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ORIGINAL ARTICLE

Selecting on age of female reproduction affects lifespan in both sexes and age-dependent reproductive effort in female (but not male) *Ceratitis cosyra*

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Abstract
The trade-off between lifespan and reproduction is central to our understanding of life-history evolution. Laboratory selection experiments have been a powerful tool for quantifying this trade-off, but these tend to be restricted in taxonomic scope, which may limit our understanding. In addition, research often focuses on the trade-off between lifespan and reproductive effort in females, and far less data test how lifespan trades off with different aspects of male reproduction (e.g. pre- and post-copulatory reproductive investment). Here, we examined the trade-off between lifespan and reproduction in females and males of the marula fruit fly, *Ceratitis cosyra* (Walker) (Diptera: Tephritidae). To do so, we selected downward or upward on age of peak female egg laying in *C. cosyra* for twenty generations. In multiple generations, we measured female and male lifespan and body size, female daily and lifetime fecundity, male courtship and mating success, as well as the number of sperm transferred at different ages and sperm storage asymmetry in spermathecae. Our selection regime appeared to achieve its aim; egg laying peaked earlier in females from downward selected lines than upward selected lines. The number of sperm transferred by males decreased in the upward selected flies, but other male reproductive traits remained the same across selection regimes. In contrast, with the wider literature, upward selection did not extend the lifespan of females or males after ten generations of selection. While lifespan in both sexes responded to selection on female egg laying schedules, it did not do so in a straightforward way. Moreover, male investment in reproductive traits was largely independent of selection regime. These counter-intuitive findings highlight the importance of working with a broad range of species and of considering the trade-off between reproduction and lifespan in both sexes.

Significance statement
The trade-off between lifespan and reproduction has been extensively studied in model species using various types of laboratory selection. A limited number of species have been considered using this approach, and the majority of the studies have focused on female, rather than male, reproductive effort. Here, we selected downwards and upwards on age of female reproduction in the marula fruit fly and measured survival, female fecundity, reproductive schedule, as well as male sperm transfer, sperm storage asymmetry, mating and calling success. We found a moderate trade-off between lifespan and early fecundity in downward selected flies, whereas no obvious trade-off was observed in upward selected lines. Regardless of the selection regime, reproductive scheduling was affected in females but not in males, while lifespan was affected in both sexes. Our results show that the timing of reproduction can evolve independently across the sexes, highlighting the importance of studying both females and males.

Keywords Experimental evolution · Lifespan · Reproduction · Trade-off · Tephritidae

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Introduction

Life-history theory considers how organisms schedule key fitness-determining events over their lives, such as how fast and large to grow, how many offspring to produce and when

to invest in maintaining the soma (Stearns 2000). Life-history traits can be connected by trade-offs, where increased expression of one trait that improves overall fitness has negative consequences for a second trait (Stearns 1989; Roff and Fairbairn 2007). Perhaps the most well-documented of these trade-offs involve the costs of reproduction, whereby increased investment in current reproductive effort results in reduced lifespan or future reproductive success (Edward and Chapman 2011). Trade-offs involving the cost of early reproduction are central to both the antagonistic pleiotropy (Williams 1957) and the disposable soma (Kirkwood 1977) theories of ageing. Accordingly, trade-offs between reproduction and lifespan have been studied in an array of species, at different levels of analyses and using a variety of approaches.

Within species, physiological trade-offs between lifespan and reproduction (i.e. those that manifest within individuals in the same generation; Flatt and Schmidt 2009) have been detected in studies quantifying phenotypic correlations between traits (e.g. McLean et al. 2019) and in studies that manipulate phenotype. For example, male Australian field crickets (*Teleogryllus commodus*) fed high protein diets invest heavily in early life reproduction (i.e. call intensely to attract a mate) even though this reduces their lifespan, whereas males fed low protein diets invest less intensely in early reproductive effort but live longer (Hunt et al. 2004). Physiological trade-offs have been identified via manipulations of the genetic pathways that are predicted to underpin those trade-offs, for example, by ablating the germ line (Barnes et al. 2006; Flatt et al. 2008). Evolutionary trade-offs (i.e. those that manifest at population level; Flatt and Schmidt 2009) between reproduction and lifespan have been identified by measuring genetic correlations between traits (Roff and Fairbairn 2007; Archer et al. 2012) or by assaying correlated responses to selection via artificial selection, experimental evolution or breeding experiments (Edward and Chapman 2011). For example, numerous classic artificial selection and experimental evolution studies using *Drosophila* as a model have shown that selecting for late life reproduction increases lifespan, and that selecting for longer lifespan usually decreases early life reproductive effort (reviewed comprehensively by Flatt 2020). Finally, comparative studies show that across species, short-lived species are slower to reach reproductive maturity and often have lower fecundity over their lifetime (Salguero-Gómez et al. 2016; Salguero-Gómez and Jones 2017). However, this comparative fast-slow pattern is by no means universal and much stronger when comparing higher taxonomic levels (Stearns and Rodrigues 2020).

While trade-offs between reproduction and lifespan are widespread, they often differ in magnitude across the sexes. For example, the trade-off between lifespan and reproduction is often more pronounced in females than in males (Bonduriansky et al. 2008; Zajitschek et al. 2009; Adler et al. 2013; Bolund et al. 2016) and this is typically attributed to sex differences in the cost of producing offspring (Zajitschek et al. 2009; Bolund et al. 2016). For example, eating a restricted diet tends to improve lifespan (Nakagawa et al. 2012; Simons et al. 2013) while reducing reproductive effort (Moatt et al. 2016). Moreover, the impacts of this dietary manipulation on reproduction generally appear to be stronger in females than in males. However, while dietary restriction studies frequently measure effects on lifespan in both sexes (Nakagawa et al. 2012), data on the effects of dietary restriction on reproduction typically comes from females, with few studies measuring effects on male reproduction in a biologically meaningful way (i.e. capturing the full costs of male reproductive investment) (Moatt et al. 2016). For this reason, apparent sex difference in the magnitude of responses to dietary restriction may reflect experimental design rather than a genuine biological signal. Similarly, artificial selection and experimental evolution studies selecting on either lifespan or reproductive scheduling in a variety of laboratory species often measure effects on lifespan in both sexes but tend to only measure effects on female age-dependent fecundity (Rose and Charlesworth 1981; Luckinbill et al. 1984; Rose 1984; Arking 1987; Tucié et al. 1990; Engström et al. 1992; Partridge and Fowler 1992; Zwaan et al. 1995; Miyatake 1997; Partridge et al. 1999; Sgrò and Partridge 1999; Stearns et al. 2000; Scannapieco et al. 2009; Anderson et al. 2011; Flatt 2011; Remolina et al. 2012; Chen et al. 2013; Carnes et al. 2015; Đorđević et al. 2015; May et al. 2019; Foucaud et al. 2020). There are some exceptions, for example, experimental evolution studies in *D. melanogaster* (Borash et al. 2007), the nematode *Caenorhabditis remanei* (Chen et al. 2016), seed beetles (Berg and Maklakov 2012) and crickets (Hunt et al. 2006) selected on lifespan, age-dependent mortality risk or reproduction and assayed effects on male reproduction. Moreover, it is important to highlight that research using alternative methods (e.g. quantitative genetic breeding designs or inbred lines) have quantified the relationship between lifespan and reproductive traits in both sexes (Hughes 1995; Zajitschek et al. 2007; Archer et al. 2012, 2013).

