M. MOYNIHAN & D. M. SHUKER

School of Biology, University of St Andrews, St Andrews, Fife, UK

Abstract

Mating systems are shaped by a species' ecology, which sets the stage for sexual selection. Males of the gregarious parasitoid wasp *Nasonia vitripennis* compete to mate virgin females at the natal site, before females disperse. Males could increase their fitness by being larger and monopolizing female emergence sites or by emerging earlier pre-empting access to females. We consider sexual selection on male body size and development time in *Nasonia*, and a potential trade-off between the two traits. We explored sex-specific patterns of larval and pupal development, finding that smaller wasps developed slower than their host-mates. Using competition experiments between brothers, we found that earlier eclosing males mated more females independently of absolute and relative body size. Our data explain the lack of relationship between fitness and body size in male *Nasonia* and reinforce the importance of protandry in mating systems where access to mates is time-limited.

Introduction

Sexual selection is expected to have acted on male traits that make them good at finding and inseminating females and then having their sperm used to fertilize eggs (Darwin, 1871; Andersson, 1994). The way in which males achieve this fitness will be shaped by the mating system, which describes the spatial and temporal predictability of females and the extent to which males can gain or monopolize access to females (Emlen & Oring, 1977). If females can be defended, then sexual selection is predicted to have resulted in adaptations, such as weapons or large body size, which increase the chance that a particular male will be successful. If females cannot be defended, this will lead to a different suite of adaptations, to increase a male's chance of locating and courting a female (Andersson, 1994).

Females are easiest to defend if they are clustered together in space or time. If females are clustered together in time, then the life history and development

times of males and females may be under sexual selection to ensure that individuals enter the mating population at the appropriate time. Development time is known to be an important component of fitness in many organisms, such as butterflies (Fagerström & Wiklund, 1982), damselflies (Plaistow & Siva-Jothy, 1999), mayflies (Rowe & Ludwig, 1991), mosquitoes (Holzapfel & Bradshaw, 2002) and salamanders (Semlitsch *et al.*, 1988). The importance of development time to fitness is apparent in species where one sex is selected to be ready to mate before the other (Morbey & Ydenberg, 2001). Morbey & Ydenberg (2001) categorize the hypotheses to explain one sex's readiness to mate before the other into two groups (Table 1). First, there are those hypotheses in which selection influences the arrival time of each sex into the mating population, but not the relative arrival time of each sex (i.e. the difference between the sexes – the extent of protandry or protogyny – is an indirect outcome of selection). Second, there are those hypotheses that consider selection directly on the relative arrival times of each of the sexes to the mating population. For the latter, the earlier sex (usually males) either gains an advantage through increased mate opportunities, or the later sex minimizes the time spent waiting for potential or suitable mates, or

Correspondence: David M. Shuker, School of Biology, University of
St Andrews, Harold Mitchell Building, St Andrews, Fife KY16 9TH, UK.
Tel: +44 1334 463 376; fax: +44 1334 463 366;
e-mail: david.shuker@st-andrews.ac.uk

Table 1 The seven hypotheses for the evolution of protandry reviewed by Morbey & Ydenberg (2001). The hypotheses are grouped in terms of direct or indirect selection on the difference between the sexes in development time.

Hypothesis		Direct/Indirect	Summary
1	Mate opportunity	Direct	Direct selection on the relative arrival of the sexes to maximize the mating opportunities of the earlier sex
2	Waiting cost	Direct	Selection on the relative arrival of the sexes in order to minimize the time spent un-mated by the later sex
3	Mate choice	Direct	Selection on the choosing sex to arrive later in order to have a wider pool to chose from
4	Outbreeding	Direct	Selection on the relative arrival of the sexes so that related individuals avoid mating
5	Rank advantage	Indirect	Selection on the territorial sex to be earlier than their competitors
6	Susceptibility	Indirect	Selection on the later sex to avoid risky conditions they would be exposed to earlier, for example bad conditions in the mating area
7	Constraint	Indirect	Stronger selection on a trait correlating with late arrival in one sex, such as prolonged feeding for larger body size

can chose a higher-quality mate from those that have been in the mating pool for longer. The mating system of a species will play a key role in the selection for either protandry or protogyny, and the seven hypotheses need not be mutually exclusive.

Obtaining and monopolizing mates when females are clustered spatially or temporally may therefore involve contest traits (such as body size) as well as developmental traits. Traits like body size and development time may well also trade-off with one another (Stearns, 1992), and it has been shown in a number of species that as development time becomes critical, for example as the end of the season approaches, individuals sacrifice the proposed benefits of a large body size in order to become reproductively mature sooner [model (Rowe & Ludwig, 1991), empirical tests (Semlitsch et al., 1988; Plaistow & Siva-Jothy, 1999)]. Many of these studies assume that large body size confers fitness benefits to males and females: this has not always been found to be true when tested though (McLachlan & Allen, 1987; Klingenberg & Spence, 2003; Burton-Chellew et al., 2007). In any case, the extent to which contest or development time traits are favoured by sexual selection is likely to depend on the details of the ecology or mating system of any given species.

For the gregarious parasitoid wasp *Nasonia vitripennis*, the mating system is best described as a mix of scramble competition and defence of the female emergence site. Female *Nasonia* lay a large clutch of eggs on dipteran pupae within the host puparium. Once hatched, the larvae feed on the host tissues, before pupating and then eclosing into adults (Whiting, 1967). Males emerge from the host puparium first, waiting near the exit hole they chewed for newly eclosed females to emerge; such defence tends to break down if many males are present, with males scrambling to find virgin females. If the patch contains multiple hosts, males may explore other hosts and can detect whether these hosts also contain female pupae soon to eclose (Shuker et al., 2005). As with many parasitoids, *N. vitripennis* has highly structured populations, with sib-mating commonly occurring at the natal site. This can result in the

so-called local mate competition (LMC) between related males, which selects for female-biased sex ratios (Hamilton, 1967; Werren, 1983; West, 2009). Despite the female-biased sex ratios arising from LMC, males usually have at least one competitor in both the wild and the laboratory (Grillenberger et al., 2008). However, although we know a lot about some of the consequences of the mating system in parasitoid wasps like *Nasonia* in terms of things like sex ratio selection, we know rather less about the sexual selection that arises under such mating systems (Godfray, 1994).

