Cross-Species Y Chromosome Function Between Malaria Vectors of the *Anopheles gambiae* Species Complex

Federica Bernardini,* Roberto Galizi,* Mariana Wunderlich,* Chrysanthi Taxiarchi,* Nace Kranjc,* Kyros Kyrou,* Andrew Hammond,* Tony Nolan,* Mara N. K. Lawniczak,† Philippos Aris Papathanos,‡ Andrea Crisanti,*,[1] and Nikolai Windbichler*,[1]

*Department of Life Sciences, Imperial College London, South Kensington Campus, SW7 2AZ, United Kingdom, †Malaria Programme, Wellcome Trust Sanger Institute, Hinxton, CB10 1SA Cambridge, United Kingdom, and ‡Section of Genomics and Genetics, Department of Experimental Medicine, University of Perugia, 06132, Italy

ORCID IDs: 0000-0001-7526-0155 (F.B.); 0000-0003-3134-7480 (R.G.); 0000-0002-8938-5335 (M.W.); 0000-0001-7720-8430 (N.K.); 0000-0002-4919-2459 (K.K.); 0000-0002-1757-5009 (A.H.); 0000-0002-2982-8333 (T.N.); 0000-0002-3006-2080 (M.N.L.); 0000-0001-8508-4554 (P.A.P.); 0000-0001-9896-1165 (N.W.)

ABSTRACT Y chromosome function, structure and evolution is poorly understood in many species, including the *Anopheles* genus of mosquitoes—an emerging model system for studying speciation that also represents the major vectors of malaria. While the Anopheline Y had previously been implicated in male mating behavior, recent data from the *Anopheles gambiae* complex suggests that, apart from the putative primary sex-determiner, no other genes are conserved on the Y. Studying the functional basis of the evolutionary divergence of the Y chromosome in the gambiae complex is complicated by complete F1 male hybrid sterility. Here, we used an F1 × F0 crossing scheme to overcome a severe bottleneck of male hybrid incompatibilities that enabled us to experimentally purify a genetically labeled *A. gambiae* Y chromosome in an *A. arabiensis* background. Whole genome sequencing (WGS) confirmed that the *A. gambiae* Y retained its original sequence content in the *A. arabiensis* genomic background. In contrast to comparable experiments in *Drosophila*, we find that the presence of a heterospecific Y chromosome has no significant effect on the expression of *A. arabiensis* genes, and transcriptional differences can be explained almost exclusively as a direct consequence of transcripts arising from sequence elements present on the *A. gambiae* Y chromosome itself. We find that Y hybrids show no obvious fertility defects, and no substantial reduction in male competitiveness. Our results demonstrate that, despite their radically different structure, Y chromosomes of these two species of the gambiae complex that diverged an estimated 1.85 MYA function interchangeably, thus indicating that the Y chromosome does not harbor loci contributing to hybrid incompatibility. Therefore, Y chromosome gene flow between members of the gambiae complex is possible even at their current level of divergence. Importantly, this also suggests that malaria control interventions based on sex-distorting Y drive would be transferable, whether intentionally or contingent, between the major malaria vector species.

KEYWORDS vector genetics; hybrid incompatibility; gene flow; Y chromosome; malaria

SEX chromosomes often play an important role in speciation, though the molecular factors that influence this process remains an area of active investigation (Ellegren 2011). Y chromosome sequence content in heterogametic animals is transmitted in a clonal manner due to the lack of crossing over with the X across some or all of its length. This absence of recombination promotes a progressive genetic degeneration including the accumulation and rapid turnover of repetitive sequences (Charlesworth 1991; Rice 1996; Charlesworth and Charlesworth 2000; Bachtrog 2013). It is generally thought that remaining Y-linked genes represent the remnants of an inexorable process of inactivation, degradation, and gene loss, and only genes with a selectable function, such as the male-determining factor, are likely to survive

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[1]Corresponding authors: Department of Life Sciences, Imperial College London, Imperial College Road, South Kensington Campus, SW7 2AZ, UK. E-mail: n.windbichler@imperial.ac.uk; and a.drcrisanti@imperial.ac.uk

on the Y chromosome. However, while this view appears to hold true for mammals, the birth of new genes on the Y has been described in *Drosophila*, and may even dominate the evolution of its Y chromosome (Vibranovski *et al.* 2008; Carvalho *et al.* 2015). This suggests an evolutionary dynamic that is particular to the Y of different phylogenetic groups.

Sex chromosomes play an important role in reproductive isolation; however, it is mostly the gene-rich X chromosome that has been implicated with three interrelated patterns of hybrid incompatibility: Haldane's rule, the large X-effect, and the asymmetry of hybrid viability and fertility in reciprocal crosses (Wu *et al.* 1996; Masly and Presgraves 2007; Turelli and Moyle 2007). In a few cases, a link between the Y and hybrid incompatibilities has been demonstrated. For example, it has been suggested that the Y chromosome contributes to reproductive barriers between rabbit subspecies (Geraldes *et al.* 2008). This can be explained by interactions involved in determining male dimorphism or fertility, between genes that diverged from allele pairs on the Y and X, and that break down in the heterospecific context. Such X–Y chromosome incompatibilities have been demonstrated to contribute to hybrid male sterility in house mice (Campbell and Nachman 2014). Additionally, the introgression of heterospecific Y chromosomes in *Drosophila* was found to affect male fertility, and alters the expression of 2–3% of all genes in hybrids (Sackton *et al.* 2011).

The varied picture of the biological role of Y emerging form work in mammals and *Drosophila* suggests the need for additional studies using other model systems. The *Anopheles* genus, which contains all human malaria-transmitting mosquito species, has received much attention in recent years, not only due to its stark medical importance but also as a model system for studying speciation and chromosome evolution. In particular the *Anopheles gambiae* species complex of eight sibling species, including the most widespread and potent vectors of malaria in sub-Saharan Africa, offers an excellent platform to further our understanding of the biology of the Y and its possible role in reproductive isolation.

To this end, we decided to focus on the two species with prime medical importance, *A. gambiae* and *A. arabiensis*, as they are the most anthropophilic members of the complex with the widest distributions. *A. gambiae* predominates in zones of forest and humid savannah, whereas *A. arabiensis* prevails in arid savannahs and steppes, including those of the South-Western part of the Arabian Peninsula. In the sympatric areas, changes in seasonal prevalence are observed showing an increase in the relative frequency of *A. arabiensis* during the dry season. In areas where the distribution of *A. gambiae* and *A. arabiensis* overlaps, hybrids are detected at extremely low frequency (0.02–0.76%) (Temu *et al.* 1997; Toure *et al.* 1998; Mawejje *et al.* 2013). However, a recent study conducted in Eastern Uganda to investigate hybridization between these species showed that 5% of the samples analyzed were hybrid generations beyond F1 (Weetman *et al.* 2014). F1 male sterility and other postzygotic isolation mechanisms have been studied in *A. gambiae* and *A. arabiensis* hybrids. In addition to mapping multiple loci contributing to male sterility, Slotman *et al.* (2004) demonstrated, by observing the absence of particular genotypes in backcross experiments, that inviability is caused by recessive factors on the X chromosome of *A. gambiae* incompatible with at least one factor on each autosome in *A. arabiensis*.