A tendency to measure reproductive effort more frequently in females rather than in males in these studies might be due to the difficulty of measuring male reproductive investment but also may reflect complications in understanding how selecting on reproductive scheduling affects males in insect models, given female capacity to store sperm for future fertilisation of eggs (Fritz 2004; Twig and Yuval 2005; Pérez-Staples et al. 2007; Schnakenberg et al. 2011; Wolfner 2011; Degner and Harrington 2016; Zajitschek et al. 2019). Without knowing how often females remate, for how long females store sperm, or the prevailing sperm precedence rules, it becomes challenging to know exactly

how selecting on reproductive timing affects males. Regardless of why our understanding of the trade-off between lifespan and reproduction is based primarily on female data, the consequence is that our understanding of sex differences in the costs of reproduction, and how these costs affect lifespan, may be somewhat skewed.

In addition to apparent sex differences in the trade-off between reproduction and lifespan, there is some evidence that the magnitude of this trade-off seems to be more pronounced in model organisms. For example, the effects of dietary manipulations on reproductive effort (Moatt et al. 2016) and lifespan (Nakagawa et al. 2012) are greater in species including yeast, nematodes, rodents and vinegar flies. This is problematic because so much of our understanding of life-history trade-offs comes from model species. Studies using a laboratory selection regime to investigate the trade-off between lifespan and reproduction, or early and late life reproduction, have typically involved *Drosophila melanogaster* (Luckinbill et al. 1984; Rose 1984; Partridge and Fowler 1992; Stearns et al. 2000) and, to a lesser extent, nematodes (Anderson et al. 2011; Carvalho et al. 2014) or beetles (Berg and Maklakov 2012). Such restricted taxonomic scope may be problematic when tackling evolutionary questions (Russell et al. 2017), and this is particularly true given the tremendous diversity of life-history strategies in nature (Jones et al. 2014). Altogether, this means that while the trade-off between reproduction and lifespan has been the subject of intense investigation for decades, there remains a paucity of data in males and non-model organisms.

Here, we used experimental evolution to examine the trade-off between lifespan and reproduction in females and males of the marula fruit fly, *Ceratitis cosyra* (Walker) (Diptera: Tephritidae). Several studies have documented the trade-off between lifespan and fecundity in females of various species of tephritids (Miyatake 1997; Carey et al. 2005, 2008a, 2008b, 2020; Fanson et al. 2009, 2012; Carey and Molleman 2010; Papadopoulos et al. 2010; Carey 2011; Fanson and Taylor 2012; Chen et al. 2013; Papanastasiou et al. 2013; Yap et al. 2015; Malod et al. 2017, 2020a, 2020b; Roets et al. 2018). Among these species, *C. cosyra* is one of the longest-lived (on average 104 to 161 days) (Roets et al. 2018; Malod et al. 2020a) and thus provides an interesting comparison to the much shorter-lived (ca., 30 to 60 days at 25 °C; Mołoń et al. 2020) model organism, *D. melanogaster*. We utilised selection regimes similar to those seen in classical *Drosophila* studies: we selected on age of oviposition such that only females that survived up to egg collection contributed to the next generation in the upward selected regime (Rose 1984; Arking 1987). In addition to survival and female fecundity, this study assayed a wide range of male reproductive traits that are associated with both pre- and post-copulatory reproductive effort (age-dependent courtship behaviour, mating success, sperm transfer and sperm asymmetry) to better quantify any trade-offs involving males. Sperm storage asymmetry was included in our assessment of male reproductive traits because biased sperm storage in the spermathecae of mated females represents an opportunity for sperm competition to occur, which can then affect paternity by the first-mated male (Perez-Staples and Aluja 2006). Finally, the experiment ran over twenty generations to track the temporal changes in each investigated life-history trait, as correlated changes between traits can disappear or be reversed over generations (Archer et al. 2003) and phenotypes can cease to evolve in response to the selection regime (Kawecki et al. 2012).

We anticipated that our selection regimes would affect reproductive scheduling in both sexes, with downward selected lines investing in reproduction at an earlier age and upward selected lines postponing reproductive effort. We also predicted that selecting on female age of oviposition would affect male reproductive schedules in a similar way to females. This is because in other tephritid species, including the close relative *C. capitata*, there are signs of sperm competition and last male precedence (Bertin et al. 2010; Shelly 2018). Therefore, males that reproduced close to the age of female egg laying should have an advantage and thus, we were also selecting on males in a similar fashion.

Materials and methods

Fly husbandry

Infested mangoes from across Mpumalanga province, South Africa, were collected and pupae of *C. cosyra* retrieved. The wild flies emerging from these pupae were used to establish a culture that was maintained at ~ 23 °C in a climate room with a 14:10 light:dark photocycle. To create optimal mating conditions, the first and last hour of the light phase simulated dawn and dusk with 8 W fluorescent tubes (T4, Eurolux, Sandton, South Africa) that were placed obliquely to the fly culture and turned on before, and turned off after, the main room lights. The remaining room lights, comprising a combination of 20 W (G5, Eurolux, Sandton, South Africa) and 58 W (58 W/840, Osram, Germany) fluorescent tubes, were also turned on for the remainder of the light period. Adults were kept in groups of ca. 200 flies in 5 L plastic cages with unrestricted access to food (hydrolysed yeast and sugar in separate dishes) and water (water-soaked cotton wool). At 15 days after emergence, wild males were crossed with females from a laboratory culture provided by Citrus Research International (Nelspruit, South Africa). This step introduced wild genetic diversity while also retaining the tendency for culture females to oviposit into an artificial substrate. The next generation was obtained by allowing laboratory females mated with wild males to lay eggs on a

125 mL plastic container (Plastilon, South Africa) covered with a layer of laboratory film (Parafilm M, Bemis, USA) pierced several times. Tissue paper soaked with 3 mL of guava juice (Hall's concentrate, Tiger Consumer Brands Limited, Bryanston, South Africa) was placed in the plastic container to encourage females to oviposit through the film. Eggs were then washed out of the artificial substrate with water and placed on 125 mL of a carrot-based larval rearing medium (Citrus Research International, Nelspruit, South Africa) in a plastic container at an approximate density of 2.5 eggs/mL of medium. The container of larval rearing medium was then placed in a 2 L plastic box with a layer of sand and a ventilated lid. After 15 days, during the pupal phase, the sand was sifted and the retrieved pupae placed in a Petri-dish (ø 65 mm) and transferred into a 5 L cage with unrestricted access to food and water for emerging adults.

Selection regime

Selection began three generations after laboratory females had been crossed with wild males and a strong culture had been established. We selected on the age of oviposition by only providing an oviposition substrate (a 125 mL plastic container with guava juice-soaked tissue paper) when flies were 5 days old (downward selected, DS), 15 days old (control, CT) or 25 days old (upward selected, US). In our laboratory, 15 days is the average age when eggs are collected from this species and is also when oviposition peaked in earlier studies (Manrakhan and Lux 2006; Roets et al. 2018). Downward selection was performed at 5 days old and not earlier in order to allow enough individuals to mate and contribute to the next generation, as Manrakhan and Lux (2006) found fewer than 5% of *C. cosyra* mating within 1 week of emergence. For similar reasons, upward selection at 25 days rather than an older age was to ensure that enough females would contribute to the subsequent generation due to a gradual decline in oviposition from 3 weeks of age (Manrakhan and Lux 2006). This was to maintain populations of ca. 200 flies per replicate and avoid the risk of a population collapse and inbreeding. More eggs were collected in a generation preceding a test generation to ensure that enough flies would remain to produce eggs for the following generation after that test flies had been taken out. In addition, after collecting eggs for the next generation, populations from the previous generation were maintained until successful hatching of the next generation's eggs as security, in case too few flies emerged to establish the next generation. For each of the three selection lines (DS, CT and US), we established five replicate populations. We maintained the selection regime for 20 generations. Lifespan and reproductive effort assays were performed for each line at generations 0 (G_0), 4 (G_4), 10 (G_{10}), 15 (G_{15}) and 20 (G_{20}). Because flies were selected on age of oviposition, selection lines inevitably differed in their assay date.