This mating system combined with the mating behaviour of the wasps allows us to make predictions about the relative importance of body size and development time to male fitness. Most females emerge from the host puparium as virgins (99%, Drapeau & Werren, 1999) and then males mount them and begin courtship; most females mate the first male they encounter and become unreceptive to further matings for several hours (Shuker et al., 2007; Leonard & Boake, 2008). On the rare occasions that polyandry does occur, there is first-male sperm precedence (Holmes, 1974; Leonard & Boake, 2008). After mating, females disperse away from the patch to search for oviposition sites, leaving the shorter-winged (brachypterous) males behind (King, 1993). The *N. vitripennis* mating system therefore appears to favour protandry in many aspects: access to females is temporally limited (Thornhill & Alcock, 2001), and females disperse after typically mating just once, with first-male sperm precedence (Holmes, 1974; Fagerström & Wiklund, 1982; Leonard & Boake, 2008).

Just as we expect mating system to shape selection on development time, it will also impact other traits, including body size. The contribution of body size to fitness in this wasp has been investigated for both sexes. Bigger females have higher fecundity in *N. vitripennis* (van den Assem & Jachmann, 1982; West et al., 2001). For males, size could confer an advantage in intra-sex competition to control the emergence site. Anecdotally, the largest male *N. vitripennis* successfully defends the emergence hole in the host puparium and consequently monopolizes the females emerging (van

den Assem & Vernel, 1979). However, no size advantage has been found in competitive environments in previous experiments (Burton-Chellew *et al.*, 2007), although larger males have higher sperm resources on eclosion which could confer fitness advantages (Clark *et al.*, 2010). In summary, both body size and development time could contribute towards male fitness in *N. vitripennis*.

In this study, we did two things. First, we tested for a relationship between body size and development time in *N. vitripennis*. We performed three experiments in order to test this relationship at pupation, adult eclosion and emergence from the host puparium. The time spent feeding will be a crucial component to any body size–development time relationship. Observing the timescale of pupation within the context of both eclosion and emergence from the puparium allowed us to see whether larger individuals spent more time feeding, and whether larval feeding time differed between the sexes. In many parasitoids, including *Nasonia*, there is evidence of adults spending time inside the host puparium and even mating before they emerge (Drapeau & Werren, 1999). To disentangle the relationships between body size and eclosion and emergence time, we opened hosts and removed the pupae to observe the eclosion times for natural clutches of wasps. Second, we tested the fitness benefits of being larger or emerging from the host earlier in a competitive mating situation that mimics the natural mating system. The mating system of *N. vitripennis* led us to predict that earlier males will have more matings than their later competitors and that this will have a greater impact on fitness than differences in body size. If this is true, and there is a trade-off between body size and development time, we would expect body size to be sacrificed for development time.

Materials and methods

Wasps

The experiment used two laboratory strains of *N. vitripennis*: AsymC and STDR-TET. Both strains are antibiotic-treated and free from *Wolbachia* and other endosymbionts. The strains differ in the allele at the eye colour locus *S* within the *R-locus* (Whiting, 1961). The AsymC line is homozygous for a black, wild-type eye colour allele; these are referred to here as the wild-type wasps. The STDR-TET wasps are homozygous for a recessive scarlet eye colour allele at the same locus; these are referred to here as red-eye wasps. We kept the wasps under controlled conditions in incubators at 25 °C and under a cycle of 16 h of light with 8 h of darkness throughout experiments and culture. Under these conditions, the generation time is approximately 14 days (also see Results). In both experiments and culture conditions, the hosts used were the pupae of the large dipteran *Calliphora vicina*.

What is the relationship between body size and development time?

To control for maternal effects, we initiated grandparental generations for our experiments. We took mated 2-day-old females from stock tubes to be the grandmothers of the experimental generation and put them through a pretreatment process. Females are synovigenic: they are born with some immature oocytes and must host-feed to gain protein for egg maturation (Rivero & West, 2005). To allow this, we started the pretreatment process with the female on a host for 24 h. We then removed the host and gave the females a piece of filter paper soaked in honey water for 24 h, before a second host is provided for oviposition. The female had this host to oviposit on for 24 h before she was removed and the parasitized host incubated.

Once the offspring of these grandmothers emerged from the hosts, they were allowed to mate with their siblings for 24 h. We used one mated female from each tube to be an experimental mother. We put these females through the same pretreatment regime as the grandmothers with one difference: mothers had their second host to oviposit on for only 6 h. All females received their host within 45 min and were removed in the same order at the same rate. This short window ensured that any within-brood differences in development time of more than 6 h cannot be attributed to differences in laying order alone. After oviposition, we removed the females and allocated the parasitized hosts randomly to an experiment and incubated them. In order to test the relationship between body size and development time, we assayed development at three transitions: larvae to pupae; pupae to adults; and adults leaving the host puparium. This allowed us to see whether relationships between development time and body size are due to differences in time spent feeding, pupating or leaving the host puparium.