Y chromosomes of *A. gambiae* and *A. arabiensis* have recently been shown to differ dramatically (Hall *et al.* 2016). This study, which, to date, remains the only in depth analysis of Y chromosome content in *A. gambiae* and its sibling species, revealed that the Y chromosomes of *A. gambiae* and *A. arabiensis* differ dramatically both in the content and abundance of sequences on the Y chromosome. Despite using long sequence reads, a high quality assembly of the estimated ~26 Mbp Y chromosome (~10% of the genome) was not achieved, but the genic and repetitive content were sufficiently characterized to paint a picture of Y chromosome evolution and provide key data for the present study. Of the five genes that were confirmed on the Y chromosome of *A. gambiae*, only one, YG2, recently shown to act as a male-determining factor (Krzywinska *et al.* 2016), was found to be present on the Y chromosome of all species of the gambiae complex. YG1, whose function remains unknown, but which physically flanks YG2 and is homologous to it, is also shared between the Y chromosome of *A. gambiae* and *A. arabiensis*. Surprisingly, the most abundant *A. gambiae* Y chromosome sequences that represent 92% of its total sequence content, namely the satellite DNAs AgY477 and AgY373 and the Zanzibar transposon, which appears to have expanded through satellite-like processes, are not abundant on the Y chromosome of *A. arabiensis*, or are completely absent, further demonstrating the rapid turnover and expansions of sequences on these Y chromosomes. Overall previous studies suggest the rapid evolution of Y chromosome in this highly dynamic genus of malaria vectors.

In the present study, we wanted to establish whether the introgression of the *A. gambiae* Y chromosome into an *A. arabiensis* genetic background is possible when selected for in a controlled laboratory setting, whether the Y contributes to reproductive isolation, and whether a heterospecific Y, as has been reported in *Drosophila*, would markedly modulate gene expression patterns, fertility, or behavior of Y hybrid males. In addition to basic biological insights, our attempt to better understand the biology of the mosquito Y is key for both the development of male-specific traits for genetic control as well as predicting the behavior of such traits in the field.

Materials and Methods

Mosquito strains and rearing

Wild-type *A. gambiae* and *A. arabiensis* mosquitoes of strains G3 and Dongola, respectively, were used. Strain G3 was

originally isolated from West Africa (MacCarthy Island, The Gambia) in 1975, and obtained from the MR4 (MRA-112). It is considered a hybrid stock with mixed features derived from both *A. gambiae s.s.* and *Anopheles coluzzii*. The Dongola strain of *A. arabiensis* was obtained from the MR4 (MRA-856), and was originally isolated from Sudan. For the introgression experiments, we used two independently generated Y-linked *A. gambiae* transgenic strains G^{Y1} [referred to as YattP in Bernardini *et al.* (2014)] carrying a Pax-RFP marker and G^{Y2} (R.G., unpublished data). Strain G^{Y2} contains a Y linked insertion of construct pBac[3xP3-DsRed]β2-eGFP::I-PpoI-124L (Galizi *et al.* 2014) also carrying a Pax-RFP marker (no expression from the inactive β2-eGFP::I-PpoI-124L locus is detectable in this strain). Inverse PCR suggests position 17757 on the Y_unplaced collection as a likely insertion site of this construct; however, no assembly of the repetitive *A. gambiae* Y chromosome exists. Strain A^{Y2} contains the *A. gambiae* Y chromosome from strain G^{Y2} within an *A. arabiensis* background as described in the *Results* section. All mosquitoes were reared under standard condition at 28° and 80% relative humidity, with access to fish food as larvae and 5% (weight/volume) glucose solution as adults. For egg production, young adult mosquitoes (2–4 days after emergence) were allowed to mate for at least 6 days, and then fed on mice. Two days later, an egg bowl containing rearing water (dH_2O supplemented with 0.1% pure salt) was placed in the cage. One or 2 days after hatching, larvae were placed into trays containing rearing water. The protocols and procedures used in this study were approved by the Animal Ethics Committee of Imperial College in compliance with United Kingdom Home Office regulations.

Genetic crosses and fertility assays

Crosses were set up in BugDorm-1 cages with size 30 × 30 × 30 cm. Generally 100 female and 100 male mosquitoes were crossed during the introgression experiment, although the number of males varied after the F3 bottleneck and was dependent on the number of male progeny that could be recovered from the previous generation. To assay fertility of the Y-introgressed males, after 11 generation of backcrossing in cage, single crosses in cups were set up. Y-Introgressed males were singularly introduced into a cup together with one *A. arabiensis* female. In parallel, the same number of cups was set up for *A. arabiensis* males and females as a control. After 6 days of mating and blood feeding of females, eggs were collected from every cup, and the hatching rate (number of larvae/number of eggs) relative to every cross was calculated.

DNA sequencing

Samples for DNA sequencing were 10 adult male A^{Y2}, and two wild-type control *A. arabiensis* males were used individually for WGS. A^{Y2} males had been mated to *A. arabiensis* females prior to DNA extraction, allowing us to establish fertility for seven of the 10 A^{Y2} males. The DNA libraries were prepared in accordance with the Illumina Nextera DNA guide for Illumina Paired-End Indexed Sequencing. AMPure XP beads were used to purify the library DNA and for size selection, after which the resulting libraries were validated using the Agilent 2100 bioanalyzer and quantified using a Qubit 2.0 Fluorometer. Sequencing runs were performed on six lanes (two samples per lane) of an Illumina flowcell (v3) on the HiSeq1500 Illumina platform, using a 2 × 100 bp PE HiSeq Reagent Kit according to the manufacturer's recommendations. Raw reads were processed using FastQC (Andrews 2010, available online at: http://www.bioinformatics.babraham.ac.uk/projects/fastqc) and trimmomatic (Bolger *et al.* 2014).

Variant calling and read coverage analysis

Reads were aligned to the *A. gambiae* PEST reference genome assembly (AgamP4) using BWA mem (Li 2013, bio-bwa v0.7.5a) and sorted using Samtools (v1.2). We used the MarkDuplicates module from Picard tools (v1.9) to remove PCR duplicates and the genome analysis tool kit (GATK, v3.3) to realign reads around indels (McKenna *et al.* 2010). First we used the GATK modules HaplotypeCaller and UnifiedGenotyper to call raw SNPs and merged them across the 10 A^{Y2} and two A male samples using GenotypeGVCFs. Biallelic SNPs were selected using GATK VariantFiltration and SelectVariants modules following the GATK best practices guideline. For the coverage analysis we used bowtie (v1.1.1), exclusively reporting alignments for reads having only a single reportable alignment and displaying no mismatches. We used the bedtools (v2.25) makewindows tool to generate a sliding window bed file with 5 kb windows overlapping by 2.5 kb. We then used Samtools bedcov to generate per-window read counts, and calculate the group means for the seven fertile A^{Y2} and two A male control samples.

