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Parasitoid developmental mortality in the field: patterns, causes and consequences for sex ratio and virginity

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RUNNING HEAD: Developmental mortality and sex ratio

Summary

1. Sex ratio theory predicts that developmental mortality can affect sex ratio optima under Local Mate Competition and also lead to 'virgin' broods containing only females with no sibling-mating opportunities on maturity.

2. Estimates of developmental mortality and its sex ratio effects have been laboratory based and both models and laboratory studies have treated mortality as a phenomenon without identifying its biological causes.

3. We contribute a large set of field data on *Metaphycus luteolus* Timberlake (Hymenoptera: Encyrtidae), an endoparasitoid of soft scale insects (Hemiptera: Coccidae), which has sex allocation conditional on host quality and female biased brood sex ratios. Developmental mortality within broods can be both assessed and attributed to distinct causes, including encapsulation by the host and larval-larval competition.

4. Thirty percent of *Metaphycus luteolus* offspring die during development with 65% of this mortality due to encapsulation and 28% due to larval competition. The distributions of mortality overall and for each cause of mortality separately were overdispersed.

5. The probability of an individual being encapsulated increased with clutch size while the probability of being killed by a brood mate declined with increasing clutch size and with increasing *per capita* availability of resources.

6. The sexual compositions of broods at emergence were influenced by both the degree and the type of mortality operating. At higher levels of mortality, single sex broods were more common and sex ratios were less precise. Overall, virginity was more prevalent than predicted and was more greatly affected by the occurrence of competition than by other sources of mortality, almost certainly because competition tended to eliminate males.

7. The reproductive and developmental biology of *Metaphycus luteolus* appears to be influenced by a complex interplay of maternal clutch size and sex allocation strategies, offspring-offspring developmental interactions, host defence mechanisms and post-emergence mating behaviour. Despite the great sophistication of sex ratio theory, it has not yet evolved to the point where it is capable of considering all of these influences simultaneously.

Key-words: Developmental mortality, encapsulation, facultative siblicide, facultatively gregarious parasitoid, field study, local mate competition

Introduction

Historically, sex ratio theory is one of the more thoroughly researched and important areas in behavioural and evolutionary ecology (e.g. Seger & Stubblefield 2002; West 2009). A major step was Fisher's (1930) development of a frequency-dependent selection explanation for the sex ratio equality observed in many outbreeding species. Subsequently Hamilton (1967) applied game-theoretic reasoning to explain the strongly biased sex ratios observed in many inbreeding species. In Hamilton's model of local mate competition (LMC), offspring are assumed to develop in groups in reproductively isolated and ephemeral patches, from which mated daughters subsequently disperse. Under these assumptions, the optimal sex ratio allocated by a 'foundress' to a patch depends on the number of contributing foundresses and it is the best response in the presence of competing strategies, *i.e.*, an evolutionarily stable strategy (ESS) (e.g. Freedberg 2002; West 2009); when small numbers of mothers contribute offspring in a patch, sex ratio optima are strongly female biased. Extensions to Hamilton's model under partial local mating (when mating occurs between, as well as within, offspring groups) yield sex ratio optima that are less female biased for a given number of foundresses (e.g. Nunney & Luck 1988; West 2009). When resource quality varies within a group and fitness effects associated with resource quality are sexually differential, sex ratios are predicted to be less female biased than under invariant resource quality conditions (Werren 1984). This prediction also holds when resources within an offspring group are uniformly poor, provided there is inter-group variation in resource quality and partial local mating (e.g. Ikawa *et al.* 1993). These latter predictions combine LMC theory with the third most historically important development of sex ratio theory: sex allocation conditional on environmental conditions such as resource quality (Trivers & Willard 1973; West 2009). Responses to a combination of LMC and environmental quality are termed a 'mixed sex allocation strategy' (Mayhew & Godfray 1997).

When just one foundress reproduces in a strictly isolated patch, Hamilton's (1967) LMC model, which heuristically assumes that offspring group sizes are infinite (continuous non-integer values), predicts an optimal sex ratio of 0.0 (proportion of offspring that are male). This implies that a minimum number of sons are required to mate with the daughters. With a single foundress, and under strictly local mating, a game-theoretic approach is not, however, required to predict sex ratio optima because no competing foundress strategists are present. A number of static optimality models have developed LMC theory for such cases,

assuming strictly local mating. Realistically, the offspring group sizes are relatively small integers, and thus constrain the set of possible sex ratios. Models for a 'discrete' number of offspring predict that sex ratios should be the reciprocal of group size, if a single male is sufficient to mate all of his sisters (Green, Gordh & Hawkins 1982; Griffiths & Godfray 1988), and the sex ratios distributed across offspring groups manifest low (ideally zero) variance, i.e., precise sex ratios (Green *et al.* 1982; Nagelkerke 1996). Limited mating capacity of males or imperfect sex allocation control by foundresses each lead to greater numbers of males expected in larger groups of offspring (Green *et al.* 1982, Nagelkerke 1996; Nagelkerke & Hardy 1994; Hardy *et al.* 1998; 2000).

A further consideration incorporated into static optimality models of single foundress sex ratio optima has been that offspring may die between sex allocation and subsequent maturity and mating. Foundresses are predicted to adjust their sex allocation decisions according to the probability of developmental mortality and the offspring group size by increasing the number of sons, as insurance against the production of unmated daughters, if mortality (risk) and/or group size (value) increases (Green *et al.* 1982). A consequence of such optimal sex allocation is that, at maturation, some offspring groups will contain no males and the maturing females within these groups will remain virgin, with zero fitness if mating is strictly local. Under a given mortality risk, the proportion of virgin broods is expected to decrease with increasing offspring in a group (Heimpel 1994). Also, increasing developmental mortality is expected to lead to increases in group sex ratio variance at offspring maturity (Hardy *et al.* 1998). Optimal sex allocation is further predicted to be influenced by the probability of male developmental mortality and its distribution across offspring groups: under strict local mating, sex ratio optima are more greatly affected when mortality variance is low and complete mortality of offspring in these groups has no effect (Nagelkerke & Hardy 1994). In contrast, under partial local mating, complete mortality influences sex allocation optima via its influence on the probability of non-local mating (Freedberg 2002).

Much of the above sex ratio theory has been developed in conjunction with experimental and comparative studies on parasitoid Hymenoptera. These have a haplo-diploid sex determination system which allows maternal control of sex allocation, and a suite of variations around a relatively simple core life-history (e.g. Godfray 1994). Locally mating parasitoids manifest female biased sex ratios, approximating the reciprocal of group size and

low variance (Green *et al.* 1982; Griffiths & Godfray 1988; Hardy *et al.* 1998). Developmental mortality also correlates with sex ratio variance and the proportion of virgin broods across and within species (Hardy *et al.* 1998). The proportion of virgin broods within species generally declines as offspring group size increases (Hardy & Cook 1995; Hardy *et al.* 1998; Kapranas *et al.* 2009b).

Such studies of relationships between developmental mortality and sex ratios have, however, had several limitations. First, most estimates of the sexual composition in parasitoid offspring groups and their developmental mortality are laboratory-based whereas those in the field may differ, thus limiting the degree to which laboratory evidence is relevant (e.g. Kapranas *et al.* 2009b). More fundamentally, the static optimality models developed by Green *et al.* (1982), Heimpel (1994) and Nagelkerke and Hardy (1994) do not consider effects of environmental (host) quality, multiple-foundress cases or the possibility of partial local mating; incorporating the latter two would require a game-theoretic approach (e.g. Nagelkerke 1996; Freedberg 2002). Further, these models treat mortality in a phenomenological manner, considering given means and distributions as fixed assumptions, and predict a foundress' optimal sex allocation response to these conditions. This approach may be appropriate for externally feeding (ectoparasitoid) species with scramble-type competition between developing siblings, such as the members of the aculeate family Bethylidae with which these models have been hitherto most tested (Hardy & Cook 1995; Hardy *et al.* 1998). However, many parasitoid species develop inside their hosts (endoparasitoids) and manifest contest-type inter-sibling interactions (e.g. Godfray 1987; 1994; Pexton *et al.* 2003; Tena *et al.* 2008; 2009; Segoli *et al.* 2010). The probability of mortality due to encapsulation or within-host contests may not be independent of the initial size and sexual composition of the offspring group (Godfray 1987; Rosenheim 1993; Mayhew & Hardy 1998; Ode & Rosenheim 1998; Pexton *et al.* 2003; Tena *et al.* 2009). Models exploring sex ratios under developmental mortality may thus require a more dynamic interplay between sex allocation strategies and the subsequent offspring mortality.

Here we address some of the above limitations via a field-based evaluation of sex ratio and developmental mortality in an endoparasitoid species, *Metaphycus luteolus* Timberlake (Hymenoptera: Encyrtidae), in which the causes of developmental mortality that operate within each offspring group can be identified. We show that mortality falls into at least three biologically different classes; encapsulation by the host, larval-larval competition

and natural (intrinsic) causes, each occurring with a different frequency. Lastly, the sexual compositions of broods are affected by both the degree and type of mortality operating.

