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Characterization and Expression of Immune Related Genes in Feral Colonies of *Apis* Species from Bengaluru

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ABSTRACT: Eco-immunological studies on feral organisms can throw insights into how host–pathogen dynamics fluctuate with selective pressure from human interventions and other natural infestations. Feral colonies are more susceptible to pathogen attack than the managed bees, in the absence of disease treatments. During the study tried to investigate the role of pathogen infections [Deformed wing virus (DWV) and *Nosema ceranae*] and gene expression of immune members (defensin-1, hymenoptaecin, pgrp-lc, pgrp-s2 and argonaute- 2) in the survival of feral bee colonies across seasons (winter and summer). We surveyed a total of 90 feral colonies over a 3-year period (2018–2021), measuring the pathogen levels and immune gene expression using quantitative polymerase chain reaction (qPCR). Our results confirmed of the positive upregulation of the immune gene members in winters where pathogen loads are found to be more. Higher pathogen levels seem to be associated with increased immune gene expression across all the species in the study. There was no significant effect seen among species, but significant effect was seen across the seasons ($p<0.05$). Our results could help in suggesting the possible indicator genes to assess the overall health of honey bee colonies and provide evidence for the role of feralization in how they cope up with pathogen landscapes.

KEYWORDS: *Apis cerana*, feral colonies, Immune related genes, Real time PCR.

INTRODUCTION

Eco-immunology studies of wild organisms can provide valuable information on how host-pathogen dynamics change as organism's transition from human-managed conditions. To nature. Honey bees provide an ideal system for studying these questions, as these social insect colonies often escape management and become established in the wild. While managed honey bee colonies have a low probability of survival in the absence of disease treatments, wild bee colonies often persist in the wild, where pathogen pressure is thought to be higher due to lack of treatment. Disease treatment.

Domesticated organisms restore themselves to the wild without anthropogenic influence through the process of feralization (Gering *et al.,* 2019a). Research on feralization typically takes place in a genetic and ecological context, understanding how environmental factors and genetic mutations affect how well feral organisms can compete with domesticated alternatives. According to some research, domesticated plants and animals show reduced fitness upon reintroduction to the wild due to genetic bottlenecks and artificial selection (Araki et al., 2009; Meyer and Purugganan, 2013). Despite this, feral species (e.g., cats, dogs, pigs) thrive, and they do not always revert to the wild (Taylor et al., 1998; Bellard et al., 2017). It is important to note that feral species often outnumber their wild counterparts, which may alter ecosystem composition by increasing competition for prey resources and potentially spreading pathogens to wild species (Bevins *et al.,* 2014). Due to the effect of the wild on conservation biology and surroundings management, there's a developing hobby in those environmental impacts.

Environmental conditions in wild running can promote disease tolerance or resistance. Domesticated pathogens are usually controlled by humans to avoid the rapid spread of disease among livestock. In contrast, transmission of pathogens in wild populations is uncontrolled. Under these conditions, host–pathogen interactions in feral populations may therefore facilitate the rapid evolution of natural mechanisms of disease tolerance or resistance (Locke, 2016). Thus, while the maintenance of traits associated with disease resistance may be impaired in domesticated species, the greater ability to mitigate the negative effects of pathogens is critical to the survival of wild organisms. (Moreira *et al.,* 2018).

During feralization, honey bee colonies (*Apis)* offer a surest version to check the speculation that not only improves bodily defences and disorder but also tolerance among the feral bees. There are a number of pests and pathogens that threaten honey

bee populations, both domesticated and feral (Mcmahon *et al.,* 2016). *Varroa* destructor is an ectoparasitic mite that acts as a vector for multiple bee-infecting RNA viruses, and is an important cause of disease and colony losses in honey bee colonies (Martin *et al.,* 2012). In managed colonies of honey bees, chemical acaricides are frequently used to decrease the number of *Varroa* mites and associated viruses. Because *Varroa* mites positively affect honey bee survival, managed colonies are usually treated multiple times per year with acaricides to control mites. In general, honey bee colonies which are not treated or managed die in less than one year (LeConte *et al.,* 2010). Reports states that feral bees survive for long in the wild even though they are devoid of beekeeper management (Locke, 2016).

Studies have confirmed that feral colonies show higher immune response than those of managed colonies (Youngsteadt *et al.,* 2015). We do not know, however, if different immune phenotypes are associated with colony survival under managed or feral conditions, or with resistance to parasites. Management likely has an effect on both types of defences used by honey bees to protect themselves from pests and pathogens (Taric *et al*., 2020).

