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RESEARCH ARTICLE

Drosophila female fertility and juvenile hormone metabolism depends on the type of *Wolbachia* infection

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ABSTRACT

Maternally inherited intracellular bacteria *Wolbachia* cause both parasitic and mutualistic effects on their numerous insect hosts, including manipulating the host reproductive system in order to increase the bacteria spreading in a host population, and increasing the host fitness. Here, we demonstrate that the type of *Wolbachia* infection determines the effect on *Drosophila melanogaster* egg production as a proxy for fecundity, and metabolism of juvenile hormone (JH), which acts as gonadotropin in adult insects. For this study, we used six *D. melanogaster* lineages carrying the nuclear background of interbred Bi90 lineage and cytoplasmic backgrounds with or without *Wolbachia* of different genotype variants. The *w*MelCS genotype of *Wolbachia* decreases egg production in infected *D. melanogaster* females in the beginning of oviposition and increases it later (from the sixth day after eclosion), whereas the *w*MelPop *Wolbachia* strain causes the opposite effect, and the *w*Mel, *w*Mel2 and *w*Mel4 genotypes of *Wolbachia* do not show any effect on these traits compared with uninfected Bi90 *D. melanogaster* females. The intensity of JH catabolism negatively correlates with the fecundity level in the flies carrying both *w*MelCS and *w*MelPop *Wolbachia*. The JH catabolism in females infected with genotypes of the *w*Mel group does not differ from that in uninfected females. The effects of *w*MelCS and *w*MelPop infection on egg production can be levelled by the modulation of JH titre (via precocene/JH treatment of the flies). Thus, at least one of the mechanisms promoting the effect of *Wolbachia* on *D. melanogaster* female fecundity is mediated by JH.

KEY WORDS: Juvenile hormone, Oocytes, Egg production, Heat stress, Precocene, Fecundity

INTRODUCTION

Wolbachia are widespread maternally transmitted endosymbiotic bacteria of invertebrates capable of affecting the host's reproduction to enhance their own spread and transmission through the host generations (O'Neill et al., 1997; Mateos et al., 2006; Werren et al., 2008; Weinert et al., 2015). *Wolbachia* symbionts cause cytoplasmic incompatibility in different insect species, including *Drosophila melanogaster*, manifested as embryonic mortality in crosses between infected males and uninfected females, often resulting in an increase in frequency of infected flies in the population (Hoffmann, 1988; Werren, 1997; Yamada et al., 2007; Ilinsky and Zakharov, 2011). However, although *Wolbachia*'s dramatic effects on *Drosophila* sperm and male fertility is well known (Serbus et al., 2008; Werren et al., 2008), its influence on female reproductive biology is slightly more puzzling. In a previous study, Charlat et al. (2004) found no effect of *Wolbachia* infection on female fertility: in crosses with both infected and uninfected males, infected females were not significantly more or less fertile than uninfected ones. In contrast, Fry et al. (2004) and Weeks et al. (2007) demonstrated enhanced fecundity in *D. melanogaster* females infected with *Wolbachia* compared with uninfected females. Weeks et al. (2007) suggested that fecundity-increasing types of *Wolbachia* infection could be polymorphic in natural *Drosophila* populations. We therefore studied the impact of five *Wolbachia* variants on female reproductive biology in *D. melanogaster*.

Six different genotypes of *Wolbachia pipientis* have been identified in *D. melanogaster* – *w*Mel, *w*Mel2, *w*Mel3, *w*Mel4, *w*MelCS and *w*MelCS2 (Riegler et al., 2005; Ilinsky, 2013) – plus the pathogenic *w*MelPop strain, which is a variant of the *w*MelCS genotype that causes early death of flies (Min and Benzer, 1997; Ilinsky, 2013). The genotypes differ in the level of host antiviral protection and their load in the host (Chrostek et al., 2013; Wong et al., 2015). To study the effects of various *Wolbachia* genotypes on *D. melanogaster* fitness, we have previously created a study system (Gruntenko et al., 2017) that consists of five conplastic lineages carrying the nuclear background of one wild-type lineage, Bi90, and cytoplasmic backgrounds with *w*Mel, *w*Mel2, *w*Mel4, *w*MelCS and *w*MelPop genotype variants of *Wolbachia*. In our previous study, the *w*MelCS genotype of *Wolbachia* intensified dopamine metabolism in *D. melanogaster*, whereas *w*Mel, *w*Mel2 and *w*Mel4 did not affect it, and *w*MelPop decreased it (Gruntenko et al., 2017). Dopamine is known to rise quickly and steeply under stress conditions, playing a significant role in the regulation of response to oxidative and heat stress, influencing survival in *Drosophila* (Gruntenko et al., 2004; Ueno et al., 2012; Hanna et al., 2015). *Wolbachia* infection is also associated with the induction of oxidative stress in *Aedes aegypti* (Pan et al., 2011) and two *Drosophila* species infected by several *Wolbachia* strains, including *w*MelCS (Wong et al., 2015). As dopamine is shown to interact with juvenile hormone (JH) in *D. melanogaster* females (Gruntenko and Rauschenbach, 2008; Gruntenko et al., 2012; Argue et al., 2013), we can expect *w*MelCS and *w*MelPop infection to affect female JH metabolism and reproduction. In insects, JH plays a 'status quo' role in larvae, providing normal growth and development and preventing premature metamorphosis, and a gonadotropic role, regulating female fertility (together with 20-hydroxyecdysone, 20E) in adults (Goodman and Granger, 2005; Riddiford, 2012; Jindra et al., 2013; Dubrovsky and Bernardo, 2014). As gonadotropins, JH and 20E induce ovarian development, initiate and maintain production of yolk

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Received 6 November 2018; Accepted 14 January 2019

proteins in the fat body and in the ovary follicular cells, and control vitellogenin uptake by oocytes (Raikhel et al., 2004). Aside from participating in the control of development and reproduction, JH and 20E are involved in the endocrine stress response (Gruntenko, Rauschenbach, 2008). The increase in the JH level under unfavourable conditions leads to the accumulation of mature eggs and ovipositional delay until the unfavourable conditions have improved (Gruntenko, Rauschenbach, 2008). In *Drosophila*, the JH titre negatively corresponds to the level of its degradation, and thus the latter can be regarded as an indicator of the hormone level (Gruntenko and Rauschenbach, 2008). *Wolbachia* is shown to establish itself in JH- and 20E-producing tissues of the host, including the fat body and the ovarian follicular cells, as demonstrated in many insect species, suggesting a specific role for the symbiont in host oogenesis, embryogenesis and moulting (Negri, 2012).

In this study, we investigated a difference in the effects of various types of *Wolbachia* infection on JH metabolism and reproduction of *D. melanogaster*. Our results suggest that at least one of the mechanisms promoting the effect of *Wolbachia* on female fecundity is mediated by JH.

MATERIALS AND METHODS

Drosophila lineages

Twenty generations prior to the start of the experiments, the lineage Bi90 was treated with tetracycline for three generations to make *Wolbachia*-free lineage $Bi90^{T}$. The experiments were performed on five *D. melanogaster* conplastic lineages ($Bi90^{Mel}$, $Bi90^{Mel2}$, $Bi90^{Mel4}$, $Bi90^{CS}$ and $Bi90^{Pop}$) carrying the nuclear background of inbred Bi90 lineage and cytoplasmic backgrounds with different types of *Wolbachia* infection (*w*Mel, *w*Mel2, *w*Mel4, *w*MelCS and *w*MelPop), produced as described in Gruntenko et al. (2017) by 20 backcrosses of $Bi90^{T}$ males with the appropriate source of *Wolbachia*. *Wolbachia* infection status was regularly verified using PCR with primers specific to the *Wolbachia* 81F/691R set for the *wsp* gene (Braig et al., 1998) and 99F/994R for the *16SrRNA* gene (O'Neill et al., 1992). The *Wolbachia* genotypes were identified according to Riegler et al. (2005) and Ilinsky (2013). The sixth, non-infected $Bi90^{T}$ lineage was used as a control.