Biology of Metaphycus luteolus

Metaphycus luteolus is a facultatively gregarious endoparasitoid of soft scale insects in the genera *Coccus*, *Lecanium* and *Saissetia* (Hemiptera: Coccidae) and is considered native to California (Bartlett 1978). The focal host of the current study, the brown soft scale, *Coccus hesperidum* L., may have an African origin but is cosmopolitan and has been associated with *M. luteolu*s, one of its most important natural enemies in northern America, for at least a century (Timberlake, 1913) and the host-parasitoid association is thus likely to be coevolved. *Metaphycus* species account for *ca.*75% of brown soft scale parasitism and around 20% of this is due to *M. luteolus* (Kapranas *et al.* 2007).

Metaphycus luteolus females are synovigenic, emerging from their hosts as adults without mature eggs and then maturing eggs upon feeding on natural carbohydrate sources or the haemolymph of their hosts (Kapranas & Luck 2008). This species is facultatively gregarious and lays its eggs as clutches that range from one to nine eggs depending on host size. Each deposited egg has a stalk-like structure that protrudes from the host's integument and is thought to facilitate respiration of the egg and early instar larvae (Maple 1947). The host's development is arrested upon parasitisation, as with other *Metaphycus* species (Lampson, Morse & Luck 1996; Bernal, Luck & Morse 1998; 1999). Some *M. luteolus* eggs are encapsulated by brown soft scale (Bartlett & Ball 1964; 1966; Kapranas *et al.* 2009a), as is commonly observed in other encyrtid-soft scale associations (Blumberg 1997). The larvae that hatch may then compete with conspecifics, including siblings, within a host and such aggressive larval interactions result in the elimination and consumption of brood mates (Bartlett & Ball 1964). Development from egg to adult takes 2-3 weeks. Adults of both sexes are winged. Under laboratory conditions, females live 4–8 weeks when fed carbohydrate (honey) and hosts whereas male longevity is 3-4 weeks, if fed carbohydrates only (Kapranas & Luck 2008). Longevity and the possession of wings are likely to promote non-local and non-sibling mating in the field while gregarious development is likely to promote local mating (Kapranas *et al.* 2008).

Materials and methods

Pre-exposure rearing of hosts

We maintained a culture of brown soft scale on excised *Yucca recurvifolia* Salisbury (Liliaceae) leaves grown hydroponically in the University of California, Riverside (UCR) insectary. The leaves for the culture were obtained from plants grown in a plot at Agricultural Operations, UCR.

Field exposure of hosts

Field experiments were carried out from February 2004 to March 2006 in eleven citrus groves scattered throughout southern California, USA, generally following methods described in Kapranas *et al.* (2007; 2008). We used yucca leaves infested with 20-28 day-old brown soft scales; each leaf typically bore 200-300 individuals of variable size and developmental stage. Because there were always substantially more soft scales present on each of the yucca leaves than the numbers that were eventually parasitized (usually 15-20% of hosts on any one leaf) our design minimises, rather than excludes, potential superparasitism (see also Mackauer & Völkl 2002). Each leaf was placed in a water-filled vial fixed on top of a one metre high stake, beneath the canopy of a citrus tree. Ants were excluded from the yucca leaves by an insecticidal tape (chlorpyrifos) wrapped around the stake mid-height above the ground. We placed three leaves in each citrus grove and replaced them approximately biweekly.

Post-exposure laboratory rearings

Upon return to the laboratory, each yucca leaf was inspected for foraging adult wasps, which were removed with an aspirator and killed. This precluded oviposition in the laboratory environment. The leaves were maintained at ~25-28˚C with naturally determined photoperiod. The leaves were then inspected for the presence of parasitized scales, which can be recognized by their mummification, i.e., their light yellow to golden colour and convex shape. Also, the developing parasitoids are easily recognized and visible through the dead scale's translucent cuticle. Each parasitized scale was isolated in a 3.0 × 0.8 mm glass vial streaked with honey on the inside wall and stoppered with a cotton plug. Each sample leaf was re-inspected for parasitized scales two to three times during the subsequent 15 days; the

period between inspections allowed immature parasitoids to develop to a stage that was clearly visible.

Parasitoid emergence

We inspected vials with the developing parasitoids every 1-2 days for emergence and emerged adult parasitoids were killed by freezing. The wasps (= brood) emerging from each host was cold-stored separately in 95% alcohol. We verified that we had all of the emerged brood, as each emerging parasitoid constructs a separate exit hole through the scale integument. The number of exit holes always equalled the number of adults collected from each host.

The species and sex of each emerged wasp was identified and recorded along with the host scale's length and width to the nearest 0.01 mm, measured using an ocular micrometer mounted in the eyepiece of a dissecting microscope. As scales are much more closely discoid than spherical and because it was impractical to measure scale height, the index of host size used was scale surface area, calculated as an ellipsoid ($\pi \times \text{width} \times \text{length}/4$).

Parasitoid developmental mortality

Estimates of developmental mortality exclude cases of complete mortality of a clutch because it is not possible to identify, to species level, dead immatures within a host. Each host issuing *M. luteolus* adults was immersed in warm water for 5-10s to remove the honeydew traces and other field debris from their cuticle and then inspected at 70×magnification. The number of stalks protruding through the integument indicated the number of eggs that had been laid in the host (= clutch size) (Fig. 1). Visual inspection of the integument of each host, followed by dissection, allowed us to determine the number of encapsulated eggs (melanized capsules) per host (Fig. 1). A small proportion of dead larvae was observed to have a melanotic appearance and these larvae were considered to have died from probably 'intrinsic causes'. Cases in which the clutch size was greater than the sum of the encapsulated eggs plus larvae dying of intrinsic causes plus the number of emerged wasps (brood size) indicated direct physical competition between brood mates resulting in the defeat and consumption of larvae (according to laboratory investigations on *M. luteolus* and its congeners, larvae that lose fights are always consumed by the winners, Bartlett &

Ball 1964; Tena *et al.* 2008; 2009). We also recorded 'mixed mortality' when several causes operated within a particular clutch.

Statistical analysis

Our general approach was to use Generalized Linear Models in which the assumed distribution of residuals is matched, via initial assumptions and empirically estimated scale parameters (which correct for over- or under-dispersion), to the structure of the data rather than the 'traditional' approach of transforming data to fit standard Gaussian assumptions (e.g. Crawley 1993; Wilson & Hardy 2002). Influences on clutch size, brood size and numbers of males in a clutch were identified using log-linear analyses, which are appropriate for small count data. Influences on sex ratios, the prevalence of all-female broods, and developmental mortalities were explored using logistic analyses, which are appropriate for proportional data. We report the percentage of deviance explained (%Dev) as an approximate analogue of r^2 for log-linear and logistic models. We used backward stepwise procedures and aggregation of factor levels to obtain the parsimonious 'minimal adequate model' by model simplification (e.g. Crawley 1993; Wilson & Hardy 2002).

We used the variance ratio, R, as a descriptive statistic to quantify the variance of brood sex ratios and developmental mortality across broods: R = 1 indicates a binomial distribution while R<1 and R>1 indicate under- and over-dispersion, respectively (Krackow *et al.* 2002). The Meelis test statistic, U, was used to assess the significance of any deviations from binomiality (Krackow *et al.* 2002).

Results

Overall, we collected 2,758 parasitized scales; 494 of these scales were parasitized by *M. luteolus*. The remaining scales were parasitized by other *Metaphycus* species or by species belonging to other genera (Kapranas *et al.* 2007). A few scales yielded both *M. luteolus* and another parasitoid species but this occurred rarely (n = 7). In some cases, scale size and/or parasitoid mortality factors could not be assessed (see below) but each part of the analysis was conducted using all suitable data available. There was a total data set of 395 clutches for assessing mortality, of which 371 were gregarious (clutch size > 1).

Pre-mortality clutch composition

The number of eggs laid in each host (= clutch size) ranged between one and nine, although 95% of clutches contained 1-6 eggs, and clutch size increased with host size (log-linear analysis corrected for underdispersion; $F_{1,403} = 289.72$, $P < 0.001$, %Dev = 41.9, Fig. 2). For clutches of one egg (solitary clutches) and without developmental mortality (N = 24) the probability of the offspring being a male (sex ratio) decreased as host size increased (simple logistic regression; $G_1 = 11.20$, $P < 0.001$, %Dev = 51.8, Fig. 3). For clutches of more than one egg (gregarious clutches) and with no developmental mortality (N = 127), the proportion of offspring that were male (sex ratio) decreased significantly with clutch size (logistic regression corrected for underdispersion: $F_{1,125} = 18.87$, $P < 0.001$, %Dev = 13.0) whereas the number of males laid in each clutch increased (log-linear regression corrected for underdispersion: $F_{1,125} = 12.25$, $P < 0.001$, %Dev = 9.0). These analyses were carried out with the caveat that subsets of broods lacking developmental mortality may not be representative of the overall primary sex ratio (Fiala 1980).

Developmental mortality

Thirty percent (428/1,449) of *M. luteolus* offspring developing in gregarious clutches died before adulthood. Of these, 64% died due to egg encapsulation by the host, 27.6% died due to intra-brood competition between larvae and 8.4% of other intrinsic causes.