Individual bees also rely on humoral immunity to control infections and provide pathogen defence (McMenamin et al., 2017) along with behavioural responses to infection (McMenamin *et al.,* 2018). When the immune system responds to viruses, bacteria, and fungi, several immune pathways are activated. Antimicrobial peptides (AMPs) such as Toll and Imd, e.g., are produced by multiple immune pathways in insects (Brutscher *et al.,* 2015). As a form of antiviral defence, RNA interference (RNAi) targets double-stranded RNA that results from virus replication (Gammon and Mello, 2015).

Here, we tried to investigate the role of pathogen infections and immune gene expression in the survival of feral bees to hypothetically conclude whether feral colonies could serve as reservoirs of pathogens and do increase in pathogen levels lead to higher expression of immune genes in feral colonies. Over a 3-year period, we sampled feral colonies in the same landscapes with the participation of beekeepers at different seasons (summer and winter).

We collected individuals from a total of 90 colonies during winter and summer seasons for a period of 3 years (2018-2021) in order to assess the Eco immunology and pathogen loads among the feral colonies out of which 72 colonies were found to be infested with the pathogen loads. We studied the expression of immune gene members among the feral colonies of *Apis* species (*A.cerana, A.dorsata* and *A.florea*).

METHODS

The study was conducted in and around Bangalore district and feral colonies were used for the study. Feral colonies were selected and used for the study at different seasons and habitats. November to January was considered as winter season and March to May was considered as summer season. The number of colonies was not uniform throughout the study, due to the loss of the colonies in the particular habitats. At an average about 5 colonies (n=5) were selected for each species (*Apis cerana, Apis dorsata* and *Apis florea*) at different seasons and habitats. Study was conducted for 2018-2021 for both the seasons mentioned.

Table 1: Table showing different habitats and the vegetation chosen for the study.

B: Botanical garden; PP: Public parks; I: Industrial area; MF: Micro forests;
R: residential area.

S.No	Code	Vegetation
1	B	Large canopy trees, orchids, weeds, herbs and shrubs
2	PP	Trees, herbs shrubs, grass and weeds
3	I	Large canopy trees, shrubs weeds and herbs
4	MF	Trees, herbs, shrubs, weeds and grass
5	R	Few canopy trees and shrubs

We approximately sampled 20 forager bees from the entrance of each colony in both the seasons (summer and winter). All the feral bee individuals were sampled with sweep nets and transferred into 50ml tubes and incubated on dry ice to preserve the quality of RNA. Further the samples were stored at 80^0C. All the sampling sites were on private property and prior permission was obtained from the site owners and no protected or endangered species were involved in the study.

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IMMUNE GENES AND PATHOGENS ARE USED IN THE STUDY

To distinguish the disease dynamics in the feral bee colonies, we quantified about two pathogens which commonly infect these feral bees and negatively impacting the overall health of colony. Deformed wing virus (DWV) is an RNA virus which is said to be seriously detrimental for the bee resulting in overwintering losses (Brutscher et al., 2016). This is mostly transmitted by *Varroa* mites leading to clinically symptomatic infections (Möckel et al., 2011). Also quantified *Nosema ceranae* (D'Alvise et al., 2019) is a common microsporidian gut parasite of honey bees causing reduced lifespans among the infected bees (Goblirsch, 2018). We quantified both these pathogens in all colonies sampled in the study.

About 5 immune gene members (argonaute-2, pgrp-s2, pgrp-lc, defensin-1 and hymenoptaecin) expression was quantified in feral colonies. The gene argonaute-2 (ago2) always was upregulated at viral infections (Brutscher et al., 2015). Gene *pgrp-s2* and *pgrp-lc* encodes for an upstream recognition receptor involved in activation of the Toll immune pathway and a transmembrane protein activator of the Imd (Immune Deficiency) pathway respectively. Both these genes are reported to be highly upregulated on infection (Evans et al., 2006; Brutscher et al., 2017). Also, we quantified genes defensin- 1 (*Def1*) and hymenoptaecin (*Hym*) produced by the Toll and Imd pathways. These type of antimicrobial peptides (AMPs) are said to play a critical role in honey bee immune responses to viruses and fungal pathogens (Yi *et al.,* 2014).

Table 2: Table showing the gene members used in the expression study with accession numbers.