Analysis of Y signature elements

Paired WGS reads were mapped using Bowtie2 (Langmead and Salzberg 2012) with standard parameters (bowtie2 -x [consensus_build] -a -1 [x_R1] -2 [x_R2] -S [x.sam]) against a collection of consensus sequences of all known Y chromosome loci of *A. gambiae* (Hall *et al.* 2016). Read counts at every locus were generated with Samtools (Li *et al.* 2009), and normalized by library size and locus length in fragments per kilobase of transcript per million mapped reads (FPKM). WGS data in the form of paired Illumina reads corresponding to the control samples from *A. gambiae* males (NCBI SRA: SRR534285) and females (NCBI SRA:SRR534286) and *A. arabiensis* females (NCBI SRA: SRR1504792) were taken from the Hall *et al.* (2016) study. We also performed a separate analysis to evaluate Y chromosome satellite DNA abundance in the introgressed male samples, in part because satellites Ag53A, B and D, due to their short length, could not be appropriately assessed using Bowtie2. We used jellyfish (Marçais and Kingsford 2011) to generate unique 25mers (kmers of 25 bp long) from each of the six known

Y satellites consensus sequences (jellyfish count -C -m 25 [stDNA-locus-x.fasta] -o [output] -c 5 -s 1000000000 -t [cores]). Using the same approach, we then generated and counted unique 25mers from each of the aforementioned WGS samples (jellyfish count -C -L 5 -m 25 [x.fastq] -o [output] -c 5 -s 1000000000 -t [cores]), and assessed the abundance of each of the stDNA specific kmers within the sample-specific kmers list, resulting in a table providing abundance of each kmer in each sample (grouped by stDNA locus). Raw kmer counts were normalized by library size (sequencing depth), and, for the WGS generated in this study (control and introgressed males), we calculated the median abundance for each kmer in these two groups.

RNA and small RNA sequencing experiments

Samples for RNA sequencing were generated using the following experimental design. Four cages were set up with 40 *A. arabiensis* wild-type males and 40 wild-type females, and four cages with 40 A^{Y2} males and their nontransgenic sibling females. After mating and blood feeding, progeny was collected from the cages. 80 freshly hatched larvae were collected from each of both sets of cages and combined in a single tray for rearing. At the pupal stage, males were sexed and screened for the fluorescent marker linked to the Y chromosome. From every tray, 18 RFP positive and 18 RFP negative males were collected and placed in separate cages to allow emergence. Three days after emergence males were dissected in order to separate three different tissues the head, the abdominal segments harboring the reproductive tissues, and the remainder of the carcass. This experiment was performed twice, resulting in a total number of eight replicates for both controls and experimental samples. Libraries for total RNA sequencing were prepared using the TruSeq RNA sample preparation kit by Illumina, and sequenced on three lanes of an Illumina HiSequation 2500 using 2 × 100 paired end reads. The samples described above were also used for the construction of libraries for small RNA sequencing. Libraries were prepared using the NEBNext Multiplex Small RNA kit for Illumina and sequenced on three lanes of a MiSeq using the 1 × 42 single-read mode.

Differential expression analysis

Reads were aligned to the *A. arabiensis* genome supplemented with the 25 contigs corresponding to known *A. gambiae* Y loci (Hall *et al.* 2016) using HISAT2 (Pertea *et al.* 2016). We then used Stringtie (Pertea *et al.* 2016) in conjunction with the *Anopheles arabiensis* reference transcriptome version AaraD1.3 to predict novel genes and novel isoforms of known genes, which were merged across samples into a combined geneset using Cuffmerge (Ghosh and Chan 2016). Expression of all transcripts was then quantified using Stringtie -B. We used the Ballgown (Pertea *et al.* 2016) suite to determine transcripts showing significantly different expression levels using a cutoff of P-value <0.05 adjusted for multiple testing, a mean expression >1 FPKM across the samples in the tissue analyzed and a log2 fold-change >1 between the introgressed and control groups. To assign a repetitiveness score, the sequence of each transcript was blasted against the *A. gambiae* PEST RepeatMasker library provided by Vectorbase.org. For the small RNA dataset. we generated a count matrix using Seqbuster and SeqCluster suites (Pantano *et al.* 2011). and used DESeq2 (Love *et al.* 2014) for differential expression analysis and log2 transformation of the count data. Only putative small RNA loci with a mean expression of >5 counts across samples were taken into account for this analysis.

Competition experiments

Matings were performed in BugDorm-1 cages. Females and competing males were allocated in cage with a 1:1:1 ratio. Every experiment was run in triplicate. Crosses were set up as follows: 50 A females × 50 A males + 50 G^{Y2} males, 50 G females × 50 G males + 50 A^{Y2} males, 50 A females × 50 A males + 50 A^{Y2} males, 50 G females × 50 A males + 50 A^{Y2} males. After 6 days mating and blood feeding, females were collected from each experimental replicate and allowed to lay singularly in cups. The number of eggs and hatched larvae was calculated for every cup in order to estimate the hatching rate values. Progeny was screened for 3×P3 RFP to assess paternity, and transgene ratio was calculated in order to identify any occurring secondary mating.

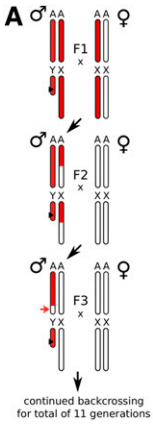
Data availability

All sequence data has been submitted to the National Center for Biotechnology Information (NCBI) short read archive, and are available under BioProject IDs PRJNA381402, PRJNA381403 and PRJNA381033. A detailed list of per-sample accession numbers is given in Supplemental Material, File S1.

Results

Experimental introgression of the A. gambiae Y chromosome into A. arabiensis

We have previously established a number of transgenic strains in which different fluorescent transgenes were inserted onto the *A. gambiae* Y chromosome (Bernardini *et al.* 2014; R.G., unpublished data). Limited recombination between the *A. gambiae* sex chromosomes has been suggested to occur in specific genetic backgrounds (Wilkins *et al.* 2007), but is generally believed to occur at very low frequencies (Mitchell and Seawright 1989; Bernardini *et al.* 2014; Hall *et al.* 2016). We concluded that, for our purpose, a Y-linked fluorescent transgene would be a reliable tool to track the Y chromosome throughout a multi-generational introgression experiment. It has previously been shown that F1 male hybrids between *A. gambiae* and *A. arabiensis* suffer from complete male sterility, which precludes Y chromosome introgression experiments in a straightforward manner. Our pilot experiments confirmed this finding (Table S1A). We employed an F1 × F0 crossing strategy (we define this as a scheme where



B

				strain G^{Y1} eggs	strain G^{Y1} progeny	strain G^{Y2} eggs	strain G^{Y2} progeny
	♂A	x	♀G		>2000		>2000
F1	♂G*	x	$♀H_{(A/G)}$		>2000		>2000
F2	♂H*	x	♀A		>2000		>2000
F3	♂H*	x	♀A	408	0	827	0
		x	$♀H_{(F2)}$	300	0	3347	0
		x	$♀H_{(F3)}$	3344	0	5210	0
	♂G	x	♀A		>2000		>2000
F1	♂G*	x	$♀H_{(G/A)}$		>2000		>2000
F2	♂H*	x	♀A		>2000		>2000
F3	♂H*	x	♀A	400	0	2095	**3**♂,4♀
		x	$♀H_{(F2)}$	6992	13♂,21♀	2795	0
		x	$♀H_{(F3)}$	2638	0	1420	7♂,8♀

