Effects of maternal age and stress on offspring quality in a viviparous fly

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Abstract

Many organisms show signs of deterioration with age, both in terms of survival and reproduction. Theory suggests that variation in such senescence patterns can be driven by resource availability or reproductive history. To test this prediction, we experimentally manipulated nutritional stress and age at first reproduction to investigate senescence in tsetse flies (Glossina). Across all treatments, offspring weight and survival followed a concave curve with mother age. Nutritionally stressed females had accelerated survival senescence, higher probability of abortion and produced smaller offspring. Despite this, there was no evidence of accelerated reproductive senescence in nutritionally stressed females and no evidence of a delay in senescence in females mated later. Offspring quality may be prioritised over somatic maintenance. Younger females who were nutritionally stressed produced offspring with the lowest starvation tolerance. As tsetse are vectors of trypanosomes, our results may have implications for population dynamics and trypanosome transmission in times of stress.

Introduction

Across the natural world, it is commonly observed that as individuals get older, they are more likely to die and invest less in their offspring (Nussey *et al.* 2013; Hoekstra *et al.* 2019; Zajitschek *et al.* 2019). Broad patterns of senescence have been well described for a wide range of taxa (Nussey *et al.* 2013; Hoekstra *et al.* 2019; Zajitschek *et al.* 2019), and extensive variation in the onset and rate of ageing, both within and across populations, has also been observed (Holand *et al.* 2016; Rodríguez-Muñoz *et al.* 2019; Cayuela *et al.* 2020). The drivers of senescence and the causes of variation in the timing and intensity of the ageing process are only just beginning to be determined (Gaillard & Lemaître 2019).

Life-history theory predicts that senescence is driven by trade-offs between reproduction and other physiological processes (Kirkwood 1977; Boggs 2009; Baudisch & Vaupel 2012; Davison *et al.* 2014). A central argument to this theory is that resources are limited and must be allocated either to reproduction or somatic maintenance (Partridge 1987; Boggs 2009). Reproductive senescence could also, however, occur through physiological damage incurred directly from reproduction, or be adaptive to prolong survival (McNamara *et al.* 2009).

Controlled experiments are useful for quantifying patterns of senescence and identifying causal factors. Experiments where the age at which females are mated and their access to resources can be varied contribute to tests of whether reproduction causes senescence, either directly or by resource allocation trade-offs. In testing various senescence hypotheses, insects are useful organisms as they have relatively short generation times, can be reared in large numbers, and access to mates and resources can be easily manipulated.

By varying the age at which females were mated, experiments with Lepidoptera have shown that delayed mating reduces fecundity but extends longevity (Unnithan & Paye 1991; Jiménez-Pérez & Wang 2009). However, virgin females still produce eggs, and in these studies, females of the same age but mated at different times were not compared. The effects of reproduction on reproductive senescence cannot therefore be fully evaluated using these data.

By manipulating nutrition, researchers have shown that, generally, females with access to fewer resources have lower overall reproductive output but longer lifespan (De Sousza Santos & Begon 1987; Ernsting & Isaaks 1991; Kaitala 1991; Chippindale *et al.* 1993; Tatar & Carey 1995; Curtis Creighton *et al.* 2009). These studies support the theory that senescence is caused, at least in part, by either direct or indirect costs of reproduction. To our knowledge, no studies have compared reproductive output of females of the same age, but mated at different ages, in the same experiment as females under nutritional stress, to compare directly the contributions of reproductive history and resource availability on reproductive senescence.

Here, we present a study on senescence in tsetse flies (*Glossina*species), where we quantify the effects of maternal age on offspring quality, under nutritional stress, delayed mating and control (standard insectary) conditions. Tsetse are vectors of human and animal trypanosomiasis in Africa. They have an unusual reproductive ecology, giving birth to a single live larva weighing the same as the mother (Hargrove & Muzari 2015), approximately every nine days and living for up to 200 days (Hargrove 2004). With immature stages that receive only energy and nutrients from the mother and a relatively long adult lifespan for their small size, tsetse present an alternative model system to study reproductive and survival senescence.

