Sexual dimorphism and sex-biased gene expression in an egg parasitoid species, Anastatus disparis

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Abstract

Background Differences in the expression of genes present in both sexes are assumed to contribute to sex differences including behavioural, physiological and morphological dimorphisms. For enriching our knowledge of gender differences in an important egg parasitoid wasp, Anastatus disparis (Hymenoptera: Eupelmidae), sex-biased differences in gene expression were investigated using Illumina-based transcriptomic analysis. Results A total of 67201 resulting unigenes were annotated, including 4206 genes differentially expressed (i.e., sex-biased genes) between females and males including 243 specific genes; the majority of the sex-biased genes (63.72%) were female-biased. Sexually dimorphic traits related to flyability and longevity were discussed at the level of gene expression, improving our understanding of those biological traits. Besides, Gene Ontology (GO) enrichment analyses showed that the functional categories in sex-biased genes were mainly related to reproduction. Conclusions Overall, this study provides comprehensive insight into the sexually dimorphic traits of parasitoid wasps, offering a basis for future research to better understand the molecular mechanisms underlying such traits and improve the application of these insects to the biological control of pests.

Background

Sex differences, including behavioural [1], physiological [2], and morphological dimorphisms [3], occur in a broad range of animal and plant species. It is often assumed that most of these phenotypic differences are mediated by differences in the expression of genes present in both sexes [4][5]. Genes that are expressed in both sexes but at a higher level in one sex have been termed sex-biased genes, which can be further separated into male-biased and female-biased genes, depending on which sex shows higher expression [6]. This differential gene expression may involve a significant proportion of the genome[7][8][9][10]. For example, when whole adult females and males of *Drosophila melanogaster* are compared, up to 57% of their genes show sex-biased expression [8] . In addition, research in *Drosophila* reveals that the genes expressed differentially tend to be male biased [11]. However, studies in *Tribolium castaneum* and *Anopheles gambiae* [12][13], have shown that many of the sex-biased genes in these species are female biased. Overall, male-biased genes evolve more rapidly than female-biased genes [14][15][16][17], indicating that males experience stronger positive selection than females [4][5]. Sex-biased gene expression has been documented in a range of different species, including brown algae [18], birds [19][20], nematodes [21], *Daphnia pulex* [10], cichlid fishes [22], guppies [23], moths [24], the pea aphid [25], and multiple insect species [8][26][27][28][29][30][31]. Studies such as those listed above provide a comprehensive overview of sex-biased gene expression in a broad range of species and offer more useful information to determine sexual dimorphism.

Haplodiploid parasitoid species are important insects that parasitize other organisms [32] and have been extensively applied to reduce the population size of pest species [33][34][35][36][37][38]. Sex determination in hymenopteran species is haplodiploid, that males develop from unfertilized eggs and are haploid, while females develop from fertilized eggs and are diploid [39]. Therefore, in haplodiploid species, females and males are nearly identical genetically [6], that most differences between females

and males are due to gene dosage. However, in most species, the male and female genomes differ by genes located on sex-specific chromosomes (such as the Y chromosome of mammals) [4][5].

Anastatus disparis (Hymenoptera: Eupelmidae) is an egg parasitoid species, which can parasitize the eggs of several noxious species of Lepidoptera, including *Lymantria dispar*, *Antheraea pernyi*, *Odonestis pruni*, and *Actias selene ningpoana* [40][41]. It has been considered a potential biological control agent of the gypsy moth in North America [40][42]. Sexual dimorphisms related to behaviour, physiology, and morphology in *A. disparis* have been extensively described. First, although wasp body size is correlated with host quality [41][43], the body size of females is always significantly larger than that of males. Second, females can live more than one month in the field and lay hundreds of eggs over their lifetime, while males live for only approximately 5-7 days and exhibit frequent and extreme fighting behaviour to acquire mating opportunities [40][43][44]. Third, females do not fly but jump very quickly, while males can fly short distances [42][44]. This study attempted to provide comprehensive insight into the sexually dimorphic traits of parasitoid wasps at the transcriptome level to improve understanding of their biological traits and thus allow better application of parasitoids to the biological control of pest species.