In 34.23% (127/371) of gregarious broods all offspring survived (brood size at emergence = initial clutch size), in 35.84% of clutches there was mortality solely due to encapsulation (mean mortality within these clutches was 46.60%; in 57.14% [76/133] of these clutches just one egg was encapsulated), in 16.98% of clutches there was mortality solely due to larval competition (mean mortality within these clutches = 34%; in 73% [46/63] cases just one larva was eliminated) and in 5.39% mortality was solely due to intrinsic causes (mean mortality within these clutches = 41.55%; in 70% [14/20] of these broods just one larva died of intrinsic causes). In some clutches (7.5%) two mortality sources were evident, with encapsulation and competition co-occurring in about 71.4% of these cases (mean mortality within these clutches = 53.1%). (All estimates of developmental mortality necessarily excluded cases of complete mortality, see Methods, and are therefore likely to be underestimates).

The overall distribution of mortality across clutches was significantly overdispersed ($R = 1.569$, $U = 5.29$, $P < 0.001$) suggesting a tendency that broods members survived or

died collectively. Analyses of the distributions of each mortality source across all 371 clutches further showed consistent overdispersion (encapsulation mortality, R = 2.351, U = 15.5, P < 0.001; competition mortality, R =1.532, U = 6.12, P < 0.001; intrinsic mortality, R = 1.71, U = 8.87, P < 0.001). Inclusion of broods with complete mortality would strengthen all of these conclusions.

The probability of mortality affecting a brood was positively related to the initial clutch size (logistic regression: $G_1 = 8.63$, P = 0.003, %Dev = 1.8): this overall relationship was due to an increase in the probability of competition mortality ($G_1 = 13.74$, P < 0.001, %Dev = 3.4) while there were no significant relationships for either encapsulation ($G_1 = 3.43$, P = 0.06 [note marginal non-significance: the trend was higher encapsulation probabilities in larger clutches], %Dev = 0.7) or for intrinsic mortality ($G_1 = 0.19$, P = 0.77, %Dev = 0.001) (Fig. 4). We calculated an approximate index for resources available per offspring within each host by dividing host size by clutch size (resource availability correlated negatively with clutch size, Spearman' rank test: $r_s = -0.52$, P < 0.001) and then repeated these analyses with resource availability as the explanatory variable. Overall, the proportion of broods suffering mortality declined as resource availability increased ($G_1 = 6.51$, P = 0.011, %Dev = 1.37). Again, the overall relationship was due solely to a decrease in the proportion of broods manifesting competition mortality ($G_1 = 22.39$, P < 0.001, %Dev = 5.62; encapsulation, $G_1 = 0.06$, P = 0.81, %Dev = 0.011, intrinsic mortality, $G_1 = 0.20$, P = 0.65, %Dev = 0.10, Fig. 4). Because physical competition only occurs during the larval stage, we explored the probability of competition mortality using host size divided by the number of eggs that issued larvae. The result was similar but stronger ($G_1 = 63.38$, P < 0.001, %Dev = 15.9). Across all broods with some mortality, the number of larvae that died declined as resource availability increased (log-linear regression corrected for underdispersion: $F_{1,241} = 43.37$, P < 0.001, %Dev = 16.4).

The likelihood of individuals dying increased weakly as the initial clutch size increased (logistic regression corrected for overdispersion; $F_{1,369} = 4.78$, P = 0.029, %Dev = 1.27) but it differed depending on the cause of death within a clutch: encapsulation was slightly more common among offspring in larger clutches whereas the proportion of immature parasitoids dying from other sources declined with increasing clutch size (logistic ANCOVA corrected for underdispersion: clutch size × mortality category interaction: $F_{3,243} = 3.24$, P = 0.023, %Dev = 3.5, Fig. 5). The increase in encapsulation as clutch size increased was not due to larger hosts, which generally contained larger clutches, encapsulating a higher

proportion of parasitoids within broods (logistic regression corrected for underdispersion: $F_{1,367} = 1.48$, P = 0.228).

Developmental mortality and sex ratio

Data from all gregarious clutches were used to explore effects of brood size and the prevalence and type of developmental mortality on the brood sex ratio at adult emergence (secondary sex ratio). Mortality type was assigned as a factor with five categorical levels (none, intrinsic causes, encapsulation, competition or mixed) and the prevalence of mortality was fitted as the proportion of eggs in a clutch that died (these analyses are unaffected by the necessary exclusion of broods with complete mortality as such broods also have no secondary sex ratios). Overall, the mean brood sex ratio was 0.262 (S.E. ± 0.01), but sex ratios decreased weakly as brood size and the prevalence of mortality increased (logistic analysis of covariance corrected for underdispersion: brood size $F_{1,355} = 22.19$, P < 0.001, %Dev = 5.55; mortality prevalence $F_{1,355} = 18.10$, P < 0.001, %Dev = 4.52, Fig. 6), indicating that, overall, males have slightly higher developmental mortality than females. The type of mortality affecting a clutch did not influence the brood's sex ratio significantly ($F_{4,355} = 0.29$, P = 0.88, %Dev = 0.28). Also there were no significant interactions between any of the fitted explanatory variables.

Developmental mortality and sex ratio variance

Metaphycus luteolus shows sex ratio precision: the sex ratios of gregarious broods were significantly underdispersed (Table 1). Calculating variances for groups of broods separately suggested that broods with no mortality had lower sex ratio variances than those affected by any of the four types of mortality (Table 1). With the caveat that analysis of relationships between variance ratios and other measured variables may not always be valid (Krackow *et al.* 2002), we explored the effect of mortality on sex ratio variance in three ways. First we correlated the variance ratio obtained within each mortality type with the mean mortality experienced within that class (Table 1): these estimates were positively correlated (Spearman's rank test: $r_s = 0.900$, P = 0.004). Second, we grouped broods by the degree of mortality they experienced (0%, 0.1-10%, 10.1-20%, etc.), irrespective of mortality type. For each group we calculated the variance ratio and regressed it against the mean mortality of the group, initially including a quadratic term to allow for potential curvilinearity and weighting

each variance ratio according to the number of broods from which it was calculated (a proxy for reliability). Variance ratios increased significantly and linearly with an increase in mean mortality ($F_{1,5}$ = 70.26, P < 0.001, r^2 = 0.92; quadratic term, $F_{1,4}$ = 2.03, P = 0.227, r^2 = 0.02, Fig. 7). We also observed that a weighted analysis gave a better fit to the assumptions of a Gaussian distribution and constant errors variances compared with the equivalent un-weighted regression. Both of these analyses lead to the same conclusion. Third, because a high sex ratio variance may be associated with a preponderance of single-sex broods, we carried out a logistic ANCOVA on whether or not broods contained just one sex of offspring in relation to i) the proportion of offspring dying within those broods and ii) the category of mortality operating (broods without mortality were excluded). Single sex broods were significantly more common when mortality was prevalent (G_1 = 79.34, P < 0.001, %Dev = 24) and this depended on the category of mortality operating via a significant interaction between proportion and category of mortality (G_3 = 10.73, P < 0.013, %Dev = 3.2). For encapsulation and competition, sex ratio variances were less sensitive to the prevalence of mortality than they were with intrinsic or mixed mortality. When overall mortality rates were low, competition tended to generate single sex broods most frequently. Collectively, these analyses show that sex ratio variance is affected both by the degree and the cause of mortality operating.

Developmental mortality and the optimal number of male eggs

We calculated the maternal optimal number of male eggs at clutch oviposition assuming single foundress clutches, strict local (within brood) mating and also that one adult male is able to inseminate all emerging females (Green *et al.* 1982; Heimpel 1994). The optimal number of male eggs, *s*, is predicted to depend on both the clutch size, *c*, and the probability of mortality, *m*, and is the number that maximises the mean number of mated daughters, *Dm*, according to Heimpel's (1994) equation $Dm = (1-m^s)(c-s)(1-m)$. Calculations first used the probability of developmental mortality estimated across all clutches and then, used clutch size specific mortality estimates because we found a relationship between mortality and clutch size (see above).

The mortality estimates derive from male and female mortality combined, although only male mortality, which is likely to be higher in *M. luteolus* (see above), is predicted to influence maternal optima (Nagelkerke & Hardy 1994). Further, Heimpel's (1994) equation assumes a binomial distribution of individual mortality across clutches, while in *M. luteolus*,

mortality is overdispersed (see above), which is predicted to reduce the influence of mortality on maternal sex ratio decisions (Nagelkerke & Hardy 1994). Our optimality calculations are therefore approximate but these two ways in which *M. luteolus* mortality does not match assumptions are expected to have opposing influences and thus may effectively cancel. Using the estimate of overall mortality, the optimal number of male eggs in clutches of 1 to 5 eggs was one, and in clutches of 6 to 9 was two. A similar result was obtained using clutch size specific mortalities but the switch to laying two male eggs occurred at clutch sizes of 7.