Evidence for age-related changes in maternal investment in field and laboratory tsetse is, to date, mixed (Jordan *et al.* 1969; Langley & Clutton-Brock 1998; McIntyre & Gooding 1998). Key limitations to existing studies are that flies were kept only under optimal laboratory conditions, not tracked individually and frequently grouped across ages.

In this study, we used a novel method of housing tsetse females to track, for individual mothers, how senescence patterns vary if we alleviate the costs of reproduction or impose nutritional stress. We manipulated nutritional stress by feeding adult females on high*vs* low quality diets and changed reproduction stress by delaying the age at which females were mated. In each treatment we measured maternal mortality, reproductive output and offspring survival. We hypothesised that offspring from older mothers would be of lower quality and would have lower starvation tolerance. If reproduction contributes to senescence, either directly by physiological damage or by resource allocation trade-offs, we hypothesised that: i) mothers mated later would experience senescence later, and potentially slower senescence; and ii) nutritionally stressed mothers would have an earlier, and potentially steeper decline in senescence. Alternatively, if senescence occurs through physiological damage during reproduction, nutritionally stressed mothers may senescence more slowly, due to an overall lower reproductive output.

Methods

Tsetse flies from a colony of *G. m. morsitans* maintained at the Liverpool School of Tropical Medicine (LSTM) were used for the experiments manipulating female nutrition and reproduction. The colony was kept at 26°C and 65 - 70% relative humidity. Flies were fed three days a week (Monday, Wednesday, Friday) on defibrinated horse blood (TSC Bioscience) which is c. 45% red blood cells.

Treatments

We set up control, nutritional stress and mating delay treatment groups, each consisting of 96 adult female *G. morsitans morsitans* (Fig. 1), (see Supporting Information, S1 File). Because reducing the amount of haemoglobin in a bloodmeal results in lower pupal wet weights (Kabayo & Langley 1985), we chose to dilute red blood cells with serum to produce a low-quality diet for the nutritional stress group. Trials testing different ratios of red blood cells to serum showed that flies fed on c. 10% red blood cells produced lighter pupae but had similar survival, over a 50 day period, compared to flies fed on c. 45% red blood cells (S2 File). For the mating delay treatment, virgin females were kept in communal cages for three weeks post-emergence. Once

mated, they were separated into individual cages, as described below. Virgin females continue to ovulate, but mature eggs eventually disintegrate (Ejezie & Davey 1977).

Selection and mating of female tsetse for the experiment

The *G. m. morsitans* colony at LSTM is maintained in 10 trays corresponding to each week of life. Female flies older than ten weeks are killed. To select females for the experiment, we collected pupae from each tray over a one-week period. There was up to four days difference in date of emergence. Only females that had taken the first blood meal that was offered were used, because our experience of rearing colony flies showed that feeding shortly after emergence is crucial for the first few weeks of survival. Newly emerged females were mated at a ratio of 2:1 (female:male) with one-week old males for 48 hours and then males were removed. We then selected 96 females for each treatment group.

Housing of individual adult females and feeding regime

Flies were housed in individual cages, which were placed on pupal collection trays, made from acrylic extrude (described in S1 File), to allow individual tracking of female reproductive output and survival. Cameras were fixed below the cages to record weekend activity and to identify exact larviposition dates. Females were monitored daily between Monday and Friday and if death occurred, we recorded the date of death. To determine the exact date of death for females found dead on a Monday morning, we reviewed video recordings from Friday evening to Monday morning (S1 File).

Experimental flies were fed following the colony schedule. To feed individual females, we placed cages on a shallow grid (S1 File). The grid ensured each female was provided with the same amount of blood and had equal opportunity for feeding, with c. 100 µl of blood provided per female. We heated the tray to 37°C and covered the blood with a silicon membrane, then allowed flies to feed for 45 minutes, which was sufficient time for most flies to feed.

Collection of pupae

Time of larviposition was recorded, including whether or not larvae produced were viable (Baldry *et al.* 1992). Production of non-viable offspring was considered a spontaneous abortion ('abortion' hereafter). It was not possible to record egg abortions as they were not visible on the pupal tray due to their small size. For pupae collected at the start of the week (Monday morning), we consulted the last 72 hours of video recordings to determine the exact day of larviposition (S1 File). For aborted larvae, however, because of the small size of early larval stages, we could not determine the abortion date for those aborted over the weekend. Hence, they could have occurred on either Friday post-feeding, Saturday, Sunday or Monday pre-feeding. They were recorded as occurring on the Friday.