Experiment one: pupation time
To investigate the relationship between body size and the onset of pupation, we scored the numbers of wasp larvae and pupae within hosts across a series of time-points. We opened 15 hosts of each strain per day from days three to six post-oviposition and counted the number of larvae. As the wasps began to pupate, we scored developmental stage more frequently: on day seven post-oviposition, we scored 36 hosts over 3.5 h, then after a 2-h gap, another 45 hosts were scored in a further 2.5-h session. On day eight, we scored 24 hosts over 2 h. The sample size for each strain is therefore n = 110, or n = 115. Any pupae were carefully removed and kept at 25 °C until they eclosed as adults. Individuals were considered pupae once legs and wings were clearly visible under a dissecting microscope, corresponding to the white pupa stage (*sensu* Whiting, 1967). A single observer (AMM) carried out this stage to standardize any

subjectivity. Once individuals had eclosed as adults, we used an Olympus microscope and digital camera set-up, using the Olympus proprietary software Cell^D to measure the hind tibia length of the wasps to 0.0031746 μm. Hind tibia length is a common measure of body size in parasitoids (Godfray, 1994). Male *N. vitripennis* are often smaller than their female conspecifics, but have longer tibia lengths due to differing allometries (Whiting, 1967; Sykes *et al.*, 2007).

Experiment two: eclosion time

In order to observe eclosion, we removed the wasp pupae from their host. To control for any potential impact of this manipulation on development time, we opened the hosts at two points: the yellow eye stage on day nine post-oviposition and the half-melanized stage on day eleven post-oviposition (Whiting, 1967). We counted any diapause or dead larvae with the pupae and included this in the final clutch size. The 33 hosts that contained more than five diapausing larvae were excluded from the analysis, as their effect on the development of their siblings is unknown. After the pupae had been removed from the host, they were returned to a glass vial and kept as a sibling-group. We set up and observed n = 72 replicates for each strain at each of the two pupal stages, at randomized positions within the incubator.

Adult wasps typically emerge around lights-on in the incubator (Bertossa *et al.*, 2010), which was 07:00 h. We commenced observations at 10:30 on day thirteen post-oviposition prior to what we expected to the commencement of emergence on day fourteen. We then started observations at 05:00 h on days fourteen and fifteen after oviposition. Observations continued until 15:00 h, by which time very few wasps per hour were eclosing. A total of 26 scans were performed across all 3 days. The times between scans varied from 23 to 231 min, with a mean of 77.07 (SE = 13.06) min; this variation was due to differing numbers of tubes requiring handling in each scan, with a desire to maximize the number of scans performed. When adult wasps were found in a tube, we removed and kept them for measuring and recording the replicate number, date and time they were collected. We followed the tibia measuring procedure described for experiment one. We also measured and recorded any individuals that did not eclose or emerge during the observation period (constituting a 27th time-point).

Experiment three: emergence from the host

Emergence occurs when the adult wasps leave the host puparium; therefore, in this experiment, we did not manipulate the hosts after oviposition. We observed n = 72 hosts for each strain. These hosts were observed, collected and measured under the same conditions, and in the same scans for adult wasps as experiment two. Again, the replicates were arranged in a random order in the incubator.

Is there sexual selection on body size and development time?

To test the prediction that males benefit from eclosing earlier and to assess the relative importance of body size and degree of protandry, we observed the eclosion time of groups of two virgin brothers together with ten virgin females and tracked the fitness of each male using a genetic eye colour marker. For all experimental wasps, we controlled maternal effects as before, giving the experimental mothers 6 h to oviposit. In order to reduce variation between the competitor males due to factors other than body size and eclosion time (for example those differences in the genetic background of the STDR-TET and AsymC strains, or those from different host environments), we used brothers that differed by genetically marked eye colour. Using brothers also allowed us to observe natural, realistic levels of variation between competitors, who in the wild will most commonly be brothers (Grillenberger *et al.*, 2008). To generate these males, we mated red-eyed females to black-eye males, so their daughters are heterozygous for the black and scarlet alleles at the eye colour locus. Half of these females' eggs will carry the scarlet allele and half the black allele due to the random assortment of chromosomes. *Nasonia,* like all Hymenoptera, are haplodiploid: males develop from unfertilized haploid eggs, and females from fertilized eggs. The male pupae in the resulting mixed-sex broods are the experimental males. These males shared a host environment and have normal relatedness between brothers, but different eye colours. At day eleven post-oviposition, the eye colour of the pupae can be discriminated. We therefore took one pair of brothers that differed from each other in eye colour from each host. The experimental females were red-eye pupae removed from their hosts on days eight and nine after oviposition and kept in groups of virgin females with nine other nonsisters. Once all the pupae had been collected, the 60 replicates were assembled in 75×10 mm glass tubes bunged with cotton wool, with each replicate containing two male pupae and ten female pupae. This set up is close to natural mating conditions: males have a competitor and two males with ten females gives a sex ratio close to those assayed under single-foundress conditions (AMM and DMS observations: 0.163 proportion male).

We recorded the number of males and females eclosed in each of the tubes every hour for 28 h. After 28 h, we removed any individuals that had not yet eclosed and excluded any replicates that did not contain two males and at least eight eclosed females. The remaining (n = 56) replicates had 24 h to mate. We then put each female on a host for 24 h, to test which males had inseminated them. Because the scarlet eye colour allele is recessive to the black, the eye colour of the daughters indicates which males, if any, inseminated the female. The hind tibia length of all individuals was recorded using the method previously described for experiment one.

The use of brothers proved successful in that we removed any strain effects found in the previous experiments, whilst retaining the two eye colour phenotypes. There was no difference in body size between the eye colours [mean red-eye male hind tibia length = 1913 μm (SE = 23.02), mean black-eye hind tibia length = 1930 μm (SE = 22.90)]. In 19 of the 53 pairs of brothers, both males eclosed in the same observation interval. Of the remaining pairs, the black-eyed brother eclosed first 21 times and the red-eyed brother 13 times. The overall proportion of females sired by different eye colours did differ: 39.3% of females laid only red-eye daughters, whereas 51.9% of females laid only black-eyed daughters with the remaining 8.8% of females laying daughters of both eye colours.