Female reproductive effort

Within a day of emergence, 50 females from each selection regime were placed into individual cages ($n = 10$ per replicate, per selection regime) for each of the five generations assayed. Each cage comprised a 125 mL plastic cup with another cup nested inside with the base removed. The containers were covered with insect screen secured by two rubber bands. The flies were provided with filtered water through the insect screen with 200 µL pipette tips loosely capped at the wide end with putty-like pressure-sensitive adhesive (Prestik, Bostik, South Africa) to minimise evaporation. Sugar and hydrolysed yeast (Yeast Extract Powder, Biolab, Merck, Germany) were provided in the lids of two 2 mL microcentrifuge tubes. Mortality was recorded daily, and food and water were replaced when close to being depleted or if the sugar liquefied (due to its tendency to absorb water vapour). The design of the cage provided an easy means of replacing food and egg dishes (see below) without females escaping (by removal of the intact bottom cup containing the food, and simultaneously sliding the other cup containing the fly onto a table).

An artificial egg laying dish was added to each of the containers. The egg laying dish comprised a black screw-top lid (volume = 5 mL, diameter = 32 mm) containing a 1:10 orange essence-water solution (Robertsons, Johannesburg, South Africa) and covered with a double layer of laboratory film, pierced ten times with an entomological pin. Every 5 days, egg dishes were removed and replaced, and the number of eggs laid by each female was counted. The total number of eggs laid by females during their lifetime was calculated as the sum of all 5-day oviposition intervals, and the average number of eggs per day as the total number of eggs divided by lifespan. The day of peak egg production was the day at the end of the 5-day oviposition interval during which the maximum number of eggs were obtained from a female.

Male reproductive effort

For each selection regime and at each generation assayed, groups of 50 males were taken from each replicate shortly after emergence and kept in separate 2 L plastic cages to prevent mating. At ages 5, 15 and 25 days (in results t_5, t_{15} and t_{25}), focal males were paired with virgin females from an unselected laboratory culture 1 h before the simulated dusk. This species only mate at dusk and new mating does not occur after darkness (personal observation). For each generation, selection regime, age and replicate, six pairs were assayed. The females used as mates were all between 10 and 20 days old to minimise the effect of female age on

male reproductive measurements. The pairs were placed into cylindrical transparent plastic containers (height = 52 mm, diameter = 35 mm) for easy observation. Pairs were observed until all lights turned off (2 h) to record if male calling and mating occurred. Due to the tendency of *C. cosyra* to mate for up to 12 h (personal observation), flies were left to mate overnight.

The following morning, females that were observed mating were dissected under a stereo microscope to analyse sperm transfer, using methods described by Roets et al. (2018). A total of 110 females were dissected (CT: $t_5 = 23$, $t_{15} = 17$, $t_{25} = 17$; US: $t_5 = 19$, $t_{15} = 13$, $t_{25} = 21$). Spermathecae of females were removed and placed individually into 15 µL drops of water on microscope slides. Each spermatheca was then crushed with an entomological pin attached to a thin wooden dowel. The crushed spermatheca was then spread by vigorous stirring for 30 s before covering it with a 22 × 22 mm cover slip. The slides were left to dry for 2–3 days before gluing the corners of the coverslip to the slide using clear nail varnish.

The number of sperm transferred was estimated using a phase contrast microscope (BX43, Olympus Corporation, Japan) and methods described in Taylor et al. (2000). In summary, a matrix of 25 fields of view at 100 × magnification (17.36% of the coverslip area) were selected. The number of sperm counted in this area was multiplied by 5.76 to estimate the total number of sperm stored per spermatheca. If no sperm were found in the 25 fields checked, the whole slide was checked to confirm absence of sperm.

Survival

For each generation assayed, within 24 h of emergence, 10 females and 10 males from each selection regime and replicate were transferred to individual cages as described for the female reproductive experiment (i.e. 50 females and 50 males for each generation). Females used for the survival experiment were not tested for reproductive effort. Mortality was recorded daily, and food and water were replaced when close to being depleted or if sugar liquefied. At death, flies from the survival experiment were individually placed in a 2 mL microcentrifuge tube and stored at – 20 °C for later determination of head width as a proxy for fly size (see below).

Head width

Head width was measured for a subset of flies from the survival experiment to assess potential effects of selection on fly size. Flies were decapitated and the heads were individually placed, face up, on the stage of a stereo microscope (SZ61, Olympus Corporation, Japan) fitted with a digital camera (Dino-eye, C-mount; AnMo Electronics Corporation, Japan). A photograph of each head was taken at 4 × magnification. A microscope stage calibration slide (0.1 mm) was also photographed during the same session to calibrate head measurements. Head width was then determined as the distance between the external side of each eye using ImageJ software (National Institute of Health, Bethesda, Maryland, USA). For each selection regime, head width was measured for 25 females and 25 males ($n = 5$ per sex, per replicate) when possible. Some selection regimes had fewer observations due to sample degradation (i.e. damaged eye).

Data analyses

Statistical analyses were performed in R (v 3.5.3, The R Foundation for Statistical Computing). Generalised linear mixed effects models were used to analyse the reproductive traits with Poisson (total eggs and sperm transfer), gamma (day of peak egg production, eggs per day), binomial (courtship or mating success) or quasi-binomial distributions (sperm storage asymmetry) with selection regime and generation included as a fixed effect. A hierarchical random structure with generation nested in replicate and replicate nested in selection (hierarchical random structure: Selectio n(Replicate(Generation))) was included as a random effect, except in models where the variance of the random structure or the random variance of one of the nested factors was null. In these cases, we removed the factor(s) with the null random variance(s) from the hierarchical structure and used a generalised linear mixed effects model, or we used generalised linear models if the entire hierarchical random structure had a null variance. The nested random effect was necessary to take into account the effect of replicate, and for the purpose of the statistical analyses, replicates of each selection regime were not assigned the same numbers. Other explanatory variables in each model are detailed below. Models were built using the functions 'glmer' or 'glm' from the 'lme4' package (Bates et al. 2015). Where appropriate, we corrected for overdispersion in generalised linear mixed effects models by adding an observation level random effect (Harrison 2014). If a significant effect was detected, post hoc pairwise comparisons tests of the estimated marginal means were performed using the function 'emmeans' (Russell 2020). Using the 'emmeans' function returns an estimate that is the difference between the two compared groups and indicates the direction of the difference; these estimates are reported below.

Because zero values prevent use of the gamma family, if the number of eggs laid per day for a female was zero, this value was replaced with the smallest value of the dataset for this trait and divided by ten. To determine the effect of selection, generation and age on the total number of sperm transferred to spermathecae, the sperm storage asymmetry and the propensity of males to call and mate, selection

regime, generation and age, as well as their interactions were included as fixed effects. No random effect was included in the model for sperm transfer and sperm asymmetry because of the null variance of the hierarchical random structure. A logistic regression was used for sperm storage asymmetry, where the spermathecae with the most and fewest sperm were combined using the 'cbind' function. We corrected for overdispersion by using a quasi-binomial (sperm asymmetry) or quasi-Poisson (sperm transferred) distributions (see Chapter 16 of Crawley (2013)). To determine which groups stored significantly more than 50% of sperm in one spermatheca (representing no asymmetry), we visually inspected whether the 95% confidence intervals of the estimated marginal means overlapped 50% (see Figure S1).