We measured the wet weight of pupae, to 0.1 mg, as an indicator of pupal size and therefore a proxy for maternal investment. Wet weight was previously found to correlate with pupal volume in field flies and does not require destructive sampling (Hargrove 1999). Of the pupae produced, we selected 70% of samples to track emergence, assigned using a random number generator in the data recording spreadsheet (Fig 1). The remaining 30% we destructively sampled for fat analysis in order to quantify how wet weight correlates with fat reserves.

Offspring fat analysis

We dried pupae in an incubator at 70°C for 48 hours, and then recorded their dry weight before placing them in ether inside a metal tray. We changed the ether each day for three days. On the fourth day, samples were again dried for 48 hours in an incubator at 70°C and then re-weighed to estimate the residual dry weight. We calculated the difference between the dry weight and residual dry weight to estimate the fat previously described (Buxton & Lewis 1934; Phelps 1973).

Offspring emergence

Pupae assigned for emergence studies were placed singly into 50 ml falcon tubes with a 3 mm hole drilled

in the centre of the screw cap to allow air flow. Tubes were given individual identification numbers and also labelled with the identification number of the mother. Pupae were observed daily for date of emergence on working days and again video recordings were used to determine the date of emergence on weekends. Pupae were observed for a maximum of 50 days. Any pupae that had not emerged by 50 days, were recorded as a failed emergence (Hargrove 2004). Each emerging fly remained in the tube until it died of starvation. Sex of emerging flies and date of death were recorded. By measuring how long a fly survived starvation, we indirectly quantified the impact of maternal investment without any effect of offspring feeding. This is an ecologically relevant measure as in natural conditions the newly emerged fly is likely to be most vulnerable to mortality from failure to find a blood meal. Therefore, the energy reserves it has available to find a host before starvation is a key fitness trait.

Statistical analyses

For each analysis described below, models were compared using Akaike's information criterion (AIC). If the ratio of the sample size to the number of model parameters was <40, we used AIC corrected for small sample size (AICc) (Burnham & Anderson 2002). For model comparison, the difference in AIC between each model and the lowest AIC model (Δ_i) and Akaike weights (ω_i) were calculated.

We analysed the data using R version 3.6 (R Core Team 2014). The data and R scripts to produce the results can be accessed at (https://github.com/jenniesuz/tsetse_senescence.git).

The survival curves for mothers in each treatment group were estimated using Kaplan-Meier survival analysis. We analysed the effect of nutritional stress and delayed mating on the survival of females in the experiment using a Cox proportional hazards model and estimated the hazard function by treatment using a kernel-based method with a bandwidth grid of 30 components (Moore 2016). For each treatment we also tested whether the risk of death increased with age by comparing exponential and Weibull parametric survival model fits to the data. For these analyses, we used the R packages 'survival', 'survminer' and 'muhaz' (Hess & Gentleman n.d.; Therneau & Grambsch 2000; Kassambara & Kosinski 2018).

For the probability of abortion, offspring wet weight, probability of offspring emergence and duration of offspring survival, we carried out statistical analyses for each treatment separately using linear and generalised linear mixed effects models implemented with the 'lme4' and 'nlme' R packages (Bates *et al.* 2015; Pinheiro *et al.*2018). For each of the models described below, we first assessed the influence of repeated measures from individual mothers by comparing: i) a full model including all explanatory variables, random intercept and slope: ii) a full model with random intercept; and iii) a full model with no random effects (Zuur *et al.* 2009). In each model, maternal age in days was incorporated as a continuous variable.

Logistic regression was used to quantify the effect of maternal age on the probability of larval abortion for each treatment. After comparing models with and without a random effect of individual mother, we compared a model including maternal age with that of the null model.

The effect of maternal age on pupal wet weight was quantified using linear mixed effects models, with the full model including a cubic effect of mother age on offspring wet weight and assuming wet weight was normally distributed. Models including mother age as a cubic, quadratic or linear effect were compared with each other and the null model.