Statistical analysis

All statistics were performed in R (R Development Core Team, 2008). Full, unsimplified models are reported throughout.

For experiment one, we wanted to analyse the relationship between body size and development. There was a negative relationship between clutch size and wasp body size for both sexes (LM: male tibia $t_{1,59} = -2.32$, $P = 0.02$; female tibia $t_{1,59} = -3.04$, $P = 0.004$), so to avoid problems associated with colinearity, we analysed body size and clutch size in separate models. We analysed the effect of clutch size and the time of observation on the proportion of the brood pupated, using a generalized linear model with a quasi-binomial error structure. The significance of each term was ascertained using a likelihood ratio test. Due to the destructive sampling for this experiment, we only knew the tibia lengths of those individuals that had already pupated by the given sampling point and were measured after they had eclosed into adults days later. We therefore analysed the relationship between the body size of those that had pupated and the proportion of the brood that had pupated within each of the hosts. A generalized linear mixed effects model (GLMM) was used to investigate the relationship between pupation times and log tibia length, using only the 81 hosts that contained pupae, with Host ID fitted as a random effect. Sex was fitted as a fixed effect in this model, because males and females have different tibia lengths, and the allometry between body size and tibia length differs between the sexes.

There was also a relationship between clutch size and sex ratio (LM $t_{1,78} = -2.76$, $P = 0.00727$), so again we analysed sex ratio and development time separately. The relationship between proportion of the brood pupated and sex ratio appeared to be nonlinear in data exploration; therefore, we used a generalized additive model (GAM) with a quasi-binomial error family (Crawley, 2005). Sex ratio was a smoothed term, with strain as a parametric term.

For experiments two and three, the between-host comparisons of eclosion and emergence using clutch size were performed using survival analysis. A mixed effect Cox's proportional hazards (MECPH) test was used within the R package 'coxme' with true time to eclosion of each individual as measured in minutes since midnight on 8th March 2009. Due to the colinearity between body size, sex ratio of the brood and clutch size, we did not include sex as a fixed effect on eclosion or emergence time. MECPH was also used to analyse the relationship between the time to eclosion or emergence relative to the first eclosed/emerged individual in the host, and the body size and sex of the wasps, with host fitted as a random effect.

For the competition experiment, we used a generalized linear model (GLM) with quasi-binomial error structure to analyse the proportion of the females inseminated by the wild-type male as a measure of his mating success. This includes any females that were inseminated by both males (i.e. polyandrous females), and the total includes any females that laid no daughters and are presumed virgins. The difference between the hind tibia length of the wild-type and red-eye males was used as the measure of relative body size in the full linear model. The limited variation in number of females available (8–10) was not associated with male success and not included in the analyses presented below. The significance of each term was tested using a χ^2 test of the full model compared with a model with the term removed.

Results

What is the relationship between body size and development time?

Experiment one: pupation time
We opened a total of 225 hosts, of which 81 contained at least one pupa. From those 81 hosts, we scored a total of 1736 pupae and 654 larvae. The mean clutch size was 29.88 (SE = 0.77). The remaining 144 hosts contained only larvae, 21 of which were opened on days when other hosts did contain pupae. The time window between the first pupae and last larvae being observed was more than 25 h, far longer than the 6-h oviposition window (i.e. variation beyond that which could be attributed to variation in time available for oviposition).

At a given point in time, a greater proportion of brood from clutches with more individuals (i.e. larger clutch sizes) had pupated (χ^2 test, $P = 0.005$), and there was no effect of strain on this relationship (χ^2 test, $P = 0.89$). Larger individuals pupated first: we found that the body size of the measured individuals was greater when a smaller proportion of their brood had pupated (Fig. 1 and likelihood ratio test, LR = 8.70, $P = 0.013$) when controlling for clutch size. There was no effect of strain on this relationship (LR = 2.66, $P = 0.26$), nor was there
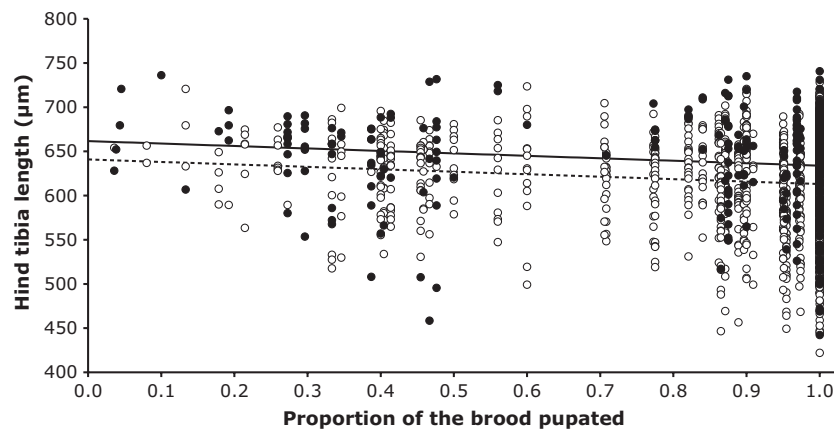
Fig. 1 Larger individuals pupate earlier generating a negative relationship between body size (measured as hind tibia length) and proportion of the brood pupated when sampled. Strains are combined. Females are represented by open circles, and males by closed circles. The lines shown are the model fits. The fitted line for males is black, and the fitted line for female is dashed [male tibia length = 661.6462 + (proportion pupated × −27.8158); female tibia length = 640.8391 + (proportion pupated × −27.8158)].

