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ORIGINAL ARTICLE

Incomplete offspring sex bias in Australian populations of the butterfly *Eurema hecabe*

DJ Kemp[1,2], FE Thomson[2], W Edwards[2] and I Iturbe-Ormaetxe[3,4]

Theory predicts unified sex ratios for most organisms, yet biases may be engendered by selfish genetic elements such as endosymbionts that kill or feminize individuals with male genotypes. Although rare, feminization is established for *Wolbachia*-infected *Eurema* butterflies. This paradigm is presently confined to islands in the southern Japanese archipelago, where feminized phenotypes produce viable all-daughter broods. Here, we characterize sex bias for *E. hecabe* in continental Australia. Starting with 186 wild-caught females, we reared >6000 F1–F3 progeny in pedigree designs that incorporated selective antibiotic treatments. F1 generations expressed a consistent bias across 2 years and populations that was driven by an ~5% incidence of broods comprising ⩾80% daughters. Females from biased lineages continued to overproduce daughters over two generations of outcrossing to wild males. Treatment with antibiotics of differential strength influenced sex ratio only in biased lineages by inducing an equivalent incomplete degree of son overproduction. Brood sex ratios were nevertheless highly variable within lineages and across generations. Intriguingly, the cytogenetic signature of female karyotype was uniformly absent, even among phenotypic females in unbiased lineages. Molecular evidence supported the existence of a single *Wolbachia* strain at high prevalence, yet this was not clearly linked to brood sex bias. In sum, we establish an inherited, experimentally reversible tendency for incomplete offspring bias. Key features of our findings clearly depart from the Japanese feminization paradigm and highlight the potential for more subtle degrees of sex distortion in arthropods.
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INTRODUCTION

Sex in most diploid organisms is diecious and anisogamous, and produces sons and daughters in a balanced 1:1 ratio. Theoretically, Fisher's principle (Fisher, 1930) explains how selection should stabilize sex ratios at unity to maximize the inclusive fitness gain from parental investment. This principle is based on a form of negative frequency-dependent selection, wherein the ultimate genetic payoff of investing in sons versus daughters depends on the frequency of each gender in the population. More formally, a unified offspring sex ratio represents the evolutionarily stable strategy (Maynard Smith, 1982) at equilibrium. Adaptive departures from unity sometimes occur, either at population levels as in parthenogenic and haplodiploid species, or at brood levels according to parental investment theory (for example, Trivers and Willard, 1973). The expectation for most species is nevertheless for a unified ratio, and departures at equilibrium present opportunities to explore the assumptions of sex allocation theory (Hamilton, 1967).

In recent decades, sex ratio has been intensively studied across a range of arthropod groups with regard to the effects of obligate microbial endosymbionts such as *Rickettsia* (Himler *et al.*, 2011), *Spiroplasma* (Jiggins *et al.*, 2000), *Cardinium* (Zchori-Fein and Perlman, 2004) and *Wolbachia* (Werren, 2008). Such microbes exist within host cells and achieve vertical transmission only by 'hitch-hiking' in the cytoplasm of female gametes. This means that the evolutionary capacity to invade and occupy host populations hinges upon realized rates of maternal inheritance (Cordaux *et al.*, 2011). Investigation across a broad range of endosymbionts has revealed the ability to manipulate host reproduction in ways commensurate with this goal. Four primary manipulations are known (Werren, 2008; Cordaux *et al.*, 2011). First, thelytokous parthenogenesis is restricted to haplodiploid hosts and causes female rather than male phenotypes to develop from unfertilized gametes. Second, cytoplasmic incompatibility (hereafter, 'CI') renders matings between uninfected males and infected females inviable; hence reducing the effective population contribution of uninfected males. Third, male killing promotes the selective death of sons in embryonic or juvenile stages (Hurst *et al.*, 1999), and may elevate the marginal fitness of infected daughters owing to factors such as reduced inter-sibling competition (Hurst and Majerus, 1993). Fourth, feminization causes genetically male hosts to develop reproductively viable female phenotypes. This manipulation is rarely documented, yet offers high evolutionary payoffs (in conventionally diploid arthropod hosts) by doubling the potential number of conduits for vertical transmission. Although not always maladaptive for hosts, these manipulations often create misalignments of evolutionary interests that, in turn, set the scene for host–endosymbiont coevolution (Riegler and O'Neill, 2007; Cordaux *et al.*, 2011). Documented host counteradaptations include nuclear

[1]Department of Biological Sciences, Faculty of Science and Engineering, Macquarie University, Sydney, New South Wales, Australia; [2]Centre for Tropical Environmental Sustainability Science and College of Science and Engineering, James Cook University, Cairns, Queensland, Australia and [3]School of Biological Sciences, The University of Queensland, Brisbane, Queensland, Australia
Correspondence: Dr DJ Kemp, Department of Biological Sciences, Faculty of Science and Engineering, Macquarie University, North Ryde, Sydney, New South Wales 2109, Australia.
E-mail: darrell.kemp@mq.edu.au
[4]Current address: Institute of Vector-borne Disease, Monash University, Clayton, Victoria, Australia.
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suppressors that restore the viability and/or natural phenotypic development of male genotypes (Hornett *et al*., 2006; Charlat *et al*., 2007b).

Lepidoptera have proven an excellent group for exploring arthropod–endosymbiont dynamics (Dyson and Hurst, 2004; Hornett *et al*., 2006; Sugimoto and Ishikawa, 2012). This group harbors bacterial symbionts such as *Spiroplasma* and *Wolbachia* collectively known to impose CI, male killing and feminization (Jiggins *et al*., 2000; Hiroki *et al*., 2004; Charlat *et al*., 2005). Studies of butterfly male-killing have yielded valuable insights into host–symbiont coadaptation, including the genetic and biogeographic basis of nuclear host suppression (Dyson and Hurst, 2004; Hornett *et al*., 2006, 2008; Charlat *et al*., 2007b, c). Lepidopterans have also informed the study of feminization, primarily via the work in *Wolbachia*-infected *Ostrinia* moths (Crambidae) and *Eurema* butterflies (Pieridae). Whereas sex bias in *Ostrinia* was initially interpreted as feminization, subsequent work has revealed a more complex effect based ultimately upon male killing (Kageyama and Traut, 2004; a manipulation also induced by *Spiroplasma* in this genus; Tabata *et al*., 2011). Data from extensive historic and contemporary studies of *Eurema* (outlined below) have nevertheless remained consistent with feminization. This system poses a rare example of this phenomenon in the Arthropoda (Cordaux *et al*., 2011), which outside of isopods is known for a mite (Weeks *et al*., 2001), a leafhopper (Negri *et al*., 2006) and a wasp (Giorgini *et al*., 2009).