Methods

Sample preparation

Anastatus disparis colonies were first established from a population reared on *Lymantria dispar* egg masses collected in the wild and subsequently maintained on *Antheraea pernyi* eggs [40][41]. Most *A. disparis* adults emerge daily in the morning, especially from 9:00 a.m. to 10:00 a.m., and we collected them during this period.

RNA extraction

Each sex had three repetitions respectively containing 15 adults, which were snap frozen in liquid nitrogen. TRIzol reagent (Invitrogen, USA) was used for extracting RNA from each sample group following the manufacturer's protocol. Non-denaturing agarose gel electrophoresis and a Nanodrop (Thermo Scientific Nanodrop 2000, USA) were used to assess the quality and quantity of the isolated RNA, respectively. The A260/280 values were all above 2.0, and electrophoresis of the RNA samples demonstrated that the 28S and 18S rRNA were intact.

Transcriptome sequencing and read assembly

3 μg of total RNA from each sample was converted into cDNA using the NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA). For resulting in raw reads, cDNA libraries were tagged with different adapters and then sequenced on the Illumina HiSeq 2000 platform by Beijing Biomarker Technologies Co., Ltd,. Then, reads containing adapter, poly-N reads and low-quality reads were removed from the raw data by FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/) yielding clean reads.

Then, all the high-quality reads from the six samples were pooled and assembled using Trinity software (v2.5.1) with the default parameters [67]. We chose the longest isoform of each gene to construct the unigene set. After the isoforms were selected, these assembled transcripts were predicted to be the unigenes produced. Bowtie was used to align the reads to the unigenes [68].

Gene expressed and functional annotation

Using our assembled transcriptome as a reference, we identified putative genes expressed in males and females by RSEM [69] using the reads per kb per million reads (RPKM) method. Functional annotation was performed by sequence similarity searching using blastx against 5 public databases, Clusters of Orthologous Groups (COG), Swiss-Prot, NCBI non-redundant protein sequences (nr), KEGG Ortholog database (KO) and GO, with an *E*-value cutoff of 10^{-5}.

Differentially expressed genes and enriched analyses

Differentially expressed genes (DEGs) were identified using the DESeq2 package (v1.6.3) in R, and RSEM reads were incorporated into DESeq2 using tximport [70]. Genes with at least 2-fold expression changes and a false discovery rate [FDR]<0.01 as found by the DESeq R package (1.10.1) were considered differentially expressed. The GOseq R package [71] was used to determine the statistical enrichment of DEGs in the GO subcategories, and an adjusted Q-value < 0.05 was chosen as the significance cutoff.

Quantitative real-time polymerase chain reaction

For checking the DEGs in transcriptomic analyses, the expressions of the selected genes were evaluated by quantitative real time polymerase chain reaction (qRT-PCR). Following abovementioned protocols, RNA from each sample group was extracted, and the concentration was measured. Then, PrimeScript RT Reagent Kit (TaKaRa; Japan) was used to synthesize the first-strand cDNA using 0.5 mg of total RNA as a template . The resultant cDNA was diluted to 0.1 mg/ml for further qRT-PCR analysis (ABI StepOne Plus; USA) using SYBR Green Real-Time PCR Master Mix (TaKaRa; Japan). Primers for the selected genes (**Table 7**) were designed using Primer Express 2.0 software. Reaction mixture contained 0.4 μL of each primer (10 μmol/μL), 10 μL of 2×SYBR Green Master Mix, and 2 μL of cDNA template to a final volume of 20 μL by adding water. The cycling parameters were 95 °C for 30 s followed by 40 cycles of 95 °C for 5 s and 62 °C for 34 s. For checking nonspecific product amplification, reaction was ended with a melting curve analysis (65 °C to 95 °C in increments of 0.5 °C every 5 s). Relative gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method, which the housekeeping gene of translation elongation factor 1-α (EF1A) was used as a reference to eliminate sample-to-sample variations in the initial cDNA samples.