a significant interaction (LR = 0.80, $P = 0.37$). Males pupated before females because as the proportion of the brood that had pupated increased, the sex ratio of those individuals that had pupated became less male-biased (Fig. 2 and GAM smooth terms, estimated d.f. = 1.54, $F = 11.24$, $P < 0.0001$) and there was no effect of strain (parametric coefficient $t_{1,80} = -1.49$, $P = 0.14$).
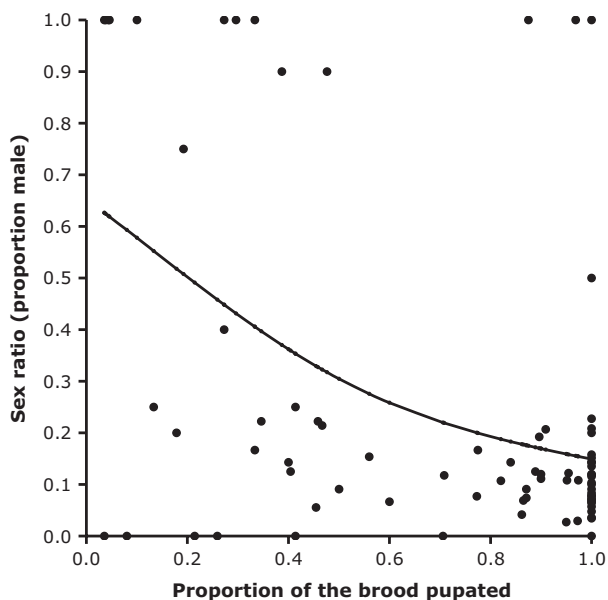

Fig. 2 Male *Nasonia vitripennis* pupate before females. As the proportion of the brood pupated increased, the sex ratio (measured as proportion male) of those that have pupated becomes less male-biased. The line shown is the model fit from the general additive model (GAM) with the strains pooled. Note that complete brood sex ratios are female-biased.

Experiment two: eclosion time

Over 3 days, we observed a total of 255 hosts, which produced a mean of 27.4 (SE = 0.5) adult wasps each. Of the broods in which all individuals eclosed within the observed period, the mean range of eclosion time was 17 h and 38 min. In 44 hosts, the entire brood eclosed between two observation points. The least synchronous host took 46 h and 21 min for all individuals to eclose. Thirty wasps eclosed after observations were completed, and these were entered as censored data into the analysis.

Wasps from larger clutch sizes eclosed sooner (MECPH $Z = 3.73$, $P < 0.0001$). The wild-type wasps eclosed later than the red-eye broods ($Z = -7.26$, $P < 0.0001$), and broods that were removed from the host on day 9 post-oviposition eclosed later than those removed on day 11 post-oviposition ($Z = -5.28$, $P < 0.0001$). This shows that opening the host earlier causes the whole brood to decelerate pupation somewhat. The clutch size effect was exaggerated for wild-type wasps ($Z = 3.92$, $P < 0.0001$).

Within hosts, larger individuals of both sexes eclosed earlier (MECPH $Z = 13.26$, $P < 0.0001$). Males were protandrous, eclosing earlier than females controlling for tibia length ($Z = 4.57$, $P < 0.0001$). Males eclosed within a smaller time window than females (Figs 3 and 4), and there was no significant interaction between body size and sex on eclosion time ($Z = -0.57$, $P = 0.64$). The bimodal pattern of eclosion observed (Fig. 4) is likely to be a result of eclosion peaking around lights-on in the incubator. Strain and the stage at which the wasp pupae were isolated had no significant effect on eclosion time within broods (strain $Z = 1.53$, $P = 0.13$; treatment $Z = 0.70$, $P = 0.48$).

Experiment three: emergence from the host

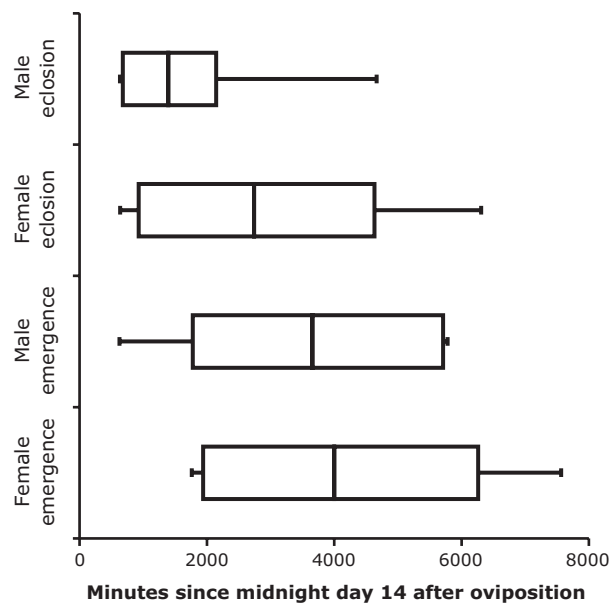A total of 144 hosts were observed, of which 134 contained wasps, producing 28.13 (SE = 0.89) adult

Fig. 3 Box and whisker plot showing the pattern of eclosion and emergence from the host puparium over true time. The box shows the range of the first and third quartile, with the median marked as a bar. The ends of the whiskers represent the full range of the observations.
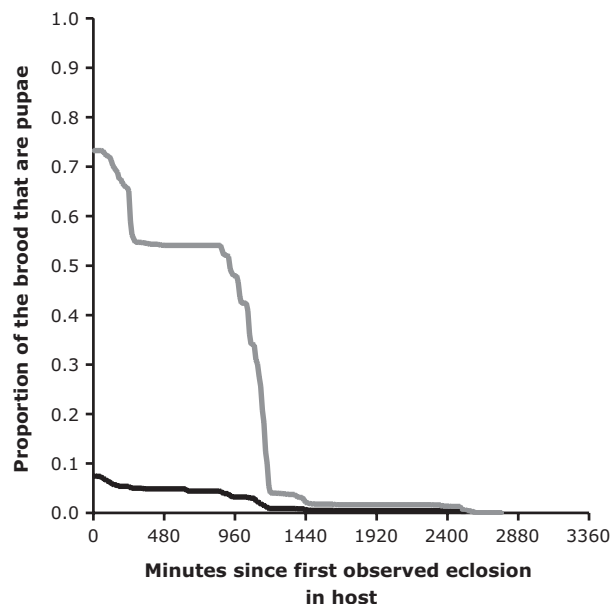