Gene Name	*Apis Cerana*	*Apis Florea*	*Apis dorsata*
ago2	XM_017065143.2	XM_031915808.1	XM_006624947.2
Hymenoptaecin	XM_017049926.1	XM_012495676.2	XM_031511975.1
defensin-1	LC331613.1	XM_003693866.3	XM_006622512.2
pgrp-lc	XM_017059285.2	XM_031916044.1	XM_006618665.2
pgrp-s2	XM_017066698.2	XM_003694445.3	XM_031509863.1
elongation factor 1-alpha	XM_017065469.2	XM_012490645.2	XM_006621994.2

RNA Extraction: About 10 bees were collected from each colony and used for the total RNA extraction. The abdomens were dissected and were pooled into 2.0ml centrifuge tubes and homogenized using a homogenizer (Eppendorf) at 6.0m/s for three 30s intervals. The RNA was then extracted from the homogenate using RNeasy spin columns (QIAGEN, Hilden, Germany), according to the manufacturers protocol and eluted into nuclease-free water. Using Nanodrop spectrophotometer assessed the quality and quantity of RNA. We then quantified the 2 pathogens and the expression of 5 immune genes through quantitative reverse-transcriptase PCR (qRT-PCR) using previously developed primer sequences (**Table 1**). According to the manufacturer's protocol (Applied Biosystems, Foster City, CA, United States) the three RNA extracts from pooled individuals per colony were individually used as templates to produce cDNA using random primers and MultiScribe RT.

cDNA synthesis: cDNA synthesis was performed using the RT-PCR kit using SuperScriptTMII Reverse Transcriptase, 200 U µLG1 (HiMedia). About 2µg of the RNA (RNA concentration 1.88µg/µL) obtained in the previous section was used as the starting reaction. So, it used about 1.23µL of the total RNA along with random primers and 1µL of RT enzyme. The contents were mixed thoroughly and incubated at 25^0C for 10 min. Following incubation at 70^0C for 45min. The cDNA thus obtained was then stored until further use for gene expression.

Quantitative real time PCR: Quantitative RT-PCR assay was performed on cDNA templates, using Realplex 4 (Eppendorf) and SYBR Green PCR master mix (Biorad) according to the manufacturer's recommendations (BioRad) with the designed primers listed in Table 1. cDNA samples obtained in the previous section were used as template in the qPCR.

Table 3: Table showing the list of primers used in the study. *elongation factor 1-alpha* was used as housekeeping gene in the study.

Gene Name		(5'->3')	Length	Tm	GC%	Product Length
ago2	FW	TTGGTGCAGACGTGACTCAT	20	59.83	55	450
	RV	TTGGATCGTGACTTGCTGCT	20	57.95	55	
hymenoptaecin	FW	ACAATGGATTATATCCCGACTCGT	24	60.04	55	189
	RV	CAATGTCCAAGGATGGACGAC	21	60.04	58	
defensin-1	FW	GGCTGCACCTGTTGAGGAT	19	59.14	55	630
	RV	TGTCCTTTGAATGAGAGAAGGTCA	24	59.97	55	
pgrp-lc	FW	TCCGTCAGCCGTAGTTTTTC	20	59.67	55	386
	RV	CGTTTGTGCAAATCGAACAT	20	60.04	50	
pgrp-s2	FW	TTGCACAAAATCCTCCGCC	19	59.73	55	246
	RV	CACCCCAACCCTTCTCATCT	20	57.91	55	
elongation factor 1-alpha	FW	GGAGATGCTGCCATCGTTAT	20	58.23	55	249
	RV	CAGCAGCGTCCTTGAAAGTT	20	59.11	55	
DWV (virus) (Ryabov et al. (2014)	FW	GTTTGTATGAGGTTATACTTCAAGG	25	58.76	55	245
	RV	GCCATGCAATCCTTCAGTACCAGC	24	59.12	58	
Nosema ceranae (Fungus) VanEngelsdorp et al. (2009)	FW	CAATATTTTATTATTTTGAGAGA	23	60.11	55	186
	RV	TATATTTATTGTATTGCGCGTGCA	24	58.96	50	

Amplification of all the gene members along with house keeping gene (*elongation factor 1-alpha*) was performed in a 25µl reaction using iQTM SYBR Green Supermix (Genei) containing 2.5µl of 10× PCR buffer with SyBr green, 2.5µl of 10mM dNTPs, 10pmoles/µl of each primer, 10.4µl of PCR water, 0.1µl of 5U Taq polymerase (Genei), and 2µl of cDNA [Malla Sudhakar, 2020]. *elongation factor 1-alpha* was used as housekeeping gene throughout the study (Lourenço *et al*.,2008).