Figure 1 F0 × F1 hybrid crossing scheme and resulting progeny. (A) Crossing scheme indicating the *A. gambiae* (red) or *A. arabiensis* (white) genomic contributions in generations F1–F3, with autosomes represented as a single pair labeled A. In the F1 cross, *A. gambiae* males of strains G^{Y1} or G^{Y2} are crossed to hybrid F1 females. Each generation, the resulting males are backcrossed to *A. arabiensis* wild-type females. In the bottleneck generation F3 hybrid males harbor an *A. arabiensis* X with a fraction (~25% on average) of autosomal regions expected to be homozygous for the *arabiensis* background (red arrow). (B) Observed number of eggs and progeny arising for each indicated cross where the F1 hybrid females had either an *A. gambiae* mother (top) or an *A. arabiensis* mother (bottom). The asterisk indicates strain G^{Y1} or G^{Y2}, respectively. In the bottleneck generation (F3), males were crossed to either pure species arabiensis females (♀A), or crossed to female hybrids with decreasing levels of *A. gambiae* genome content ($♀H_{(F2)}$, $♀H_{(F3)}$).

first generation female hybrids are crossed to pure species males) in which transgenic Y *gambiae* males were backcrossed to F1 hybrid females, which were in turn generated using either wild-type *gambiae* or *arabiensis* mothers (Figure 1A). We used two different transgenic Y strains, herein referred to as G^{Y1} and G^{Y2}, that both express an RFP reporter gene driven by the neuronal 3×P3 promoter from different insertion sites on the Y chromosome. In the F2 cross, the hybrid males containing the labeled Y are crossed again to wild-type *arabiensis* females, and then third generation hybrid males are crossed to both hybrid females and wild-type *arabiensis* females in the attempt to recover offspring. Using this crossing scheme, we encountered a severe bottleneck at generation F3 when males are predicted to have inherited a predominantly *A. gambiae* autosomal genome from their fathers in conjunction with pure *A. arabiensis* genome (including the X chromosome) from their mothers (Figure 1A). We recovered no larvae from >13,000 eggs when backcrossing of either G^{Y1} or G^{Y2} males was carried on from F1 hybrid females originated from *A. gambiae* mothers. In the reverse cross, using F1 hybrid females from *A. arabiensis* mothers, we managed to recover seven larvae (three males and four females) with strain G^{Y2} in a direct cross to wild-type *A. arabiensis* females (Figure 1B). These three hybrid males obtained were used to progress the introgression, and we continuously maintained backcross purification by crossing hybrid males recovered each generation to wild-type *arabiensis* females for a total of 11 generations to establish the introgressed strains A^{Y2} used for all subsequent experiments in this study. The occurrence of fertile phenotypes in these crosses is a rare event. In additional experiments where either G^{Y1} or G^{Y2} males were crossed to F2 or F3 hybrid females (Figure 1B) the majority of the resulting male progeny (15 larvae, of which seven were male, and 34 larvae, of which 13 were male) failed to develop into fertile adults.

Fertility of males carrying a heterospecific Y chromosome

We first asked whether A^{Y2} males showed reduced levels of fertility when compared to wild-type *A. arabiensis* males due to the presence of the heterospecific Y chromosome. In order to assay individual males, rather than a population average, we performed single-copula mating experiments of strain A^{Y2} or wild-type males mated to *A. arabiensis* females, measuring the mating rates, and oviposition and egg hatching rates of single females. Every generation, the males of the largest family were used for establishing the next round of single-copula backcrosses, and, over the course of seven generations, a total of 344 wild-type *A. arabiensis* and 350 A^{Y2} males were assayed. The rationale for this design was to exclude the possibility of *A. gambiae* fertility loci having been retained by selection in introgressed males, because such loci would be expected to segregate in this design. Figure 2 shows a summary of these experiments. Single copula matings are inefficient, in fact <25% of females would mate and oviposit under these condition (Figure 2A). No significant difference in the rate of mating was observed between A^{Y2} (22%) and control *A. arabiensis* males (15.3%). Power analysis suggested that the sample size of the successfully mated males would allow for the reliable detection of an effect of medium size ($P = 0.79$ for a two-sided t-test, P-value $= 0.05$, $d = 0.5$). We observed that females mated to A^{Y2} males and females mated to *A. arabiensis* wild-type males laid a comparable numbers of eggs (Figure 2B) that had comparable hatching rates (Figure 2C). This analysis indicates that, under laboratory conditions, and, in the absence of mate choice and male–male competition, and taking into account the above considerations on power, A^{Y2} males show no significant difference in fertility when compared to wild-type *A. arabiensis* males that retain their native Y chromosome. As an additional control, we back-crossed A^{Y2} males to *A. gambiae* females. Despite the presence of the *A. gambiae* Y chromosome in these males, we expected this experiment to recreate hybrid incompatibility in the form of male infertility in the resulting progeny. Indeed, we found hybrid males to be fully sterile (Table S1B), thus confirming that X-A incompatibilities are sufficient to explain this phenotype (Slotman *et al.* 2004).

Genomic analysis of males with a heterospecific Y chromosome

After $n = 11$ generations of backcrossing, assuming no selection for sections of the *A. gambiae* genome, the expected

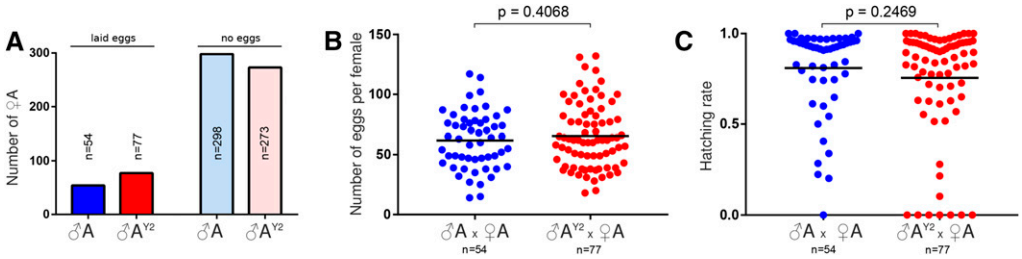


Figure 2 Single copula mating experiments. (A) Number of females ovipositing following single matings with A or A^{Y2} males. Counts of eggs (B) and hatching larvae (C) for each individual family.