Developmental mortality and the prevalence of virgin broods

For each clutch size we calculated the expected proportions of 'virgin broods', containing only females at emergence, using the optimal number of male eggs and the mortality estimates as above (this proportion is equal to m^s, Heimpel 1994; Hardy & Cook 1995). The expected proportion of virgin broods was 0.295 for clutches of 1-5 eggs, and 0.087 for clutches of 6-9 using the overall mortality estimate. Predictions were similar when using clutch size specific mortality estimates, except that virginity was predicted to be more prevalent in broods developing from larger clutches (Fig. 8a). There was no relationship between the probability of virginity and clutch size (simple logistic regression, with each brood entered as virgin or non-virgin; $G_1 = 1.57$, $P = 0.211$, %Dev = 0.33, overall proportion of virginity = 0.334, Fig. 7a). Observed virginity was significantly more prevalent than expected (χ^2-test: Using overall mortality, $\chi^2 = 43.2$, d.f. = 7, $P<0.001$; Using clutch size specific mortality, $\chi^2 = 33.3$, d.f. = 7, $P < 0.001$).

We next explored whether there was an effect of mortality category (see above) on the proportion of broods that were virgin, using logistic ANCOVA, initially including mortality as a factor with five levels and clutch size as a variate. The type of mortality experienced had a significant effect on the probability of virgin broods ($G_4 = 32.10$, $P < 0.001$, %Dev = 27.1) and there was a significant interaction with clutch size ($G_4 = 12.4$, $P = 0.042$, %Dev = 2.1). Attempts at model simplification by progressively aggregating factor levels showed that the 'encapsulation', 'intrinsic' and 'mixed' categories of mortality did not differ significantly in their effects on virginity, but 'competition' and 'no mortality' could not be merged with other categories. Virginity decreased as clutch size increased for competition and other sources of mortality, but among broods without mortality, virginity was almost absent irrespective of the clutch size (Fig. 8b).

Finally, using logistic ANCOVA, we explored the relationship between the proportion of virgin broods and the proportion of offspring in those broods that died. Broods were also classified by the type of mortality that was observed (i.e., intrinsic mortality, encapsulation, competition, and mixed mortality; broods lacking mortality were excluded). Virginity increased significantly with the prevalence of mortality ($G_1 = 32.81$ $P < 0.001$, %Dev = 10.0) and aggregation of factor levels showed that virginity in broods with mortality due to encapsulation, competition and intrinsic causes did not differ significantly in their relationships with increasing mortality, but broods with 'mixed' causes of mortality were significantly more sensitive to the prevalence of mortality (interaction term: $G_1 = 7.19$, $P < 0.007$), indicating a synergistic effect of mortality causes (Fig. 9).

Discussion

The observed reproductive biology of *M. luteolus* is typical of facultatively gregarious endoparasitoids of soft scale insects. Their offspring generally develop in small broods, the size of which depends on host size (e.g. Kapranas *et al.* 2008). In single egg clutches, sex allocation was host size dependent, as has been observed in many solitary parasitoids, including *Metaphycus* species (e.g. Lampson *et al.* 1996; Bernal *et al.* 1999; Godfray 1994; West & Sheldon 2002; Kapranas *et al.* 2008; West 2009). However, this relationship was not detected in a previous field study of *M. luteolus* sex allocation (Kapranas *et al.* 2008). The sex ratios of larger broods lacking developmental mortality decreased with brood size, as observed within and across other parasitoid species (e.g. Griffiths & Godfray 1988; Hardy *et al.* 1998; Smart & Mayhew 2009; see Abe *et al.* 2009 for a recent exception) and also showed low variance. Sex ratio precision is achieved by non-random sequences of sex allocation, with one or two male eggs usually laid at the end of an oviposition sequence (Kapranas *et al.* 2009b), again in common with many other species of parasitoids experiencing LMC (e.g. Hardy 1992). Thus, *M. luteolus* females control clutch size and sex allocation, with sex ratio optima apparently influenced by a combination of host-size dependence and some degree of LMC, i.e., a 'mixed sex allocation strategy' (Mayhew & Godfray 1997).

Developmental mortality

At least thirty percent of *M. luteolus* offspring died before maturity under field conditions. This concurs with laboratory based-estimates for *M. luteolus* mortality (≈40%, including broods with complete mortality, Kapranas *et al.* 2009b) and alleviates concerns that laboratory estimates of parasitoid mortality may be generally inaccurate (e.g. Hardy *et al.* 1998; Kapranas *et al.* 2009b). In particular, the relatively minor difference in these estimates suggests that the complete mortality of broods is not extremely common in *M. luteolus* and thus, that the exclusion of field data from such broods will not greatly affect our major conclusions (note also that estimates of virginity and secondary sex ratio are unaffected by exclusion of broods in which both males and females have all died).

Encapsulation was the most common cause of mortality (≈70% of all mortality) and its rate increased slightly with increasing clutch size, although there was no significant relationship. This might be due to the fact that larger clutches tend to be laid in larger hosts but larger hosts are generally more resistant to parasitism via encapsulation (Blumberg 1997). The absence of any significant relationship between encapsulation rates and either host size or clutch size could also be because data from broods with complete encapsulation could not be included. Complete encapsulation was less frequent when clutch sizes were larger in the solitary encyrtid *Comperiella bifasciata* Howard (Rosenheim & Hongkham 1996; Ode & Rosenheim 1998) and when clutch sizes were increased by superparasitism in *Metaphycus flavus* (Howard) (Tena *et al.* 2008). From the host's perspective, complete encapsulation is the only successful outcome of the post-parasitism host-parasitoid interaction.

The second most common cause of mortality (≈22%) was intra-brood larval competition and intrinsic causes accounted for ≈8%. In contrast to trends for encapsulation, mortality due to competition and intrinsic causes was less prevalent overall among offspring developing from larger clutches. Although the likelihood that at least some competition mortality occurred was greater among larger clutches, the number of larvae that were killed was smaller. Competition mortality was clearly dependent upon the availability of resources to developing offspring, only commonly occurring when resources were relatively scarce (see also Tena *et al.* 2009). Intrinsic mortality showed no such pattern, suggesting that this category of mortality was not due to a relatively cryptic form of inter-larval resource competition such as physiological suppression.

Mortality was not randomly distributed across broods. Members of broods tended to survive or die collectively, as observed in other parasitoid species and across a range of other taxa (Hardy *et al.* 1998; Freedberg 2002). Although statistical overdispersion is commonly

caused by probability variation across categories within a data set (e.g. Krackow *et al.* 2002; Wilson & Hardy 2002) and identified causes of *M. luteolus* mortality each had different means, separate analyses for each mortality type showed that mortality was overdispersed regardless of whether it was due to encapsulation, competition or intrinsic causes. This was true even though the modal number of offspring dying was just one when mortality occurred. These estimates further suggest that prior reports of overdispersed parasitoid mortality represent biologically-based overdispersion rather than statistical mixtures of binomially or sub-binomially distributed biological effects.

Sex ratio consequences of developmental mortality

Developmental mortality had a weak effect on mean brood sex ratio, indicating that mortality is slightly more common among males than among female offspring. Candidate explanations for sexually differential mortality include intrinsic differences between the sexes (e.g. the expression of deleterious mutations in male haploids, Smith & Shaw 1980) and sexually-asymmetric susceptibility to extrinsic factors such as competition or encapsulation (discussed below).

Previous field-based estimates of parasitoid brood sex ratio variances have variously reported under-dispersion, binomiality and over-dispersion but were either unable to provide associated estimates of developmental mortality (Mackauer & Völkl 2002) or were constrained to laboratory estimates of the mortality of broods collected in the field (Hardy *et al.* 1998). Laboratory estimates, within and across species of gregarious parasitoids, indicate that sex ratio variances are increased by mortality, as would be expected if mortality has a random component (Hardy *et al.* 1998). In *M. luteolus*, mortality clearly affected sex ratio variance in the field; despite sex ratio precision (R values were consistently < 1), variances generally increased with increasing mortality. The occurrence of single-sex broods (which were mostly all-female broods, as also found by Kapranas *et al.* 2008) further suggests that variance was not solely affected by random mortality because, for a given proportion of developmental mortality, brood compositions at emergence were differentially affected by the cause of mortality. In particular, when the overall proportion of mortality was low, single-sex broods were observed with relatively high frequency when the mortality was due to competition. This suggests that small numbers of male *M. luteolus* were eliminated by females in these broods.

Virginity consequences of developmental mortality

Virginity (broods containing no males at emergence) is an aspect of variation in brood sex ratio for which optimality theory provides explicit predictions (Heimpel 1994). Virginity was virtually absent among those gregarious broods in which developmental mortality did not occur. Among broods with some mortality, the probability of virginity was higher when the proportion of mortality was higher (tallying with findings for the occurrence of single-sex broods). These findings indicate that mortality, rather than factors such as limited maternal control of sex allocation, is the predominant cause of virginity in *M. luteolus,* matching the conclusions of other recent studies (Kapranas *et al.* 2008; 2009b).

