Sex Differences in Immune Gene Expression in the Brain of a Small Shorebird

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Research Article

Keywords: sex-biased gene expression, avian immunity, RNA-seq, brain, mating system

Posted Date: May 25th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-536989/v1

Abstract

Background: Males and females often exhibit different behaviour, life histories and ecology, and sex differences are typically reflected in their brains. Neuronal protection and maintenance include complex processes led by the microglia that also interact with metabolites such as hormones or immune components. Despite increasing interest in sex-specific brain activation in laboratory animals, the crucial significance of immune function protecting in the brain of wildlife is widely lacking. Here, we study sex-specific expression of immune genes in the brain of a small shorebird, the Kentish plover (*Charadrius alexandrinus*), that is an emerging model of mating system evolution and speciation. We compare immune gene expression patterns between adult males and adult females in two wild breeding populations in contrasting habitats: a coastal sea-level population and a high-altitude inland population in China.

Results: Our analysis yielded 379 genes associated with immune function. We show a significant male-biased immune gene upregulation, which is in line with ecological studies that showed higher survival in males than in females. Immune gene expression in the brain did not differ in upregulation between the coastal and inland populations.

Conclusions: We discuss the role of dosage compensation in our findings and their evolutionary significance mediated by sex-specific survival and neuronal deterioration. Similar expression profiles in the coastal and inland populations suggest comparable pathogen pressures between the habitats. We call for further studies on gene expressions of males and females in wild population to understand the implications of immune function for life-histories and demography in natural systems.

Background

Sex differences in behaviour, morphology, and physiology are widespread in nature, with most dioecious animals thought to present at least some degree of sexual dimorphism. The brain – the centre of the nervous system in many organisms – is an example of one of these sexually dimorphic structures as it may differ in size, function and gene expression [1–3]. Sex differences (or sexual dimorphism as is usually termed) in the brain is being increasingly investigated using humans and laboratory animals. Although in wild species these topics have been considerably less explored, recent evidence suggests sex differences in neural organisation and gene expression in the brain [4–9].

With evidence suggesting the importance of functional and structural differences in the male and female brain, it is possible that complementary processes such as disease risk and its defence response may also differ between the sexes. Indeed, sex differences are found in neuronal diseases such as Parkinson disease, where males exhibit a greater reduction in global cognition and language than females, or in Alzheimer disease, presenting women with faster rates of brain atrophy than males [10, 11]. Understanding the causes of these differences are important, since many diseases – including the recent COVID-19 – have different mortality rates in males and females, and ultimately, producing sex-biased

mortalities in several organisms [12, 13]. Sex differences in microglial and astrocytic cells, such as their heightened sensitivity to inflammatory stimuli and their anatomical distribution [14–18], have been postulated to mediate sex differences in cognition and memory in rodent models [19, 20]. However, research addressing the main system responsible for inflammatory responses and pathogen defence, i.e. the immune system, in the brain of wild animals is widely lacking, despite showing important differences in various immune parameters between the sexes [21–23].

In the nervous system, immune function seems to be under particularly intense modulation since an insufficient response may result in infection, but an excessive response could result in prolonged inflammation and tissue damage [24]. Also, in tissues like the brain, general immune factors seem to serve a variety of non-immunological functions [25, 26]. Furthermore, many variables may influence the immune response, including biotic and abiotic factors or the combination of both, such as seen in birds in the tropics that seem to upregulate aspects of their immune function in the wet season, presumably as defence mechanism against increased pathogen pressure that emerges from increased rainfall [27, 28].

The Kentish plover (*Charadrius alexandrinus*) is a small shorebird that is emerging as an ecological model system of sexually dimorphic reproduction and speciation [29, 30]. Kentish plovers are widely distributed along coastal and inland waterbodies across Eurasia and North Africa [31]. Previous studies of Kentish plovers have found that males generally survive better than females [32, 33]. Though the causes of this female-biased mortality in Kentish plover are still unknown, previous studies addressing parasite burden suggest that males and females share comparable infection rates of blood parasites and pathogenic bacteria [34–36].