autosomal genome proportion of the *A. gambiae* donor would be $1/2^n$ or <0.05%. Given that *gambiae* genomic regions contributing to hybrid incompatibilities would be selected against in males, this is likely an underestimation due to such detrimental haplotypes being selectively removed. To confirm that our backcrossing scheme had eliminated the *A. gambiae* autosomal genome but retained the *A. gambiae* Y chromosome, we performed DNA WGS from 10 A^{Y2} males and two wild-type control males of our *A. arabiensis* laboratory colony. To determine whether introgressed males were fertile, they were singularly mated to wild-type arabiensis females before their genomic DNA was extracted. WGS reads were mapped to the *A. gambiae* genome to identify genomic regions with fixed allele differences between introgressed and control groups by confining our analysis to biallelic SNPs. No assembly of the *A. gambiae* Y exists; however, the PEST assembly includes the Y_unplaced sequence collection that includes ~230 kb of unscaffolded contigs that have been assigned to Y. Our analysis (Table 1) showed that the autosomes of introgressed-Y and pure species contained a small number of differentially represented SNPs comparable in number to the X chromosome, which, since it is replaced in every backcross generation, serves as a background control. In contrast, the majority of differentially represented SNPs (74.7% of the total number of differential fixed SNP and 35.6% of the total number of SNPs on the Y) arose from reads mapping to the Y_unplaced portion of the *A. gambiae* genome that represents <0.1% of the total genome assembly. Within the Y_unplaced collection, we found that most SNPs mapped to the largest contig, which also contains the male-determine gene (Figure S1). In addition 12.7% of the total number of SNPs arose from the UNKN collection (unassigned contigs) that is also expected to contain a number of unassigned Y sequences and repetitive elements. We performed an additional sliding-window analysis, where we considered only reads mapping uniquely to the *A. gambiae* genome and allowed for no mismatches. The rationale was that, given the observed levels of divergence between these genomes, perfectly matching reads are expected to predominantly map to the genome of origin. When comparing the mean number of reads of A^{Y2} fertile introgressed males and the samples of the *A. arabiensis* control group, we find that the Y_unplaced collection experiences significant coverage only in A^{Y2} males, as do parts of the UNKN collection. For the autosomes and the X, few windows accrue a significant number of reads, and we find no substantial differences between the groups in the direction of A^{Y2}, with the possible exception of an intergenic region on chromosome 2L (Figure S2). This suggests that, apart from the Y, both groups have a similar *A. arabiensis* background, and we concluded that the transgenic *A. gambiae* Y had been successfully purified in an *A. arabiensis* genomic background, although our data cannot rule out that some fraction of *A. gambiae* genomic DNA other than the Y chromosome persists in the A^{Y2} strain.

Analysis of Y chromosome sequence content in introgressed males

To assess whether introgression of the *A. gambiae* Y chromosome into the *A. arabiensis* genome coincided with any detectable structural rearrangements of the Y, for example, the selective elimination of sequences that would be detrimental in hybrids, we mapped DNA reads from all A^{Y2} individuals as well as pooled control datasets from Hall *et al.* (2016) against a collection of known Y chromosome genes and repeats of *A. gambiae*. This collection includes consensus sequences of all known and putative genes and repetitive elements, as well as satellite DNA. We assessed normalized read depth at each locus (FPKM) as a proxy for copy number of each sequence element in a given background. This analysis is complicated by the occurrence of autosomal copies of many of these elements, as well as possible variation between Y chromosome isolates. Figure 3 shows normalized read counts for these elements in strain A^{Y2} plotted *vs.* males and females of both *A. gambiae* and *A. arabiensis*. We observe an excellent correlation in the representation of these Y signature sequence elements between *A. gambiae* males and A^{Y2} males (Figure 3A). Because these signature elements are derived from, and, to some degree, specific to the *A. gambiae* Y chromosome, they were under-represented in *A. arabiensis* control males and in females of both species. Interestingly, the AgY280 satellite shows an ~15× higher coverage in *A. gambiae* males compared to the A^{Y2} strain. However this satellite is known to be highly variable between Y isolates (Hall *et al.* 2016). In order to better assess the content of DNA satellites, in particular satellites Ag53A, B, and C, which are too short for standard read mapping, we performed an additional analysis based on kmer counts in the read libraries. This analysis showed that A^{Y2} males resemble *A. gambiae* males with regards to the content of DNA satellites attributed to the Y (Figure S3). Together, these analyses therefore confirm the presence of the *A. gambiae* Y chromosome in the introgressed

Table 1 Analysis of single nucleotide polymorphisms between A and A^{Y2} males

Chromosome	Base pairs	Total number of variants	Biallelic SNPs without missing data	Differentially fixed SNPs	
				Number	%
X	24,393,108	1,040,639	583,061	27	0.0046
2L	49,364,325	2,180,463	1,464,616	42	0.0029
2R	61,545,105	2,233,941	1,573,367	12	0.0008
3L	41,963,435	1,702,574	1,159,627	27	0.0023
3R	53,200,684	2,480,254	1,653,367	6	0.0004
Mt	15,363	23	21	0	0
Unknown	42,389,979	749,098	351,870	115	0.0327
Y_unplaced	237,045	4,180	1,902	677	35.5941

strain, and suggest that the introgression experiment did not impact in any evident way the original content of this Y chromosome.

Transcriptomic analysis of males carrying a heterospecific Y chromosome

We next asked to what extent the presence of a heterospecific Y chromosome would alter the expression of autosomal or X-linked genes of the *A. arabiensis* background. This could occur by (i) the action of *gambiae* Y *trans*-acting factors or absent *A. arabiensis* Y *trans*-acting factors, (ii) by Y sequences that recruit cellular *trans*-acting factors and/or modulate the chromatin structure and epigenetic state of other chromosomes, or (iii) by the activation of mobile genetic elements present on the *A. gambiae* Y that trigger a cellular response in X or autosomal sequences. We focused our analysis on three tissues, the head, the terminal abdominal segments containing the reproductive tissues and the remainder of the carcass. We performed RNA sequencing of a total of eight control and eight experimental samples for each tissue from A^{Y2} and wild-type males that had been reared in the same larval tray and that were sexed and separated at the pupal stage. Paired-end reads were mapped against the 1214 *A. arabiensis* genomic scaffolds supplemented by 25 consensus sequences corresponding to known *A. gambiae* Y loci previously mentioned, as well as the reporter gene construct. Due to the incomplete annotation of the *A. arabiensis* genome we performed an isoform level analysis, where we first predicted novel genes and novel isoforms of known genes across all samples. Gene-level expression of the experimental groups as well as sample relationships are summarized in Figure S4 and File S1. Finally, in order to indicate whether differentially expressed transcripts potentially represented known mobile elements or repetitive DNA arising from the Y (but matching paralogous sequences present on the *A. arabiensis* scaffolds, which could thus be misreported as differentially expressed) we blasted each predicted transcript to the *A. gambiae* repeat library and assigned a repetitiveness score. We then predicted differential expression of transcripts between A^{Y2} and wild-type males (Figure 4 and File S2). Few transcripts were expressed significantly lower in A^{Y2} males. This is partially expected because *A. arabiensis* genomic scaffolds are derived from the DNA of females (Neafsey *et al.* 2015), and it is thus not possible to identify any *A. arabiensis* Y-linked genes that would have fallen into this class. However, it also indicates that few, if any, endogenous genes are downregulated as a result of the presence of the heterospecific Y chromosome. In contrast, a number of transcripts displayed significantly higher levels of expression in A^{Y2} males. However, the majority of these correspond to the known *A. gambiae* Y loci (purple triangles in Figure 4). The expression of these genes, not present on the *A. arabiensis* Y, and hence absent from *A. arabiensis* wild-type males, is also summarized in Figure S5. Of the remaining upregulated *A. arabiensis* transcripts (colored circles in Figure 4) the majority had a high repetitiveness score *i.e.*, significant homology to *A. gambiae* repeats. A manual homology search using all *A. arabiensis* transcript sequences passing the significance threshold and cut-off for expression confirmed that virtually all differentially expressed transcripts are related to repetitive DNA. In addition to the above analysis, we also measured small RNA expression in these tissues, finding no evidence for the differential expression of small noncoding RNAs between A^{Y2} and wild-type males (Figure S6). While it is possible that such effects could occur in other developmental stages or under specific environmental conditions, we find little evidence that the heterospecific Y-chromosome markedly affects expression of the nonrepetitive, autosomal, or X-linked gene repertoire of the *A. arabiensis* genome.