Under strict LMC and optimal sex allocation, the overall degree of developmental mortality observed in *M. luteolus* is predicted to lead to a lower proportion of virginity when offspring develop from larger clutches. In fact, the prevalence of virginity was independent of clutch size; this contrasts with laboratory data on *M. luteolus,* in which virginity declined with clutch size (explaining only 6.5% of the deviance) but not with results for two other *Metaphycus* species (Kapranas *et al.* 2009b). The prevalence of *M. luteolus* virginity was also greater than expected overall. The latter observation could be explained by a higher incidence of mortality among males than is assumed in our calculations.

Prior laboratory studies on species in other parasitoid families have, in contrast, generally found a qualitative fit to the expected overall prevalence of virginity and the predicted relationship with clutch size. However, in those species, sexually differential mortality appears to be absent (Hardy *et al.* 1998; 2000). Prior studies have not been able to explore the effects of different sources of mortality on the prevalence of virginity; doing this showed that in *M. luteolus*, virginity declined with increasing clutch size, provided some mortality occurred. The relationship was strongest for mortality due to larval competition; as above, this suggests that in small broods, initially containing high proportions, and small numbers, of males, these males tended to be eliminated when competition occurred (as found in *Metaphycus flavus* and other parasitoid species, Tena *et al.* 2009).

Conclusions and implications

Our findings indicate that *M. luteolus* foundresses laid larger clutches into larger hosts; this response to host size results in less resource per offspring in larger clutches and consequently, a higher probability of competition occurring between larvae within larger broods. The probabilities of other causes of mortality operating within a brood are unrelated

to clutch size or resource availability. Within broods with competition, the proportion of offspring dying averaged *ca.* 0.3-0.4 and declined with increasing clutch size. Sex ratios were initially female biased and precise but developmental mortality lead to a high proportion of single-sex (including 'virgin') broods at offspring maturity. Virginity was more prevalent than predicted from the overall mean mortality but this is at least partially explained by higher male *versus* female mortality. Virginity did not decline with increasing clutch size overall, but declined among broods experiencing some mortality and according to the cause of mortality. When competition mortality occurred in small clutches, virginity frequently resulted; in larger clutches all males initially present were rarely eliminated by competition.

Our data, along with results from recent studies of *M. luteolus* and its congeners (Kapranas *et al.* 2008; Tena *et al.* 2009), indicate that competition mortality is higher among males than among females. Two candidate, and mutually non-exclusive, explanations for sexually asymmetric competitive outcomes within small clutches are that: i) male larvae are physically less able competitors than females or even that females are more aggressive than males (Tena *et al.* 2009) and ii) females benefit more from siblicide than do males, due to the genetic relatedness asymmetries between male haploids and female diploids (e.g. Godfray 1987; 1994; Mayhew & Hardy 1998; Ode & Rosenheim 1998; Giron *et al.* 2004; Gardner *et al.* 2007). The latter requires the ability of immatures to assess relatedness within a host, which has been demonstrated in other endoparasitic encyrtids (Giron & Strand 2004; Giron *et al.* 2004) and seems likely in *M. flavus* (Tena *et al.* 2008; 2009). Eliminating brood mates via competition increases the *per capita* resources available to survivors but carries inclusive fitness costs which are higher when eliminated competitors are more closely related and more numerous (e.g. Mayhew & Hardy 1998). The incidence of competition mortality is thus expected to be brood-size dependent; in particular, siblicide should be rare when clutch sizes are greater than *ca.* 3-4 eggs, especially when sex ratios are female biased (Godfray 1987; Segoli *et al.* 2010). The vast majority of *M. luteolus* broods showed these characteristics and while the occurrence of at least one contest per brood is greater among larger broods, the proportion of individuals killed declines. The incidence of siblicide may also be reduced in single sex broods with high intra-brood relatedness (Rosenheim 1993; Ode & Rosenheim 1998). In *M. luteolus*, single sex broods are common, but only as a result of (usually male) mortality, as very few broods are single sex at oviposition (Kapranas *et al.* 2009b). Models of siblicide, however, assume that offspring within a host are full siblings but superparasitism may not be entirely absent in *M. luteolus* (see also Tena *et al.* 2008 for *M.*

flavus), which could explain a high incidence of competitive elimination in *M. luteolus* (Godfray 1987) while attachment of early instar larvae to the hosts integument (Maple 1947) could help to explain why several (usually female) members of a brood often survived when competitive elimination of (usually male) brood mates occurred (Pexton *et al.* 2003; Tena *et al.* 2009).

A further aspect of *M. luteolus'* life history that could promote within-host inter-sexual competition is non-local mating (e.g. Gardner *et al.* 2007). Little is known about the mating structure of *M. luteolus:* males are winged and long-lived so they are likely able to disperse from the host from which they emerged and mate with females from other broods, favouring less biased sex ratios than would be observed under strictly local (within brood) mating (Nunney & Luck 1988; Hardy 1994; Gardner *et al.* 2007; West 2009). In our study, developmental mortality resulted in 32.8% of gregarious broods being all-female. Under strict LMC, 30.6% of the females developing from all gregarious broods would remain unmated and would consequently be constrained to produce only male offspring. However, production of all-male *M. luteolus* clutches is rare (4% of all gregarious clutches, see also Kapranas *et al.* 2008), indicating that most ovipositing females are mated and thus, that females from all-female broods mate non-locally. Non-local mating would reduce the cost of all-female brood production, but would also decrease the advantage of female-biased sex ratios and consequently the selective advantage of sex ratio precision (Nunney & Luck 1988; Hardy 1994; Kapranas *et al.* 2008; West 2009).

The reproductive and developmental biology of *Metaphycus luteolus* appears to be influenced by a complex interplay of maternal clutch size and sex allocation strategies, offspring-offspring interactions and host defence. Despite considerable developmental mortality and probable partial local mating, brood sex ratios have low variance yet, because of considerable mortality, virgin broods are common, particularly due to sexually asymmetric resource competition. Despite the great sophistication of sex ratio theory (e.g. Godfray 1994; West 2009) it has yet to be developed sufficiently to consider all of these influences simultaneously. A tangible next step might be to develop predictive models of sex ratio strategies that take into account differences in the causes of mortality as well as its sex specific means and distributions.

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Table 1. Variance of brood sex ratios among clutches with different classes of mortality

Clutch types	Initial number of gregarious clutches	Mean egg to adult mortality (%)	Number of gregarious broods (≥ 2) for variance analysis	Sex ratio variance		
				R	U	p
All clutches	371	28.7	293	0.387	-7.4	< 0.001
Without mortality	127	0	127	0.090	-7.91	< 0.001
With some mortality	244	43.7	167	0.619	-3.42	< 0.001
Intrinsic mortality	20	41.6	12	0.373	-1.43	NS
Competition mortality	63	34.0	56	0.549	-2.65	< 0.01
Encapsulation mortality	133	46.6	78	0.640	-2.39	< 0.05

Figures legends

Fig. 1. A brown soft scale parasitized by *Metaphycus luteolus*. The scale host has had 7 parasitoid eggs laid into it. Four eggs have been encapsulated (black arrows). Three eggs have hatched and larval intestines are visible (light yellowish areas, white arrows). protruding white-coloured stalks of parasitoid eggs are also visible.

Fig. 2. Influence of host size on clutch size.

Fig. 3. Influence of host size on the sex of offspring emerging from solitary clutches.

Fig. 4. Influence of clutch size and resource availability on the probabilities of different types of mortality. Each fitted line is the parsimonious description of the relationship derived from a separate logistic regression of the probability of a particular source of mortality occurring in a brood (non-significant relationships are plotted as means). Data are binary and not shown.

Fig. 5. Influence of clutch size on the prevalence of different types of mortality. Regression lines for each cause of mortality are shown separately; the categories 'Intrinsic' and 'competition' did not, however, differ significantly ($F_{2,243} = 0.80$, P = 0.45). Data points are shown slightly vertically displaced to indicate sample sizes. The fan shape of the data is due to necessary exclusion of broods with complete survival and complete mortality, and the limited range of proportions possible from small integer data.

Fig. 6. Influences of brood size (panel a) and developmental mortality (panel b) on brood sex ratio. Data points are shown slightly vertically displaced to indicate sample sizes.

Fig. 7. Relationship between brood sex ratio variance and developmental mortality. Numbers above data points indicate the number of broods from which each variance ratio is estimated.

Fig. 8. Relationships between the prevalence of virgin broods and clutch size. Panel a shows the proportion of virginity predicted using both the overall mean mortality and the clutch size specific estimates. Observed proportions are illustrated by the proportions of virginity at each clutch size (data points) and the overall mean (line with slope of zero, which is the minimal adequate regression model). Panel b shows relationships between the proportion of virginity (data are binary as individual broods either were or were not virgin, but are shown slightly vertically displaced to indicate sample sizes) and clutch size according to the class of mortality experienced by members of the brood.

Fig 9. Relationships between the probability of a brood being virgin and the degree and source of developmental mortality experienced by brood members.