Fig. 4 Male *Nasonia vitripennis* eclose before females. The black line represents male eclosion (in terms of proportion still pupae), and the grey line, female eclosion. Time is measured as the minutes since the first observation of an experimental host.

wasps each. When we opened the host puparia after the observation period had ended, we found 83 wasps that had not emerged and these were again included as censored data. Of those broods from which all individuals emerged within the observation period, the maximum range of emergence time was 39 h and 31 min. Two broods emerged entirely between two observation points. The mean range of emergence times was 11 h and 19 min (Fig. 3).

Individuals from larger clutches emerged sooner (MECPH $Z = 4.57$, $P < 0.0001$). There is no effect of strain ($Z = -1.155$, $P = 0.25$), nor an interaction of clutch size with strain ($Z = -1.31$, $P = 0.19$). Larger individuals of both sexes also emerged sooner ($Z = 8.49$, $P < 0.0001$). Males of a given size were protandrous, emerging before females ($Z = 3.48$, $P = 0.0005$), and there was no difference between the strains in emergence time ($Z = -1.23$, $P = 0.22$). The relationship between body size and emergence time did not differ between the sexes ($Z = -1.89$, $P = 0.058$).

## Is there sexual selection on body size and development time?

The earlier a male eclosed relative to his competitor, the higher his mating success (Table 2 and Fig. 5). The mating success of a male was not affected by the eclosion time of the females (Table 2). The difference in male size did not predict male fitness in this competitive environment (Table 2). This finding is robust: if we remove the minutes until the red-eye competitor male ecloses from the model, none of the effects are significant. Also, if we used the actual tibia lengths of both males instead of the difference, there was still no influence of body size on mating success (GLM $F_{2,10.5} = 0.77$, $P = 0.46$). If we just fit the difference in tibia to the proportion of females inseminated, we find that body size still has no significant effect (GLM, $F_{1,13.9} = 0.58$, $P = 0.45$). We also found no significant quadratic term for the relative eclosion time squared ($F_{1,10.34} = 1.57$, $P = 0.22$) or the difference in body size squared ($F_{1,10.08} = 0.19$, $P = 0.66$) on male mating success. A wild-type male inseminated 55.7% of the females that laid daughters.

Discussion

Highly structured populations in which mating and competition for mates commonly occur between kin have been studied in detail in terms of traits such as sex allocation, but much less so in terms of the sexual selection arising from this competition (Hardy *et al.*, 2005a,b). In *N. vitripennis*, mating populations are not only highly spatially structured, but reproductively receptive females are also extremely clumped temporally, following emergence from their host. Using an experiment that mimics these mating conditions, we have found that the time a male enters the mating population is under sexual selection, resulting in selection for protandry (Table 2, Fig. 5). We propose that male *N. vitripennis* prioritize early arrival to the mating pool over

Table 2 The relationship between wild-type male success, body size and eclosion time. The full model is shown here. The significance of each term was calculated using model comparison.

	Coefficient	Standard error	P
Intercept	6.659	5.235	
Minutes until the red-eye male ecloses	1.752×10^{-03}	5.905×10^{-04}	0.001829
Minutes until first female ecloses	-2.655×10^{-05}	8.220×10^{-04}	0.9742
Minutes until the last female ecloses	1.132×10^{-04}	8.684×10^{-04}	0.8799
Minutes until median female ecloses	-1.726×10^{-04}	8.461×10^{-04}	0.8383
Mean female tibia length	-1.136×10^{-04}	8.688×10^{-05}	0.1900
Difference in tibia length between the black and red-eye male	1.709×10^{-05}	1.977×10^{-05}	0.3865
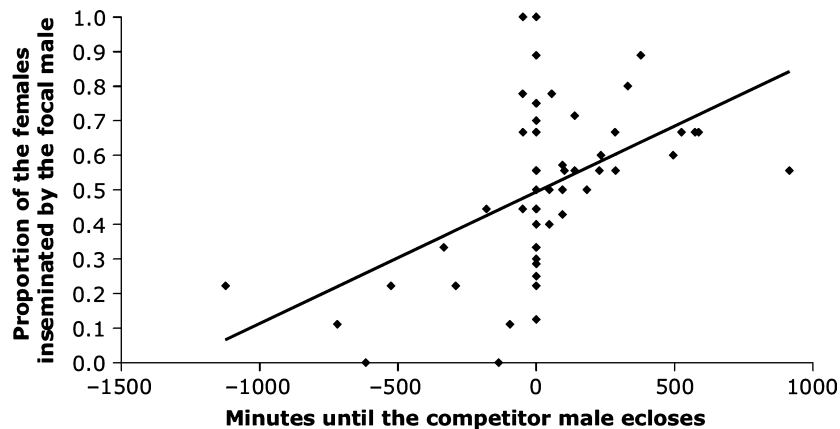
Fig. 5 Protandry is under sexual selection in *Nasonia vitripennis*. There is a positive relationship between the difference in emergence time of focal and competitor males and the mating success of the focal (wild-type) male. The line shown is the model fit from a generalized linear model with a quasi-binomial structure (proportion of females inseminated by the focal male = 0.4937 + 0.0003808 × min until competitor male ecloses).

body size, beginning pupation as soon as their host-mates do. The contribution of protandry to male fitness has been under-appreciated in previous parasitoid studies, where body size has been the focus (e.g. Burton-Chellew *et al.*, 2007; Reece *et al.*, 2007). An exception to this is the parasitoid wasp *Melittobia acasta*, where eclosion order predicts winning the lethal male–male fights typical of this species to a greater extent than being the larger male (Innocent *et al.*, 2007). When males were age-matched post-eclosion, size played a more important role (Innocent *et al.*, 2007). Our study is perhaps the first to clarify that it is the early development, and not the body size, that confers the competitive advantage to males, when males emerge both earlier and larger under controlled laboratory conditions.