Longevity assay

For conducting the longevity assays, males and female (1 day old) were selected at 10:00 a.m. Each male was isolated individually in a cylindrical box (height: 5 cm, diameter: 10 cm) and received honey

water daily (honey: water = 2:3 vol/vol). Each male was inspected twice daily, at 10 a.m. and 10 p.m., and the date of death of each male was recorded.

Statistical analysis

Prior to analysis, the raw data were tested for normality and homogeneity of variances using Kolmogorov-Smirnov and Levene's tests, respectively, and the data were transformed where necessary. The qRT-PCR data comparing gene expression in male and female were analysed with an independent t-test. Survival analysis was applied to analyse the difference of sex on longevity. All statistical analyses were performed using SPSS software (version 20).

Results And Discussion

Transcriptome sequencing and read assembly

We constructed 6 cDNA libraries derived from three *A. disparis* female and male adult samples. Approximately 8.6 Gb of paired-end reads were produced for each RNAseq sample. After removing reads containing adapter sequences, poly-N reads and low-quality reads from the raw data, approximately 7.24 Gb of clean reads were obtained from each sample. The Q30 percentage was higher than 88.72% in each sample, showing that the sequencing of each sample was of high quality. All high-quality reads from the six samples were pooled and assembled using Trinity with the default parameters. A total of 338,400 transcripts with lengths longer than 200 bp were generated. In total, 43.96% of the transcripts were longer than 500 bp in length, and the N50 size was 3,533 bp. Then, these assembled transcripts were predicted to be produced from a total of 225,389 unigenes. The N50 size of the unigenes was approximately 715 bp, and their mean length was 570.38 bp (**Table 1**).

Functional annotation

For annotation, the mapped unigenes were searched using blastx against 5 public databases with an *E*-value cutoff of 10^{-5}. A total of 67205 unigenes were successfully annotated, as shown in **Table 2**, including 23642 genes in GO, 18222 genes in COG, 51186 genes in nr, 25021 genes in Swiss-Prot, and 20966 genes in KEGG. In the GO analysis, 18222 unigenes were successfully annotated and classified into three major GO categories: Molecular Function (MF), Cell Component (CC) and Biological Process (BP). The dominant subcategories for the classified genes were catalytic activity and helicase activity for the MF category, nucleoplasm part and nuclear pore for the CC category, and tRNA aminoacylation for protein translation for the BP category.

Sex-biased genes

Using our assembled transcriptome as a reference, we identified putative genes expressed in males and females using the reads per kb per million reads (RPKM) method. Genes with at least 2-fold expression changes and false discovery rate (FDR) < 0.01 were defined as differentially expressed genes (DEGs). By

comparing female to male transcriptomes, 4206 DEGs in *A. disparis* were found (**Table 2**). Genes that were expressed in both sexes but at a higher level in one sex were termed sex-biased genes, which could be further separated into male-biased and female-biased genes, depending on which sex showed higher expression [6]. Consistent with previous results in *T. castaneum* and *A. gambiae* [12][13], many of the annotated sex-biased genes in our study were female-biased; respectively, 2680 and 1526 were female- and male-biased genes.

Sex-biased differences in flyability

Transcriptome data suggested that a fact that males can fly short distances, while females do not fly [42] [40][44]. The protein-coding genes *vestigial* (**Figure 1a,** c42845.graph_c0; qRT-PCR: t=-4.799, df=4, p<0.05) and *nubbin* (**Figure 1b,** c72605.graph_c2; qRT-PCR: t=-4.507, df=4, p<0.05) were highly expressed in males; both have been identified as important for the physical development of wings [45]. Simultaneously, the highly expressed genes coding *trehalase* (involved in the conversion of trehalose to glucose [46]) (**Figure 1c**, c23296.graph_c0; qRT-PCR: t=-3.169, df=4, p<0.05) and *seryl-tRNA synthetase* (involved in tRNA metabolic processes [47]) (**Figure 1d,** c66701.graph_c0; qRT-PCR: t=3.396, df=5, p<0.05) in females have been demonstrated to be related to flightless morphs.