Here we investigate immune aspects in the male and female brain of Kentish plovers in two contrasting environments in China: the Bohai Bay located on the East coast, and the Qinghai Lake located inland at high elevation in the Qinghai-Tibetan Plateau. We focus on expression patterns of immune system genes, since a healthy immune system in the brain is essential for protecting the animal against infection and maintaining cognitive ability [37–40]. We quantify the expression of genes annotated with immune functions in four brain regions (hypothalamus, medial extended amygdala, nucleus accumbens and septum), with the aim of evaluating sex differences in immune genes [41]. We test two specific hypotheses. First, based on sex differences in gene expression in the brain in various bird species [e.g. 7, 8, 9] and survival demographic analyses of Kentish plover, we expect increased upregulation of immune genes in the male brain. Despite both Bohai Bay and the Qinghai Lake being stopovers of the East Asian-Australasian Flyway and the Central Asian Flyway, respectively, these environments show contrasting differences that could determine patterns of pathogen exposure, namely increased interaction with waterbird and marine diversity in the coastal site, *versus* the high-altitude inland site. Because local environmental factors such as pathogen pressure, can shape immune function [e.g. 42], we expect differences in immune gene expression between the sites, with a possible upregulation of immune genes in the coastal population.

Results

Differential expression analysis

We identified 403 genes associated with immune processes, and after filtering out low counts (≥ 5), 379 immune-related genes were further processed. Of these, 11 genes had significant differences in expression pattern between the sexes, with 10 exhibiting male-biased expression and one having female-biased expression (Fisher's exact test, *P*-value = 0.001, Table 1 and Table S1; Fig. 1a and 2). The male-biased immune genes were involved in the activation of various components of the immune system (details in Table S2), whereas the female-biased gene was involved in T-cell upregulation, consistent with a role in the activation of adaptive immunity.

Comparing profiles of immune system gene expression between the two populations, we found 17 differentially expressed genes (Fig. 1b). The number of differentially upregulated immune genes did not differ between Bohai Bay and Qinghai Lake (9 and 8 genes, respectively; Fisher's exact test, *P*-value = 0.807; Table 1).

Interaction analysis showed a significant effect of sex on environment, where in both the coastal and inland location immune genes were significantly overexpressed in males but not in females (Table 1 and S1; Figure S1).

Chromosomal location

Out of 379 immune genes, 341 genes were located on the autosomes, 12 in the Z chromosome, with the remaining 26 genes not yet assigned to any chromosome. Nine out of 10 male-biased genes were linked to the Z chromosome and one gene was located in the autosomes (Fig. 1). The location of the female-biased gene was in the autosomes (Fig. 1; Table S2). In contrast, all differentially expressed genes identified when comparing the coastal and the inland environments (9 and 8 genes, respectively) were located in the autosomes (Fig. 1; Table S3). Four out of 5 significantly male-biased overexpressed genes in the interaction analysis were located in the Z chromosome and one gene in the autosomes (Figure S1; Table S2).

Table 1
Number of genes with biased expression according to (a) sex, (b) habitat and (c) their interaction in brain tissues of 24 Kentish plovers. The total number of genes investigated was 379. *P*-values refer to Fisher's exact test.

a) Sex biases	**Up males**	**Up females**	***P*-value**
	10	1	0.001
b) Habitat biases	Up Bohai Bay	Up Qinghai Lake	*P*-value
	9	8	0.807
c) Sex*habitat	Up males	Up females	*P*-value
Bohai Bay	5	0	0.024
Qinghai Lake	5	0	0.020

Discussion

Here we showed that the vast majority of immune genes have similar expression levels between males and females, and between two ecologically different habitats. We identified 10 immune genes which had higher expression in males and one gene that was more highly expressed in females. Though we note that the magnitude of the differential gene expression was small, in all cases no larger than − 1/+1 Log2 fold change.

Recent studies are addressing specific aspects of immune defence using transcriptomes in birds [e.g. 43, 44, 45], although despite the growing importance of sex-specific research across disciplines [46, 47], only the work of Wang et al. [48] has so far explored sex differences using blood samples in captive Eurasian magpies (*Pica pica*), finding important sex-biases in expression of genes related to stress resistance, immunity, energy metabolism, reproduction and lifespan regulation. Genomic methods are powerful since they introduce a more holistic perspective in regards to a functional gene group, but it should be noted that the correlation between mRNA and protein concentrations could be inconsistent [49, 50] and that tissue type may influence gene expression profiles [51].