The amplification conditions for cDNA of ago2, defensin-1, *pgrp-lc*, *pgrp-s2* were 95°C for 10min for initial denaturation, followed by 35 cycles of amplification at 95°C for 30sec, 58°C for 40sec, and 72°C for 50sec. A final extension at 72°C for 10min was followed at the end of the run. All the samples were run in Duplicates and samples with no cDNA served as negative control.

The amplification conditions for cDNA of *hymenoptaecin*, *elongation factor 1-alpha*, *DWV (virus)* and *Nosema ceranae (Fungus)* were 93°C for 10min for initial denaturation, followed by 35 cycles of amplification at 93°C for 45sec, 61°C for 50sec, and 73°C for 45sec. A final extension at 73°C for 10min was followed at the end of the run. All the samples were run in Duplicates and samples with no cDNA served as negative control. The relative expression of the target genes in the different samples were calculated according to the following formula: ΔCt = Ct (target gene) - Ct (housekeeping gene) where Ct (target gene) indicates the value of the threshold cycle for the gene of interest, and Ct (housekeeping gene) indicates the value of the threshold cycle for the housekeeping gene used as normalizer.

And the relative expression of all samples was calculated as follows: $RE = 2^{-\Delta\Delta Ct}$[where ΔΔCt = ΔCt (sample) – ΔCt (control)] where RE indicates the relative expression, ΔCt (sample) indicates the difference between Ct values of the target gene and the housekeeping gene(s) calculated for the test sample, ΔCt (control) indicates the difference between the Ct values of the target gene and the Ct values of the housekeeping gene(s) obtained from the corresponding with control [Sudhakar Malla, 2020].

RESULTS AND DISCUSSION

Pathogen Levels

Our study states that mean DWV levels were significantly higher in feral colonies in winter than in summer at all years ($P < 0.05$). Levels of DMV was found to be significantly more than the *N. ceranae* throughout the study. All the colonies tested positive for the presence of DWV and *N. ceranae* at all timepoints but significantly higher during the winter than summer. Out of the 72 colonies tested, *N. ceranae* was detected in about 58 feral colonies and DMV was detected in 61 feral colonies.

A two-way ANOVA between the seasons and species was conducted to compare the DMV prevalence. There was a significant effect of the prevalence between the seasons remembered at the $p<0.05$ level. The significance effect of the seasons on the DMV prevalence was reported to be [$F(5,17) = 96.4889$, $p = 2.86E-05$]. However, there was no significant effect of DMV prevalence among the species [$F(2,17) = 60.722$, $p = 0.136807$]. There was a significant effect of the prevalence among the species remembered at the $p<0.05$ level.

A two-way ANOVA between the seasons and species was conducted to compare the *N. ceranae* prevalence. There was a significant effect of the prevalence between the seasons remembered at the $p<0.05$ level. The significance effect of the seasons on the *N. ceranae* prevalence was reported to be [$F(5,17) = 611.122$, $p = 0.000358$]. However, there was no significant effect of *N. ceranae* prevalence among the species [$F(2, 17) = 120.0556$, $p = 0.11966$]. There was a significant effect of the prevalence among the species remembered at the $p<0.05$ level.

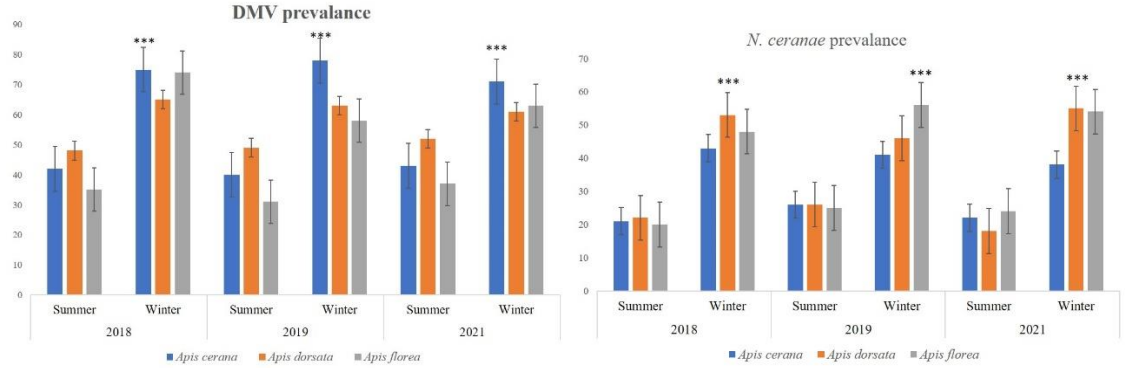

Figure 1: Graph showing the pathogen prevalence of DMV and *N. ceranae.* Prevalence of pathogens in feral colonies. Deformed wing virus (DWV) and *N. ceranae* were detected in all colonies at all times of sampling ($P<0.05$). All the values are average of triplicates. Values are expressed as mean ± SD.