Sex-biased differences in longevity

Consistent with many other parasitoid species, the lifespan of females is longer than that of males rearing indoor (**Figure 2a**, *Wilcoxon*=34.646, df=1, p<0.001). Furthermore, *A. disparis* females usually survive more than one month, but males live only approximately a week in wild [40]. A superoxide dismutase (SOD) (**Figure 2b**, c61117.graph_c0; qRT-PCR: t=2.694, df=6, p<0.05), which has been hypothesized to play a key role in ageing and is associated with longevity in *Drosophila* [48], was annotated and upregulated in *A. dispais* females. In addition, we also found that 2 genes (**Figure 2c&2d**, c67249.graph_c0: qRT-PCR, t=3.955, df=6, p<0.05; c68140.graph_c0: qRT-PCR, t=2.831, df=4, p<0.05) encoding *Vitellogenin*, which is a yolk protein believed to play an important role in egg production, was upregulated in females [49]. In addition, *vitellogenin* also plays an important role in antioxidant function related to queen longevity and is strikingly upregulated in the queens of diverse eusocial insect taxa [50] [51][52][53].

Male-biased genes

In the GO enrichment analyses, 11 subcategories were enriched in male-biased genes (**Table 3)**, which may be mainly related to male reproductive traits, such as sperm. Their enrichment in subcategories (transport GO:0006810; transmembrane transport GO:0055085; transporter activity GO:0005215) involving genes encoding transport proteins are consistent with the motile nature of sperm [11]. The high over-representation of gene products associated with membranes (integral component of membrane GO:0016021; membrane GO:0016020; Golgi cisterna membrane GO:0032580) is likely due to the requirements of the sperm axoneme structure [11]. In addition, the enriched subcategories (oxidation-reduction process GO:0055114; oxidoreductase activity GO:0016491) included many annotated genes

involved in oxidation, which in males are needed to provide an environment supportive of sperm viability and motility and counter the oxidative effects of sperm catabolism [54][55].

Female-biased genes

In the GO enrichment analyses, 21 subcategories were enriched in female-biased genes, as shown in **Table 4**. As previous studies have reported in flies, mosquitoes and *Daphnia* [8][9][10], many subcategories of BP were assigned to genes encoding proteins involved in ribosomal function, translation initiation, and DNA replication, which are probably expressed to produce eggs in females [11] [56][57]. Genes encoding translational initiation complexes and ribosomal content are highly expressed in females and are associated with maternal loading into the egg. Indeed, mutations in many ribosomal protein genes result in female sterility due to defective egg formation [57]. The over-representation of transcripts from genes required for DNA replication may be required for nurse cell polyploidization or for the rapid division of embryonic cells, which rely on maternally deposited gene products [11][56].

Sex-specific genes

Among the genes with sex-biased expression, some are expressed exclusively in one sex, and we term this phenomenon sex-specific expression [6]. In most species, the male and female genomes differ by genes located on sex-specific chromosomes (such as the Y chromosome of mammals) [4][5]. Because sex determination in hymenopteran species is haplodiploid, females and males are nearly identical genetically [6]. However, a total of 243 sex-specific genes were found in our species (**Table 2**), accounting for 5.78% of the sex-biased genes, and 211 and 32 were female- and male-specific genes, respectively. Unquestionably, many sex-specific genes are related to reproductive traits, for example, male-specific genes encoding ejaculatory bulb-specific protein (c52066.graph_c0), which are specifically expressed in the ejaculatory bulb and seminal fluid, and female-specific genes encoding S phase kinase-associated proteins (c67498.graph_c0), which are related to oocyte meiosis (**Table 5**).

These sex-specific genes included additional useful candidate genes for further study. For example, an annotated *toll* gene was specifically expressed in females; this gene is important during embryogenesis to establish the dorsal-ventral axis and is required for innate immunity in both mammals and invertebrates [58][59]. As parasitoids can propagate on or in other arthropods, parasitoid species can be applied to reduce pest species population sizes. The venom of parasitoid wasps, which is injected into a host by a female wasp before or at oviposition, is important for the successful development of the progeny. Parasitoid venoms have diverse physiological effects on their hosts, including developmental arrest, alteration in growth and physiology, suppression of immune responses, induction of paralysis, oncosis or apoptosis, and alteration of host behaviour [60][61][62][63]. The transcriptome data showed that 6 female-specific genes annotated as venom proteins (see details in **Table 6**) were related to known insect venoms from *Nasonia vitripennis* belonging to previously known insect venom families, such as serine proteases and endonucleases [64][65]. Despite the large diversity of parasitoid wasp species, only a small number of venom proteins have been described from wasps. A wealth of unexplored biomolecules is present in parasitoid venoms; these proteins are of value in basic