Evidence for *Eurema* feminization has arisen from studies of Japanese *E. hecabe/mandarina*. The effect was first established for *E. mandarina* (then considered a subspecies of *E. hecabe*; Hiroki, 2002; Hiroki *et al*., 2004; Narita *et al*., 2007a, c), but has since been extended to the species distinguished as *E. hecabe* (Narita *et al*., 2011). Each species is subject to infection by the same two genetically distinct strains of *Wolbachia*. The first strain (*w*CI) occurs at near fixation in *Eurema* populations throughout Japan (Narita *et al*., 2011). It has been established to impose strong cytoplasmic incompatibility across this range, but is also known for male killing in a single mainland population (Narita *et al*., 2007b). The second strain (*w*Fem) is restricted to islands in the southern Japanese archipelago, where it occurs as a low frequency coinfection alongside *w*CI (Hiroki, 2002; Hiroki *et al*., 2004; Narita *et al*., 2011). Doubly infected females from these populations are known to produce broods consisting entirely of daughters, yet produce 100% sons if treated with antibiotics (Hiroki, 2002; Hiroki *et al*., 2004). This implies that such individuals in fact possess male sexual karyotypes and are capable of engendering normal development of male phenotypes in the absence of feminization. Cytogenetic study has corroborated this view because doubly infected phenotypes lack the signature of female (W) chromatin readily visualized in genetic females (Hiroki, 2002; Narita *et al*., 2007a, 2011). More recently, Kern *et al*. (2015) used Z-linked genetic markers to reveal that doubly infected female *E. mandarina* possess just one paternally inherited Z chromosome, and raised the possibility that *w*Fem may mimic W chromosomes in such individuals.

This study examines sex ratio distortion in *E. hecabe* on the Australian continent. Offspring bias is not presently known for this species beyond Japan, nor has it been noted in Australian *Eurema* despite decades of research across the genus (for example, Jones *et al*., 1987; Jones, 1992). Interestingly, more recent pedigree-based study of *E. hecabe* from tropical North Queensland revealed the overproduction of daughters in a small fraction of lineages (DJK, unpublished data). Although offspring biases may arise for diverse reasons (Hurst *et al*., 1996), the paradigm established in Japanese conspecifics lends precedent to a hypothesis of *Wolbachia* causality. The most straightforward extension of this paradigm offers two basic scenarios. The first is that Australian butterflies experience feminization simply in the manner of their oriental counterparts. This would imply a Western Pacific distribution of *w*Fem/*w*CI that extends (or has recently extended) well beyond the known limits of subtropical Japanese islands (for example, Narita *et al*., 2011). The second is that either or both strains (or their variants) reside in Australia but engender male killing (as seen rarely in Japan; Narita *et al*., 2007b). Either case would present opportunities to examine the broader biogeographic and/or coevolutionary context of host–symbiont interaction. Of course, sex distortion could arise from any number of variants on these scenarios, or entirely different scenarios involving novel *Wolbachia* strains, other symbionts and microorganisms (Kageyama *et al*., 2012), or nuclear factors such as epistasis and meiotic drive (Jiggins *et al*., 1999; Weeks *et al*., 2002).

We first aimed to establish the incidence of offspring sex bias in Australian *E. hecabe*, as expressed among broods of two tropical populations obtained over successive years. We next sought to test whether sex biases exhibit the maternal inheritance and antibiotic sensitivity characteristic of endosymbiotic bacteria. Experimental antibiotic treatments were applied selectively to lineages each year to test whether broods revert either to unity (1:1), as indicative of male killing, or to the all-male phenotypes evidenced in feminized Japanese conspecifics (Narita *et al*., 2011). Last, we explored offspring sexual karyotypes using cytogenetic assays and investigated the presence of *Wolbachia* (specifically, the strains *w*CI and *w*Fem) using diagnostic PCR and sequencing.

MATERIALS AND METHODS

General husbandry protocols

We used standard husbandry protocols throughout. All work was conducted in a single climate-controlled laboratory (temperature = 26.0 ± 1.0 °C, humidity = 60 ± 5%, photoperiod = 14L:10D (2008) and 16L:8D (2009)). Overhead fluorescent and metal-halide lights provided bright, full-spectrum illumination (Kemp, 2008) and hostplant (*Sesbania cannabina*) was grown in an outdoor greenhouse.

Lines were established each year using females captured at Cairns (16.90° S, 145.75° E) and Port Douglas (16.48° S, 145.46° E) in North Queensland, Australia. Females were stored in 60 ml specimen jars with access to 15% honey water, and at ~9:00 h the next day, placed individually in blindly labeled oviposition cups containing a hostplant cutting (Figure S1). This was repeated across days until females had laid >35 eggs or expired, when they were frozen at −20 °C. Hatchlings developed communally until the fourth and fifth instars, when they were separated into groups of 8–10 and 4–5, respectively. Hostplant was provided throughout in oversupply. Adults were sexed using the presence of male-limited ventral androconial brands.

Females designated as dams for subsequent generations were marked with identifying numbers on both ventral hindwings (using colored Sharpie markers). They were released along with an equivalent number of males into 600 mm^3 mating cages (aluminium-fiberglass mesh construction; Kemp, 2008). The cages were monitored at 15 min intervals during light hours to identify mating pairs, which were removed to individual gauze-topped containers (Figure S1).

2008 Breeding experiment

This experiment was designed to inform the incidence and magnitude of sex bias, establish whether it is inherited and provide preliminary insight into antibiotic effects. We started by rearing 1449 F1 offspring from females caught at Cairns (N = 45; 14 February 2008) and Port Douglas (N = 38; 13 February 2008; Figure 1a). At eclosion, we identified the 10 most female-biased families, mated daughters from each family with random wild-caught males and reared their offspring to adulthood. Twenty F1 females produced a total of 160 F2 individuals (Figures 1b and c). Following eclosion, we randomly assigned daughters from the three most female-biased F2 families among two treatments (Figure 1c):

(1) Control: housed for 3 days in a gauze-topped cup (Figure S1) with *ad libitum* access to a cotton-wool bud soaked with 15% honey water;
(2) Treatment: housed as controls but with 1 mg tetracycline hydrochloride supplemented per 1 ml of honey water (as shown to invert sex ratio in Japanese *Eurema*; Hiroki, 2002; Hiroki *et al.*, 2004; Narita *et al.*, 2007b).

Females were subsequently mated to wild-caught males and used to rear an F3 generation (comprising $N=229$ adults from 13 control dams and 217 adults from 15 treatment dams; Figure 1d).