Female mate-choice and male competition experiments

Although we find no strong effect of the heterospecific Y chromosome on the transcriptome and fertility of individual A^{Y2} males, it is possible that Y-linked sequences do play a role in male fitness or female choice that would also limit the practical use of introgressed Y-linked traits *e.g.*, for vector control. In order to test this hypothesis, we set up a panel of competitive mating experiments, where two strains of males were allowed to compete for mating with females in population cages (Figure 5). Both wild-type *A. arabiensis* and A^{Y2} males performed substantially worse (winning only 13.7 and 11.1% of matings, respectively) than *A. gambiae*

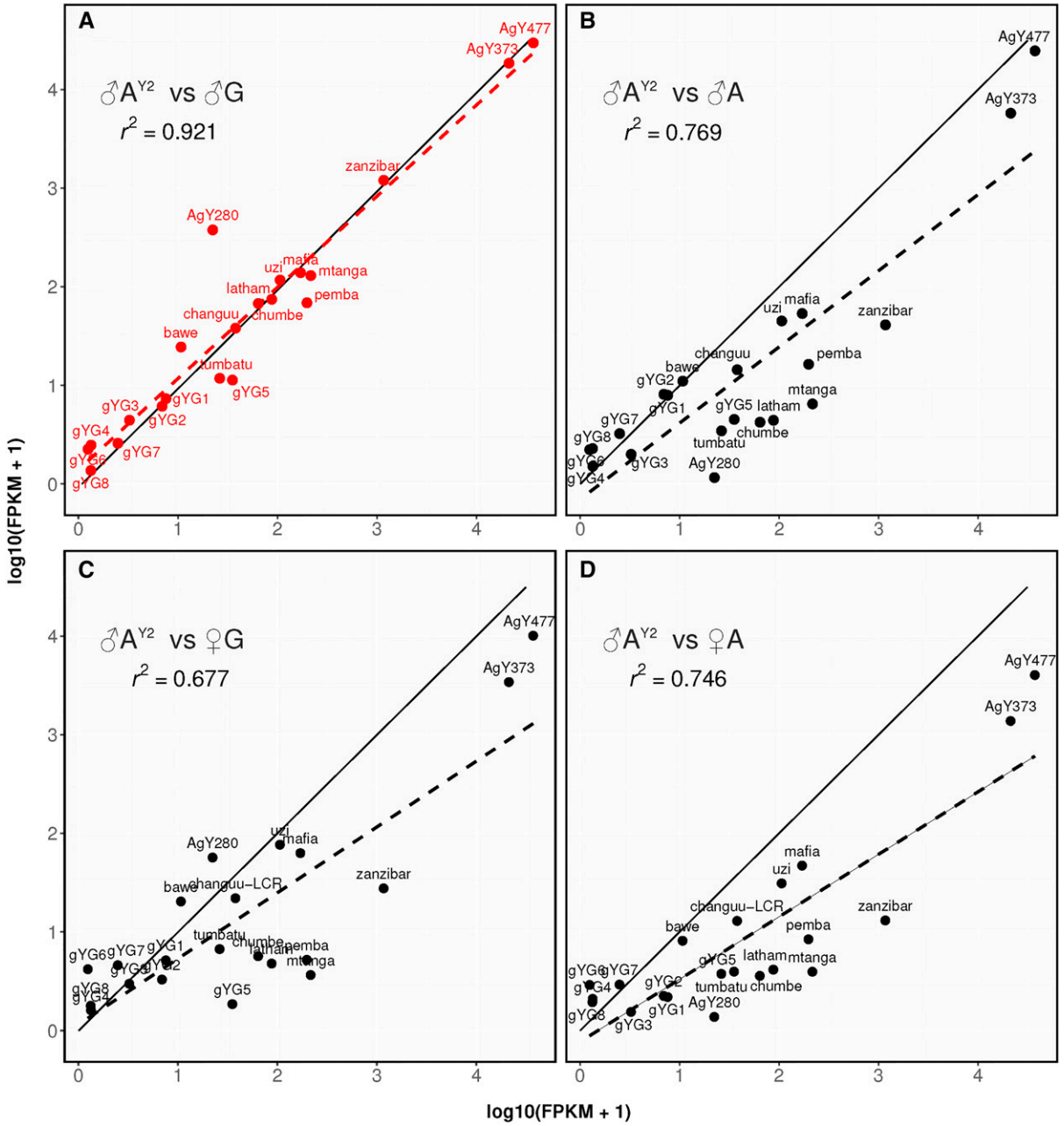


Figure 3 Analysis of the content of the introgressed Y chromosome. The plots show the number of normalized reads mapping to the *A. gambiae* Y chromosome reference loci calculated as $\log_{10}$ transformed FPKM values for A^{Y2} males on the *x*-axis compared to either wild-type *A. gambiae males* (A), wild-type *A. arabiensis* males (B), *A. gambiae* females (C) and wild-type *A. arabiensis* females (D) on the *y*-axis. The dashed linear regression line and associated r^2 coefficient indicate the best correlation in read counts of signature Y elements between A^{Y2} and *A. gambiae males*.

males in competition experiments for either *A. gambiae* or *A. arabiensis* females. These findings confirms previous observations (Schneider *et al.* 2000), and demonstrate that, irrespective of the female type, *gambiae* males are superior to *A. arabiensis* males in the laboratory setting. In contrast, A^{Y2} males were only slightly less competitive compared to wild type *arabiensis* males, winning ~40% of matings with *arabiensis* females ($P = 0.0146$), and no significant difference was observed when they competed against arabiensis males for *gambiae* females ($P = 0.156$). The particular set up of the experiments also allowed to score for secondary mating measured by the percentage of transgenic male progeny. With the possible exception of one case, remating (showing a significant deviation from a 50% transgene ratio in the progeny) was not observed in these experiments. Overall, a slight reduction in male competitiveness was observed which could relate, in addition to the effect of the heterospecific Y, to inbreeding or fitness costs associated with expression of the fluorescent marker gene. Importantly, in all cases no significant