evolutionary studies, venom biology, host-parasite interactions, and the study of the evolution of life strategies, and they may potentially contain components that could be used in biological control and pharmacology [66]. Thus, the female-specific genes annotated for venom proteins may be candidate genes for further study.

Conclusions

In this study, we studied sex differences at the transcriptional level in an egg parasitoid species, *A. disparis*, by carrying out an Illumina-based transcriptomic analysis, which revealed a large set of genes showing sex-biased expression, including a few sex-specific genes. The majority of sexually dimorphic traits are assumed to arise from differences in the expression of genes present in both sexes [4][5]. This study provides comprehensive insight into the sexually dimorphic traits of parasitoid wasps (e.g., flyability, longevity) at the transcriptome level to improve understanding of those biological traits. In addition, this study also provides a large amount of useful information and candidate genes for future research to better understand the molecular mechanisms underlying biological traits. For example, venom proteins are of value in many aspects of biology and could be used in biological control and pharmacology.

Abbreviations

Gene Ontology (GO)

Molecular Function (MF)

Cell Component (CC)

Biological Process (BP)

reads per kb per million reads (RPKM)

false discovery rate (FDR)

Differentially expressed genes (DEGs)

quantitative real time polymerase chain reaction (qRT-PCR)

translation elongation factor 1-α (EF1A)

Declarations

Ethical approval and consent to participate

For this type of study formal consent is not required. This article does not contain any studies with human participants or animals performed by any of the authors.

Consent for publication

Not applicable.

Availability of data and materials

The data sets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Conflicts of Interest

The authors declare that they have no conflict of interest.

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Contributions

PCL conceived and performed the experiments, and wrote the paper. HYH analysed the data, and JRW provided the experimental material. PCL and DJH reviewed the manuscript. All authors read and approved the final manuscript.

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Tables

Table 1. Statistics of transcriptome assembly and predicted unigenes

Length Range	Transcripts	Unigenes
200-300 bp	107,627(31.80%)	96,604(42.86%)
300-500 bp	82,020(24.24%)	69,412(30.80%)
500-1000 bp	55,088(16.28%)	37,422(16.60%)
1000-2000 bp	33,674(9.95%)	13,172(5.84%)
>2000 bp	59,991(17.73%)	8,779(3.90%)
Total Number	338,400	225,389
Size of data (bp)	425,770,709	128,558,149
N50 length (bp)	3,533	715
Mean length (bp)	1258.19	570.38

Table 2. Functional annotation of assembled unigenes, sex biased genes and sex specific genes

Annotation database	Annotated unigenes	Number of sex biased genes	Number of sex specific genes
COG	18222	1259	14
GO	23642	1598	28
KEGG	20966	1445	24
Swiss-Prot	25021	2164	57
Nr	51186	3275	110
Total	67205	4206	243

Table 3. Male-based genes in enrich GO subcategories

Ontology	Item	Adjusted *p*
Molecular Function	oxidoreductase activity	0.000331
	transporter activity	0.007652
	alpha-1,4-glucosidase activity	0.025888
	sequence-specific DNA binding transcription factor activity	0.032643
Cellular Component	integral component of membrane	0
	Membrane	4.49E-05
	Golgi cisterna membrane	0.039127
Biological Process	Transport	0.00012
	G-protein coupled receptor signaling pathway	0.006367
	transmembrane transport	0.011418
	oxidation-reduction process	0.019337