To determine the effect of selection, generation and sex on survival, a Cox proportional hazards model with a hierarchical structure was performed using the 'coxme' function from the 'survival' package (Therneau 2015). The function 'cox.zph' from the same package was used to test the proportional hazards assumption. Selection regime, generation, sex and their interaction were fixed effects in the model, while a hierarchical structure with generation nested in replicate and replicate nested in selection was included as a random effect (hierarchical random structure: Selection(Replicate(Generation))). The nesting structure was used to differentiate the replicates between selection regimes. If a significant effect was detected, post hoc pairwise comparisons tests of the estimated marginal means were performed. In this particular case, the 'emmeans' function returns the estimated proportional hazards for each group and a contrast comparison that is the ratio between the two compared groups. A ratio of 1 indicates that both groups have equal mortality risk, whereas a ratio significantly superior or inferior to 1 indicates that first group has a lower (ratio < 1) or higher (ratio > 1) risk of mortality than the second one.

Head width was analysed using an analysis of variance with selection regime, generation and sex as fixed effects. Random effect of replicate was not included due to the random factor having a null variance. Step-wise selection of the minimal adequate model was performed using the 'step' function. If a significant effect was detected, post hoc pairwise comparisons tests of the estimated marginal means were performed. A series of Pearson's product moment correlations were performed to evaluate the association between head width and mean lifespan within each selection regime.

Results

Female reproductive effort

The timing of peak egg laying (i.e. the greatest number of eggs laid in a 5-day window) was affected by an interaction between selection regime and generation (Table 1) (Fig. 1a). Prior to G_{15}, the timing of oviposition was consistent across lines, and then differences began emerging in the direction predicted. At G_{15}, DS females oviposited earlier than the CT and US females (CT vs DS: estimate = 0.50, $p = 0.006$; DS vs US: estimate = −0.83, $p < 0.001$), while no significant difference was observed between CT and US flies. At G_{20}, oviposition was still significantly earlier in DS females than in CT or US females (CT vs DS: estimate = 0.65, $p < 0.001$; DS vs US: estimate = −1.07, $p < 0.001$). In addition, egg laying peaked later in US than in CT females at G_{20} (estimate = −0.41, $p = 0.041$).

The effects of selection on female lifetime fecundity differed between generations (Table 1) (Fig. 1b): fecundity rose from the unselected flies (G_0) to G_4, where US females laid significantly more eggs than the unselected population they originated from (G_0 vs G_4: estimate = −1.18, $p < 0.001$). Fecundity rose higher still until G_{10} where all selection lines had higher fecundity than the unselected starting population (G_0 vs G_{10}: CT: estimate = −0.86, $p = 0.009$; DS: estimate = −0.79, $p = 0.019$; US: estimate = −1.40, $p < 0.001$). However, after G_{10}, there was a decline in fecundity in US females only, with more eggs being produced at G_{10} than at G_{15} and G_{20} (G_{10} vs G_{15}: estimate = 0.73, $p = 0.044$; G_{10} vs G_{20}: estimate = 0.92, $p = 0.004$). Although this was not significant, we observed fewer eggs being laid by US females than by CT ones at G_{20} (CT vs US: estimate = 0.55, $p = 0.098$).

A selection by generation interaction also affected daily egg production (Table 1) (Fig. 1c). Significant differences in daily fecundity emerged from G_{15}. At G_{15}, DS females laid the most eggs per day (CT vs DS: estimate = −0.66, $p = 0.031$; DS vs US: estimate = 1.29, $p < 0.001$) and no difference was observed between CT and US females. However,

Table 1 Analyses of female reproductive effort using generalised linear and generalised linear mixed models in control, downward-selected or upward-selected flies

Effects	χ^2	df	p
Lifetime egg production			
Selection	2.44	2	0.295
Generation	8.02	4	0.091
Selection × Generation	32.41	8	**< 0.001**
Eggs per day			
Selection	5.63	2	0.059
Generation	19.14	4	**< 0.001**
Selection × Generation	22.73	8	**0.004**
Peak egg production			
Selection	0.65	2	0.721
Generation	4.75	4	0.313
Selection × Generation	33.32	8	**< 0.001**

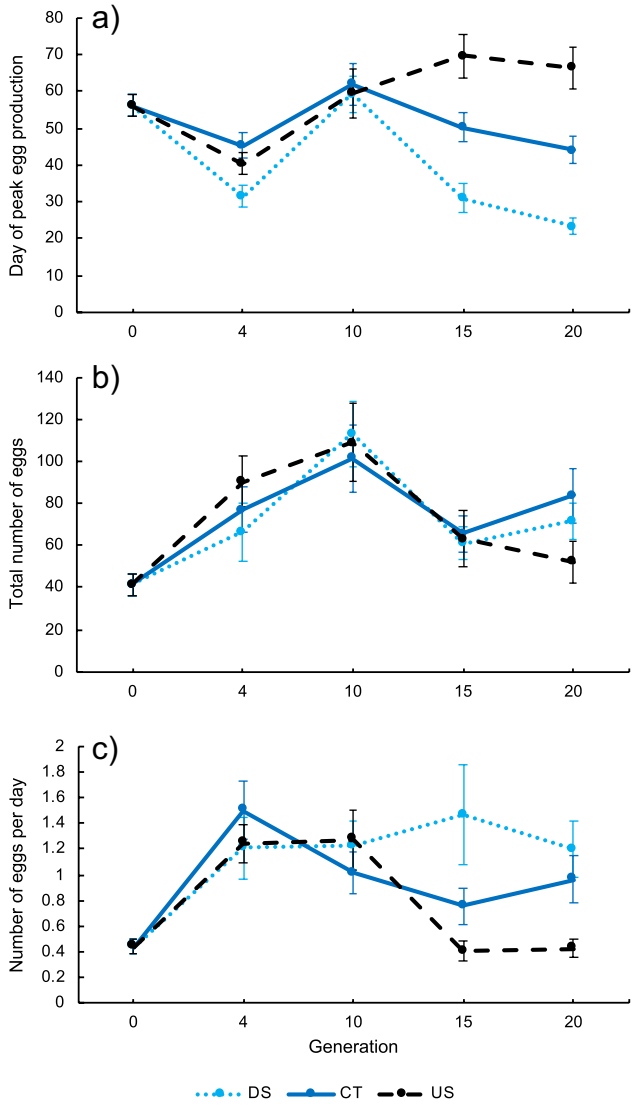

Fig. 1 Reproductive effort of *C. cosyra* females issued from control (CT), downward- (DS) and upward-selected (US) lines across five generations. The values displayed are the average day of peak egg production (**a**), average daily egg production (**b**) and average lifetime egg production (**c**) of virgin females individually kept in a container. The error bars represent the standard error of the mean

US females produced fewer eggs per day than CT and DS females at G_{20} (CT vs US: estimate = 0.82, $p = 0.005$; DS vs US: estimate = 1.04, $p < 0.001$), while daily fecundity of DS lines did not differ from the CT lines.