Real time PCR:

From the real time quantification, we show that overall feral bee colonies have higher levels of DWV and fungal parasite (*N. ceranae*) loads which was seen across the seasons, but significantly lesser load than the winter ($P<0.05$). Though we didn't study the mode and way of transmission of these two pathogens, but still we could find feral bees, could serve as reservoirs for DWV and *N. ceranae*. We found a strong correlation between the gene members and seasons, which was obvious from the loads of pathogens ($p<0.05$). Almost all the gene members got over expressed in winter seasons more than the summer seasons. This was uniform across the years of study. We also found higher immune gene expression in feral colonies across the winter seasons even at time points when DWV levels are almost similar.

Specifically, we found hymenoptaecin was highly expressed in winter seasons, where the supposed parasite load was higher in winters than in summers. Many studies report that this gene expression is strongly correlated to increased colony survival (Wu *et al.,* 2020). Hymenoptaecin is generally considered as a good AMP which protects the host from harmful pathogens like DWV and *Varroa* mites (Kuster *et al*, 2014) reported that this gene was stably over expressed when bees were exposed to pathogens and wounding events (Galbraith et al., 2015). Hence from our study and other previous reports, we could strongly suggest this gene to be used as biomarker of honey bee health.

Further analysis of gene members also revealed that levels of defensin and Ago2 also were positively correlated to the expression of hymenoptaecin in the winter seasons. The strength of correlations was high which might be again due to genetic features and environmental conditions which decide the phenotype of an individual. Both defensin and Ago2 genes were found to be positively over expressed ($p<0.05$) with the parasite load, suggesting of their role in immune protection. Both these genes

were identified to be differentially expressed in bees with virus infestation, which is again a positive correlation to the hosts reduced host mortality (Ryabov *et al.,* 2014). Though we didn't relate our study to the survival rates of the colonies, still our data suggests of the strong role of the gene members in expression, while the host is under viral stress.

PGRP-LC and PGRP-s2 were also over expressed in winter season alone, which is again in accordance to our previous findings. But interestingly, the expression levels seem to be more or less similar for the member, PGRP-s2 wherein the levels seem too low or negligible (P<0.05). No reports were observed on similar findings of these gene members, and needs further correlation study and validation.