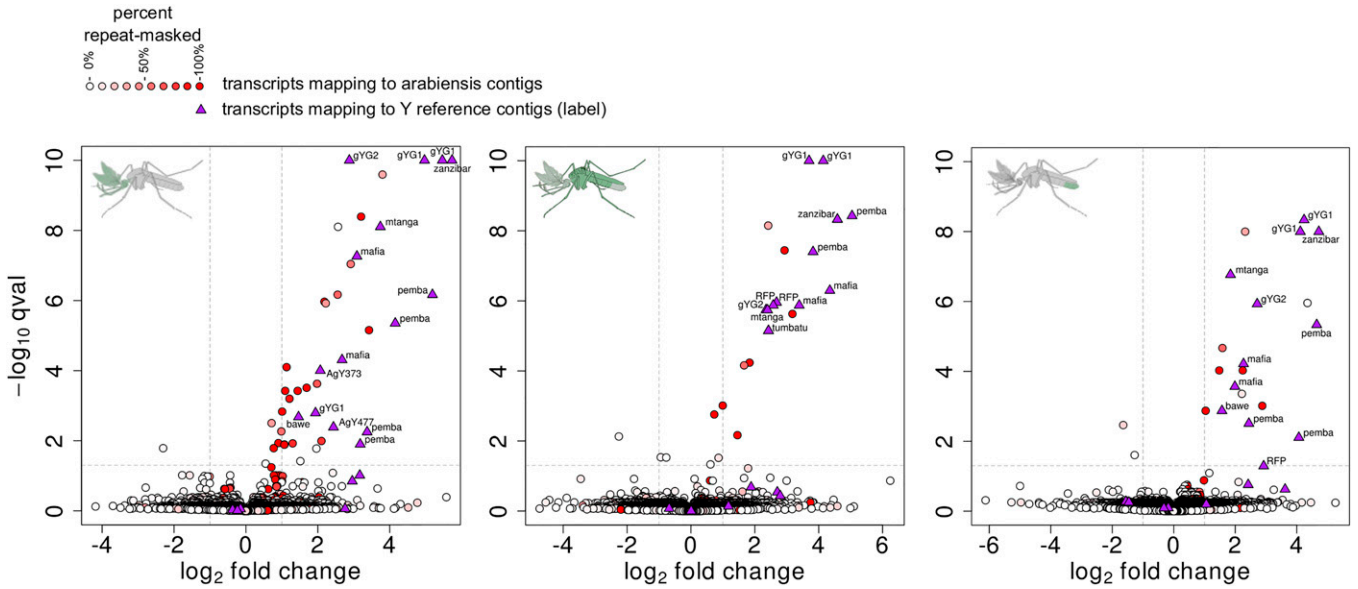


Figure 4 Differential expression analysis by RNA-seq. Volcano plots showing $\log_2$ fold-change values (*x*-axis) by $-\log_{10}$ corrected *P*-values (*y*-axis) for all transcripts between introgressed A^{Y2} males and *A. arabiensis* control males. The analysis was performed separately for the head (left panel), the carcass (middle panel), and the abdominal segments containing the reproductive tract (right panel). Transcripts derived from *A. arabiensis* scaffolds are represented as circles, and colored based on the percentage of their sequence masked by sequences in the *A. gambiae* repeats library. Transcripts from the reference set of *A. gambiae* Y loci are indicated by purple triangles and the name of the signature locus. Dashed lines represent the thresholds used for adjusted *P*-value ($P < 0.05$) and $\log_2$ fold change (>1.0).

difference to the wild-type *A. arabiensis* males was observed in terms of the number of laid and hatching eggs from matings with A^{Y2} males, confirming our previous analysis.

Discussion

In a classic study, Slotman *et al.* (2004) mapped quantitative trait loci related to male sterility in hybrids between *A. gambiae* and *A. arabiensis*, and at least five or six sterility factors were detected in each of the two species. The X chromosome was found to have a disproportionately large effect on male hybrid sterility (Slotman *et al.* 2004), which is likely related to divergent alleles present within multiple fixed chromosomal rearrangements on the X. A possible role of the Y chromosome in hybrid incompatibilities was suggested by the authors but not followed up on experimentally. Using an F1 × F0 crossing scheme, we generated F3 males with an *arabiensis* X chromosome, and a set of *arabiensis* autosomes, as well as an *A. gambiae* Y chromosome. The second set of autosomes is expected to contain, on average, 75% *A. gambiae* sequences. The majority of such F3 males were expected to be sterile; however, we hypothesized that it should be possible to select a small fraction of fertile males that lacked the *A. gambiae* incompatibility loci that cause sterility when interacting with the *A. arabiensis* background. Indeed, we recovered 56 larvae out of a total 29,776 eggs laid (0.18%) from pooled backcrosses of ~600 F3 males sampled.

Surprisingly, after multiple generations of backcross purification, we found that the *A. gambiae* Y chromosome does not markedly influence male fertility, fitness, or gene expression in Y hybrids. This rules out the Y chromosome as a major factor contributing to hybrid incompatibilities, and, more importantly, it is in stark contrast to findings in the *Drosophila* model (Sackton *et al.* 2011). Despite the relative paucity of genes in the fly Y, even intraspecific Y chromosome variants profoundly affect the expression a substantial number of genes located on the X or the autosomes. The introgression of heterospecific Y chromosomes in *Drosophila*, to the extent that it is even possible (Johnson *et al.* 1993), has consequently been found to markedly affect male fertility and gene expression in interspecific hybrids. In a *D. simulans* background, the *D. sechellia* Y has little effect on viability, but it reduced male fecundity by 63% as well as sperm competitiveness (*D. simulans/sechellia* divergence time has been estimated at only 0.25 MY). Y introgression differentially affected genes involved in immune function and spermatogenesis, suggesting a trade-off in investment between these processes. However, it has also been suggested that a significant part of the observed effect in *Drosophila* may be attributed to the ribosomal DNA (rDNA) clusters that are abundant on both the X and the Y in the fly. Paredes *et al.* (2011) have shown that deletions within the Y-linked rDNA arrays of *Drosophila* result in the differential expression of hundreds to thousands of unlinked genes due to a decreased heterochromatic composition of the genome. The affected genes significantly overlap with those affected by natural polymorphisms on Y chromosomes, suggesting that rDNA copy number variation is an important determinant of gene expression diversity in natural populations, and contributes to biologically