2009 Breeding experiment

This experiment expanded upon the 2008 design by nesting antibiotic treatment within lineages designated as either female-biased or unbiased (1:1) sex ratio. We also added a third dam feeding treatment based on rifampicin, which is a bacteriocidal compound of greater potency than tetracycline (Charlat *et al.*, 2007a). Females caught from Cairns ($N=63$) and Port Douglas ($N=40$) on 27 February 2009 (Figure 2a) were used to rear 1661 F1 adults. Daughters from each of five female-biased and five unbiased lineages were randomly assigned to control (C), tetracycline (T) or rifampicin (R) feeding treatments (Figure 2b). The latter treatment consisted of both tetracycline and rifampicin dissolved in honey water, each at a concentration of 1 mg ml^{-1}. Females were subsequently mated with wild-caught males and contributed an F2 generation comprising 2354 total offspring (Figure 2c). Finally, we undertook a limited series of crosses between control F2 individuals (Figure 2d) to further investigate the inheritance of sex bias; these are outlined in the results.

Molecular assays

Diagnostic PCR assays were used to test the presence of *Wolbachia* DNA in selected experimental subjects (specified in the results) in both years. Single butterflies were homogenized for 90 s in a 2 ml screw cap tube containing three sterile glass beads (2 mm) and 600 µl of squash buffer (10 mM Tris, 1 mM EDTA, 50 mM EDTA, pH 8.2), using a bead beater (Biospec, Bartlesville, OK, USA). The homogenate was centrifuged for 10 min at 20 000 *g* and the DNA was extracted from the supernatant using phenol/chloroform/isoamyl alcohol followed by a chloroform/isoamyl alcohol, and precipitated using sodium acetate and ethanol. DNA was resuspended in 50 µl TE buffer, and 1 µl of a 1/20 dilution was used for the PCR. We used the forward primer *wsp*81F and the reverse primer *wsp*691R to amplify a portion of the *wsp* gene, which codes for an outer membrane protein in *Wolbachia* (Zhou *et al.*, 1998). We also tested for the presence of *w*CI and *w*Fem, the two strains known for Japanese *E. hecabe* (Narita *et al.*, 2011), using the following primer pairs:

(1) *wsp*81F (5′- TGGTCCAATAAGTGAAGAAAC - 3′) (Zhou *et al.*, 1998) and *w*HecFem1 (5′-ACTAACGTCGTTTTTGTTTAG-3′) (Hiroki *et al.*, 2004) that amplify a 232 bp section of the *wsp* gene characteristic of strain *w*CI;
(2) *w*HecFem2 (5′-TTACTCACAATTGGCTAAAGAT-3′) (Hiroki *et al.*, 2004) and *wsp*691R (5′-AAAAATTAAACGCTACTCCA-3′) (Zhou *et al.*, 1998) that amplify a 398 bp section of the *wsp* gene characteristic of *w*Fem.

To confirm successful DNA extraction in each sample, we also performed PCRs using the 28S ribosomal DNA primer set (28sF3633+28sR4076; Rugman-Jones *et al.*, 2010) or 18S ribosomal DNA (18sF+18sR; Duplouy *et al.*, 2013), both of which amplify insect DNA. All molecular work was undertaken at the University of Queensland's *Wolbachia* laboratory using a proof-reading Elongase mix (Life Technologies, Carlsbad, CA, USA), following the manufacturer's instructions. PCR cycling conditions were as follows: 95 °C for 3 min, followed by 95 °C for 30 s, 53 °C for 30 s and 68 °C for 1 min for 35 cycles, then 68 °C for 10 min. PCR products were run on agarose gels and stained with ethidium bromide. PCR products obtained used the primer combination *wsp*81F+*w*HecFem1 or *wsp*691R+*w*HecFem2, were purified using a gel extraction kit (Qiagen, Hilden, Germany), cloned into pGEM-Teasy (Promega, Madison, WI, USA) and sequenced at AGRF using T7 primers.

Cytogenetic assays

Sexual karyotype was investigated using a standard cytogenetic technique for visualizing female W chromatin (Traut *et al.*, 2007). We assayed male and female phenotypes from selected lineages and as fully specified in the results. Unfortunately, live Japanese conspecifics were not available for comparison, however, we assayed several additional butterfly species (*Belenois java*; Pieridae and *Hypolimnas bolina*; Nymphalidae) as positive controls for the technique.

Statistical procedures

We conducted discrete contrasts of sex ratio according to the absolute frequencies of males and females. Exact binomial tests were used to assess departures from unity (that is, the nominal null hypothesis for butterflies), and likelihood-based G-tests were used to test 'intrinsic' hypotheses for sex ratio

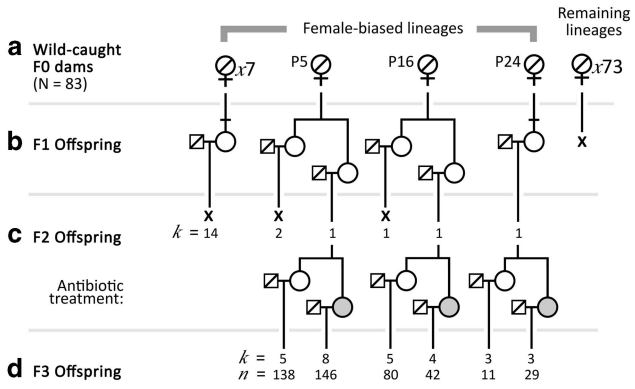

Figure 1 Schematic for the 2008 experiment. Dams (circles) and sires (squares) are depicted with a diagonal slash if wild-caught, and duplicate crosses are collapsed to aid presentation. (**a**) Wild-caught females contributed an F1 generation of 1449 individuals in $k=83$ families. (**b**) Daughters from the 10 most female-biased F1 families were then outcrossed with wild-caught males; (**c**) F2 offspring ($n=160$ in $k=20$ broods) were reared to adulthood. Daughters from three female-biased families (tracing from F0 lineages 'P5', 'P16' and 'P24') were randomly assigned to control versus tetracycline (shaded) feeding treatments, then outcrossed with wild males; (**d**) F3 offspring (total $n=446$ in $k=28$ broods) were subsequently reared for the assessment of adult sexual phenotype.