Male reproductive effort

Courtship was affected by an interaction between generation, selection regime and male age (Table 2) but there was no consistent pattern in these effects (Fig. 2a, b, c). The only differences between selection regimes were found at G_4, where at 15 days of age, CT and DS males called less than US males (CT: estimate = −2.69, $p = 0.039$; DS: estimate = −3.10, $p = 0.005$) (Fig. 2b) and at 25 days of age, CT males called less than DS males (estimate = −2.26, $p < 0.001$) (Fig. 2c). There was no significant main effect of selection regime, generation or age (Table 2).

Table 2 Analyses of male reproductive effort using generalised linear and generalised linear mixed models in control, downward-selected or upward-selected flies

Effects	χ^2	df	p
Courtship			
Selection	4.85	2	0.088
Generation	4.55	4	0.337
Age	0.78	2	0.675
Selection × Generation	13.71	8	0.089
Selection × Age	7.79	4	0.112
Generation × Age	25.84	8	**0.001**
Selection × Generation × Age	41.17	16	**< 0.001**
Mating			
Selection	0.79	2	0.672
Generation	1.15	4	0.885
Age	0.14	2	0.932
Selection × Generation	13.34	8	0.101
Selection × Age	3.14	4	0.535
Generation × Age	8.16	8	0.417
Selection × Generation × Age	45.19	16	**< 0.001**
Sperm transfer			
Selection	8.89	2	**0.011**
Generation	7.03	4	0.134
Age	113.88	2	**< 0.001**
Sperm storage asymmetry			
Selection	0.55	2	0.758
Generation	0.35	4	0.986
Age	30.34	2	**< 0.001**

*The minimal adequate model included only the main effects of selection regime, generation and age without their interactions

Mating success was affected by a three-way interaction between male age, selection regime and generation (Table 2) (Fig. 2d, e, f). As for the propensity of males calling, there was no consistent pattern. Differences between selection regimes were only found at two generations and were age dependent. At G_4 for the 15-day-old groups, more males mated in the CT and DS lines than in the US lines (CT: estimate = 2.63, $p = 0.003$; DS: estimate = 3.18, $p < 0.001$) (Fig. 2e), whereas 25-day-old CT males mated less than US counterparts of the same age (estimate = −1.56, $p = 0.015$) (Fig. 2f). Moreover, mating success was lower in CT than in DS lines at G_{20} and 25 days old (estimate = −1.60, $p = 0.037$) (Fig. 2f).

Selection regime and male age affected sperm transfer (Table 2). CT males transferred significantly more sperm than US ones (estimate = 0.19, $p = 0.012$), but there were

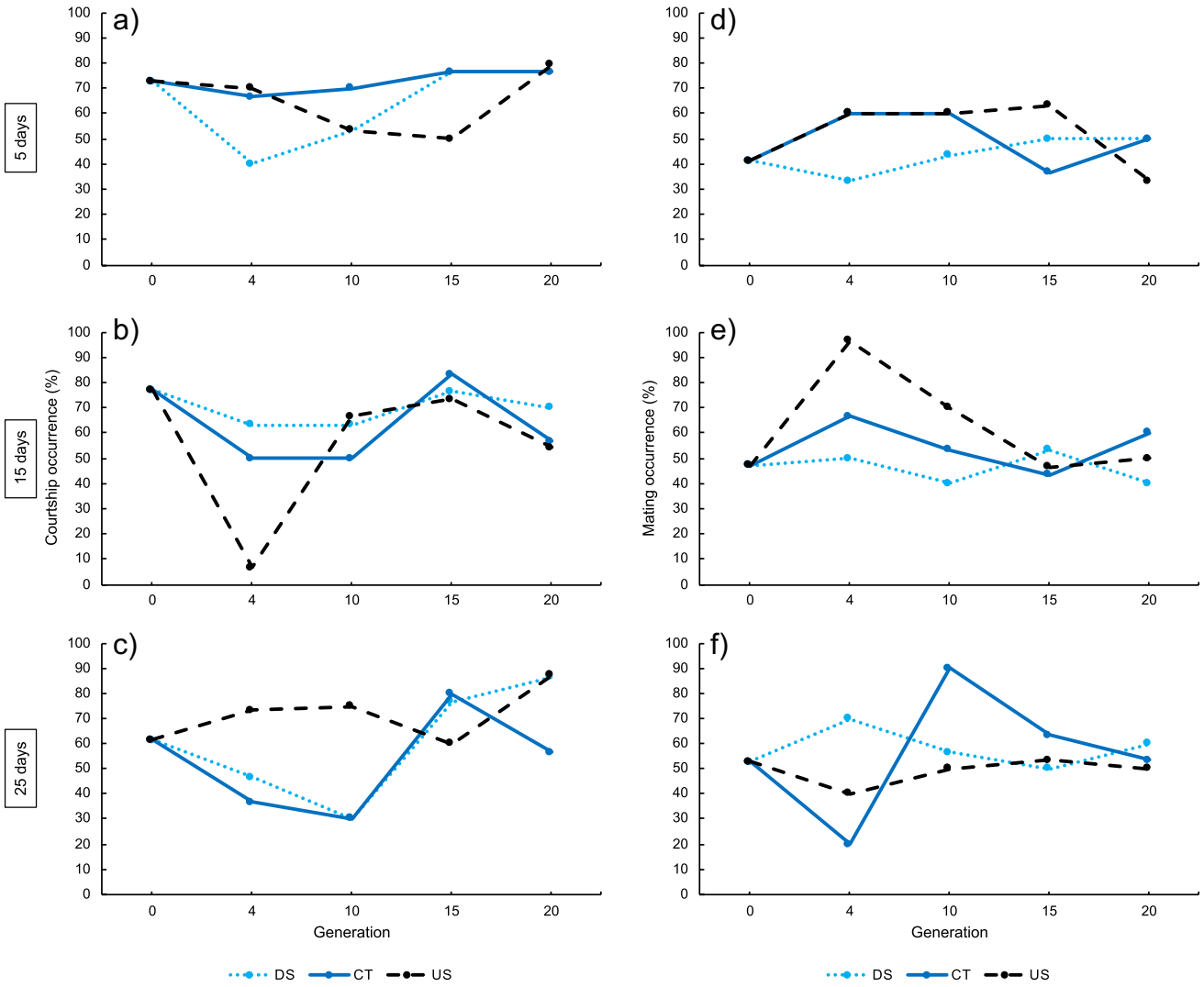

Fig. 2 Reproductive behaviour of *C. cosyra* males issued from control (CT), downward- (DS) and upward-selected (US) lines at three different ages and across five generations. The values displayed are the proportions of males that called (**a**, **b**, **c**) and mated (**d**, **e**, **f**). At ages 5 (**a**, **d**), 15 (**b**, **e**) and 25 (**c**, **f**), males were individually paired with a virgin female of 10 to 20 days of age from an unselected laboratory culture

no significant differences between CT and DS males or DS and US males (Fig. 3a). In addition, 5-day-old males transferred significantly fewer sperm than their older counterparts (15 days: estimate = −0.56, $p < 0.001$; 25 days: estimate = −0.69, $p < 0.001$), and no significant difference was observed between 15- and 25-day-old males (Fig. 3a) regardless of the selection regime.