1 FIGURE 1

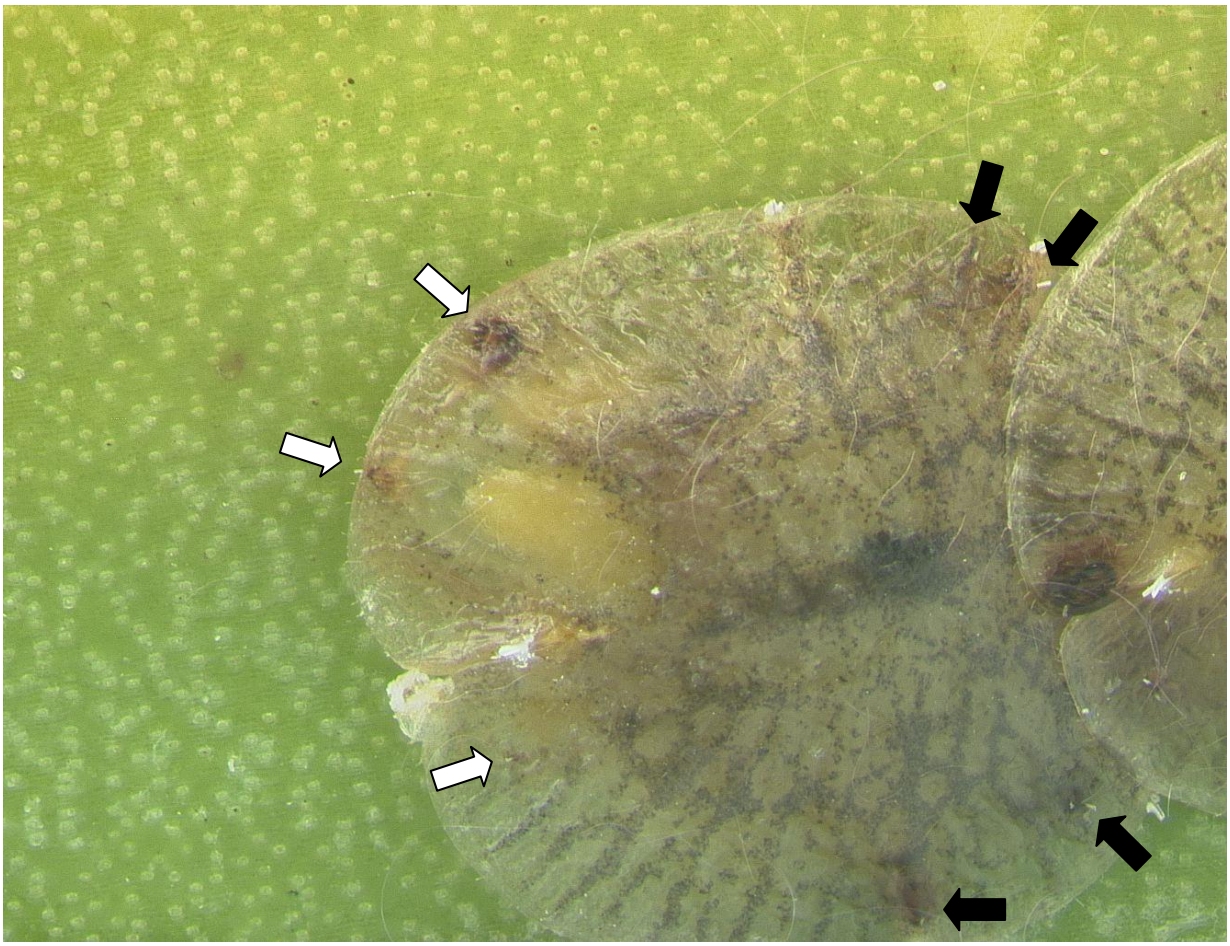


FIGURE 2

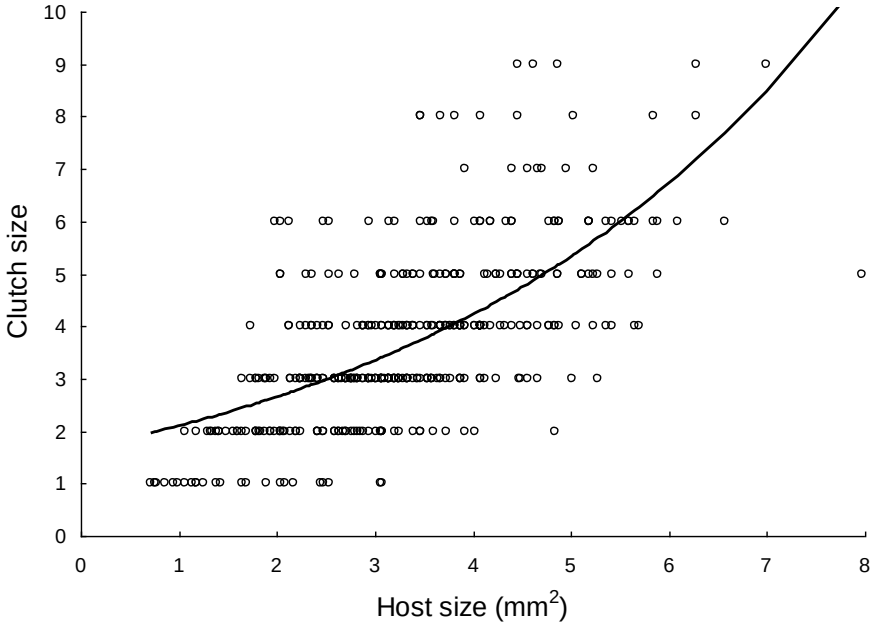


FIGURE 3

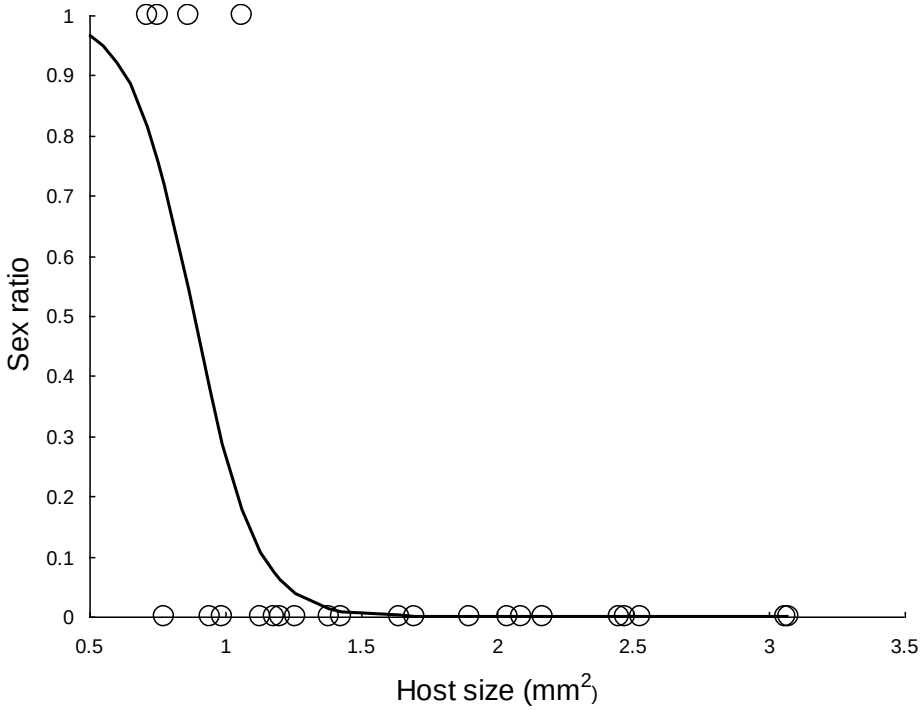

Sex ratio
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0.5
1
1.5
2
2.5
3
3.5
Host size (mm2)

FIGURE 4

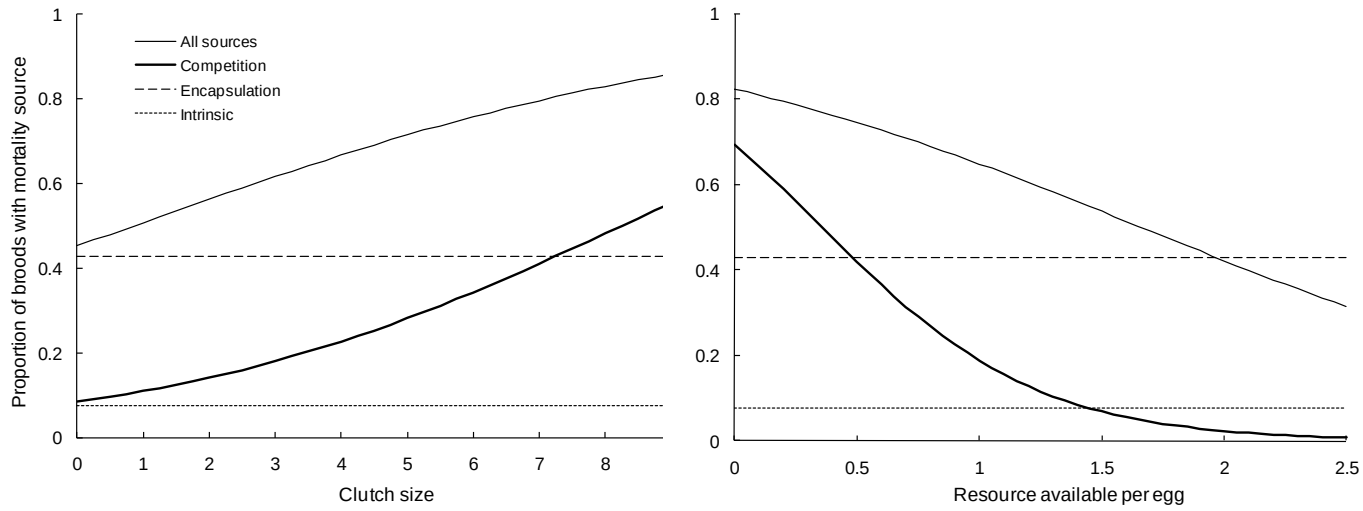

All sources
Competition
Encapsulation
Intrinsic
Proportion of broods with mortality source
Clutch size
Resource available per egg