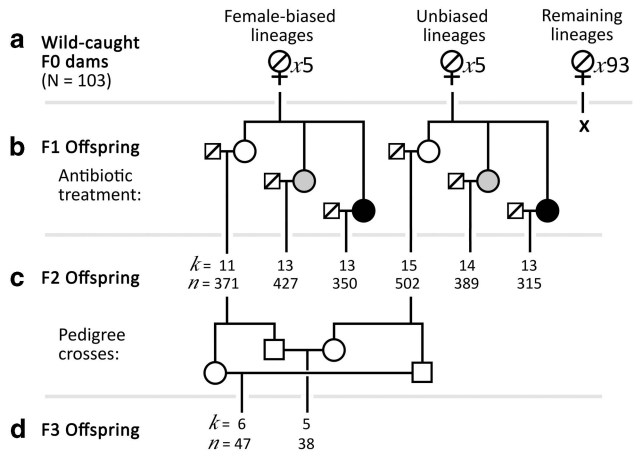

Figure 2 Schematic for the 2009 experiment, presented as per Figure 1. (**a**) We reared the broods of 103 wild females, and identified 5 female-biased and 5 unbiased F1 families; (**b**) Daughters in each F1 family were randomly assigned to control, tetracycline or rifampicin feeding treatments (open, shaded and solid circles, respectively), then outcrossed with wild-caught males; (**c**) F2 offspring ($n=2354$ in $k=79$ broods) were reared to adulthood. Finally, we performed reciprocal crosses between biased and unbiased control F2 individuals, and (**d**) reared F3 offspring ($n=85$ in $k=11$ broods).

variation among different groups. The aim to assess biases among multiple individual broods introduced the potential for type II error; hence, we conducted such tests using replicated goodness of fit procedures (Sokal and Rohlf, 1995; pp. 715–724). Overall goodness of fit (to a 1:1 ratio) was partitioned into components owing to deviance for all offspring pooled versus deviance contributed by individual broods. Significantly biased broods were then identified using the *a posteriori* simultaneous test procedure.

General linear models were used to analyze the experimental data sets in each year. We tested main and interactive effects, as well as *a priori* contrasts for specific treatment differences in 2009, as specified below. All models analyzed daughter frequency (dependent variable) as a function of total brood size (random covariate); hence, sex ratio was parameterized as the marginal frequency of daughters. This was desirable because broods varied greatly in offspring number and simple sex ratio indices (such as % daughters) inflated the variance contribution of smaller families. Data of daughter and offspring frequencies were normalized prior to analysis using the square-root transformation, which is appropriate for data consisting of counts (Sokal and Rohlf, 1995).

All models included treatment as a fixed effect, and in 2009, included sex ratio pedigree (that is, whether broods derived from putatively 1:1 or female-biased pedigree). We also tested in both years for differences among broods of different F0 lineage, specified as a fixed factor because lineages were chosen *a priori* (Sokal and Rohlf, 1995). In 2009, we specified three planned contrasts for the effect of antibiotic treatment on F2 sex ratio. The first was designed to test prediction for no effect of experimental group in 1:1 lines (that is, C ~ T ~ R).

Table 1 Summary of F1 offspring of female *E. hecabe* caught from Cairns and Port Douglas in each study year

Year	*2008*			*2009*		
Sampling population	*Cairns*	*Port Douglas*	*Total*	*Cairns*	*Port Douglas*	*Total*
Wild-caught (F0) dams	45	38	83	63	40	103
F1 offspring						
Males	318	324	642	472	311	783
Females	408	399	807	521	357	878
Total	726	723	1449	993	668	1661
F1 sex ratio (%female)	56.2	55.2	55.7	52.5	53.4	52.9

The next two contrasts were designed to test specific effects in female-biased lines: (a) for divergence of controls from both treatment groups (that is, C≠T +R; where we predicted female bias in C) and (b) for divergence among treatment groups (that is, T≠R).

All analyses were performed using Statistica version 7.1 (Statsoft, Tulsa, OK, USA). Means are reported with standard errors throughout, with sex ratio described as %females (that is, F/(M+F)) unless otherwise specified.

RESULTS

Incidence of offspring sex bias in Australia

Wild-caught females produced F1 offspring comprising 642 sons versus 807 (55.7%) daughters from 83 broods in 2008, and 783 sons versus 878 (52.9%) daughters from 103 broods in 2009 (Table 1). Overall, sex ratio exhibited a female bias in each year (exact binomial $P<0.0001$ (2008) and $P<0.05$ (2009)), which did not vary among years ($G_1=2.50$, $P=0.114$) or populations (that is, Cairns versus Port Douglas; $G_1=0.150$, $P=0.698$ (2008); $G_1=0.153$, $P=0.696$ (2009)).

Sex ratio varied significantly among F1 broods in both 2008 ($G_{82}=145.0$, $P<0.0001$) and 2009 ($G_{102}=176.3$, $P<0.0001$). The simultaneous test procedure identified five 2008 broods as significantly biased. These comprised between 79.2 and 90.0% daughters (mean = 85.0 ± 4.8%; Figure 3). Five biased broods were identified in 2009, of which four were female-biased (range = 78.8–100% daughters; mean = 87.1 ± 9.5%) and one comprised 92.3% sons (Figure 3). Only one of 186 F1 broods across the sample consisted entirely of daughters.

2008 Breeding experiment

In 2008, we reared offspring from 10 female-biased F1 lineages (Table 2). The overall F2 sex ratio deviated from unity (exact $P<0.0001$) and more greatly than evidenced for the broader F1 generation (that is, 65.6% versus 55.7% females; $G_1=5.90$, $P<0.05$). This supports a maternal component of inheritance because the nonrandom selection of F1 dams engendered a more strongly female-biased offspring generation. The degree of bias was however weaker than expressed across the 10 contributing F1 families (that is, the broods from which founding dams were selected; $G_1=5.17$, $P<0.05$). Although F2 family sizes precluded broader analysis, there was no categorical evidence for the production of 100% daughters. Sex

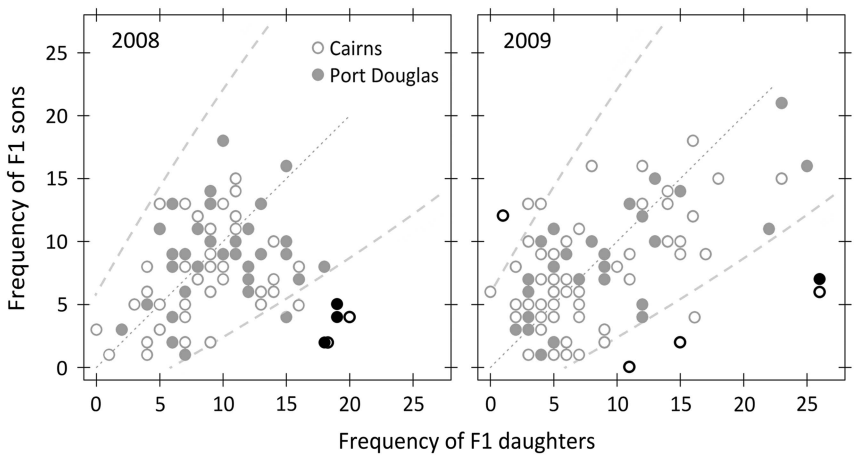

Figure 3 Frequencies of sons versus daughters among the broods of wild-caught females in 2008 and 2009. Each point represents a single F1 family. Points are presented in halftone except those determined as deviating significantly from 1:1 sex ratio (the dotted central line) according to the replicated goodness of fit G-test. The outer dashed lines indicate the 95% confidence bounds for binomially expected outcomes under a hypothesis of 1:1 sex ratio, which is a less statistically conservative criterion for detecting bias.

ratio was instead varied considerably, even within lineages (as shown notably by the two large broods in the 'P5' lineage that comprised 52% versus 92% daughters; Table 2).