Sperm storage asymmetry was only affected by male age (Table 2). Asymmetry was greatest in spermathecae of females mated with 15-day-old males (5- vs 15-day-old: estimate = −0.14, $p < 0.001$; 15- vs 25-day-old: estimate = 0.17, $p < 0.001$) (Fig. 3b). There was no significant difference in sperm asymmetry in spermathecae of females mated to 5- and 25-day-old males. Sperm storage asymmetry significantly differed from 50% at 15 days of age in all selection regimes and generations except for US flies at G_4. There was also significant asymmetry in CT flies at 5 days of age in all generations. Significant asymmetry was also observed in all selection regimes in 5-day-old flies at G_{20}, and at the same generation, asymmetry was significant in 25-day-old flies from the CT lines.

Survival

Selection regime, generation and sex had a significant effect on lifespan (Table 3) (Fig. 4a, b) and survival (Fig. S2). The effect of the selection regime on survival differed across generations as indicated by the significant interaction between selection and generation (Table 3, Fig. 4a, b). Post hoc analyses indicated

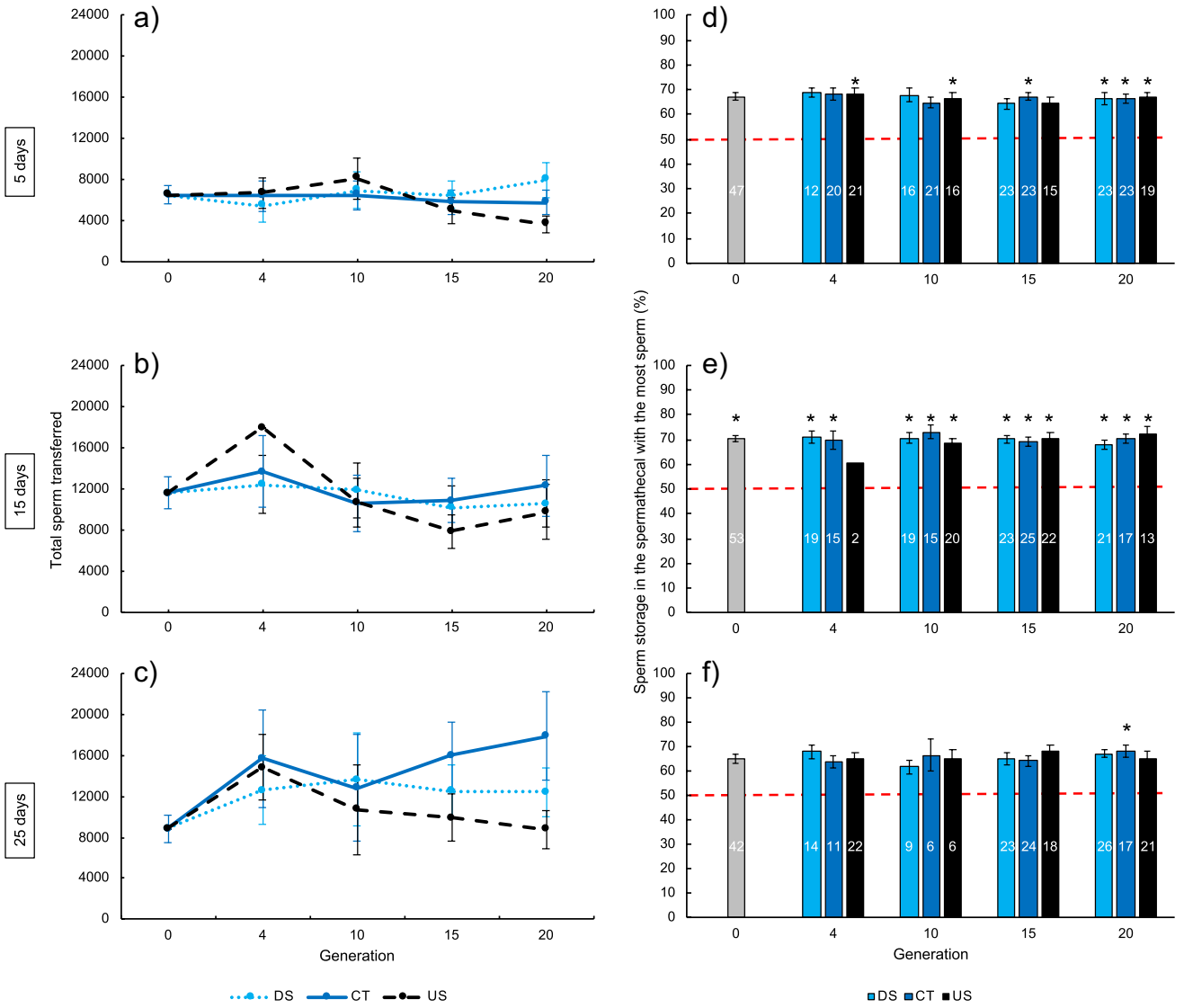

Fig. 3 Sperm transfer and sperm storage asymmetry of *C. cosyra* from control (CT), downward- (DS) and upward-selected (US) lines at three different ages and across five generations. The values displayed are the average of sperm transferred by males to females' spermathecae (**a**, **b**, **c**) and the average proportion of sperm stored in the spermatheca with the most sperm (**d**, **e**, **f**). At ages 5 (**a**, **d**), 15 (**b**, **e**) and 25 (**c**, **f**), males were individually paired with a virgin female of 10 to 20 days of age from an unselected laboratory culture. The error bars represent the standard error of the mean. Error bars at G_4 for the US males at 15 days of age are not represented because only two males transferred sperm, group sizes are indicated above the bars in panels **d** to **f**. The asterisks in panels **d** to **f** indicate which bars significantly differ from 50%

that significant differences between selection regimes started from G_{10} (see Table S1 for the estimated proportional hazards of each group). At G_{10}, the risk of dying was greatest in the DS lines and lowest in the US lines (CT/US: ratio = 2.21, $p < 0.001$; DS/US: ratio = 4.05, $p < 0.001$; CT/DS: ratio = 0.547, $p = 0.014$). At G_{15}, CT lines had the lowest risk of dying (CT/US: ratio = 0.49, = 0.003; CT/DS: ratio = 0.35, $p < 0.001$) while the US and DS lines were indistinguishable. At G_{20}, differences between CT and US as well as DS lines remained (CT/US: ratio = 0.35, $p < 0.001$; CT/DS: ratio = 0.21, $p < 0.001$), but the risk of dying was significantly greater in the DS than in the US lines (DS/US: ratio = 1.68, $p = 0.044$).

A significant interaction between generation and sex (Table 3) indicated that sex differences in survival varied across generations. Post hoc analyses indicated that prior to G_{10} sex differences were negligible (see Table S2 for the estimated proportional hazards of each group). At G_{10} and G_{15}, mortality risk was higher in females than in males (G_{10}: ratio = 1.71, $p < 0.001$; G_{15}: ratio = 1.28, $p = 0.038$), but the difference was not significant when flies reached G_{20}.

Table 3 Analyses of survival and head width using Cox proportional hazards and linear models in control, downward-selected or upward-selected flies

Effects	χ^2	df	p
Survival			
Selection	6.77	2	**0.034**
Generation	68.27	4	**< 0.001**
Sex	13.34	1	**< 0.001**
Selection × Generation	57.47	8	**< 0.001**
Selection × Sex	2.49	2	0.287
Generation × Sex	10.65	4	**0.031**
Selection × Generation × Sex	4.90	8	0.767
Head width			
Selection	0.01	2	0.087
Generation	0.52	4	**< 0.001**
Sex	0.31	1	**< 0.001**
Selection × Generation	0.50	8	**< 0.001**

Head width

Differences in head width between different selection regimes were generation dependent (Table 3) (Fig. 5a, b).