We hypothesised that the effect of maternal age on the probability of emergence and the subsequent survival time would be determined largely by energy reserves, as indirectly measured by offspring wet weight. Heavier offspring would have more reserves and therefore would be more likely to emerge and then survive longer before starvation. Maternal age could also, however, affect offspring survival directly. The effect of mother age and offspring wet weight on the probability of emergence was therefore quantified using logistic regression. We assessed the effect of repeated measures from the same mothers, including a quadratic effect of mother age in the full model.

For the survival data, there was no censoring and the data were approximately normally distributed (S3 Fig.). The relationship between offspring wet weight, sex, mother age and survival time was therefore modelled

using linear mixed effects models for each treatment. We included mother age, as a quadratic or linear effect, and compared model fits with sex to the fit of models including only wet weight and the fit of the null model. To visualise our results, we used fitted models to predict the effect of maternal age on emergence and survival for each wet weight quartile.

Results

Survival of adult females

Of the 96 females in each treatment group, 94% in the control, 92% in the mating delay, and 65% in the nutritional stress group survived the 100 days of the experiment (Fig. 2a). The hazard of death for adult females in the nutritional stress group was 6.7 times greater than that of the control (95% C.I. 2.82 – 16), according to Cox proportional hazards analysis. The hazard of death for the mating delay group was 1.3 times that of the control (95% C.I. 0.46 – 3.8) (Tables S3.1, S3.2). The smoothed hazard function indicated increasing risk of death with age for the nutritional stress group (Fig. 2b) and this was supported by a better fit to the data using a Weibull survival model in the parametric analysis, compared with exponential (Weibull $\omega = 1$). Parametric analysis also provided evidence that the risk of death increased with age in the mating delay group (Weibull $\omega = 0.996$), but not the control group (Weibull $\omega = 0.348$) (Tables S4.3, S4.4).

Abortions

There was evidence of variation between individual mothers in the probability of abortion for the nutritional stress group (model with random intercept $\omega = 0.985$; model without $\omega = 0.015$), but not for the control or mating delay groups, based on comparison of models with and without random effects (Table S4.5). The predicted probability of abortion increased with age across all treatments, up to c. 0.2 for the mating delay group and c. 0.5 for the control group by 100 days (Fig. 3, Table S4.5). Females in the nutritional stress group had higher expected probability of abortion than those in the control or mating delay groups for any given age, increasing to c. 0.7 by 100 days (Fig. 3, Table S4.6).

Offspring wet weight and fat

Over the duration of the experiment, 570 pupae from the control group, 301 from the mating delay group and 369 from the nutritional stress group were produced. Of these 1209 were weighed, as 31 were damaged before weighing. There was an average of six pupae larviposited per individual mother in the control group (range one to eight) and four from both the mating delay (range one to six) and nutritional stress (range one to seven) groups.

For all three treatment groups, the wet weight of pupae increased as mothers aged up until c. 60 days and then declined until the end of the experiment at 100 days (Fig. 4, Tables S4.7-9). The fitted curves and coefficients were similar between treatment groups, but pupae from nutritionally stressed mothers were overall smaller than those from the control or mating delay groups (Fig. 4, Table S4.9). For all three treatments, there was evidence for individual variation among mothers in the weight of their pupae and the effect of age (models with random intercept and slope: control $\omega = 0.992$; mating delay $\omega = 0.909$; nutritional stress $\omega = 0.993$). This variation was more pronounced in the mating delay group compared to the other two treatments with a random intercept variance of 31.6, compared with 8.1 for the control group and 2.0 for the nutritional stress group (Table 1, Table S4.9).

In general, offspring fat increased linearly with offspring wet weight (Fig. S6), thus for brevity we focus on wet weight as our trait of interest but the patterns are qualitatively similar if offspring fat is considered instead.

Offspring emergence and subsequent survival

During the 50-day pupal observation period, 96% of 355 pupae from the control, 93% of 187 pupae from the mating delay, and 91% of 232 pupae from the nutritional stress group emerged successfully. There was no evidence of variation in the probability of emergence between pupae from different mothers across

treatments and no convincing evidence of an effect of pupal wet weight or mother age on the probability of emergence for pupae from the control or mating delay groups (null models: control$\omega = 0.612$; mating delay $\omega = 0.492$) (Table S4.10). However, there was evidence of an effect of both wet weight and mother age for the nutritional stress group (model with weight and age $\omega = 0.602$). Pupae in the nutritional stress group from older mothers had a lower probability of emergence and this was exacerbated for pupae in the lowest weight quartile (Fig. S7, Table S4.11).