The cultures were maintained on standard *Drosophila* medium (agar-agar, 7 g l^{-1}; corn grits, 50 g l^{-1}; dry yeast, 18 g l^{-1}; sugar, 40 g l^{-1}) at 25°C under a 12 h:12 h light:dark cycle, and the adults were synchronised at eclosion (flies were collected every 3–4 h).

Syto-11 staining

Syto-11 staining of DNA was performed as described in Casper-Lindley et al. (2011) to visualise *Wolbachia* and host nuclei in the ovarian tissues. Female flies were examined in their reproductive peak at the age of 6 days after eclosion. Ovarian tissues were stained with Syto-11 to label *Wolbachia* DNA, and then the bacteria nucleoids were imaged by confocal microscopy in stage 10 oocytes. The ovaries of five mated females from each *Drosophila* lineage were dissected in a Petri dish in ice-cold PBS, and transferred to a large coverslip in a drop of PBS. Individual ovarioles and individual egg chambers were separated, and mature eggs were removed from the coverslip. PBS was removed with a pipette and a drop of Syto-11 (1:100; Molecular Probes, Invitrogen) was applied. Slides were placed in a dark, moist chamber and incubated at room temperature for 20–25 min. Specimens were then covered with a smaller coverslip, which was placed upon strips of tape glued to both sides of the lower coverslip to prevent the crushing of tissues. Immediately after they were analysed by laser scanning microscope (LSM 780 NLO) based on the inverted microscope AxioObserver Z1 (Carl Zeiss, Oberkochen, Germany) at the Institute of Cytology and Genetics Microscopy Center (Novosibirsk, Russia) for no more than 20–25 min.

ImageJ 2.0 Fiji software (National Institutes of Health) was used to quantify the oocyte *Wolbachia* titre. Before quantification, stacks of confocal images were examined to identify the deepest possible focal plane where *Wolbachia* were clearly visible across all samples of the replicate (Serbus et al., 2015), and images were manually processed to remove extraneous signal outside the oocyte. For fluorescence quantification, the selection tool was used to isolate the oocyte and then the brightness/contrast tool was used to set the threshold on the image to remove background noise. A mean intensity of fluorescent signals per pixel was measured for each oocyte studied. Three to eight experimental replicates were performed for all *Drosophila* lineages examined.

The relative amount of *Wolbachia* genomic DNA

DNA was extracted from the ovaries of ten 6-day-old mated females of each *Drosophila* lineage for each biological replicate using the CTAB DNA Extraction Protocol (Huang et al., 2000) with modification. Briefly, females were dissected in physiological saline and 10 ovaries per sample were ground in liquid nitrogen. These pools were incubated at 56°C for 1 h with 30 µl of lysis buffer (BioSilica, Russia) with 2 µl of proteinase K. Then, 450 µl of CTAB extraction buffer (20% CTAB, 100 mmol l^{-1} Tris–HCl pH 8.0, 20 mmol l^{-1} EDTA pH 8.0, 1 mol l^{-1} NaCl and 1.5% β-mercaptoethanol) and 450 µl of chloroform were added, shaken and centrifuged for 5 min at 2300 ***g***. The aqueous phase was transferred to a new tube and an equal volume of isopropanol was added to precipitate DNA. The samples were then incubated for 10 min at 4°C and centrifuged for 10 min at 5900 ***g***. Isopropanol was removed and DNA was washed with 70% ethanol, centrifuged for 5 min at 5900 ***g***, dried, dissolved in TE buffer and stored at −20°C. Quantitative real-time PCR was performed using a CFX96 Real-Time PCR system (BioRad Laboratories) with a SYBR Green I R-402 kit (Syntol, Russia) as per the manufacturer's instructions. Each reaction was performed in triplicate with five biological replicates. Specific primers for measuring *Wolbachia* titer in *D. melanogaster* by qPCR technique were designed previously: Wolb2-F 5′-TCACAGACCT-GTATTTGGTTACA-3′ and Wolb2-R 5′-ACTAAGCCCAACAGT-GAACATA-3′ (I. Mazunin, personal communication). The *Drosophila Rpl32* gene was used to normalise the quantitative PCR data (Rpl32-F1 5′-CAGCATACAGGCCCAAGATC-3′, Rpl32-R 5′-CGATGTTGGGCATCAGATACTG-3′) as described in Pérez-Moreno et al. (2014). The following thermal cycling protocol was applied: 3 min at 95°C, and 45 cycles of 15 s at 95°C, 15 s at 56°C, 15 s at 62°C and 5 s at 78°C. Melting curves were examined to confirm the specificity of amplified products. Cycle threshold (C_t) values were obtained using Bio-Rad CFX Manager software with default threshold settings.

Fecundity analysis

For the egg production analysis, newly eclosed full-sib flies (three females and three males) carrying the same infections were placed into a vial with filter paper soaked in nutritional medium as described in Rauschenbach et al. (2014). The sample size was 20 to 40 vials (60–120 females) for each lineage under study. The nutritional medium contained 0.5% sucrose and 0.1% yeast. The flies were transferred to vials with fresh medium daily. Fecundity was calculated as the number of eggs per female per 24 h.

JH and precocene treatment

To study the effect of JH or precocene on egg production, 1-day-old females were treated with acetone, JH or precocene as described below, and their fecundity was evaluated. For the precocene (JH inhibitor) treatment, females were collected soon after eclosion and placed in vials with standard medium (three females and three males in a vial) for 1 or 5 days. After that, flies were anaesthetised with ether, and 0.5 µl of JH-III (Sigma-Aldrich) or 0.2 µl of Precocene I (Sigma-Aldrich), diluted in acetone to a concentration of 2 or 1 mg ml^{-1}, correspondingly, was applied to the abdomen of each female. Control females were treated with an equal amount of drug vehicle (acetone) (the treatment with pure acetone does not affect fecundity; Rauschenbach et al., 2017). The sample size was 10 vials (30 females) for each group.

JH-hydrolysing activity assay

For the JH hydrolysis measurement each fly was homogenised in ice-cold 0.1 mol l^{-1} sodium phosphate buffer, pH 7.4, containing 0.5 mmol l^{-1} phenylthiourea. The homogenates were centrifuged for 5 min at 13,300×***g***, and samples of the supernatant were taken for the assay. A mixture consisting of 0.1 µg of unlabelled JH-III (65%, Sigma-Aldrich) and 0.1 µg [3H]JH-III (2.2 Ci $mmol^{-1}$, radiochemical purity 95%, prepared as described in Romanova et al., 2017) was used as a substrate. The reaction was carried out for 30 min and then was stopped with the addition of 5% ammonia, 50% methanol (v/v). Unhydrolysed JH was extracted with heptane. The tubes were shaken vigorously and centrifuged at 13,300 ***g*** for 10 min. Samples of both the organic and aqueous phases were placed in vials containing dioxane scintillation fluid and counted. All components of dioxane scintillation fluid were purchased from Sigma-Aldrich. The fresh fluid was prepared prior to conducting the actual experiments. Control experiments showed a linear substrate–reaction product relationship; the activity measured is proportional to the amount of the supernatant (i.e. enzyme concentration) (Gruntenko et al., 2000). Before measurement, half of the flies in each group under study were exposed to heat stress by transferring vials containing them from a 25°C incubator to a 38°C incubator for 2 h. The sample size was 20–40 flies per each control or experimental group. JH stress reactivity was calculated as the percent change in JH-hydrolyzing activity following heat stress relative to the value of the corresponding parameter obtained under normal conditions (each value obtained at 38°C was compared with the average value obtained at 25°C).