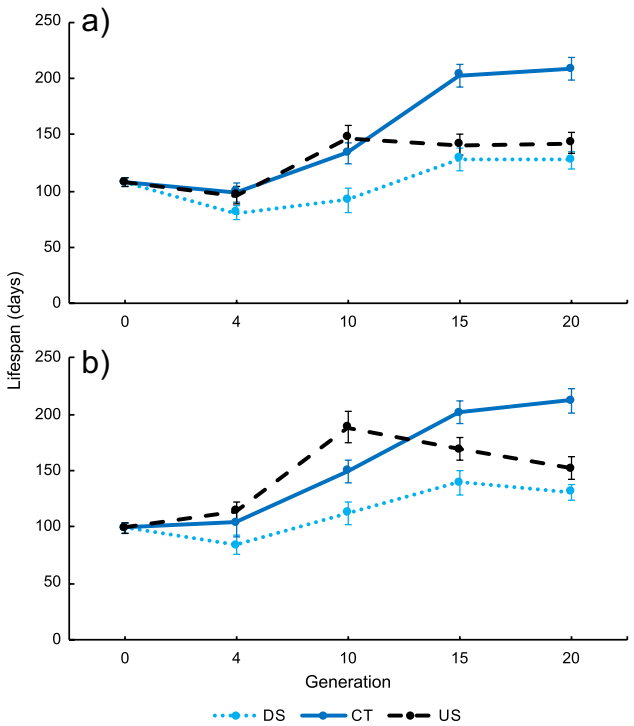

Fig. 4 Average lifespan of females (**a**) and males (**b**) *C. cosyra* across five generations and issued from control (CT), downward- (DS) and upward-selected (US) lines. The error bars represent the standard error of the mean. Downward-selection was performed by allowing females to oviposit only at 5 days after adult emergence, upward selection at 25 days after emergence, whereas eggs were collected from controls at 15 days

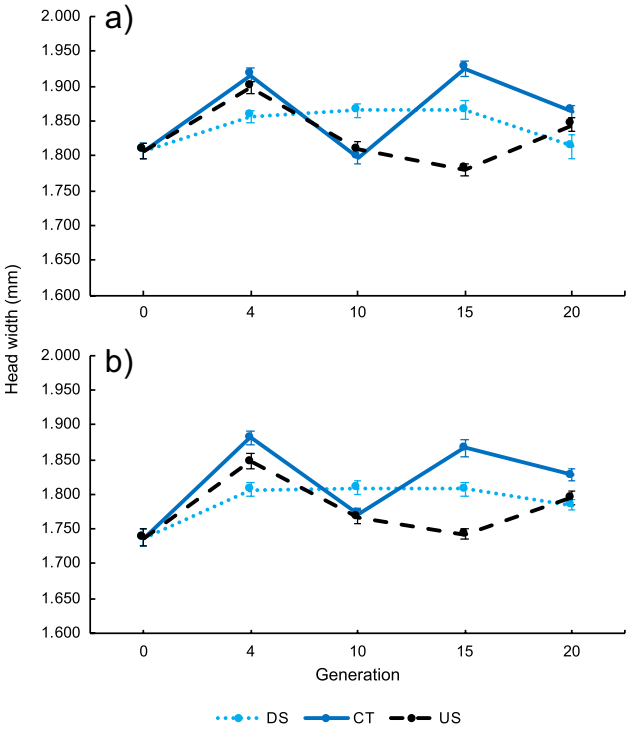

Fig. 5 Average head width of females (**a**) and males (**b**) *C. cosyra* across five generations and issued from control (CT), downward- (DS) and upward-selected (US) lines. The error bars represent the standard error of the mean

CT flies had larger heads than both DS and US lines in G_4, G_{15}, and G_{20} (Table S3). At G_4, DS flies had narrower heads than US ones, but from G_{10} to G_{15}, DS flies had larger heads than US ones and no difference between them was found at G_{20} (Table S3). In addition, there was a significant effect of sex (Table 3) as males had smaller heads than females (coefficient = −0.04, $p < 0.001$) (Fig. 5a, b). There was no or only weak correlation between head width and mean lifespan within DS ($r = 0.14$, $n = 10$, $p = 0.690$), CT ($r = 0.10$, $n = 10$, $p = 0.790$) or US ($r = -0.45$, $n = 10$, $p = 0.190$) flies.

Discussion

Selecting downwards and upwards on age of female oviposition in *C. cosyra* affected the timing of peak egg production in the direction expected: females selected for earlier reproductive investment laid more of their eggs earlier in life, whereas oviposition peaked later in life in upward selected females. In contrast, the other traits assayed did not respond to the selection regimes in the manner predicted. There were modest changes in lifetime egg production between selection lines, with small declines in fecundity for upward-selected lines in the last generation, but overall, females responded to the selection regimes by producing a similar

number of eggs but adjusting the scheduling of egg production. Unlike observations in females, most male reproductive traits remained unaffected by selection regime and the few differences observed were generation dependent. An exception to this was an overall decrease in sperm transfer in lines selected upwards in comparison with the control lines. Finally, we predicted that selection upwards on age of female egg laying would increase lifespan in both sexes. While upward-selected lines benefited from a lifespan extension in the early generations of the selection regime, these increases in lifespan subsequently plateaued in the upward-selected lines, while they continued to rise in control lines. In consequence, the later generations of control lines were the longest lived. Altogether, our results indicate that our selection acted similarly on both female and male lifespan (albeit not entirely in the manner predicted), whereas reproductive effort of both sexes appears to have evolved differently with limited changes in males.

Selecting upwards on the age of egg laying resulted in a postponed peak of egg production and decreased daily fecundity. Moreover, in the last generation of our selection regime (i.e. G_{20}), we started to observe a tendency for upward-selected females to produce fewer eggs in their lifetime than control females. Lifetime fecundity could perhaps have continued to decrease in the subsequent generations, in much the same way that the number of sperm transferred by males from these lines showed a gradual decline (see below). This might reflect a late response to our selection regime with females investing less in reproduction to allocate more to somatic maintenance. Gamete production is energetically costly in females, and to a lesser extent in males (Hayward and Gillooly 2011); therefore, upward-selected flies might have adapted to the late availability of the oviposition substrate by lowering investment in gonadic tissues to prioritise the soma until a reproductive opportunity is given.

With the exception of fewer sperm being transferred by upward-selected males than control flies, male reproductive effort remained largely unaffected by the selection regime. Apart from a few exceptions with no clear pattern in a specific generation and at a given age, courtship and mating behaviour were broadly unaffected by selection, and sperm was stored similarly by females (i.e. no differences in sperm asymmetry between selection regimes). Sperm storage asymmetry may be regarded as a signature of post-copulatory selection on males in tephritid flies (Pérez-Staples et al. 2007, 2014), because it provides a mechanism whereby females can store sperm from different males separately after remating. We speculate that the lack of differences in sperm storage or asymmetry between selection regimes means that sperm competition levels were similar across selection regimes. For example, if females from a given selection regime were more likely to remate than their counterparts from other lines, we predict that they may have stored sperm unevenly between spermathecae to discriminate between males. This is particularly true if second-male sperm precedence (i.e. sperm of the second male is more likely to be used for fertilisation of the eggs) were to occur in *C. cosyra*, as is the case in a related species, *C. capitata* (Scolari et al. 2014). Countering the potential for our selection regime to affect male reproductive traits is the presence of strong female remating inhibition. Female *Ceratitis* flies are unlikely to remate in large numbers when males have access to the nutritional resources provided in our study (Gavriel et al. 2009; Costa et al. 2012).