We reared an F3 generation using dams from three female-biased families (tracing from three separate lineages) that were assigned across control (honey water) versus treatment (tetracycline) groups. General linear models analysis revealed that brood sex ratio was affected by feeding treatment ($F_{1,21}=26.2$, $P<0.001$), but did not differ among lineages ($F_{2,21}=0.235$, $P=0.793$). There was no significant interaction between treatment and lineage ($F_{2,21}=0.238$, $P=0.790$); hence, the effect of treatment was consistent across all lineages. In sum, control F3 offspring expressed a female bias (79 sons: 150 daughters; exact $P<0.0001$), whereas treatment offspring were male-biased (147 sons: 70 daughters overall; exact $P<0.0001$; Figure 4a). The absolute departure from 1:1 was indistinguishable among groups ($G_1=0.251$, $P=0.616$), which suggests that antibiotic treatment inverted an otherwise heritable female bias to an equivalent degree of son overproduction (Figure 4a).

Table 2 Sex ratios of F1 and F2 broods in the 10 lineages that were perpetuated for the 2008 experiment (labelled according to F0 dam ID)

Lineage ID	*F1 offspring M:F (%F)*	*F2 broods*		
		N	*M:F (%F)*	*Sum*
(a) Port Douglas population				
P5	**5:19 (79.2)[a]**	3	**2:24 (92.3)**	**14:36 (72.0)**
			9:10 (52.6)	
			3:2 (60.0)	
P14	4:19 (82.6)[a]	3	3:7 (70.0)	6:14 (70.0)
			1:6 (87.5)	
			2:1 (33.3)	
P16	**1:7 (87.5)**	2	**1:16 (94.1)**	**2:16 (88.9)**
			1:0 (0.0)	
P19	7:16 (69.6)	2	13:12 (48.0)	16:13 (44.8)
			3:1 (25.0)	
P21	4:15 (78.9)[b]	2	1:4 (80.0)	3:4 (57.1)
			2:0 (0.0)	
P24	**8:12 (60.0)**	1	**2:9 (81.8)**	**2:9 (81.8)**
P32	2:18 (90.0)[a]	2	2:3 (60.0)	3:4 (57.1)
			1:1 (50.0)	
(b) Cairns population				
C15	7:16 (69.6)	1	1:0 (0.0)	1:0 (0.0)
C25	4:20 (83.3)[a]	3	3:3 (50.0)	4:5 (55.5)
			0:2 (100)	
			1:0 (0.0)	
C20	6:14 (70.0)	1	4:4 (50.0)	4:4 (50.0)
Sum	48:156 (76.5)	20		55:105 (65.6)

Abbreviations: 'M' = male, 'F' = female. Lineages highlighted in bold type contributed to subsequent generations. For F1 broods.
[a]$P<0.05$ according to the simultaneous test procedure.
[b]$P<0.05$ according to the exact binomial test.

As in previous generations, there was no evidence for 100% sex-biased offspring. Treated F2 dams exclusively produced more sons than daughters, but brood sex ratios spanned a practical range of 20–45% females (Figure 5; see Table S1 for full details). Control offspring expressed a similar spread of female bias except for one brood in the 'P5' lineage comprising 30 sons versus 13 (30.2%) daughters. This contrasted notably against two clearly female-biased broods generated by control sisters (for example, 5:32 and 6:24 sons: daughters; Figure 5). Overall, control offspring exhibited a weaker female bias than their founding F2 families (that is, 65.5% versus 90.7% females; $G_1=15.8$, $P<0.0001$).

2009 Breeding experiment

In 2009, we reared an F2 generation using dams of 1:1 versus female-biased F1 pedigree that were allocated across control, tetracycline and rifampicin treatments. General linear models analysis indicated that F2 brood sex ratio did not vary according to pedigree ($F_{1,72}=1.53$, $P=0.220$), but there was an effect of treatment ($F_{2,72}=8.00$, $P<0.001$), and an effect of the interaction between pedigree and treatment ($F_{2,72}=3.30$, $P<0.05$; Figure 4b). The significant interaction effect revealed that sex ratio responded differently to antibiotic treatment in the female-biased versus the 1:1 pedigree. We further informed the nature of this effect via three planned contrasts. The first contrast revealed no effect of antibiotic treatment among broods of unbiased pedigree ($t=0.915$, $P=0.363$). Offspring sex ratio did not deviate from unity either within each experimental group or across all groups pooled (control: exact $P=0.177$; tetracycline: $P=0.384$; rifampicin: $P=0.630$; all groups: $P=0.976$). The remaining two contrasts dealt only with the female-biased pedigree, indicating that: (a) brood sex ratios in both treatment groups deviated from controls ($t=4.86$, $P<0.0001$) and (b) sex ratios were equivalent under tetracycline

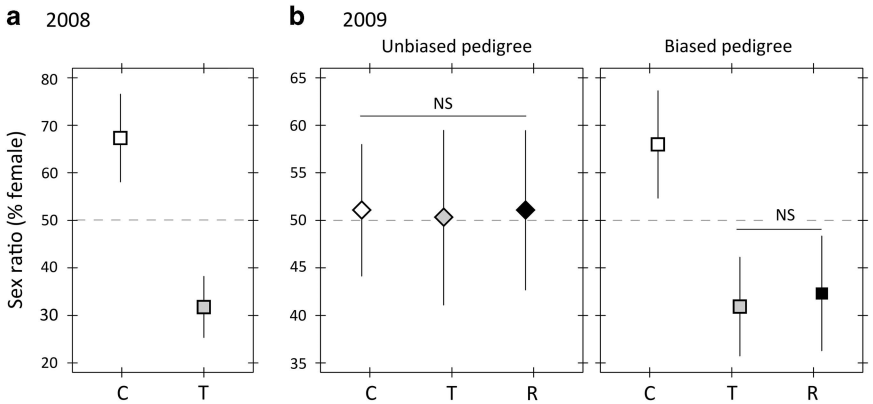

Figure 4 Summary of experimental antibiotic effects for (**a**) F3 offspring in 2008 and (**b**) F2 offspring in 2009. Dams of either female-biased (squares) or unbiased (diamonds) pedigree were assigned across control (C), tetracycline (T; halftone) and rifampicin (R; solid) treatment groups. Mean ± 95% confidence interval for sex ratio is indicated. Groups within panels differed significantly ($P<0.05$) except as shown for 2009. NS, not significant.