Table 4. Female-based genes in enrich GO subcategories

Ontology	Item	Adjusted *p*
Molecular Function	DNA binding	5.83E-08
	nucleic acid binding	7.06E-05
	translation initiation factor activity	0.00266
	exonuclease activity	0.003988
	RNA polymerase II transcription cofactor activity	0.013837
	structural constituent of ribosome	0.028774
	zinc ion binding	0.031526
Cellular Component	Nucleus	1.39E-07
	mediator complex	0.017532
Biological Process	negative regulation of protein import into nucleus	1.13E-06
	negative regulation of smoothened signaling pathway	5.18E-06
	positive regulation of apoptotic process	8.18E-06
	regulation of proteolysis	8.18E-06
	positive regulation of JNK cascade	1.26E-05
	establishment of ommatidial planar polarity	4.15E-05
	Cellularization	0.000899
	cell division	0.003532
	DNA duplex unwinding	0.004103
	DNA replication initiation	0.016483
	transcription, DNA-templated	0.023857
	fucose metabolic process	0.039563

Table 5. Sex-specific genes in enrich GO subcategories

	Ontology	Item	Adjusted *p*
Female-specific	Molecular Function	ribosomal protein S6 kinase activity	0.049
	Biological Process	negative regulation of protein import into nucleus	0.003
		negative regulation of smoothened signaling pathway	0.004
		positive regulation of apoptotic process	0.005
		regulation of proteolysis	0.005
		positive regulation of JNK cascade	0.005
		establishment of ommatidial planar polarity	0.006
		Cellularization	0.011
		Digestion	0.023
		collagen catabolic process	0.045
		positive regulation of macroautophagy	0.045
Male-specific	Biological Process	developmental process	0.005
		single-organism process	0.009

Table 6. Female-specific genes involving encoding venom proteins

ID	FDR	log_2FC	nr_annotation
c63272.graph_c0	7.29E-15	9.031764	venom protein Z precursor [Nasonia vitripennis]
c59938.graph_c0	6.18E-13	8.874451	venom protein V precursor [Nasonia vitripennis]
c63922.graph_c0	0.003566	5.202852	serine protease 33 precursor [Nasonia vitripennis]
c67361.graph_c0	1.38E-07	7.155023	serine protease 137 precursor [Nasonia vitripennis]
c17999.graph_c0	1.17E-05	6.680388	PREDICTED: venom peptide SjAPI-like [Nasonia vitripennis]
c71471.graph_c0	7.63E-08	7.441613	endonuclease-like venom protein precursor [Nasonia vitripennis]

Table 7. Primer pairs used for expression analysis using qRT-PCR

Gene name	Primer sequences
c42845.graph_c0	Forward: 5’- CGACCTGTCGTGACACTTTC-3’
	Reverse: 5’- ATGAGCACTTGCTGAAGCTG-3’
c72605.graph_c2	Reverse: 5’- TAGCAAGACCAACGTCACCT-3’
	Forward: 5’- CCGTTAGTCCAGCCAAATCC-3’
c23296.graph_c0	Forward: 5’- CCGTTAGTCCAGCCAAATCC-3’
	Reverse: 5’- AGAGGCTTGCTACTCTGTGG-3’
c66701.graph_c0	Forward: 5’- TGATGTGTTTCACAAACTGCAA-3’
	Reverse: 5’- TGACATTGAGGCTTGGATGC-3’
c61117.graph_c0	Forward: 5’-GGGCTCGAGTGCCTTATAGT -3’
	Reverse: 5’- CTGCCAAGCGTGCTATTGTC-3’
c67249.graph_c0	Forward: 5’- ATTCCGTGATGCTGTTGCTC-3’
	Reverse: 5’-AACTTGGGTGGAATCCGCTA -3’
c68140.graph_c0	Forward: 5’- ATGAACGCGAACGTAAGCAA -3’
	Reverse: 5’- TCAACGTCGCTCATTCCAAC -3’
EF1A	Forward: 5’- ACCACGAAGCTCTCCAAGAA-3’
	Reverse: 5’- AATCTGCAGCACCCTTAGGT-3’