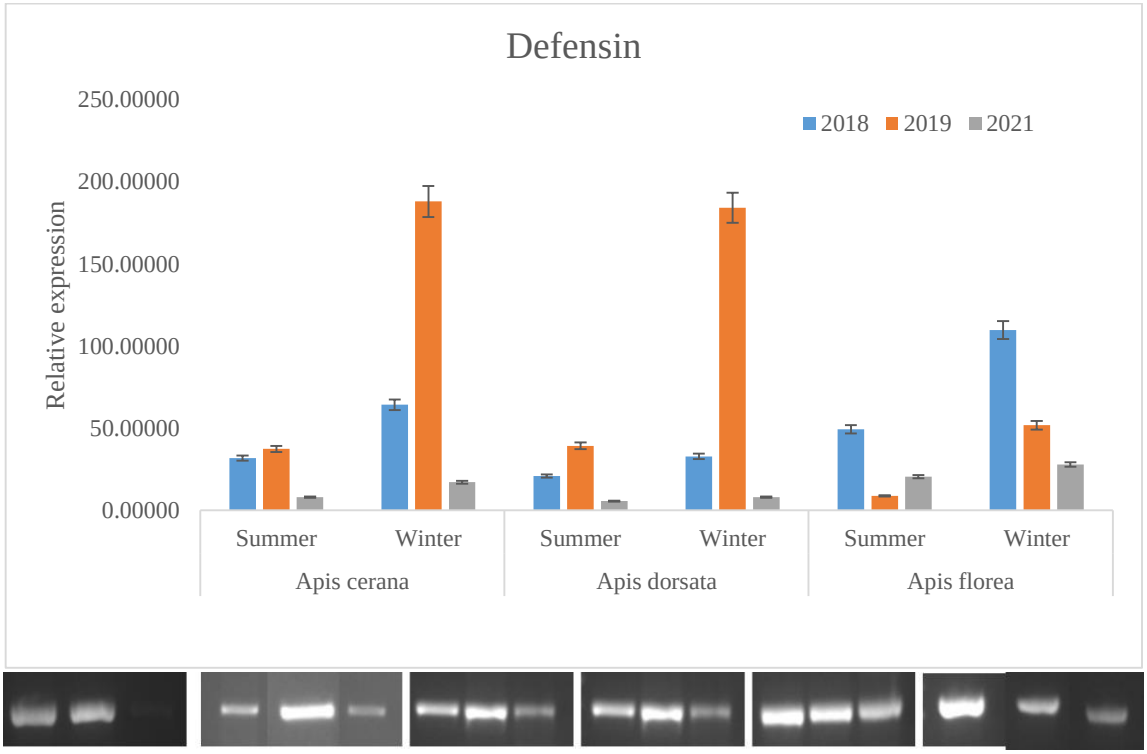

Figure 2: Relative expression levels of defensin gene member across the seasons and among the species. All the values are average of triplicates. Values are expressed as mean ± SD. 1.2% agarose gel with the expressed gene members can be seen in the below image.

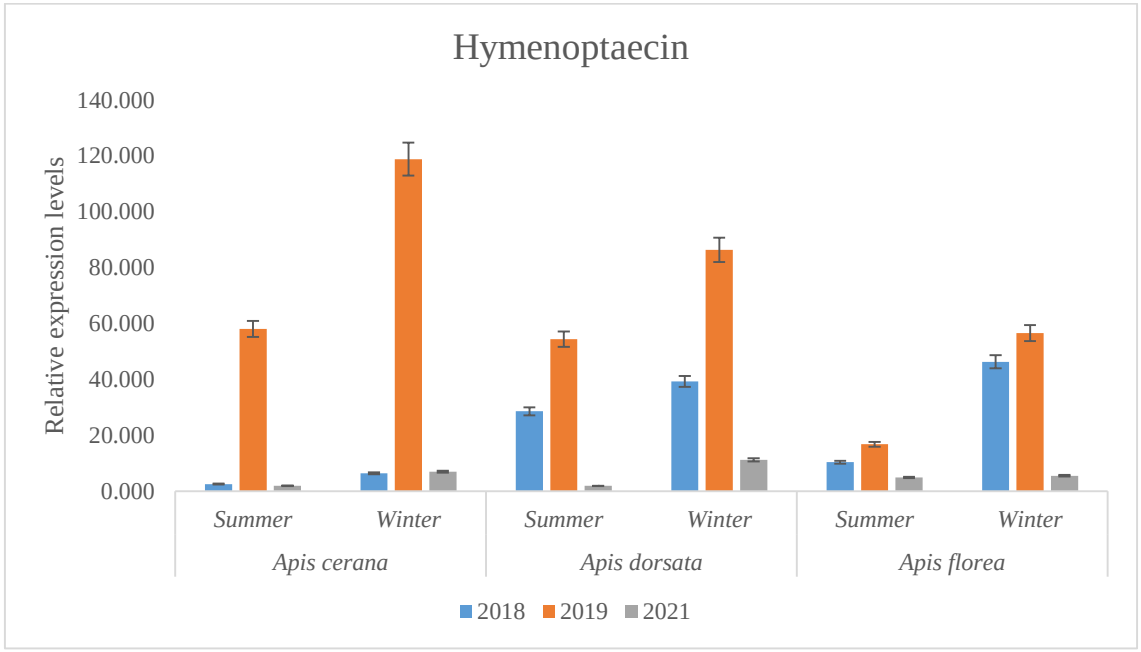

Figure 3: Relative expression levels of *hymenoptaecin* gene member across the seasons and among the species. All the values are average of triplicates. Values are expressed as mean ± SD.