All starved offspring were dead by 15 days post emergence. There was only evidence for an effect of individual mother on survival in the nutritional stress group (model including random intercept: control$\omega = 0.347$; mating delay $\omega = 0.287$; nutritional stress$\omega = 0.916$) (Table S4.12). Although there was no evidence for a difference in wet weight between female and male offspring (Fig. 5a), across all three treatments there was evidence that on average female offspring survived slightly longer than males (Table S4.13, Fig. 5b). For all three treatment groups, there was a quadratic effect of mother age on offspring survival and offspring from young mothers in the nutritionally stressed group were particularly vulnerable to early starvation (Fig. 5b, Table S4.12 – 14).

Discussion

There is substantial evidence of survival senescence in insects and some evidence for reproductive senescence. Here we manipulated both age at mating and nutrition to quantify senescence patterns in tsetse flies. The decline in offspring weight and starvation tolerance with mother age, after the peak at c. 60 days, was similar across treatments. Contrary to predictions from life history theory, therefore, neither changes in reproductive investment nor resources affected the timing and rate of reproductive senescence, in terms of offspring quality.

We did observe a steeper increase in the hazard of mortality with age for nutritionally stressed mothers. This is despite nutritionally stressed mothers having higher probability of abortion and producing smaller offspring at any age compared with mothers in the control and mating delay groups. The increase in the hazard of death with age for nutritionally stressed females contrasts with findings from studies of other insects where nutritional stress reduced reproductive output but either maintained or extended lifespan relative to a control group (De Sousza Santos & Begon 1987; Ernsting & Isaaks 1991; Kaitala 1991). Taken together, it may be that factors other than reproduction cause reproductive ageing. Alternatively it may be that, given the extreme maternal investment in tsetse, even though females produce relatively smaller offspring they still pay a high cost of reproduction in terms of physiological damage; and females on a poor quality diet experience this cost to a greater extent in terms of impact on mortality. In an analysis of tsetse caught in the field, smaller females were shown to invest relatively more of their fat in their offspring, even though their offspring were smaller (Hargrove *et al.* 2018).

We find that females experiencing nutritional stress have a relatively higher rate of spontaneous abortion, particularly at later ages. Hargrove and Muzari (Hargrove & Muzari 2015), using field collected *G. pallidipes* , showed that transfer of the majority of fat to the larva occurs only after c. 80% pregnancy has been completed. Therefore, a female could potentially abort a larva if there are not enough fat reserves for a full-term pregnancy.

Evolutionary models tailored to tsetse life-history, with high investment in single offspring across multiple reproductive bouts, could yield insights into whether such spontaneous abortion is an adaptive strategy to retain reserves for future reproduction, or a result of physiological constraints that limit the reserves available (McNamara*et al.* 2009).

Our study highlights the benefits gained from individual-level data to understand senescence. The concave pattern observed here is strikingly similar in shape across treatments, reaching a peak at c. 60 days and declining thereafter, and reflects the general concave pattern of reproductive senescence observed across diverse taxonomic groups e.g. (Velando *et al.* 2006; Sharp & Clutton-Brock 2010). The concave relationship of offspring quality with age may have contributed to the relatively small effects of age evident in previous studies where grouped ages and mean values were used, rather than tracking reproductive output from indi-

vidual females (Langley & Clutton-Brock 1998; McIntyre & Gooding 1998). In addition, tracking individual mothers provided insights into individual variation in reproductive investment. There was marked variation in offspring weight between mothers and variation in senescence patterns for this trait, particularly for nutritionally stressed mothers. There was more variation in offspring weight between individual mothers for the mating delay group relative to the control, predominantly contributed from mothers producing smaller than average offspring. There was less variation in offspring weight for young nutritionally stressed mothers, but this increased as they aged. These observations suggest that variation in offspring quality cannot be explained by variation in mother size alone. The large amount of variation in offspring size in this study was unexpected, suggesting that future studies quantifying the relative roles of mother size and condition on offspring size would be valuable.