Figures

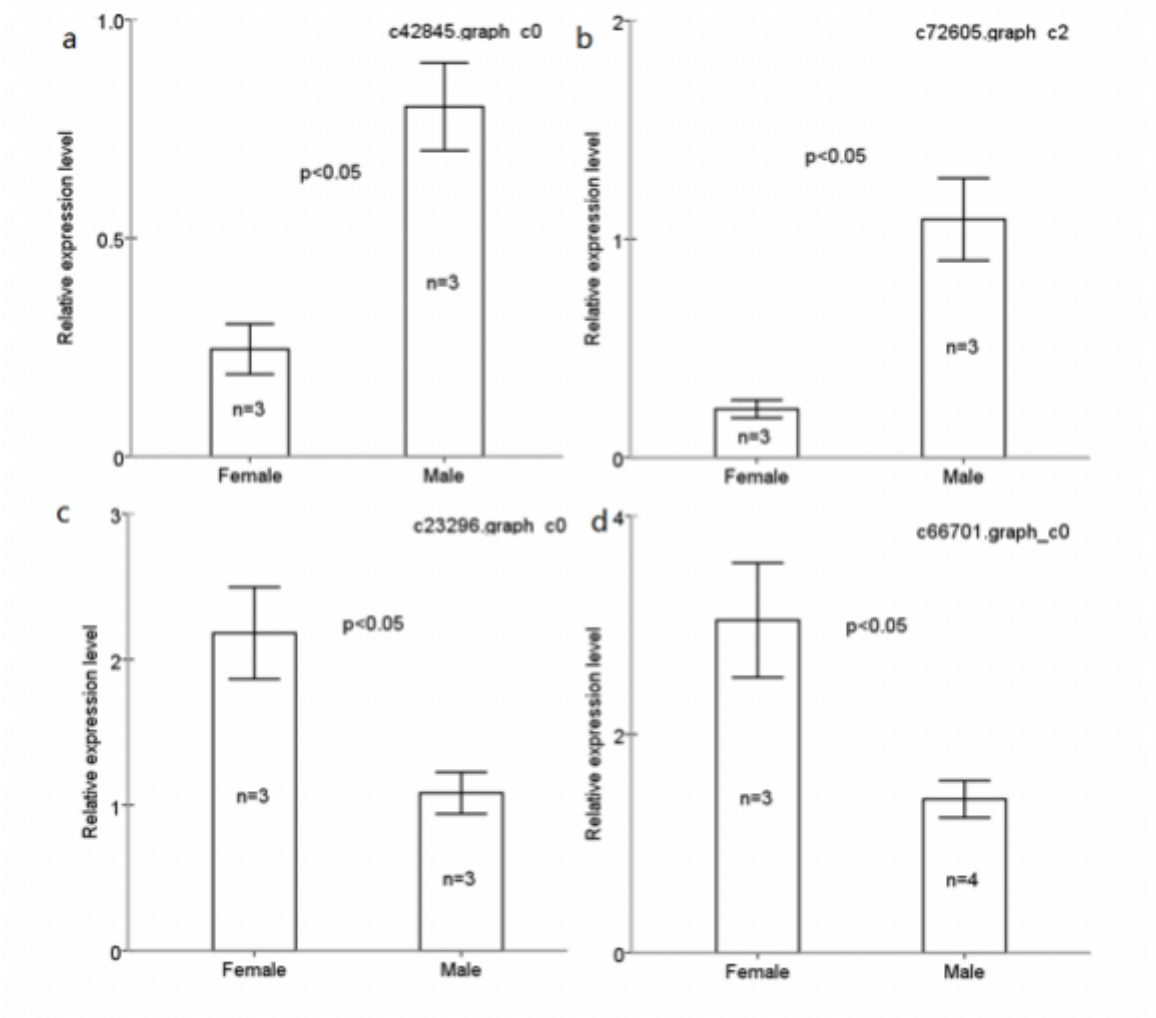

Figure 1

Sex differences in expression of annotated genes involving in flyability from qRT-PCR. The expression of genes determined through qRT-PCR was calculated by the 2-ΔΔCt method using the housekeeping gene EF1A as a reference to eliminate sample-to-sample variations in the initial cDNA samples.