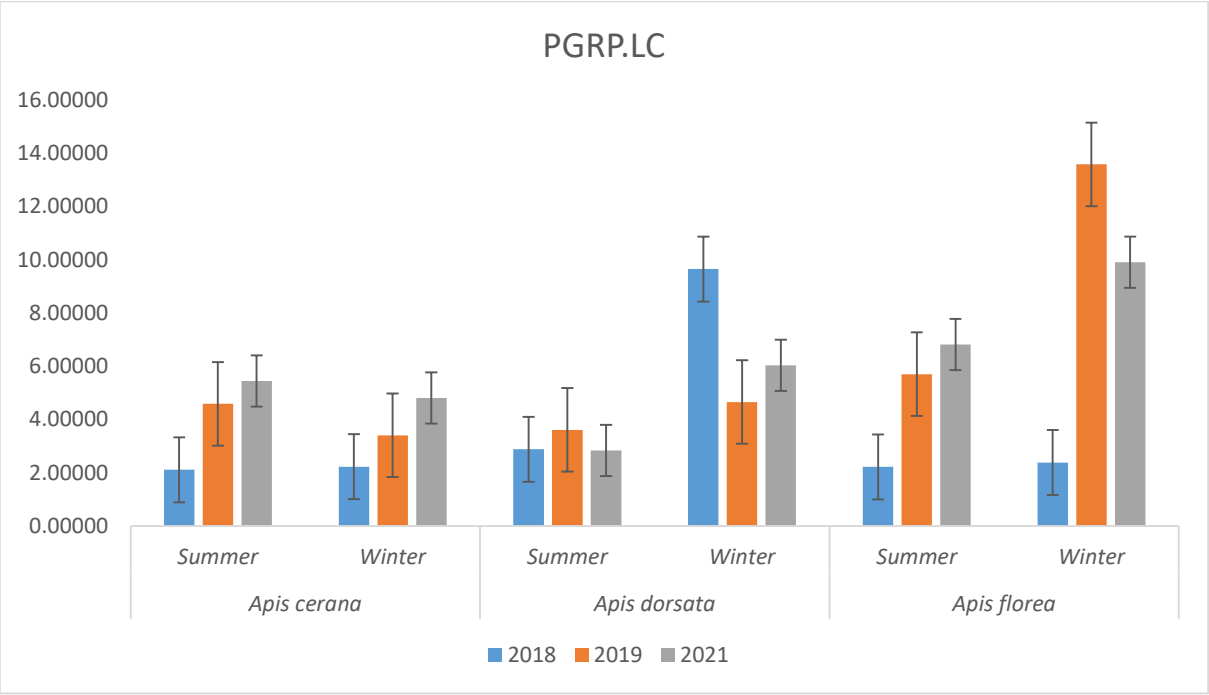

Figure 4: Relative expression levels of *PGRP.LC* gene member across the seasons and among the species. All the values are average of triplicates. Values are expressed as mean ± SD.