Statistical analysis

All data are presented as means±s.e.m. The false discovery rate corrections for multiple comparisons were made when appropriate. The Kolmogorov–Smirnov test was used to determine whether it was appropriate to use parametric tests. Data were subjected to arcsine or power transformation prior to analysis when appropriate. Data on fecundity (number of eggs per day per female) were analysed via two-way mixed-design ANOVA (with day after eclosion as the within-subjects factor; and infection, precocene treatment or JH treatment as the between-subjects factors). Data on the JH degradation level and the intensity of its responses to stress (JH stress reactivity) were analysed via two-way or one-way ANOVA, respectively (with infection and heat stress, or infection as the between-subjects factors, respectively). Data on the relative amount of *Wolbachia* were analysed via one-way ANOVA (with infection as the between-subjects factor). The comparison of the group means was performed with the Benjamini–Hochberg stepwise *post hoc* test. The results were considered significant at $P<0.05$.

RESULTS

The conplastic lineages differed by *Wolbachia* load in ovarian tissues

To find out whether various types of *Wolbachia* promote different effects on female host reproduction, we first tested the bacterial load of different genotypes in *D. melanogaster* ovaries and quantified this using ImageJ (Serbus et al., 2015). This analysis revealed that oocytes of $Bi90^{CS}$ females carried more bacteria nucleoids than oocytes of $Bi90^{Mel}$ females, but less than oocytes of $Bi90^{Pop}$ females (infection: $F_{3,14}=36.81$, $P\ll0.0001$; Fig. 1).

The data on the relative amount of *Wolbachia* genomic DNA suggested that bacterial load in the ovaries of 6-day-old mated $Bi90^{Pop}$ females was twice as high as that of $Bi90^{Mel}$ and $Bi90^{CS}$ females (infection: $F_{3,16}=18.97$, $P<0.0001$; Fig. 2). No significant difference in bacterial load was found between $Bi90^{CS}$ and $Bi90^{Mel}$ females.

The effects of various *Wolbachia* genotypes on the host fecundity

Because we found variability in the number of *Wolbachia* nucleoids in the ovaries of *D. melanogaster* lineages infected with various *Wolbachia* genotypes, we next tested the effects of different types of *Wolbachia* infection on egg production as a proxy for fecundity in comparison with uninfected controls (Fig. 3). No difference in fecundity was found between uninfected $Bi90^{T}$ flies and $Bi90^{Mel}$, $Bi90^{Mel2}$ and $Bi90^{Mel4}$ flies (Fig. 3A). The egg production of $Bi90^{CS}$ flies was significantly lower in the beginning of the oviposition (days 3–5 after eclosion) and significantly higher from the sixth day after eclosion in comparison with that of uninfected $Bi90^{T}$ flies (age: $F_{9,522}=205.20$, $P\ll0.0001$; infection×age: $F_{9,522}=9.63$, $P\ll0.0001$; Fig. 3B). On the contrary, the $Bi90^{Pop}$ flies demonstrated an initial increase in egg production compared with uninfected $Bi90^{T}$ controls during the 3–5 days after the start of egg laying and a later subsequent decrease compared with $Bi90^{T}$ flies from the eighth day after eclosion (age: $F_{9,612}=244.37$, $P\ll0.0001$; infection×age: $F_{9,612}=10.38$, $P\ll0.0001$; Fig. 3B).

As *Wolbachia* is known to affect male fertility (Serbus et al., 2008; Werren et al., 2008), we tested whether the difference in the fecundity of *Drosophila* $Bi90^{Mel}$ and $Bi90^{Pop}$ lineages is due to the changes in male or female reproductive function (Fig. 4). Comparing the fecundity level of the uninfected $Bi90^{T}$ lineage, the $Bi90^{Mel}$ lineage and hybrids from both directions of crossing between these lineages, we found that the fecundity of females in the ♀$Bi90^{Mel}$×♂$Bi90^{T}$ cross did not differ from that of the $Bi90^{Mel}$ and $Bi90^{T}$ lineages, whereas the ♀$Bi90^{T}$×♂$Bi90^{Mel}$ cross showed a strong decrease in the number of eggs laid in days 3–5 after eclosion (age: $F_{7,336}=210.09$, $P\ll0.0001$; infection×age: $F_{7,336}=16.21$, $P\ll0.0001$; Fig. 4A). The comparison of fecundity levels of hybrids from both directions of crossing between the $Bi90^{Pop}$ and $Bi90^{T}$ lineages showed a similar pattern: the fecundity of females in the ♀$Bi90^{T}$×♂ $Bi90^{Pop}$ cross was significantly lower than the fecundity of the uninfected $Bi90^{T}$ lineage in days 3–6 after eclosion (Fig. 4B). Two-way mixed design ANOVA (day after eclosion as the within-subjects factor; infection as the between-subjects factor) revealed significant effects for infection ($F_{1,38}=85.47$, $P\ll0.0001$) and age ($F_{7,266}=159.50$, $P\ll0.0001$). A significant interaction of these factors ($F_{7,266}=18.50$, $P\ll0.0001$) was also found. However, the most interesting finding of this experiment is that the fecundity of females in the ♀$Bi90^{Pop}$×♂$Bi90^{T}$ cross did not differ from that of the $Bi90^{Pop}$ lineage, but it differed from the fecundity of the $Bi90^{T}$ lineage in the same way as the $Bi90^{Pop}$ lineage differed from the $Bi90^{T}$

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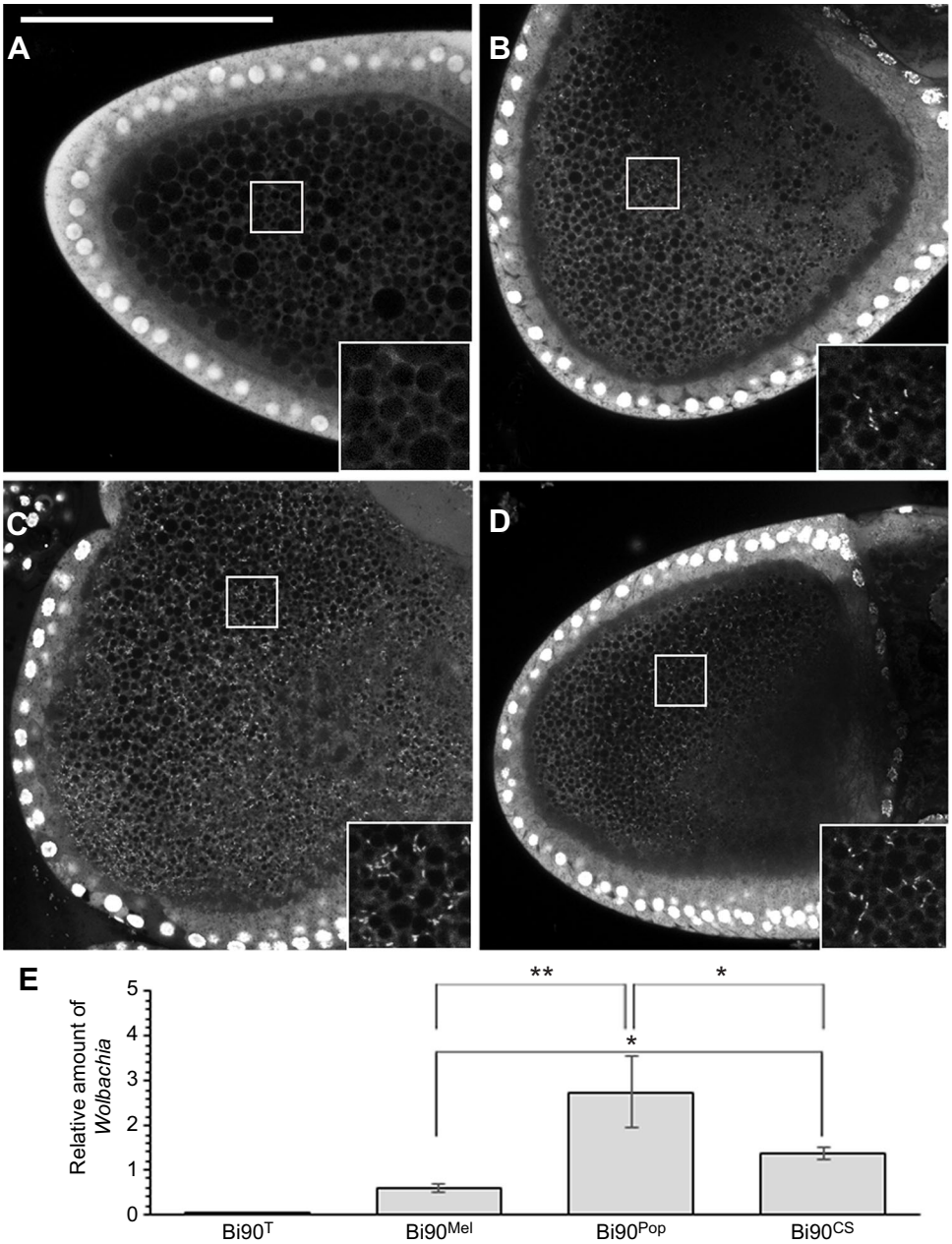