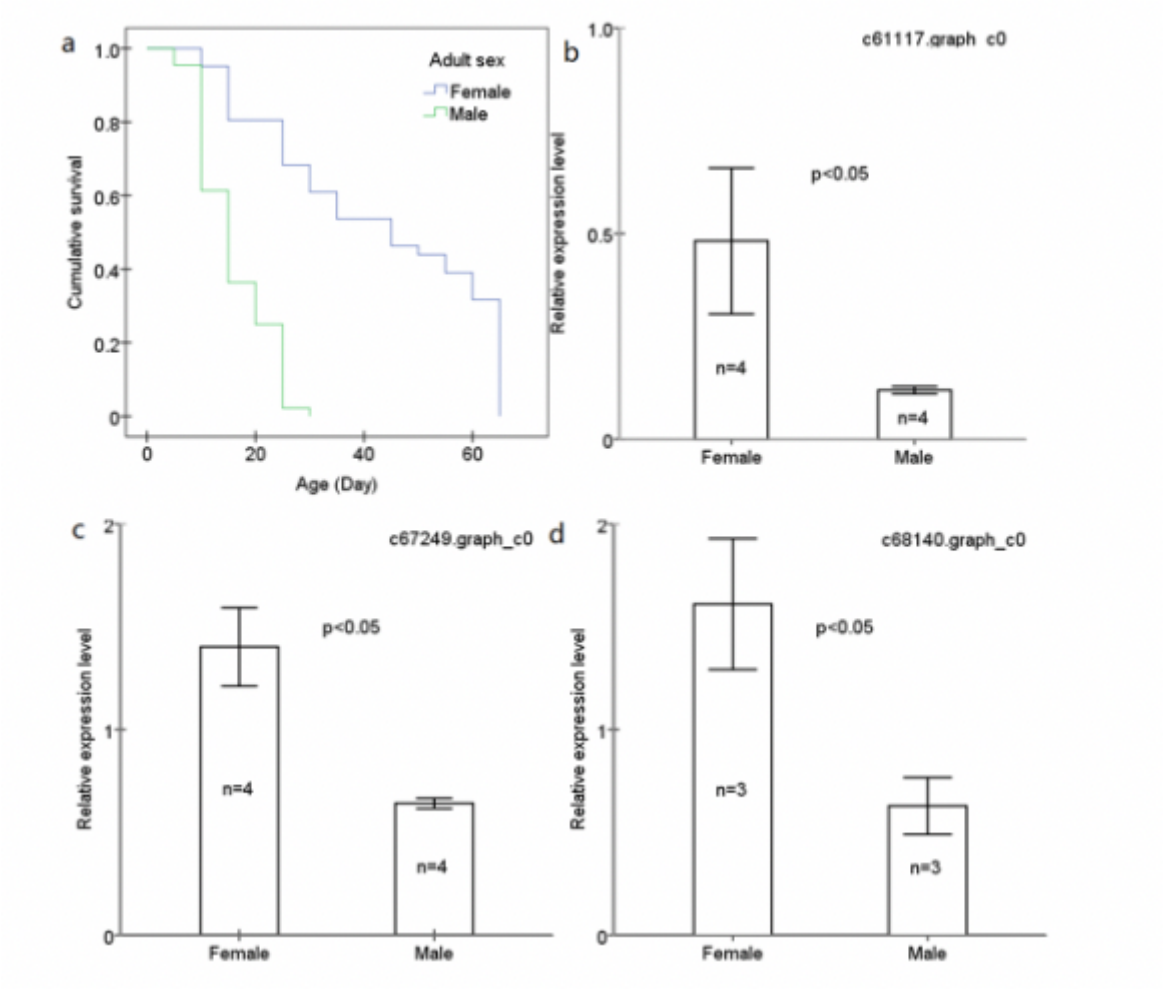

Figure 2

Sex differences in longevity (a) and expression of annotated potential genes encoding superoxide dismutase (b) and Vitellogenin (c, d)from qRT-PCR. The expression of genes determined through qRT-PCR was calculated by the 2-ΔΔCt method using the housekeeping gene EF1A as a reference to eliminate sample-to-sample variations in the initial cDNA samples.