Offspring from young mothers that were nutritionally stressed had the lowest survival under starvation. That our analyses showed an effect of mother age on offspring survival separate from its effect via energy reserves, as measured by wet weight, indicates that there may be other factors associated with mother age that influence the quality of offspring. While the resource-allocation framework of senescence focuses on absolute resources, there may be more subtle effects of mother age on the quality of those resources transferred to offspring that warrant further investigation in tsetse and other species. For tsetse, during late stages of pregnancy females not only transfer fat but also amino acids. It may be that young nutritionally stressed females are limited in these amino acids. Cmelik et al. (Cmelik *et al.* 1969) observed high amounts of tyrosine in the gut contents of third instar larvae, which is involved in tanning of larval and adult cuticles. They reasoned that the tyrosine and phenylalanine obtained from a single bloodmeal is unlikely to be sufficient to meet the amount required by offspring and that a surplus stored from previous bloodmeals may be required. This suggests that reserves of essential nutrients could also regulate the size of the offspring – if there are only reserves sufficient for offspring of a given size. It also demonstrates that the resource allocation theory is likely more complex, and more nuanced studies on the effects of the quality of resources as well as quantity may be required to understand the ageing process.

Evidence that smaller pupae do not survive starvation for as long as larger pupae is in contrast to studies showing that smaller sized insects are potentially more competitive, or survive longer under times of stress because their overall energy requirements are lower than larger offspring (De Sousza Santos & Begon 1987).

We focused on reproductive senescence in this study. One limitation is, therefore, that we did not continue the experiment beyond 100 days to quantify more fully mother survival for all three treatments. Our evidence of survival senescence in the nutritionally stressed group supports analysis of mark-recapture studies of *G. m. morsitans* in the field (Hargrove *et al.* 2011) which also showed an increase in mortality as a function of age. Considering this, an additional experiment to test whether females mated later experience a delayed onset in survival senescence would be informative, given the similar rates of reproductive senescence observed across treatments.

With respect to tsetse as vectors of trypanosomes, an increase in the hazard of death of an insect vector, with age, affects pathogen transmission rates and may therefore need to be accounted for in quantitative assessments of control strategies (Bellan 2010; Ryan*et al.* 2015). Moreover, older mothers often produce compromised offspring, in terms of their own lifespan (Lansing 1947) or immunity (Clark *et al.* 2017). For tsetse, it has been shown that nutritionally stressed individuals can be more susceptible to trypanosome infection (Kubi *et al.* 2006). Maternal age effects on offspring nutritional status therefore has consequences for modelling the dynamics of the spread of tsetse populations and the pathogens they transmit (English *et al.* 2020). At present, we still understand very little about the extent of senescence in insect vector populations and the underlying drivers of variation in these life history patterns.

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Figure 1 Overview of experiments. M – mating.

Figure 2 Kaplan-Meier survival curves (a) and smoothed hazard function (b) for adult females by treatment. For survival curves in (a), shading indicates 95% confidence intervals.

Figure 3 Predicted probability of larval abortion as a function of mother age, by treatment. Predicted probabilities from generalised linear mixed effects model fits to the data and 95% confidence intervals. Plots of raw data are provided in S5 File.

Figure 4 Offspring wet weight as a function of mother age and treatment. Showing model fits to the data: thick line – population level, thinner lines – individual level. Points – average wet weights for 10-day intervals and 95% confidence intervals. Plots of raw data are provided in S5 File.

Figure 5 Effect of sex, wet weight and mother age on the number of days a newly emerged fly can survive starvation. a) Wet weight as a function of offspring sex by treatment; b) Predicted survival time based on linear mixed effects model. Days adults survived starvation is plotted against mother age. Prediction for each wet weight quartile shown. Plots of raw data are provided in S5 File.

Supplementary Files

S1 File. Additional methods.

S2 File. Nutritional stress pilot.

S3 Figure. Histogram of offspring survival time by treatment.

S4 File. Statistics.

S5 File. Raw data plots.

S6 Figure. Offspring fat as a function of wet weight.

S7 Figure. Probability of pupal emergence as a function of mother age for each wet weight quartile.