versus rifampicin treatment ($t=0.305$, $P=0.762$). As in 2008, control broods retained a female bias (exact $P<0.0001$), but antibiotic treatment induced male bias (tetracycline: exact $P<0.001$; rifampicin: $P<0.005$). Additional analyses conducted separately for each pedigree group indicated no significant variation among constituent lineages and no significant treatment × lineage interaction (details provided in Table S2).

F2 broods in this experiment again provided no indication for 100% sex biases (Figure 6; Tables S3–S4). Control broods of sex-biased pedigree (Figure 6a) also echoed earlier results by indicating a weaker female bias than their founding F1 families ($G_1=26.07$, $P<0.0001$).

Last, we performed limited crosses between control F2 individuals to inform the paternal inheritance of sex bias. Full siblings from four

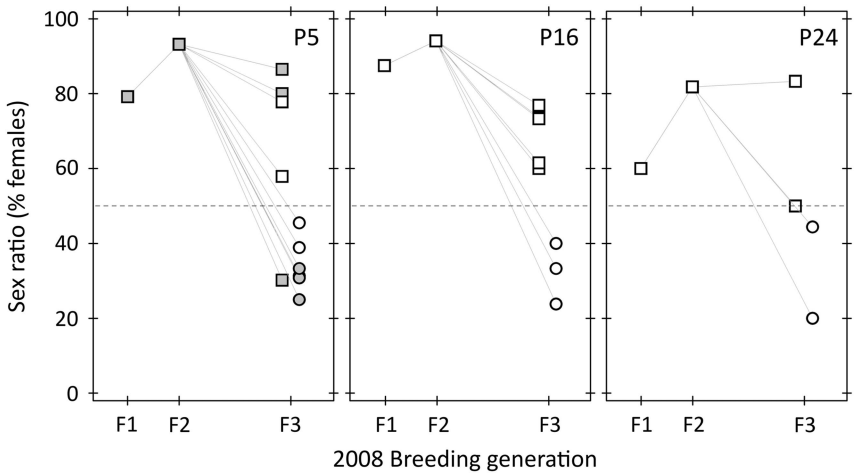

Figure 5 Brood offspring sex ratios across each of three lineages perpetuated in the 2008 experiment. Squares indicate broods derived from unmanipulated (honey water-fed) dams and circles indicate F3 broods derived from tetracycline-fed dams. Panels are labeled for matriline identity (and trace to Table 2). Shaded broods in matriline 'P5' contributed individuals for diagnostic *Wolbachia* PCR assays and sequencing.

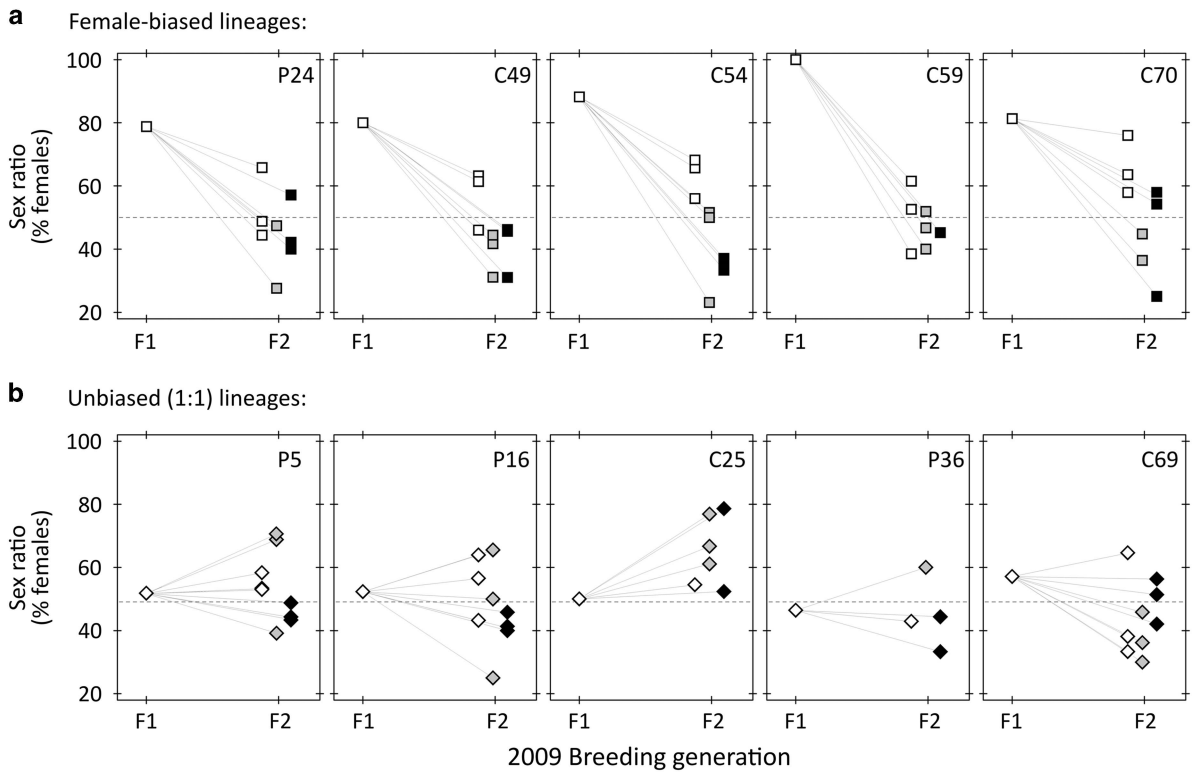

Figure 6 Brood sex ratios across the F1 and F2 generations for (**a**) female-biased and (**b**) unbiased lineages in the 2009 experiment (indicated as squares and diamonds, respectively). Symbols indicate broods from untreated (open), tetracycline-treated (shaded) and rifampicin-treated (solid) F1 dams. Panels are labeled according to matriline identity. Brood sex ratios are fully outlined in Tables S3–S4.