Fig. 1. Infection status of the *Drosophila melanogaster* lineages significantly impacts *Wolbachia* load in ovaries. Syto-11 staining indicates *D. melanogaster* nuclei as large circles and *Wolbachia* as small puncta. (A) Typical stage 10 oocyte uninfected with *Wolbachia*. (B) Typical stage 10 oocyte infected with the *w*Mel genotype of *Wolbachia*. (C) Typical stage 10 oocyte infected with the *w*MelPop pathogenic strain of *Wolbachia*. (D) Typical stage 10 oocyte infected with the *w*MelCS genotype of *Wolbachia*. Scale bar (applies to A–D): 100 μm. (E) *Wolbachia* relative quantification in the ovaries of *D. melanogaster* lineages $Bi90^{T}$ (*N*=6), $Bi90^{Mel}$ (*N*=6), $Bi90^{CS}$ (*N*=3) and $Bi90^{Pop}$ (*N*=3) with the use of ImageJ 2.0. $Bi90^{T}$, uninfected $Bi90^{T}$ lineage; $Bi90^{Mel}$, $Bi90^{CS}$ and $Bi90^{Pop}$, Bi90 lineages carrying the *w*Mel, *w*MelCS and *w*MelPop *Wolbachia*. Data are means±s.e.m. Asterisks indicate significant differences between lineages (one-way ANOVA; **P*<0.05; ***P*<0.01).

lineage. The fecundity in the ♀$Bi90^{Pop}$×♂$Bi90^{T}$ cross compared with the uninfected $Bi90^{T}$ control increased in the beginning of oviposition (days 3 and 4 after eclosion) and decreased from the seventh day after eclosion (age: $F_{7,266}$=90.92, P≪0.0001; infection×age: $F_{7,266}$=13.64, P≪0.0001; Fig. 4B).

Effects of various *Wolbachia* genotypes on JH degradation levels in *D. melanogaster* females under normal and heat stress conditions

To determine whether *Wolbachia* infection status and/or type affect female fecundity via JH, which is required for oogenesis (Goodman and Granger, 2005; Riddiford, 2012), we studied the levels of JH degradation in 1- and 6-day-old females of the $Bi90^{Mel}$, $Bi90^{CS}$ and $Bi90^{Pop}$ lineages in comparison with $Bi90^{T}$ under normal conditions and under heat stress (Fig. 5A,C). We also tested whether such stress (38°C, 2 h) eliminates *Wolbachia* infection in flies, and found out that it does not. Under normal conditions, $Bi90^{Mel}$ flies did not differ from the uninfected $Bi90^{T}$ in the level of JH degradation; in $Bi90^{CS}$ and $Bi90^{Pop}$ flies, the JH degradation level, as well as the level of egg production, changed in opposite directions compared with the $Bi90^{T}$ control. At the very beginning of oviposition, the JH degradation level was increased in 1-day-old $Bi90^{CS}$ females and decreased in $Bi90^{Pop}$ females compared with $Bi90^{T}$ (infection: $F_{3,191}$=11.11, P<0.0001; Fig. 5A). At the age of 6 days, $Bi90^{CS}$ females demonstrated a decreased JH degradation level and $Bi90^{Pop}$ females an increased one (infection: $F_{3,132}$=24.05, P≪0.0001; Fig. 5C) under normal conditions.

Heat stress affected the levels of JH degradation in both 1-day-old (heat stress: $F_{1,191}$=171.71, P≪0.0001; Fig. 5A) and 6-day-old (heat stress: $F_{1,132}$=411.20, P≪0.0001; Fig. 5C) females of all lineages such that JH degradation was lower under heat stress than in normal conditions. The ability of the JH degradation activity of infected flies to respond to heat stress differed between lineages either on day 1 (infection×heat stress: $F_{3,191}$=6.47, P=0.0004;

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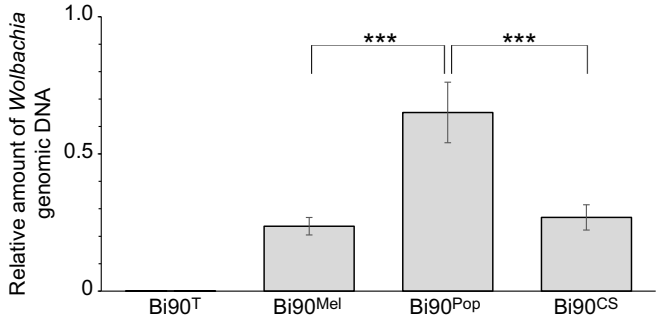

Fig. 2. Quantification of the relative amount of *Wolbachia* genomic DNA in the ovaries of *D. melanogaster* lineages Bi90^{T}, Bi90^{Mel}, Bi90^{CS} and Bi90^{Pop}. Bi90^{T}, uninfected Bi90^{T} lineage; Bi90^{Mel}, Bi90^{CS} and Bi90^{Pop}, Bi90 lineages carrying the *w*Mel, *w*MelCS and *w*MelPop *Wolbachia* (*N*=5 per each group). Relative amount of *Wolbachia* genomic DNA was calculated using host *Rpl32* as a reference gene. Data are means±s.e.m. Asterisks indicate significant differences between lineages (one-way ANOVA; ***P<0.001).

Fig. 5A) or on day 6 (infection×heat stress: $F_{3,132}$=10.90, P<0.0001; Fig. 5C). In particular, the stress reactivity of JH metabolic system was higher in 1-day-old Bi90^{CS} females and lower in 1-day-old Bi90^{Pop} females in comparison with Bi90^{Mel} and uninfected Bi90^{T} females of the same age (infection: $F_{3,107}$=11.90, P<0.0001; Fig. 5B). In contrast, the stress reactivity of the JH metabolic system was lower in 6-day-old Bi90^{CS} females and higher in 6-day-old Bi90^{Pop} females in comparison with Bi90^{Mel} and Bi90^{T} females of the same age (infection: $F_{3,71}$=16.90, P≪0.0001; Fig. 5D).