Differential gene expression between the male and female brain has been demonstrated in several species, but an emphasis on immune genes is rarely seen, hence the little knowledge about possible causes and consequences of this sex difference. Studies not distinguishing for sex in murine models describe an association between increased activation of innate immune genes in the brain and neuronal aging [52], alterations of social behaviour [53], and the risk of developing schizophrenia [54]. Note that the microglia, the major source of immune genes in the brain, is known to regulate the brain functional connectivity and behaviour [55–58]. Moreover, immune-related genes, such as the major histocompatibility complex (MHC) molecules, complements and their receptors, are known to be expressed in the brain and regulate brain structural and functional plasticity, either directly or indirectly by

controlling microglial or immune activation [59–61]. Perhaps sex-specific effects could be linked to the sex hormones, since the main female sex hormone, oestrogen, has been associated with several roles in the microglia, thought to drive behavioural differences during development in mammalian and avian models [9, 17–19]. Also, because interspecific variation of the microglia is of common occurrence in several neurodegenerative diseases in mammals, our findings could entail sex-specific mortality costs for the animals [14, 16, 17]. Unfortunately, research on sex-specific neurodegenerative diseases in wild birds is in its infancy thus limiting us from drawing further conclusion.

Our results showed that upregulated immune genes in the brain in the coastal environment was similar to the high-altitude habitat in the Qinghai-Tibetan plateau. Because many immune genes expressed in the brain are believed to regulate behaviour and other cellular functions, this suggests that the genetic control of these processes seems rather uniform between environments. Furthermore, our findings also suggest that despite their striking differences in landscape, the pathogen pressure in both environments were comparable [62, 63].

We found that most male-biased genes were located on the Z chromosome. Interestingly, when comparing differential immune gene expression between the two environments, all upregulated genes belonged to the autosomes. This could suggest that the sex-biases in immune gene expression in Kentish plover are linked to the sex chromosomes, possibly expected because of the absence of dosage compensation in birds [64–67]. Unfortunately, there is not yet an assembly available for the W chromosome in Kentish plover [68], which truncates further conclusions. Wang et al. [48] found in Eurasian magpies that, in general, males show more upregulated genes than females in the blood, although some specific immune pathways were found to be upregulated in females. Unfortunately, Wang et al. [48] did not characterise the chromosomal location of these genes.

Conclusions

The use of genomic methods is a convenient approach for addressing a wide range of specific questions. Though caution at interpretation is required because often single genes have several biological functions and these can differ between tissue type. Here we showed that, though small, sex but not environment has an effect on the upregulation of immune gene expression in Kentish plover. We do not know the impact that such differences could have on the bird's life trajectory, but we nevertheless note that the direction of the sex-bias in immune genes met the expected predictions based on higher survival rate of male Kentish plover over females in the wild [69]. We are aware, however, that sex-specific mortality can be multifactorial and future studies should further the knowledge of sex differences in immune gene expression in this shorebird.

Methods

Sampling

Fieldwork took place at two sites in China where the Kentish plover is a locally abundant breeder (Figure S2): Bohai Bay at sea level [39° 7'6.22"N, 118°11'49.84"E; more details of the site are described in 70], and Qinghai Lake (Koko Nor), the largest lake in China at 3,200 m above sea level [36°45'56.86"N, 100°43'21.68"E; 71]. Fieldwork was conducted during the breeding period in May 2015, capturing 24 incubating adults on the nest (6 males and 6 females at each site) followed by morphometric measurements according to a standard protocol [see 72]. To obtain blood and brain tissues, the birds were sacrificed conforming to the regulations of ethical conditions by the Chinese Animal Welfare Act (20090606), and of the Animal Welfare & Ethical Review Body of the University of Bath and the Institutional Ethical Committee of Animal Experimentation of Sun Yat-sen University (2005DKA21403-JK). This study did not involve endangered or protected species.

Tissue samples were collected from four brain regions: the hypothalamus, medial extended amygdala, nucleus accumbens and septum. Using a stainless-steel rat brain matrix, brain samples were dissected by first cutting the brain coronally in order to rostrocaudally standardise the tissue slabs, then each region of interest was manually dissected using a portable dissection microscope following a chicken brain atlas [73]. The Kentish plover and the chicken brain differ in morphology, thus we used visible anatomical landmarks to define the approximate margins of the dissections. Brain tissue samples were stored in RNAlater (Qiagen) and kept cold (in the field) using either liquid nitrogen or cold blocks. At the end of each day samples were stored in freezers (-20°C) and then once at the lab samples were stored at -80°C prior RNA extraction.