Table 3 Overview of F2 crosses performed in 2009

F2 cross details		*F3 Offspring*		
Parental lineages (total %F)	*Total broods*	*M:F (%F)*	*Deviation from unity (1:1)*	*Deviation from parental bias*
Biased sires (65.9)×1:1 dams (55.7)	5	21:17 (44.7)	$P=0.627$	$G_1=6.16$, $P<0.05$
1:1 sires (56.7)×biased dams (65.5)	6	19:28 (59.6)	$P=0.243$	$G_1=0.585$, $P=0.444$

Sex ratio, given as %females, is summed for the F2 families from which sires and dams were chosen, and also for F3 broods obtained from each group of crosses (a full breakdown of individual broods is provided by Supplementary Table S5). Deviation from unity (1:1) was assessed using exact binomial tests.

biased families were used as sires and dams in crosses with offspring from several unbiased lineages (as indicated by Figure 2c and detailed in Table S5). Overall, F3 broods obtained from sex-biased sire crosses comprised 44.7% daughters, whereas those obtained from sex-biased dam crosses comprised 59.6% daughters. Neither sex ratio deviated significantly from unity; however, sires but not dams failed to engender the strength of female bias expressed among founding F2 lineages (Table 3).

Molecular assays

In 2008, we screened for the presence of *Wolbachia* DNA among individuals in the 'P5' lineage according to the sampling scheme shown by Figure 5. We tested an F1 female, six of her F2 daughters (three control and three tetracycline-treated, post-oviposition), and five F3 male and female offspring per daughter. All 67 tested individuals were seen to amplify positively for *Wolbachia* DNA. This included the three control F2 daughters and their progeny, even despite highly divergent F3 brood sex ratios. More notably, this also encompassed tetracycline-treated F2 daughters and their progeny across three male-biased F3 broods (Figure 5). *Wolbachia* DNA was detected in all of 16 wild females sampled in 2009, which included the 5 dams that produced biased progeny, (Figure 3) as well as 11 that produced unbiased broods. Nine rifampicin-treated F2 females and four untreated F2 females of biased pedigree likewise tested positive. Strain-specific PCRs amplified positively for *w*CI, but never *w*Fem in all tested specimens across both years. Cloned 602 bp sequences of the *wsp* gene obtained from all 67 individuals in 2008 proved 100% identical to *w*CI.

Cytogenetic assays

In 2009, we examined over 60 male and female phenotypes from groups of 1:1, female-biased and male-biased F2 broods (the latter two groups arising from control versus antibiotic-treated lineages of sex-biased pedigree). In no case did we detect the W-chromatin characteristic of female karyotype. The assay however proved 100% accurate for sexual phenotype in *B. java* and *H. bolina* (Figure S2).

DISCUSSION

Eurema butterflies are an exemplar for genetic feminization, yet this paradigm is confined to a cluster of Japanese Islands (Narita *et al*., 2011). Here we reveal the incidence of sex distortion well beyond this biogeographic range. We show that a small fraction of Australian *E. hecabe* produce incompletely female-biased broods, a feature that is both maternally heritable and disrupted by antibiotic treatment. Although consistent with the action of *Wolbachia*, we only detected one (non-feminizing) Japanese strain. We also found no evidence of female (WZ) karyotypes in any individual, which is noteworthy, given the prominence of cytogenetic assays in supporting lepidopteran feminization. In sum, our findings implicate bacteria as an agent of sex bias, yet neither demonstrate nor discount the role of *Wolbachia*, and clearly depart from the Japanese paradigm.

Incidence of offspring sex bias

Studies of lepidopteran sex distortion have largely addressed male-killing effects, where female bias occurs at or close to 100% (for example, Hurst *et al*., 1999; Jiggins *et al*., 2000; Charlat *et al*., 2005). Complete brood biases have also been reported virtually without exception in feminized *Eurema* (Hiroki, 2002; Hiroki *et al*., 2004; Narita *et al*., 2011). Against this background, our findings are notable in revealing fractional degrees of daughter overproduction. This was evident among broods obtained from wild-caught females (Figure 3), as well as F2–F3 offspring across treatment groups (discussed below). The total observed incidence of all-female F1 broods (1/186) contrasts starkly with reports for feminized *E. mandarina* (3/14 and 2/10; Hiroki, 2002; Hiroki *et al*., 2004) and *E. hecabe* (3/15; Narita *et al*., 2011).

The continuous nature of offspring sex ratio posed important analytical consequences. Most broadly, it relegated judgements of bias to statistical inference, and thereby dependent upon sample size and subject to sampling variance. Power for concluding bias scaled with offspring number (and was effectively 0 for $n<10$). This implies the likelihood of imprecision in our assignment of biased F1 broods. Further, a reliance on statistical criteria inevitably conflates estimation of within-brood bias strength (that is, average effect size) with the population incidence of such broods. Whereas we conclude a conservative 5% incidence of ~85% female F1 broods, more relaxed statistical criteria identify nearly twice the number of slightly less-biased broods (indicated by Figure 3). Ultimately, this disposed a greater emphasis on analyses at lineage and pedigree/group levels.

The inheritance of bias

We perpetuated each years' pedigree by outcrossing females with wild-caught males, which prioritized the test of maternal inheritance. At the group level, biased lineages indicated daughter overproduction across all generations, whereas unbiased lineages engendered 1:1 offspring. This established the inheritance of sex bias through maternal lines, a critical assumption of endosymbiont causality (Werren, 2008; Cordaux *et al*., 2011). Although our design precluded a detailed assessment of paternal effects (discussed further below), several crosses in 2009 supported a stronger vector of maternal inheritance.

Although variation at brood levels offered a limited basis for (*a posteriori*) statistics, several points were notable. First, there was inconsistency among lineages in whether and how strongly F1 sex bias carried through to the F2 generation (for example, lines P19, P32 and C25; Table 2). This contrasts with the perfect inheritance of 100% bias reported for Japanese *E. hecabe* (Narita *et al*., 2011), but incomplete transmission of sex-distorting symbionts is not unusual for lepidopterans. Second, there was great disparity in brood sex ratio within biased lineages. This was clearly evident in the 2008 P5 lineage, where

daughters from an F1 brood of 5:19 (M:F) produced both unbiased (9:10) and biased (2:24) families, with F3 broods from the latter family ranging from female to male bias (5:32 and 6:24 vs 30:13; Figure 5). Hence, strong offspring bias in this lineage persisted alongside high inter-brood variation over two generations of inheritance. This could either reflect inconsistent transmission of a sex distorter or a variable efficacy of sex distortion depending upon host nuclear genome (*sensu*. Kageyama *et al*., 2009). The latter possibility includes a potential role of paternal genotype, which could be informed by pedigree-based approaches such as backcrossing.