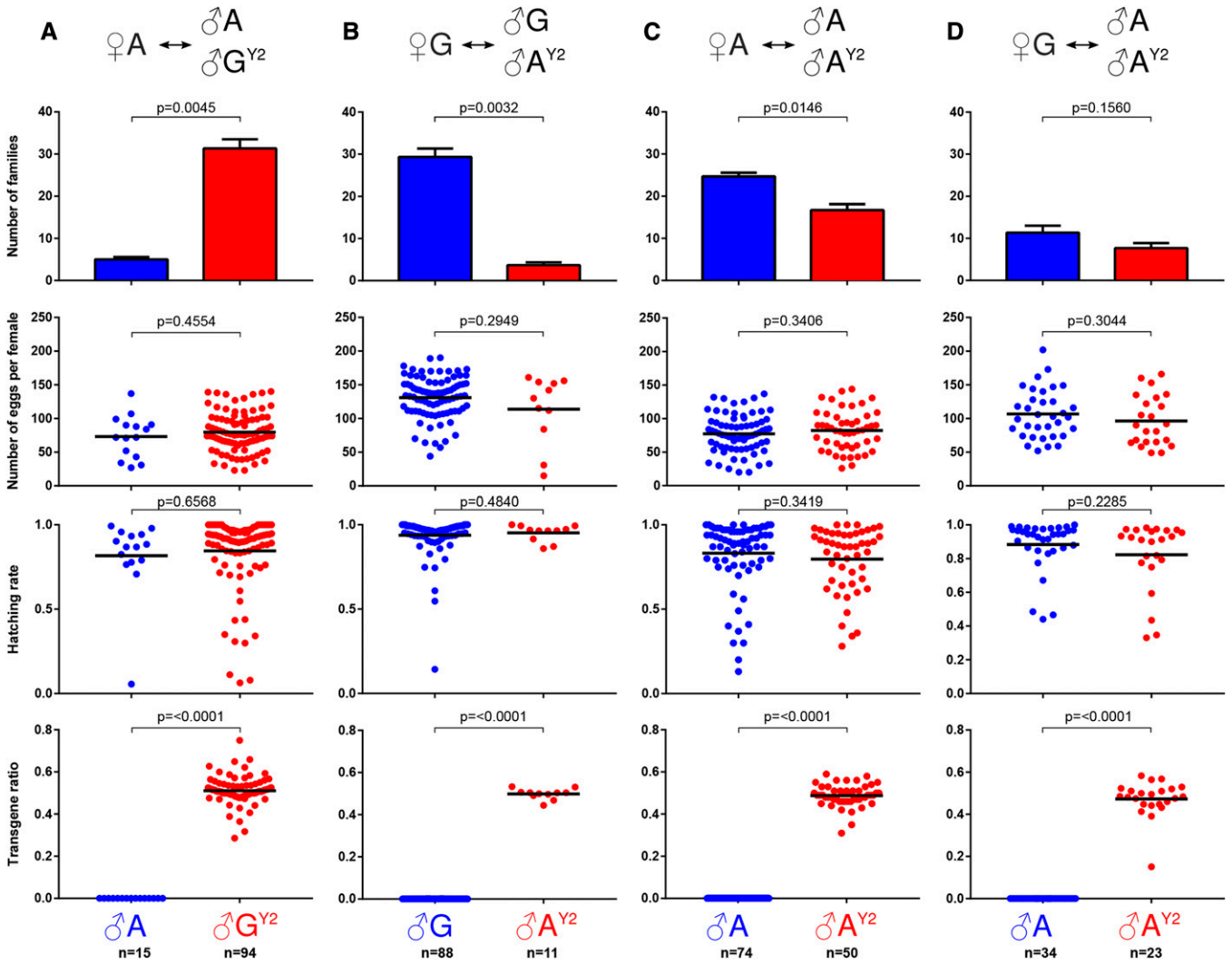


Figure 5 Mating competition experiments. The genotypes of the females and the two types of competing males is indicated at the top for each experiment (A–D). The second, third, and fourth row of panels show the number of eggs laid by individual females, the hatching rate for each family, as well as the ratio of transgenic to wild-type larvae respectively. *P*-values were calculated using Welch's two-tailed *t*-test.

relevant phenotypic variation (Paredes and Maggert 2009; Paredes *et al.* 2011). Importantly, in the *A. gambiae* strain G3 we used for this study [unlike some strains such as those used by Wilkins *et al.* (2007)] and in *A. arabiensis*, the rDNA is located exclusively on the X-chromosome, and thus does not confound introgression experiments the same way. Our results may thus be more indicative than similar experiments in *Drosophila* of the true effect a gene-poor heterospecific Y exerts on the genome of a related species.

It is possible that more subtle effects of the introgressed Y, not detectable in our experimental setup, exist. Future work could involve hybrid performance testing in mating swarms or under semi-field conditions. However, the fact that, despite its radically different structure and an estimated divergence time of ~1.85 MY or >7 times that between *D. simulans* and *D. sechellia* (Fontaine *et al.* 2015), the *A. gambiae* Y seems to be able to fully replace the *A. arabiensis* Y, suggests that, in Anopheles, the Y either carries no functionally important factors or that such factors have not undergone functional divergence between these two species. Although, early work had implicated the Anopheline Y chromosome in mating behavior in a study using *A. labranchiae* and *A. atroparvus* species (Fraccaro *et al.* 1977), our work is thus more in line with a recent study that leveraged long single-molecule sequencing to determine the content and structure of the Y chromosome of the primary African malaria mosquito, *A. gambiae* in comparison to its sibling species in the gambiae complex (Hall *et al.* 2016).

The role of gene flow between *A. gambiae* and *A. arabiensis* in leading to adaptive introgression, and the implications for vector control has been highlighted by Weetman *et al.* (2014). Post-F1 gene flow occurs between *A. gambiae* and *A. arabiensis*, and, especially for traits under strong selection, could readily lead to adaptive introgression of genetic variants relevant for vector control. Introgression of the Y chromosome between species is generally viewed as unlikely, and

markers found on the Y generally show restricted gene flow relative to other loci. However, this contrasts with the recent hypothesis (Neafsey *et al.* 2015; Hall *et al.* 2016) of Y gene flow between *A. gambiae* and *A. arabiensis*. Contrary to the established species branching order, the YG2 gene tree suggested that Y chromosome sequences may have crossed species boundaries between *A. gambiae* and *A. arabiensis* (Hall *et al.* 2016) and the authors suggested that Y chromosome introgression could have predated the development of male F1 hybrid sterility barriers that exist between this pair of species (Hall *et al.* 2016). Our study lends support to this hypothesis as it experimentally demonstrates the possibility of a cross-species Y chromosome transfer, and shows that the Y is functional in this context.

Our findings thus also suggest that Y-linked genetic traits generated in *A. gambiae* could be transferred to its sister species. For example, strain A^{Y1} carries a site-specific docking site that now also allows the generation of male-exclusive genetic traits in *A. arabiensis*. Recent progress toward the elusive goal of efficient sex-ratio distortion by a driving Y chromosome (Bernardini *et al.* 2014; Galizi *et al.* 2014) could lead to the development of invasive distorter traits in *A. gambiae* that may then be transferred to sibling species. This could be done deliberately in the laboratory as we have demonstrated here, but it could also occur contingently in the wild after a large-scale release of transgenic males. While one should always be wary extrapolating laboratory studies to conditions in the field, the notion that the Y chromosome does not represent a genetic barrier for gene flow between two members of the *A. gambiae* species complex (*A. gambiae* and *A. arabiensis*) should inform the design and implementation of genetic control interventions based on transgenic mosquitoes.

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