In theoretical models, reduced male dispersal and female monandry, such as we see in *N. vitripennis*, contribute to the evolution of protandry (Fagerström & Wiklund, 1982). Males can benefit from protandry through many routes (Morbey & Ydenberg, 2001); the relative weight of these benefits will be a product of the mating system. As discussed earlier, these benefits fall into two broad groups: those where the arrival of each

sex into the breeding pool is under selection independently and those where the arrival time of one sex relative to the other is under selection. Of the seven hypotheses reviewed by Morbey & Ydenberg (2001), our experiment is less able to explore the effects of relative emergence times between the two sexes central to hypotheses 1–4 (Table 1), because we restricted female dispersal. In our experiment, once females had eclosed, they spent an average of 35.5 h with males, which is much higher than we would expect from an observed dispersal rate of one female every 180 s from more realistic artificial patches (C. Doughty & D.M. Shuker, unpublished). This restriction would have removed any disadvantage to males eclosing later than females; the females were unable to disperse un-mated, and as such, we found no effect of female eclosion time on male fitness (Table 2). Although we have not considered these possibilities here, there is evidence that such direct selection on the relative arrival time of the sexes occurs in species of migratory birds (Coppack *et al.*, 2006).

That said, of the direct selection hypotheses, we can perhaps rule out three of them for other reasons though. As *N. vitripennis* commonly mates with siblings as a result

of LMC (Grillenberger *et al.*, 2008), inbreeding avoidance (H4, Table 1) is an unlikely hypothesis to explain protandry. Mate choice (H3, Table 1) is another unlikely explanation of protandry in *N. vitripennis* as females usually mate with the first male they encounter (R. Watt, A.M. Moynihan & D.M. Shuker, unpublished), although there is some suggestion of assortative mating by eye colour (Figueredo & Sage, 2007). Waiting costs for females (H2, Table 1) are also unlikely to explain protandry, not least as female *Nasonia* do not need to spend time in a comparatively risky breeding environment in order to gain a mating, as they can gain some fitness as virgins (Steiner & Ruther, 2009a). Also some females eclose before males (Fig. 3). The mating opportunity hypothesis (H1, Table 1) proposes that protandry allows males to maximize their mating opportunity, and this is consistent with the mating system of *N. vitripennis*, as earlier males will have more females to mate with. Evidence for the mate opportunity hypothesis has been found in other species, including Waved Albatross (*Phoebastria irrorata*) (Huyvaert *et al.*,2006).

Of the three remaining (indirect selection) hypotheses, the constraint hypothesis (H7, Table 1) can be discounted. If this were explaining protandry in *N. vitripennis*, we would expect to have found that later eclosing females were larger; however, the opposite relationship was found. The susceptibility hypothesis (H6, Table 1) is harder to reject, but is not compelling either: female *Nasonia* disperse soon after their first mating, and so poor conditions in the mating pool are unlikely to influence emergence timing. The rank advantage hypothesis (H5, Table 1), which has had the greatest support from migratory bird studies (Kokko *et al.*, 2006; Sergio *et al.*, 2007), is also the most supported by the data presented here. The earlier males will win contests for the best territories. Males emerge and defend a hole in the host puparium, with territory 'quality' being determined by gaining access to the earliest females that eclose. Earlier females of *N. vitripennis* and other parasitoid wasps are typically larger and have more oocytes and therefore represent a more valuable resource for males (van den Assem & Jachmann, 1982; West *et al.*, 2001). Moreover, the first male has sperm precedence on the rare occasions when females mate more than once (Holmes, 1974), compounding the advantages for an early male. Mechanistically, early-eclosing males will be fully sclerotized with a full complement of pheromones to attract females before they appear (Ruther *et al.*, 2009). The male's rectally produced female-attract-and-arrest pheromone is likely to be important in mate acquisition (Steiner & Ruther, 2009b), and an earlier male could potentially have more opportunities to deposit pheromone and attract females. Protandry in *N. vitripennis* is therefore favoured by both mate opportunities (H1) and rank advantage (H5) (Table 1).

Although our data indicate a larger role of development time than previously appreciated, selection is still likely to have influenced male body size. Our assay detected no advantage to larger body size within the naturally generated range of sizes observed (Table 2), consistent with previous studies (Burton-Chellew *et al.*, 2007). Selection on male body size may therefore be stabilizing, with a lower limit due to the need to produce enough sperm, and few benefits to being very large. Sperm depletion is potentially an important constraint for *Nasonia* male fitness as they do not produce spermatozoa as adults (Clark *et al.*, 2010) and can have very high numbers of females available to mate with in the wild (Werren, 1983; Grillenberger *et al.*, 2008). This can be problematic for females too because males could continue to mate after they are sperm depleted, as is the case for the parasitoid *Trichogramma evanescens* (Damiens & Boivin, 2005). Whereas mated females with little or no sperm can gain some fitness in haplodiploids, sperm limitation is nonetheless costly. Male body size may also be influenced by selection on female body size through correlated selection (Lande & Arnold, 1983). Males have a smaller body size overall compared with females, but they can overlap to some extent. The dimorphism between male and female *N. vitripennis* is otherwise large: females are fully winged so they can disperse to find new hosts, whereas males are brachypterous with vestigial wings that prevent flight (Whiting, 1967). This sexual dimorphism, with different allometries for different body parts, suggests that males and females have evolved in response to different selective pressures on components of body size.