Bi90^{Mel} females did not differ from uninfected Bi90^{T} females in the levels of JH degradation and stress reactivity of the JH metabolic system in both ages studied (Fig. 5). Bi90^{CS} females significantly differed from Bi90^{Pop} females in the levels of JH degradation and stress reactivity of the JH metabolic system either on day 1 or on day 6 after eclosion (Fig. 5).

The effect of JH on the influence of *Wolbachia* on female fecundity

To determine whether the changes in the reproductive function of *D. melanogaster* Bi90^{Pop} and Bi90^{CS} females are associated with the altered JH level, we investigated their egg production following an artificial increase or decrease of JH titre. The decreased JH degradation level in young (just starting to lay eggs), 1-day-old Bi90^{Pop} females (Fig. 5A) and mature (having a reproduction peak),

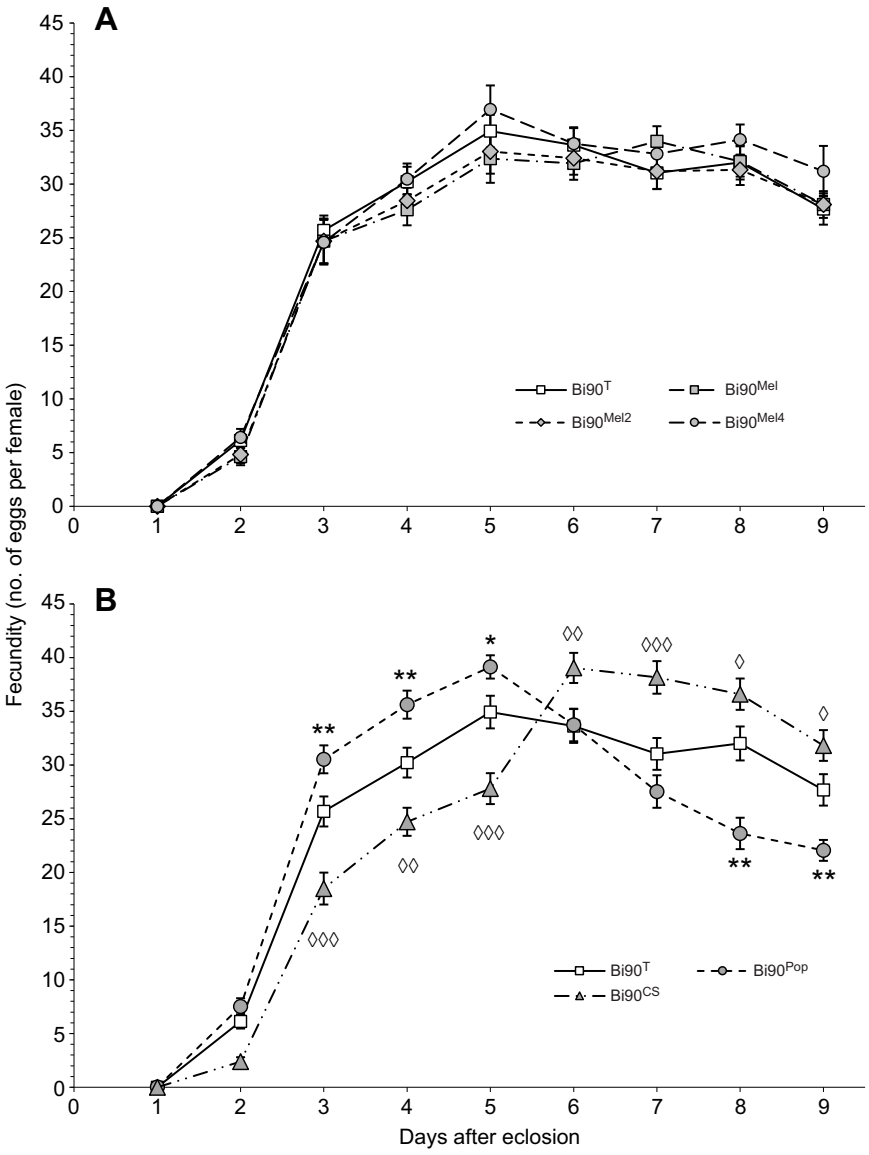

Fig. 3. The effect of various types of *Wolbachia* infection on *D. melanogaster* fecundity in comparison with uninfected (tetracycline-treated) controls. (A) Bi90^{T}, uninfected Bi90^{T} lineage; Bi90^{Mel}, Bi90^{Mel2} and Bi90M^{el4}, Bi90 lineages carrying *w*Mel, *w*Mel2 and *w*Mel4 *Wolbachia*. (B) Bi90^{T}, uninfected Bi90^{T} lineage; Bi90^{CS} and Bi90^{Pop}, Bi90 lineages carrying *w*MelCS and *w*MelPop *Wolbachia*. (*N*=20 for Bi90^{Mel}, Bi90^{Mel2} and Bi90^{Mel4}, *N*=30 for Bi90^{T} and Bi90^{CS}, *N*=40 for Bi90^{Pop}.) Data are means±s.e.m. Diamonds indicate significant differences between uninfected Bi90^{T} control and Bi90^{CS} flies; asterisks indicate significant differences between Bi90^{T} and Bi90^{Pop} flies (two-way mixed-design ANOVA; ◊/*P<0.05; ◊◊/**P<0.01; ◊◊◊/***P<0.001).

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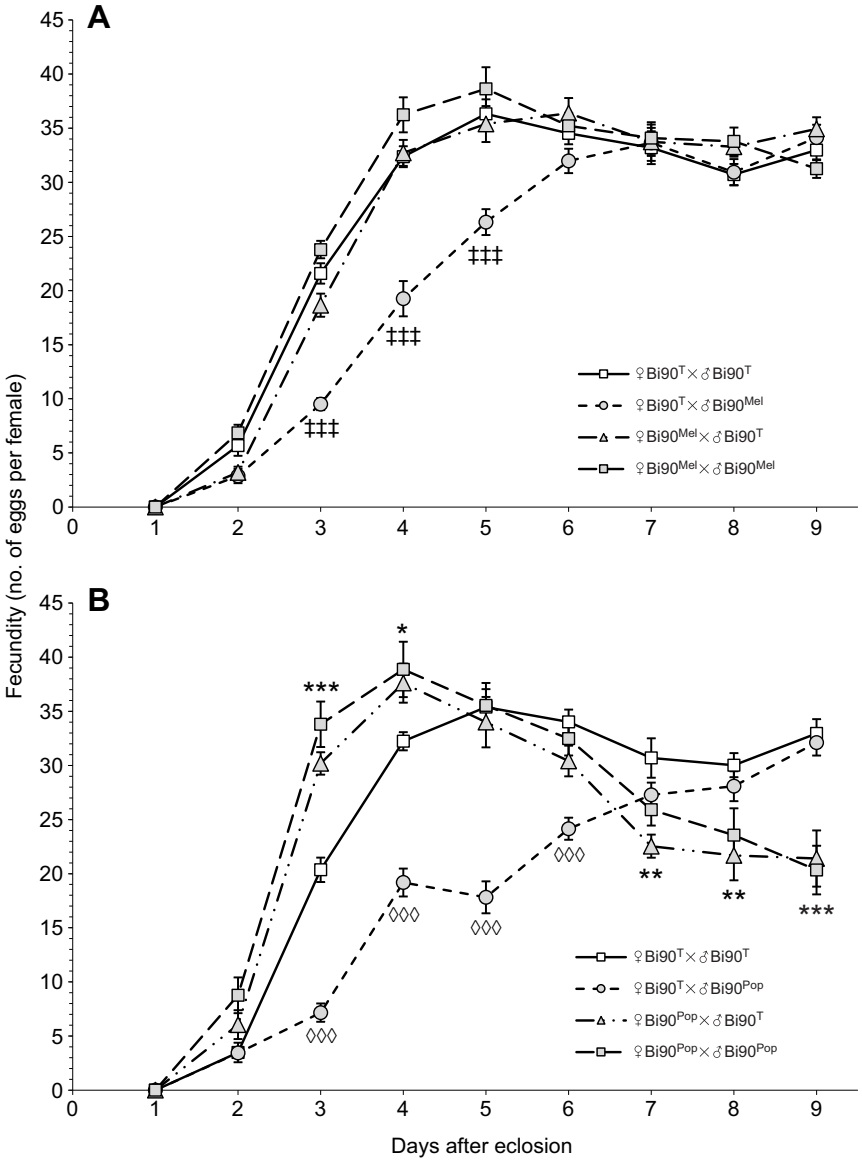