While we found that sperm storage asymmetry was unaffected by the selection regime, it was affected by age. This was similar to previous observations in *C. cosyra* (Roets et al. 2018), with the highest sperm storage asymmetry found at 15 days of age in our laboratory adapted flies, regardless of generation. However, asymmetry was not the norm at other ages. Although there was no difference in mating propensity between ages, and males transferred as much sperm at 15 days as at 25 days of age, it appears that there is a factor triggering post-copulatory selection by females when mating with 15-day-old males. As sperm quantity did not differ, perhaps this was triggered by a difference in ejaculate quality and future investigations should look at male accessory glands and seminal fluid proteins (Scolari et al. 2012).

Contrary to expectations, upward selection did not result in the evolution of longer lives in either sex. Females from lines selected for earlier reproductive effort did live the shortest lives overall after twenty generations, which may be the signature of a trade-off between lifespan and early reproductive effort. In another tephritid species, *Zeugodacus cucurbitae*, where selection on age of reproduction was applied, a trade-off between lifespan and early fecundity was also observed (Miyatake 1997). This trade-off has been observed in various populations of *D. melanogaster* and can represent a physiological cost of reproduction on survival, or indicate a negative genetic correlation between both traits (Flatt 2011).

Had we stopped the experiment at generation ten, we would have concluded that selecting upwards on the timing of female reproduction improved lifespan. However, there was an inflection point between G_{10} and G_{15}, where the differences in lifespan between selection regimes changed direction. From the fifteenth generation onwards, while all lines lived longer than the starting unselected population (i.e. the starting stock population from which lines originated), flies from control populations lived longer than flies from either selection regime. The general rise in lifespan across all lines could reflect laboratory adaptation; even without any selection regime being applied, laboratory adaptation alone can increase lifespan and fecundity as shown in *D. melanogaster* (Sgro and Partridge 2000) and *Bactrocera tryoni* (Meats et al. 2004; Gilchrist et al. 2012).

This may explain our results in part, given that all selection regimes began in flies that had only been in the laboratory for three generations (although wild males were crossed with laboratory-adapted females). One possible explanation for the generational changes of direction of survival (i.e. where lifespan plateaued in upward-selected lines and was best in the control lines) is that so few individuals successfully reproduced later in life in the upward selection regime that the population suffered from a low effective population size and this led to inbreeding and fitness reduction. Similarly, this could explain poor survival in the downward-selected lines if few flies had matured by day 5 to contribute to the next generation. However, this seems unlikely, as lifespan of both sexes for all lines was much longer than the age at which we selected upwards, and so was the age of peak egg production. In addition, males assayed at 25 days were still showing high mating success (> 50%) and transferring large numbers of sperm. Variation in body size (i.e. head width) detected in our study did not vary consistently with lifespan regardless of selection regime. As body size is associated with fitness in many tephritid flies (e.g. Rodriguero et al. 2002; Ekanayake et al. 2017; Tejeda et al. 2020), this result also suggests that inbreeding and fitness reductions are unlikely to explain lack of lifespan improvement in upward-selected *C. cosyra*. Therefore, it seems improbable that the decline in lifespan we observed in the upward selected lines can be explained by the costs of inbreeding alone, although we cannot dismiss variation in effective population sizes as playing a role in outcomes.

The absence of lifespan extension in the upward-selected lines could potentially indicate that the selection regime was not strong enough, although the strength of the selection could not be determined here as we did not assay the phenotype of the parents and offspring (i.e. we did not test two consecutive generations). Nevertheless, the counter-intuitive observations on *C. cosyra* selected upwards on age of reproduction could also reflect that offspring quality can decline as a function of parental age. Indeed, several studies have found a negative relationship between parental age and offspring fitness (reviewed in Monaghan et al. 2020). These reductions in offspring fitness could reflect declines in the quality of gametes produced by older parents or for example, deterioration during storage (Moore and Moore 2001; Schroeder et al. 2015; Monaghan et al. 2020). While we lack data showing if such effects on the phenotype can be cumulative, there are signs that there is potential for the phenotype costs of parental age to be magnified across generations (Monaghan et al. 2020). This is one potential explanation for the poor fitness estimates for these upward-selected lines, although this idea needs further testing, particularly because the selection for reproductive success at older ages employed here should select against these costly effects of parental age. Alternatively, these results could also simply reflect standing genetic variation and more work is needed to determine the causes of the outputs of our selection regime on the flies selected upwards.

Finally, it is interesting to note that responses of lifespan to selection were broadly similar across the sexes. This may reflect selection acting in a similar manner directly on female and male age of reproduction. However, because female remating is typically low in *Ceratitis*, it is likely that females in our selected lines mated prior to an egg laying substrate being provided and then used stored sperm to oviposit. This would relax the direct selection acting on the age of male reproductive effort. Effects on male lifespan may instead represent correlated responses to selection on females (e.g. if there is a positive intersexual genetic correlation for lifespan). While this idea is untested, it seems likely that in the field selection acts similarly on lifespan in both sexes. This is because a similar lifespan in females and males could represent an adaptation to seasonal host availability in *C. cosyra*, which is known to have a distribution closely associated with one of its preferred hosts, the marula tree (*Sclerocarya birrea*) (De Villiers et al. 2013). As the species does not undergo diapause, it would be advantageous if a fraction of the population, both females and males, survive until the next fruiting season. A previous study supports this idea, showing that *C. cosyra* has a particularly long lifespan among other tephritid flies (Malod et al. 2020a). Nevertheless, in *C. capitata*, courting has a direct cost on male lifespan (Papadopoulos et al. 2010), and in *D. melanogaster*, reproductive activity alters gene expression in males, which then results in reduced lifespan (Branco et al. 2017). Therefore, further investigations are needed to determine how selection on age of female reproduction affects male lifespan in mated individuals. While lifespan responded similarly across the sexes to selection, reproductive scheduling did not. This may suggest that age-dependent reproductive effort is free to evolve independently across the sexes, although once again, this idea needs to be tested further.

Conclusion

In conclusion, our results in downward selected flies show the potential for a trade-off between lifespan and reproduction in females, where late survival could be decreased due to a stronger early reproductive effort as a sign of an antagonistic pleiotropic effect (Flatt 2020). In contrast, we did not observe a trade-off in males, at least not on the measured reproductive traits, but male lifespan responded to the selection regime on female age of oviposition. Our results are not clear cut and do not follow much established theory; the usual lifespan extension observed with upward selection only occurred in the early generations, and upward-selected lines lived shorter lives than control lines for reasons yet to

be elucidated. Regardless of the selection regime, our results suggest that female and male lifespans evolve together in *C. cosyra*, and that it is linked to female reproductive schedule, but that most male reproductive traits and schedule are dissociated from these evolutionary changes. Due to this, we suggest that a wider range on non-model organisms and males be included in future assessment of the trade-off between lifespan and reproduction using laboratory selection studies.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00265-021-03063-8.

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Author contribution CRA and CWW conceived and designed the study. CRA, CWW, PDR and HB conducted the experiments and data collection. KM performed the statistical analyses. KM, CRA and CWW wrote the manuscript and all authors contributed to the final version.

Data availability Data are available from the University of Pretoria online repository: https://doi.org/10.25403/UPresearchdata.14915046.v2

Declarations

Competing of interests The authors declare no competing interests.

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