We found a negative relationship between body size and time spent in development for both sexes. This is contrary to the findings of two other parasitoid studies: larger individuals take longer to develop for *Microplitis demolitir* and *Apanteles carpatus* (Harvey *et al.*, 2000) and *Muscidifurax raptorellus* (Harvey & Gols, 1998). However, in other invertebrate orders, a negative relationship like that found here for *N. vitripennis* has been observed (Gebhardt & Stearns, 1993; Blanckenhorn & Fairbairn, 1995; Castillo & Nunez-Farfan, 1999; Maklakov *et al.*, 2004). Larger individuals were developing faster at all three of the transitions we measured: larvae to pupae, pupae to eclosed adult and the emergence of adults from the host. This shows that the relationship we have observed here starts with differences in time spent feeding and then follows through the rest of the developmental schedule.

Although our data could support the idea that the body size–development time relationship is under selection in *N. vitripennis*, we cannot rule out several alternative explanations. Differences in individual- or resource quality could generate such a negative relationship between body size and time spent in development. It has been shown that differences in resource availability during development can result in an absence of predicted trade-off between life history traits (van Noordwijk & de Jong, 1986). Such differences in resource quality can

arise through several routes. For instance, differing growth rates continuing through size-triggered molt period result in larger individuals reaching the final instar sooner (Klingenberg & Spence, 2003). Alternatively, the resources available for growth can differ: for parasitoids, clutch sizes can differ on equal-sized hosts due to differences in the mother's body size and egg availability or the presence of a previous female's eggs (Hardy *et al.*, 1992; Flanagan *et al.*, 1998). Indeed, when we look at relationships between broods, we find that individuals from larger clutches are both smaller in body size and the whole clutch develops faster than smaller broods. This is in stark contrast to the within-clutch relationships where individuals are sharing resources, and we see that the larger individuals are the faster developers.

These mechanisms for creating negative relationships between life history traits might explain the disagreement between our study and those from other parasitoid studies. We propose that small *N. vitripennis* males sacrifice the greater sperm reserves associated with larger body size (Clark *et al.*, 2010) in order to arrive in the mating pool at the same time as their competitors, because early development confers more advantages in competition between host-mates than body size (Table 2). The aforementioned *Microplitis demolitir* and *Apariteles carpatus* study (Harvey *et al.*, 2000) looked at solitary parasitoids: such individuals do not share host resources; hence, we would not expect males to trade-off body size in favour of development time as there are no males to compete against in the host patch. Similarly, the between-host comparisons of Harvey & Gols (1998) with *Muscidifurax raptorellus* would also not show such a trade-off.

Two other studies have measured protandry, body size and fitness. In the grasshopper, *Sphenarium purpurascens*, there was a negative relationship between body size and arrival time to the mating pool (Castillo & Nunez-Farfan, 1999). For the grasshopper, male fitness depended on both early arrival and large body size, leading Castillo and Nunez-Farfan to suggest that the correlation between development time and body size is under selection. For the spider *Stegodyphus lineatus*, larger males were found to reach maturity later: the opposite relationship to that found here in *Nasonia* (Maklakov *et al.*, 2004). *Stegodyphus lineatus* males benefited from early maturity as virgin females are less choosy than previously mated females. Mated females then choose larger males for second mates, leading to benefits in being large. Maklakov *et al.* (2004) suggest that being early is most important, and if a male cannot be early, he should at least be large to gain fitness as a second male. The spider and grasshopper studies assayed the body size–development time relationship under ambient conditions and as such they could not control for lay date or temperature effects and consequently could not rule out artefacts of different growth rates explaining the relationships they observe between body size and development time, unlike our study under laboratory conditions. These two previous studies, and our work here with *N. vitripennis*, represent different outcomes of selection acting on the links between body size, time taken to develop and fitness for males. The spiders either develop early or compensate by spending longer in developing to become larger, whereas the negative relationship between body size and development time we found in *Nasonia* rules out this sort of compensation that could have masked body size benefits in previous studies that did not control for age. The grasshoppers also appear to be selected to be early and large.

Finally, if development time is so crucial for male fitness, can larvae alter their developmental schedules depending on the developmental stages of other larvae in the host puparium? Although we do not know the mechanism, there is some evidence that individuals can modulate their developmental rate. When more than one female parasitizes a host (superparasitism), if the second brood is laid within 48 h of the first, the two broods will eclose more or less synchronously (Werren, 1980). The cuticular hydrocarbon profile of a wasp (*Vespula germanica*) has been shown to change on pupation (Brown *et al.*, 1991), suggesting a possible mechanism for detection. Experiments that manipulate larval developmental plasticity, and explore how it is controlled, are therefore an obvious next step.

In summary, our experiments have shown that within the size range tested, development time is more important to male fitness than body size. Our study highlights the importance of considering details of the mating system when investigating the effects of sexual selection: despite a number of previous experiments focusing on body size in parasitoids, it has proved to be less important than development time when variation in an important aspect of the mating system, protandry, varies amongst males. As such, we suggest that development time rather than body size is under sexual selection in *N. vitripennis* males. Moreover, male mating behaviour and the selection arising from variation in that behaviour also influences sex allocation in species such as *Nasonia*. When LMC is extreme (i.e. females lay eggs alone), the number of males produced is expected to be influenced by the ability of a given male to fertilize a given number of its sisters before they disperse (Godfray, 1994). However, selection for success in competitive mate acquisition may not be the same as selection for efficient mating of females when mate competition is reduced (under female-biased sex ratios). The extent to which sexual selection and sex allocation therefore interact in *Nasonia* is currently being explored.

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