Fig. 4. The effect of the crosses between *Wolbachia*-infected males and uninfected females (and vice versa) on *D. melanogaster* fecundity in comparison with uninfected and infected maternal lineages. $Bi90^{T}$, uninfected $Bi90^{T}$ lineage; $Bi90^{Mel}$ and $Bi90^{Pop}$, Bi90 lineage carrying *w*Mel and *w*MelPop *Wolbachia*. (A) Double daggers indicate significant differences between $♀Bi90^{T}×♂Bi90^{Mel}$ hybrids and uninfected $Bi90^{T}$ lineage; N=30 for $Bi90^{T}$ and N=20 for $Bi90^{Mel}$ and both hybrids. (B) Diamonds indicate significant differences between $♀Bi90^{T}×♂Bi90^{Pop}$ hybrids and $Bi90^{T}$ control; asterisks indicate significant differences between uninfected control and $♀Bi90^{Pop}×♂Bi90^{T}$ hybrids; N=10 for $Bi90^{Pop}$ and N=20 for $Bi90^{T}$ and both hybrids. Data are means±s.e.m. (two-way mixed-design ANOVA; ‡/*P<0.05; **P<0.01; ‡‡‡/◊◊◊/***P<0.001).

6-day-old $Bi90^{CS}$ females (Fig. 5C) compared with $Bi90^{T}$ females of the same age corresponds to an increased JH level in these flies (Gruntenko and Rauschenbach, 2008). Therefore, we studied the impact of the JH inhibitor precocene (Wilson et al., 1983; Argue et al., 2013) on young $Bi90^{Pop}$ and mature $Bi90^{CS}$ females to test the role of JH in the fecundity differences of these lineages. Young $Bi90^{CS}$ females and mature $Bi90^{Pop}$ females, characterized by an increased JH degradation (Fig. 5A,C) and presumably reduced JH level, were treated with a JH-III on the same days. The data are provided in Fig. 6.

The treatment with precocene decreased egg production in both young $Bi90^{Pop}$ females (precocene: $F_{2,27}$=24.03, P<0.0001; age: $F_{3,81}$=159.44, P≪0.0001; precocene×age: $F_{6,81}$=14.16, P≪0.0001; Fig. 6A) and mature $Bi90^{CS}$ females (precocene: $F_{2,27}$=29.60, P<0.0001; age: $F_{4,108}$=52.14, P≪0.0001; precocene×age: $F_{8,108}$=8.42, P≪0.0001; Fig. 6D) compared with the acetone-treated groups, and abolished the differences between the *Wolbachia*-infected and uninfected lineages.

The increase of the JH level via its application raised the egg production of both young $Bi90^{CS}$ females (JH: $F_{2,27}$=17.16, P<0.0001; age: $F_{3,81}$=165.39, P≪0.0001; JH×age: $F_{6,81}$=4.98, P=0.0003; Fig. 6B) and mature $Bi90^{Pop}$ females (JH: $F_{2,27}$=32.30, P≪0.0001; age: $F_{4,108}$=24.87, P≪0.0001; JH×age: $F_{8,108}$=2.93, P=0.0054; Fig. 6C) compared with the acetone-treated groups, and eliminated the differences between the *Wolbachia*-infected and uninfected lineages.

DISCUSSION

In our previous work (Gruntenko et al., 2017) and the present study, we try to reveal the mechanism by which the genetic background of the endosymbiont can affect host fitness by investigating how different types of *Wolbachia* modulate survival and fecundity of *D. melanogaster* lineages with the same genetic background. Here, we report that the *w*Mel and *w*MelCS genotypes of *Wolbachia* cause different effects on gonadotropic (JH) metabolism and egg production, differently contributing to the fitness of the host. The effect of the *w*MelPop strain differs from the effects of both the *w*Mel and *w*MelCS genotypes. Our data suggest that the fecundity level of *Drosophila* lineages carrying various types of infection depends on how *Wolbachia* affects the reproductive biology of