RNA extraction and sequencing

RNA extraction and sequencing was performed by Novogene Beijing. RNA degradation and contamination was monitored using 1% agarose gels and RNA purity was assessed using a NanoPhotometer spectrometer (IMPLEN, CA, USA). RNA concentration and integrity were measured using a Qubit 2.0 Flurometer (Life Technologies, Carlsbad, CA, USA) and an Agilent Bioanalyzer 2100, respectively. Sequencing libraries preparation used 3µg RNA per sample and cluster formation was conducted following manufacturer's recommendations (TruSeq PE Cluster Kit v3-cBot-HS, Illumina). Sequencing was performed on an Illumina HiSeq platform. The outcome generated paired-end reads with a fragment length of 300-500bp and an average paired end length of 150bp, for a total of 519 millions of raw paired-end reads sequenced.

Transcriptome profile annotation

Reads were cleaned using Trimmomatic v0.35. All further analysis used these high-quality reads. Cleaned reads were aligned to the Kentish plover genome using HISAT2 v2.1.0 [74]. We used Samtools v1.2 [75] to clean HISAT2 outputs in order to remove multi-mapped reads, unmapped reads and unmapped mates (-q 40, -f 2, -F 12). Counts of raw read were extracted from cleaned HISAT2 outputs using HTSeq-count v0.11.2 [76].

Differential expression analysis

We examined the clustering of the samples gene expression patterns using principal component analysis. This revealed little distinction in expression between the regions except for the nucleus accumbens (Figure S3). Thus, we pooled all brain regions together (averaging counts of the four regions) for further analysis. The analysis of differential gene expression was performed between the sexes and populations using the function *DESeq* from the R statistical package *DeSeq2* [77]. This function calculates differential expression based on the negative binomial (i.e. Gamma-Poisson) distribution, which is an advantage over earlier binomial models because it makes fewer type I errors [78]. The function operates estimating size factors, then estimating dispersion of each gene, to finish fitting negative binomial generalised linear models and Wald statistics (see *DESeq* documentation for further details).

Differential expression analysis was performed on two different groupings of the dataset, i.e. population (Bohai Bay *versus* Qinghai Lake), sex, and the two-way interaction of population and sex. Genes were filtered and removed if they had below 5 counts per million mapped reads. Analyses were conducted in R version 3.3.2 [79].

Gene set enrichment analyses

Gene ontology enrichment categories and the extraction of GO terms followed methods previously described in Wang et al. [68]. We evaluated GO terms from the domain biological processes and then the subcategory immune system processes. The gene enrichment analyses were performed using a hypergeometric test for overrepresentation and corrected for false positives with the Benjamini and Hochberg false discovery rate (FDR) correction [80]. Functional groups with a FDR P-value < 0.05 were regarded as statistically significantly overrepresented. Cases where the expected number of genes less than one were only classified as significantly enriched if the observed number of genes was higher than one.

Sex- and population-specific gene expression

We used Fisher's exact tests to examine for differences between males and females in the proportion of up and downregulated sex-biased genes. The same method was used in statistical comparisons between population, and in the sex*population interaction.

Declarations

Acknowledgements

We thank Naerhulan Halimubieke, Xuejing Wang, Zhenhua Liu and Mengjie Jin for their support during fieldwork, and Chenqing Zheng for coordinating sample transportation and RNA sequencing.

Authors' contributions

AU, TS, KHM and YL designed the study. JOV wrote the manuscript and did the data analysis. KHM conducted the laboratory work and bioinformatics. GZ did the brain dissections. All authors contributed substantially to revisions of the paper.