FIGURE 5

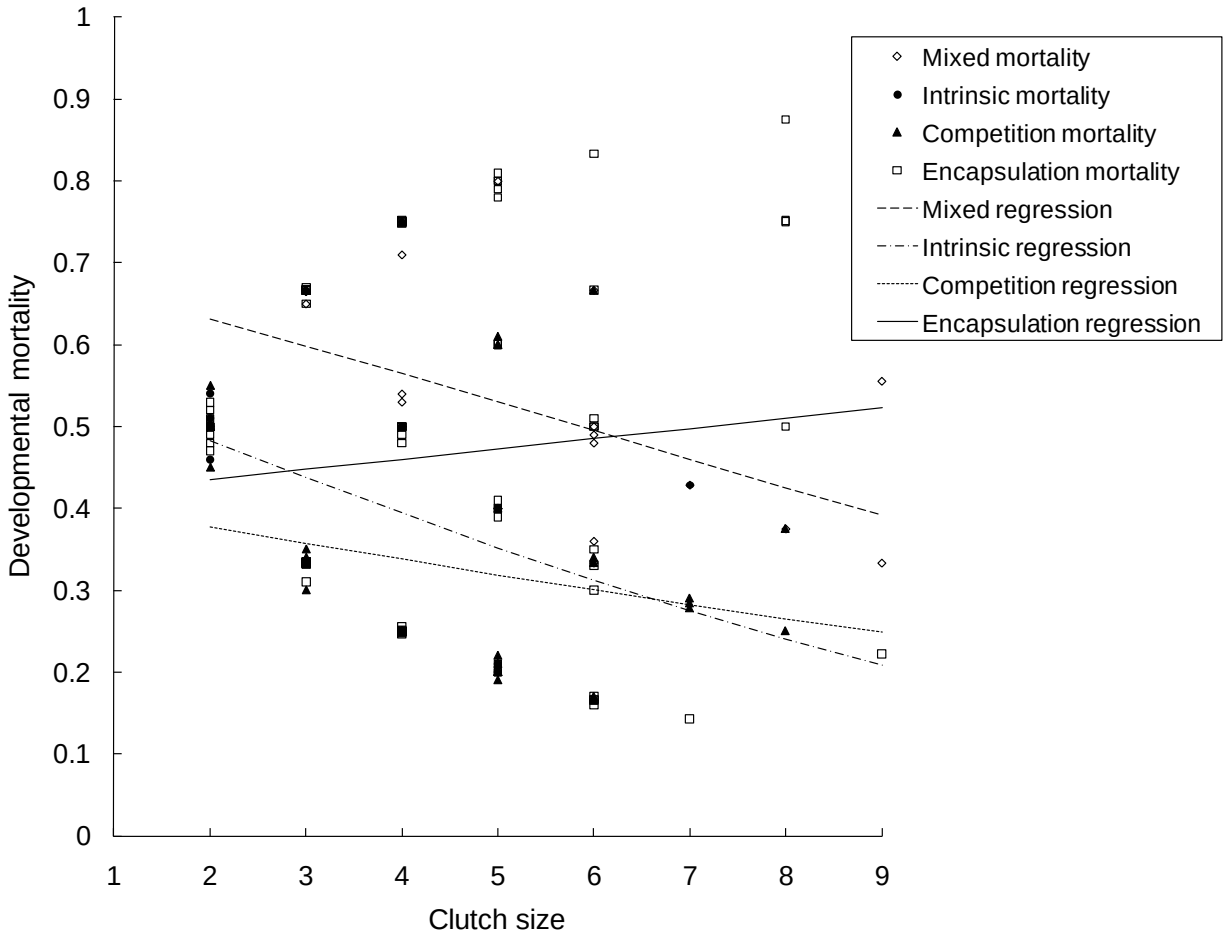

Developmental mortality
Clutch size
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
1
2
3
4
5
6
7
8
9
Mixed mortality
Intrinsic mortality
Competition mortality
Encapsulation mortality
Mixed regression
Intrinsic regression
Competition regression
Encapsulation regression