Journal of Experimental Biology (2019) 222, jeb195347. doi:10.1242/jeb.195347

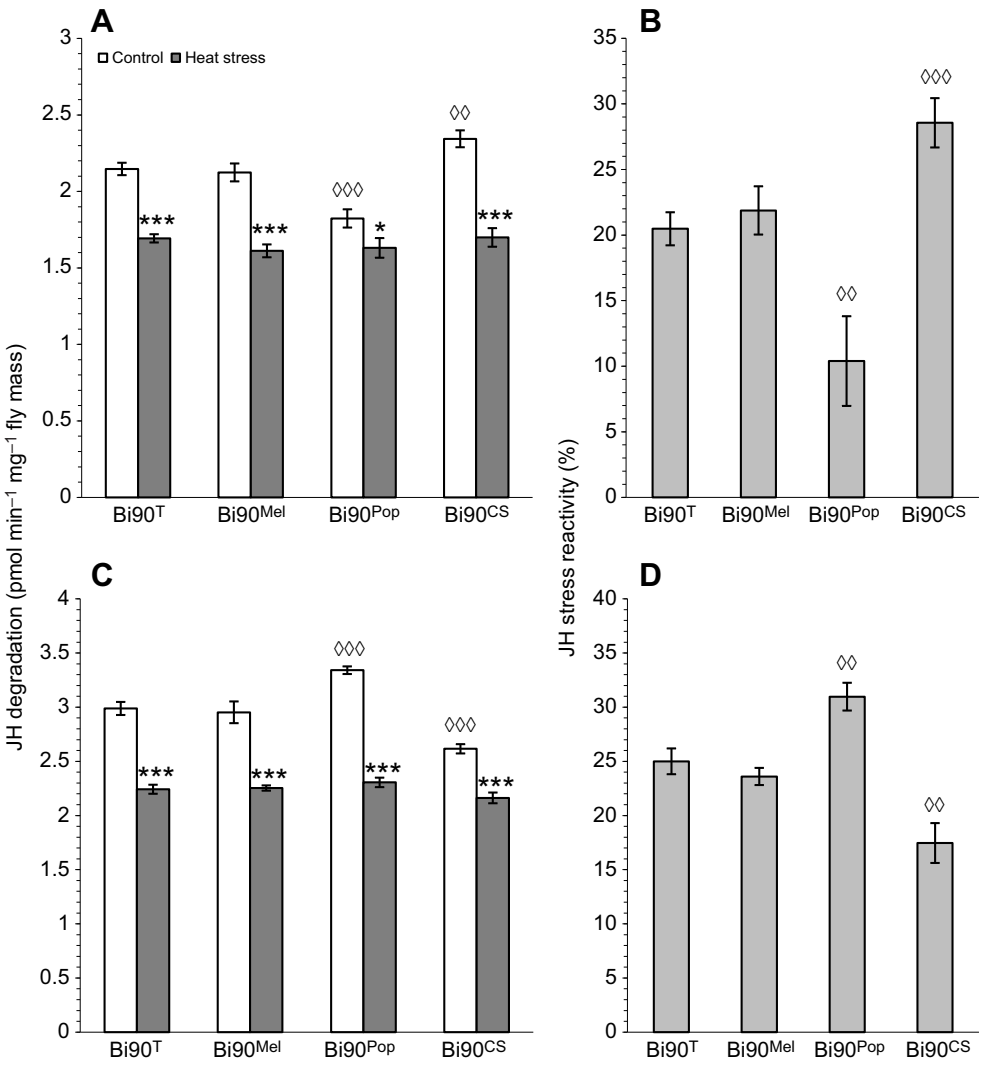

Fig. 5. The effect of various *Wolbachia* infections on juvenile hormone (JH)-hydrolysing activity in *D. melanogaster* females in comparison with uninfected controls. Bi90^{T}, uninfected Bi90^{T} lineage; Bi90^{Mel}, Bi90^{CS} and Bi90^{Pop}, Bi90 lineage carrying *w*Mel, *w*MelCS and *w*MelPop *Wolbachia*. (A,C) JH degradation in (A) 1-day-old females under normal conditions (*N*=29 for Bi90^{T}, *N*=19 for Bi90^{CS}, *N*=23 for Bi90^{Mel} and *N*=15 for Bi90^{Pop}) and upon heat stress (38°C; *N*=45 for Bi90^{T}, *N*=30 for Bi90^{CS}, *N*=23 for Bi90^{Mel} and *N*=15 for Bi90^{Pop}) and (C) 6-day-old females under normal conditions (*N*=20 for Bi90^{T} and *N*=15 for Bi90^{CS}, Bi90^{Mel} and Bi90^{Pop}) and upon heat stress (38°C; *N*=20 for Bi90^{CS}, Bi90^{Pop} and Bi90^{T}, *N*=15 for Bi90^{Mel}). Data are means±s.e.m. Diamonds indicate significant differences between infected and uninfected females; asterisks indicate significant differences between heat treated and control females of the same lineage (two-way ANOVA). (B,D) JH stress reactivity in (B) 1-day-old females (*N*=44 for Bi90^{T}, *N*=29 for Bi90^{CS}, *N*=23 for Bi90^{Mel} and *N*=15 for Bi90^{Pop}) and (D) 6-day-old females (*N*=20 for Bi90^{CS}, Bi90^{Pop} and Bi90^{T}, *N*=15 for Bi90^{Mel}). Diamonds indicate significant differences between infected and uninfected females; double daggers indicate significant differences between *w*MelCS- and *w*MelPop-infected females (one-way ANOVA). ◊/*P<0.05; ◊◊P<0.01; ‡‡‡/◊◊◊/***P<0.001.

females. It was shown earlier that the effect of *Wolbachia* on host fitness and stress resistance is dependent on the genomic background of the *Drosophila* lineage (Fry et al., 2004; Capobianco et al., 2018). Based on these data, Capobianco et al. (2018) concluded that the effect of *Wolbachia* on fitness is unpredictable across the individual genetic backgrounds of host animals. We can make an addition to this statement: the effect of *Wolbachia* on the host fitness depends not only on the genetic background of the host, but also on that of the symbiont.

We have found that *Wolbachia* of different genotypes are present in *Drosophila* oocytes in different titres (Fig. 1). The higher load has been shown earlier for the pathogenic *w*MelPop strain, which overproliferates and shortens host lifespan (Min and Benzer, 1997; McGraw et al., 2002; Chrostek et al., 2013). Chrostek et al. (2013) found a higher relative amount of *w*MelCS *Wolbachia* compared with *w*Mel in *D. melanogaster* males from 2 weeks after eclosion. However, they did not detect any difference in bacterial load between *w*MelCS- and *w*Mel-infected males at the age of 3–6 days, which is in agreement with our data on the relative amount of *Wolbachia* genomic DNA in 6-day-old females (Chrostek et al., 2013). Perhaps the variation in bacterial load among different types of *Wolbachia* infection partly explains the diversity in the effects they caused in the host. Such a correlation between *Wolbachia* titre and cytoplasmic incompatibility was discovered earlier in the parasitoid wasp *Asobara japonica* and the mosquito *Aedes albopictusi* (Kraaijeveld et al., 2011; Calvitti et al., 2015).

Our results show that *Wolbachia* of the *w*Mel group (*w*Mel, *w*Mel2 and *w*Mel4) do not affect the fecundity of infected *Drosophila* lineages, whereas bacteria of the *w*MelCS group decrease the egg production in young females, just starting oviposition, and increase the egg production in mature females at their reproductive peak (Fig. 3). The pathogenic *w*MelPop strain causes the opposite changes in fecundity compared with *w*MelCS *Wolbachia*, although they are indistinguishable in terms of genetic markers (Riegler et al., 2012), except the Octamom copy number (Chrostek and Teixeira, 2015). These findings correspond well with our previous data on the effects of these *Wolbachia* variants on host survival under stress (Gruntenko et al., 2017); the survival of *D. melanogaster* lineages infected with the *w*Mel, *w*Mel2 and *w*Mel4 genotypes do not differ from the survival of uninfected flies, whereas *w*MelCS increases the survival and *w*MelPop decreases it. One would expect that the positive fitness effects caused by *Wolbachia* infection would help to promote its spread throughout a host population; thus, there is still no explanation to the fact that the *Wolbachia* of the *w*MelCS group, having higher fecundity and stress resistance, is the rare variant in natural *D. melanogaster* populations compared with the bacteria of the *w*Mel group (Riegler et al., 2005; Ilinsky and Zakharov, 2007; Nunes et al., 2008).