Funding

Funding was provided by the Chilean National Agency for Research and Development (ANID, former CONICYT), BECAS CHILE 72170569 to JOV. KHM was supported by a NERC GW4+ studentship (NE/L002434/1) and a Korner Travelling Award. KHM, ZZ, YL and AOU were supported by a British Council and Chinese Scholarship Council Newton Fund PhD Placement. The National Natural Science Foundation of China funded PQ and ZZ (No.31572288), and NKFIH-OTKA K116538 research grant to GZ, and TS. The Raymond F. Schinazi International Exchange Program supported AOU, LJY and TS. TS was also funded by the Royal Society (Wolfson Merit Award WM170050, APEX APX\R1\191045) and by the National Research, Development and Innovation Office of Hungary (ÉLVONAL KKP-126949, K-116310). Royal Society Dorothy Hodgkin Research Fellowship (DH071902) funded AOU.

Ethics approval and consent to participate

Birds were sacrificed conforming to the regulations of ethical conditions by the Chinese Animal Welfare Act (20090606), and of the Animal Welfare & Ethical Review Body of the University of Bath and the Institutional Ethical Committee of Animal Experimentation of Sun Yat-sen University (2005DKA21403-JK).

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Availability of data and materials

The full dataset can be found in the following link: https://figshare.com/s/153d63db7e798269aa5d.

Upon acceptance the status of the dataset will be changed to publicly available.