Antibiotic inversion of brood sex ratio

Both experiments demonstrated antibiotic effects only in broods of sex-biased pedigree. At the group level, treated dams overproduced sons to a degree equivalent to the overproduction of daughters by untreated cousins. This implicates a causal role for intracellular bacteria, as opposed to protozoans, viruses or fungi (Kageyama *et al*., 2012). It also discounts causality based purely upon nuclear agents such as meiotic drivers (without excluding their interactive role; *sensu*. Kern *et al*., 2015). Here, we discuss treatment effects in relation to bacterial sex distortion, given that two candidate mechanisms for diplodiploid arthropods—male killing versus feminization—make fundamentally different predictions.

Under a simple male-killing scenario, antibiotic treatment is expected to restore the viability of sons and thereby 1:1 sex ratios in subsequent broods. This is supported by studies of Lepidoptera generally (for example, Hurst *et al*., 1999; Charlat *et al*., 2007a; Tabata *et al*., 2011) as well as one *w*CI-infected *Eurema* population (Narita *et al*., 2007b). Alternatively, treatment of hosts subject to genetic feminization should completely invert offspring sex ratio because such individuals possess karyotypes that are either genetically male (ZZ) or require *Wolbachia* to achieve female phenotypic development (Kern *et al*., 2015). All-male broods have been repeatedly induced in doubly infected Japanese lines (Hiroki, 2002; Narita *et al*., 2007c, 2011). Against this background, our findings are intriguing because rather than generating categorically 50 or 100% sons, antibiotic treatment instead shifted offspring phenotypes quantitatively in the direction of male bias. This was equivalent, albeit with high brood-level variation, across experiments and despite antibiotic treatments of varied known potency (Charlat *et al*., 2007a).

We consider two explanations for this finding. First, son overproduction in antibiotic lines may reveal an underlying signature of host counteradaptation. This could arise via adaptations in feminized matrilines to compensate for their overproduction of daughters. Second, as reported in *Ostrinia* (for example, Kageyama and Traut, 2004), selected matrilines of Australian *E. hecabe* may harbor a male-killing endosymbiont that is also essential for the development of viable female phenotypes. Antibiotic treatment of these lines would rescue sons yet kill daughters, thereby generating male bias. However, to explain our results, mortality would have to be lower than 100% both for sons in female-biased lines and then daughters under antibiotic treatment.

Ultimately, distinguishing between male killing and feminization will hinge upon whether phenotypes from biased lines actually possess W chromosomes. An absence of cytogenetically-visible heterochromatin is uncharacteristic for *Eurema* except in putatively feminized Japanese matrilines (Hiroki, 2002; Narita *et al*., 2011; Kern *et al*., 2015). Such phenotypes are thought to either possess a degraded version of the W chromosome (that is, a W'Z karyotype) or lack the chromosome altogether (that is, 0Z; Kern *et al*., 2015). Our findings raise the possibility for a broader loss/degradation of the W chromosome in Australian *E. hecabe*, which could result from the population-wide invasion of a feminizer (Cordaux *et al*., 2011). This is known for isopod populations, wherein sexual phenotype is entirely determined by the presence or dosage level of feminizing *Wolbachia* inherited by host individuals (Rigaud *et al*., 1997).

Potential causality of Australian sex bias

On the basis of the absence of *w*Fem, we conclude that sex bias in Australian *E. hecabe* does not arise simply as a geographic extension of Japanese feminization. The *Wolbachia* strain that we did identify (*w*CI) induces cytoplasmic incompatibility in *Eurema* and *Colias* (Narita *et al*., 2007c, 2009, 2011). This *wsp* sequence is also indistinguishable from a resident of *H. bolina* known formerly for varied degrees of male killing across the Western Pacific (Charlat *et al*., 2005). Notably, *w*CI has also been linked to incomplete male killing in one Japanese *Eurema* population (resulting in 69–89% daughters; Narita *et al*., 2007b). However, antibiotic treatment reverted sex ratio in such lines to 1:1, unlike the male-biased outcomes we observed, and W chromatin is clearly observable (Narita *et al*., 2007b).

The omnipresence of *w*CI across years regardless of variation in sex ratio argues generally against its sex-biasing role. In particular, this strain would neither be expected among parents and progeny of unbiased lines, nor to have persisted post-antibiotic treatment (given the effect of such treatment on sex ratio). The latter result may reflect incomplete curing, which is known for *Wolbachia*-infected lepidopterans, given one generation of adult tetracycline exposure (Kageyama *et al*., 2003; Charlat *et al*., 2007a). Yet under this scenario, we should have observed a qualitatively different effect of tetracycline versus rifampicin, either in the incidence of *w*CI or in offspring sex ratio (for example, Charlat *et al*., 2007a). One possibility is that the *wsp* sequence has become incorporated into the *E. hecabe* genome (*sensu*. Hotopp *et al*., 2007). This would explain why molecular assays proved universally positive, while simultaneously obscuring the actual incidence of *w*CI itself (if present) based on *wsp* PCR/sequencing. Beyond *w*CI, the candidates for sex bias range from novel strains of *Wolbachia* to additional sex distorters such as *Spiroplasma*, *Cardinium*, *Rickettsia* and *Flavobacteria* (Kageyama *et al*., 2012). More detailed molecular investigation will be necessary to assess the full complement and potential role of bacterial agents in Australian *E. hecabe*, and to further explore the possibility of lateral gene transfer.

CONCLUSION

Feminized Japanese *Eurema* emphasize a dichotomy of all-female versus 1:1 offspring, but our data indicate a skewed continuous distribution of sex ratio in Australia. We found an incidence of incompletely biased broods that persisted across generations and despite reversal by antibiotic treatment. Resolving the causal basis will require dedicated microbial study (as above) plus more detailed consideration of factors such as incomplete transmission, variable virulence and host nuclear effects (*sensu*. Kern *et al*., 2015). Heuristically, our data are novel for the study of arthropod sex distortion (as summarized comprehensively by Werren, 2008; Cordaux *et al*., 2011; Kageyama *et al*., 2012). The mechanisms and consequences of complete sex biases are well-documented, including their ability to drive females to near-fixation frequencies in lepidopteran populations (Charlat *et al*., 2005). More subtle degrees of sex distortion — as revealed in this study — are by definition less likely to draw attention, yet may exist more broadly than appreciated.

DATA ARCHIVING

These data have instead been placed in the Dryad repository. The DOI for this submission is: doi:10.5061/dryad.44t2n.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies this paper on Heredity website (http://www.nature.com/hdy)