Journal of Experimental Biology (2019) 222, jeb195347. doi:10.1242/jeb.195347

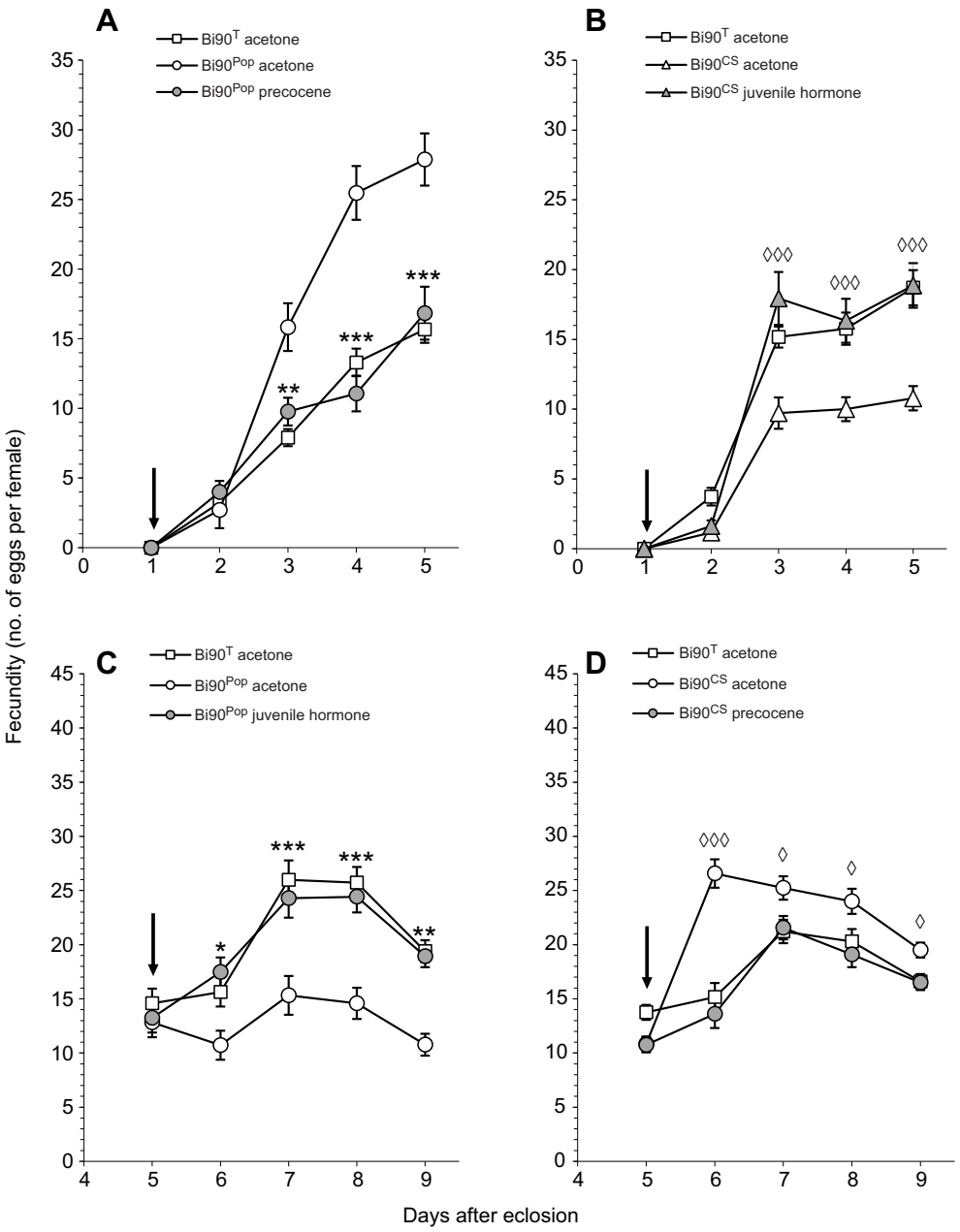

Fig. 6. The effect of JH or precocene treatment on the fecundity of *D. melanogaster* lineages carrying different types of *Wolbachia* infection in comparison with uninfected controls. $Bi90^{T}$, uninfected $Bi90^{T}$ lineage; $Bi90^{CS}$ and $Bi90^{Pop}$, Bi90 lineage carrying *w*MelCS and *w*MelPop *Wolbachia*. *N*=10 for each experimental group. (A,C) Fecundity level of uninfected flies and flies infected with *w*MelPop *Wolbachia* following precocene (A) or JH (C) application (shown by arrows). (B,D) Fecundity level of uninfected flies and flies infected with *w*MelCS *Wolbachia* following JH (B) or precocene (D) application (shown by arrows). The control groups of flies were treated with equal amounts of acetone (JH and precocene vehicle). Asterisks indicate significant differences between treated and untreated $Bi90^{Pop}$ flies; diamonds indicate significant differences between treated and untreated $Bi90^{CS}$ flies (two-way mixed-design ANOVA; $^{◊/*}P<0.05$; $^{**}P<0.01$; $^{◊◊◊/***}P<0.001$).

Previously, Fry et al. (2004) demonstrated that crosses between infected males and uninfected females resulted in a decrease in egg production, and suggested that the phenomenon could promote the spread of infection just like cytoplasmic incompatibility. Our findings support these results, showing that uninfected females crossed with males infected with *w*Mel or *w*MelPop produced many fewer eggs than infected or uninfected females crossed with uninfected males (Fig. 4). We also found that the fecundity pattern of *w*MelPop-infected females does not depend on the infection status of males crossed with them. Therefore, *Wolbachia* not only modify host sperm and affect fertility of their male hosts (Serbus et al., 2008; Liu et al., 2014), but affect female reproductive biology as well. This could provide some explanation as to the maintenance of *Wolbachia* in *D. melanogaster*, which does not seem to be associated with cytoplasmic incompatibility because it is weak or absent in this species (Fry et al., 2004).

Our findings support and extend previous studies showing positive correlations between an increase/decrease in JH degradation (accompanied by a decrease/increase in JH level) and a decrease/increase in the fecundity of *D. melanogaster* females (Gruntenko and Rauschenbach, 2008; Gruntenko et al., 2010; Rauschenbach et al., 2014). Thus, in young *w*MelCS-infected females, the fecundity decrease (compared with uninfected females) is accompanied by an increase in JH degradation activity; and vice versa, in mature *w*MelCS-infected females, the fecundity increase is accompanied by a decrease in JH degradation (Figs 2 and 4). The increase/decrease in the fecundity of young/mature *w*MelPop-infected females also correlates with decrease/increase in the JH degradation in these females (Figs 2 and 4). At the same time, females infected with the *w*Mel genotype of *Wolbachia* do not differ from uninfected females in terms of both JH metabolism and fecundity level (Figs 2 and 4). Importantly, our data indicate that the effect of *Wolbachia* infection on the stress response of the JH

metabolic system also depends on the type of infection and age of the flies. The results of the influence of *w*MelCS and *w*MelPop on JH metabolism correspond well with the data by Zheng et al. (2011) and Liu et al. (2014), who demonstrated an interaction between *Wolbachia* and the JH signalling pathway in the testes of male *D. melanogaster*. Zheng et al. (2011) found a 10-fold increase in the transcription level of the gene for JH-induced protein 26 (*JhI-26*) caused by *Wolbachia* infection. *JhI-26* expression is shown to be triggered rapidly and specifically by JH-III (Dubrovsky et al., 2000). Liu et al. (2014) showed that *Wolbachia* significantly increased the expressions of genes that play key roles in the JH signalling pathway: *Jhamt* (encoding JH acid methyltransferase, a key regulatory enzyme of JH biosynthesis) and *Met* (encoding JH receptor). These data support our conclusion concerning the influence of *Wolbachia* on JH signalling in *Drosophila*.

JH/precocene treatment of young and mature females infected with *w*MelCS and *w*MelPop *Wolbachia* produced a pronounced fecundity response in both treated lineages (Fig. 6). This treatment was aimed at a shift in the JH level in young or mature *w*MelCS- and *w*MelPop-infected females to the level typical for uninfected controls. Egg production curves from infected females with a corrected JH level coincide with the curves from uninfected flies both in the beginning and at the peak of oviposition. It is worth noting that the influence of JH on the egg production of *w*MelCS- and *w*MelPop-infected females is strain-specific in contrast to the JH effect on stress resistance: the same JH treatment induces a decrease in the survival under heat stress in *Drosophila* females infected with *w*MelCS, *w*Mel and *w*MelPop as well as in uninfected controls (Rauschenbach et al., 2018). These results indicate that JH plays a mediating role in the effect of *Wolbachia* on host fecundity.

In conclusion, we found that the influence of *Wolbachia* on female reproductive biology in *D. melanogaster* depends on the genetic background of the symbiont, and JH mediates the *Wolbachia* modulation of female fecundity.

Acknowledgements
We thank Dr Luis Teixeira for sharing the valuable *iso w*MelPop strain, and Darya Pirozhkova for assistance with the quantification of the relative amount of *Wolbachia* genomic DNA.

Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: N.E.G., Y.Y.I., I.Y.R.; Methodology: Y.Y.I.; Validation: P.N.M.; Investigation: E.K.K., N.V.A., O.V.A., E.V.B., R.A.B.; Data curation: P.N.M., I.Y.R.; Writing - original draft: N.E.G.; Writing - review & editing: N.E.G., Y.Y.I., P.N.M., I.Y.R.; Supervision: N.E.G., I.Y.R.; Project administration: N.E.G.; Funding acquisition: I.Y.R.

Funding
This work was supported by the Russian Foundation for Basic Research grant 16-04-00060 and by The Ministry of Science and Higher Education of the Russian Federation (Budgeted Project 0324-2019-0041).

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