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Figures

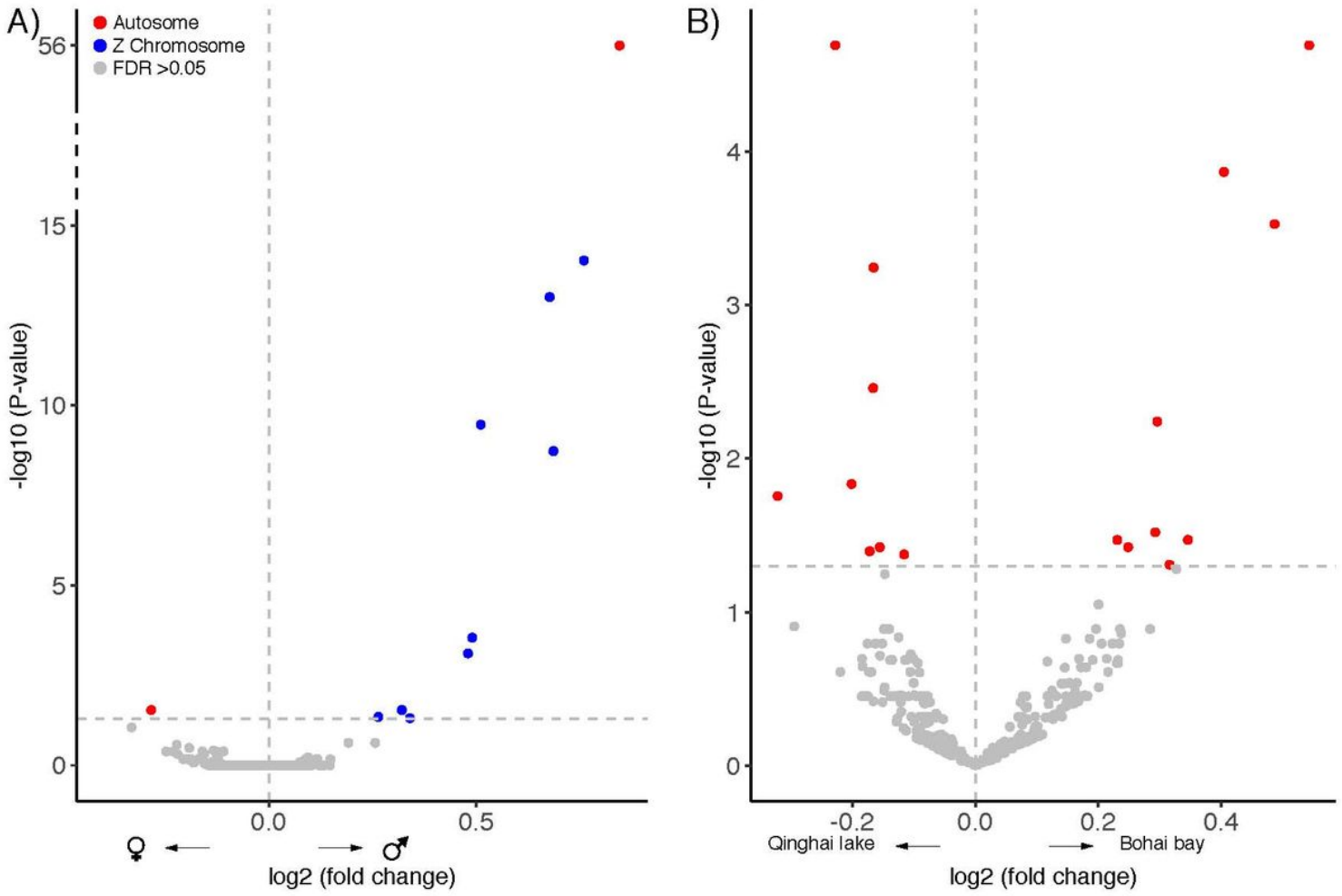

Figure 1

Biases in immune gene expression in brain tissue in Kentish plover. a) Shows sex-specific immune gene expression where positive values indicate male-biased expression and negative a female bias. b) Immune expression bias in relation to habitat. Colours indicate chromosomal location of the differentially expressed genes. The horizontal dashed line indicates a false discovery rate (FDR) threshold of 0.05.

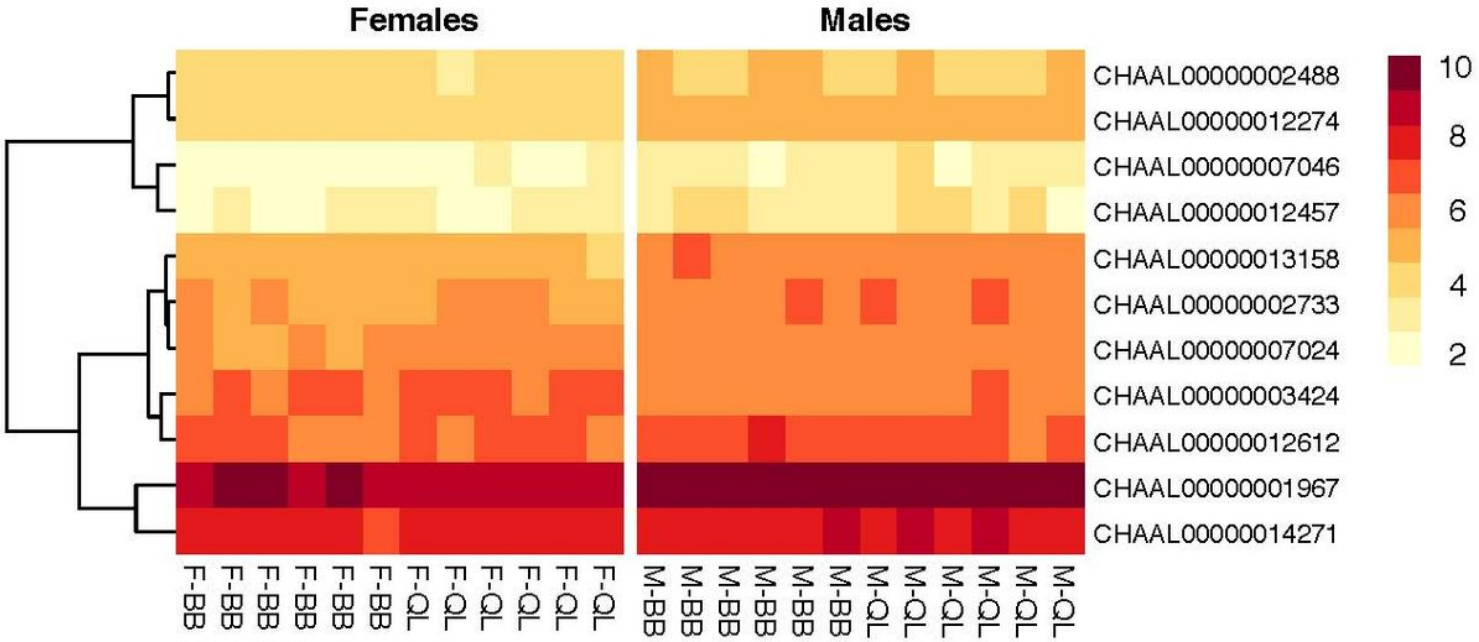

Figure 2

Heatmap of 11 significantly differentially expressed immune genes in 24 male and female Kentish plovers sampled in China. Colours represent normalised average expression counts (log2(n+1)). Bottom column names refer to females (F), males (M) from Bohai Bay (BB) and Qinghai Lake (QL).

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