FIGURE 6

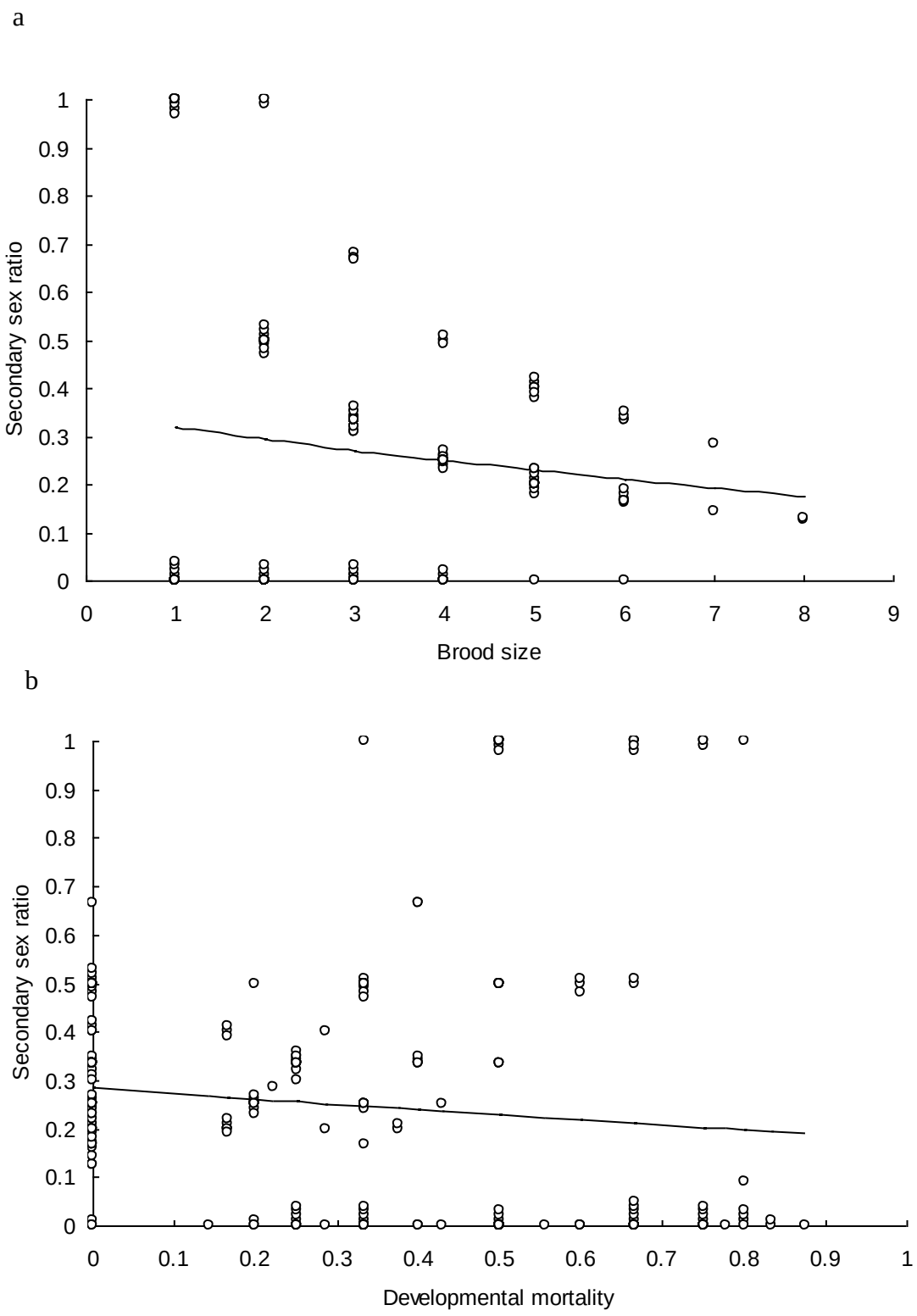

a
Secondary sex ratio
Brood size
b
Secondary sex ratio
Developmental mortality

FIGURE 7

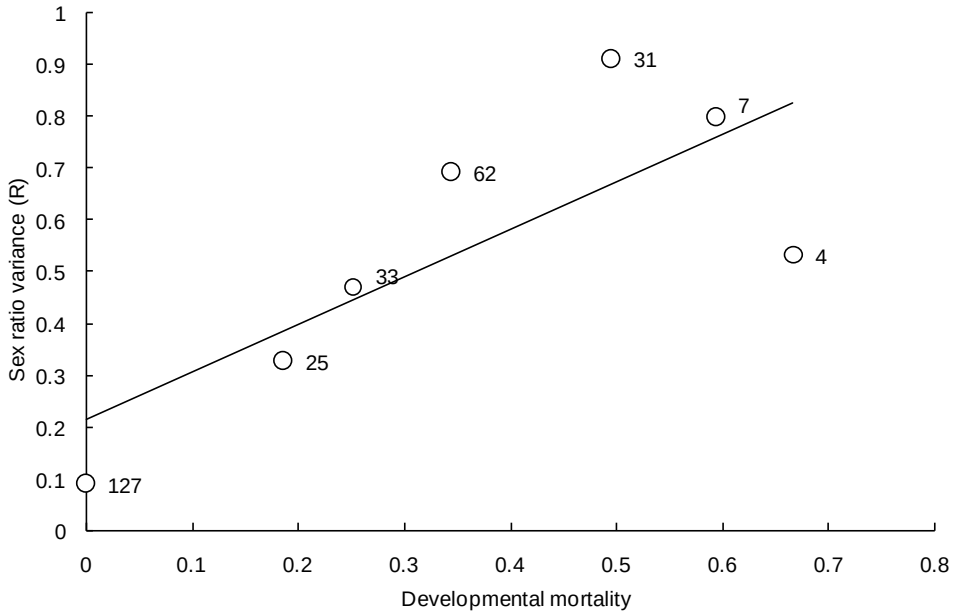

Sex ratio variance (R)
Developmental mortality
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
31
7
62
4
33
25
127

FIGURE 8

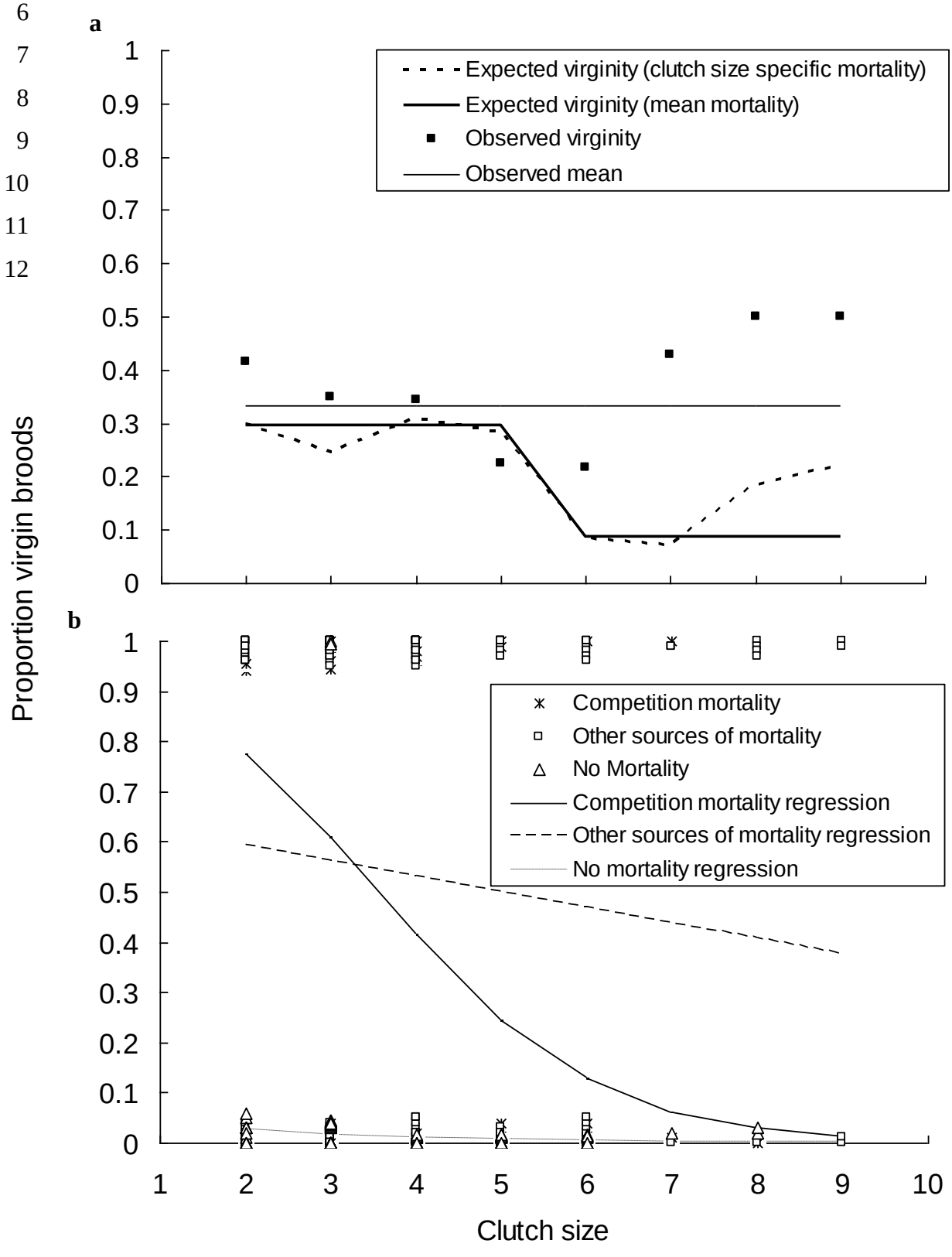
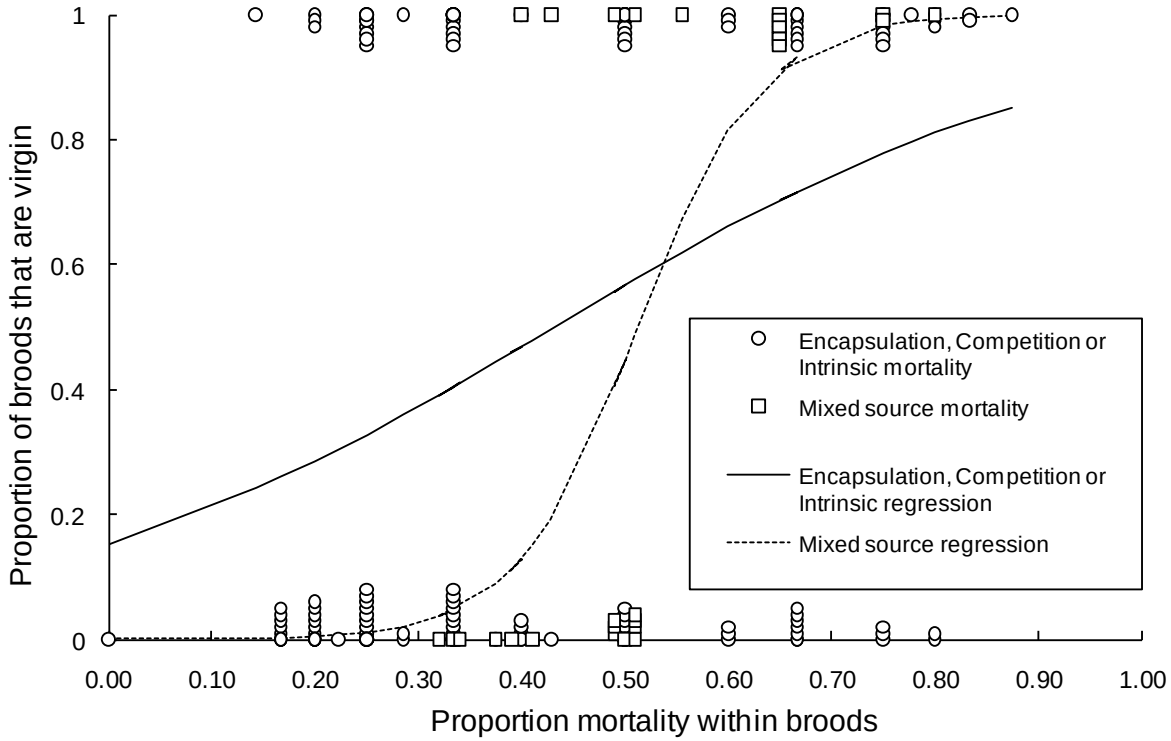

a
b
Expected virginity (clutch size specific mortality)
Expected virginity (mean mortality)
Observed virginity
Observed mean
Competition mortality
Other sources of mortality
No Mortality
Competition mortality regression
Other sources of mortality regression
No mortality regression
Proportion virgin broods
Clutch size
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
1
2
3
4
5
6
7
8
9
10

FIGURE 9


Proportion of broods that are virgin
1
0.8
0.6
0.4
0.2
0
0.00
0.10
0.20
0.30
0.40
0.50
0.60
0.70
0.80
0.90
1.00
Proportion mortality within broods
Encapsulation, Competition or Intrinsic mortality
Mixed source mortality
Encapsulation, Competition or Intrinsic regression
Mixed source regression