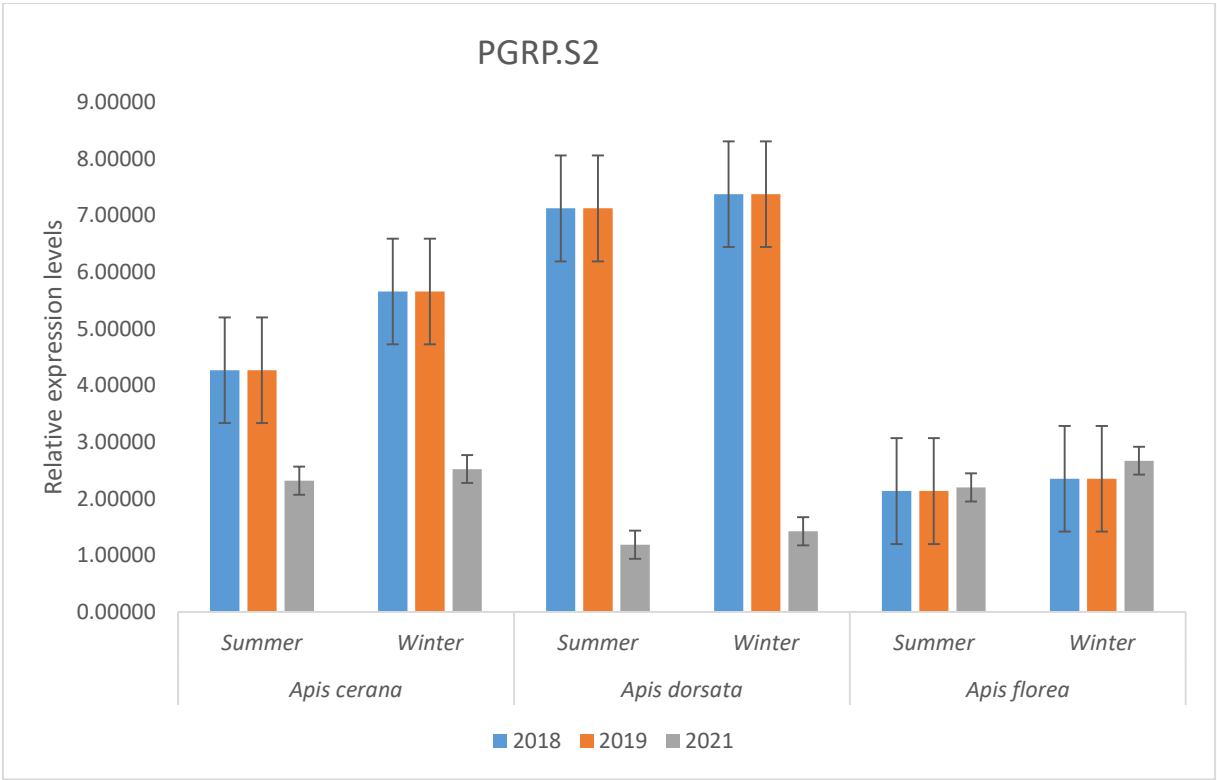

Figure 5: Relative expression levels of *PGRP.S2* gene member across the seasons and among the species. All the values are average of triplicates. Values are expressed as mean ± SD.

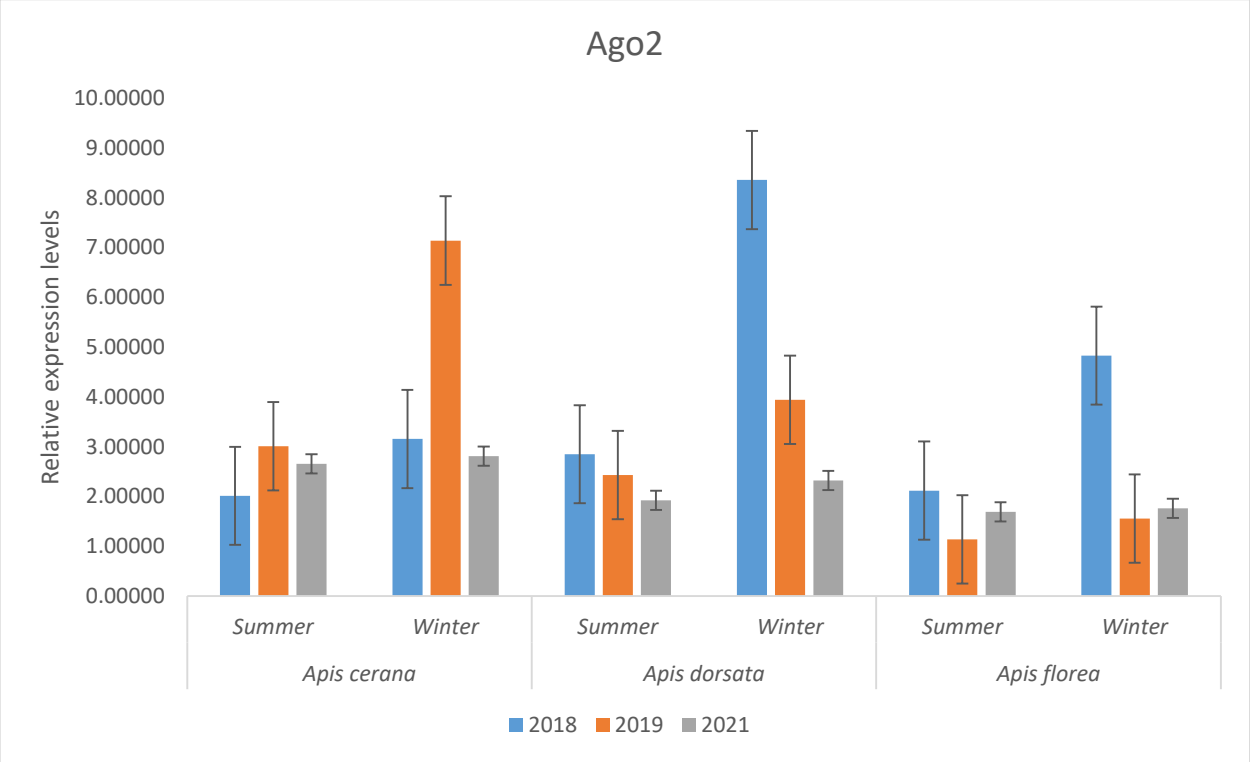

Figure 6: Relative expression levels of Ago2 gene member across the seasons and among the species. All the values are average of triplicates. Values are expressed as mean ± SD.

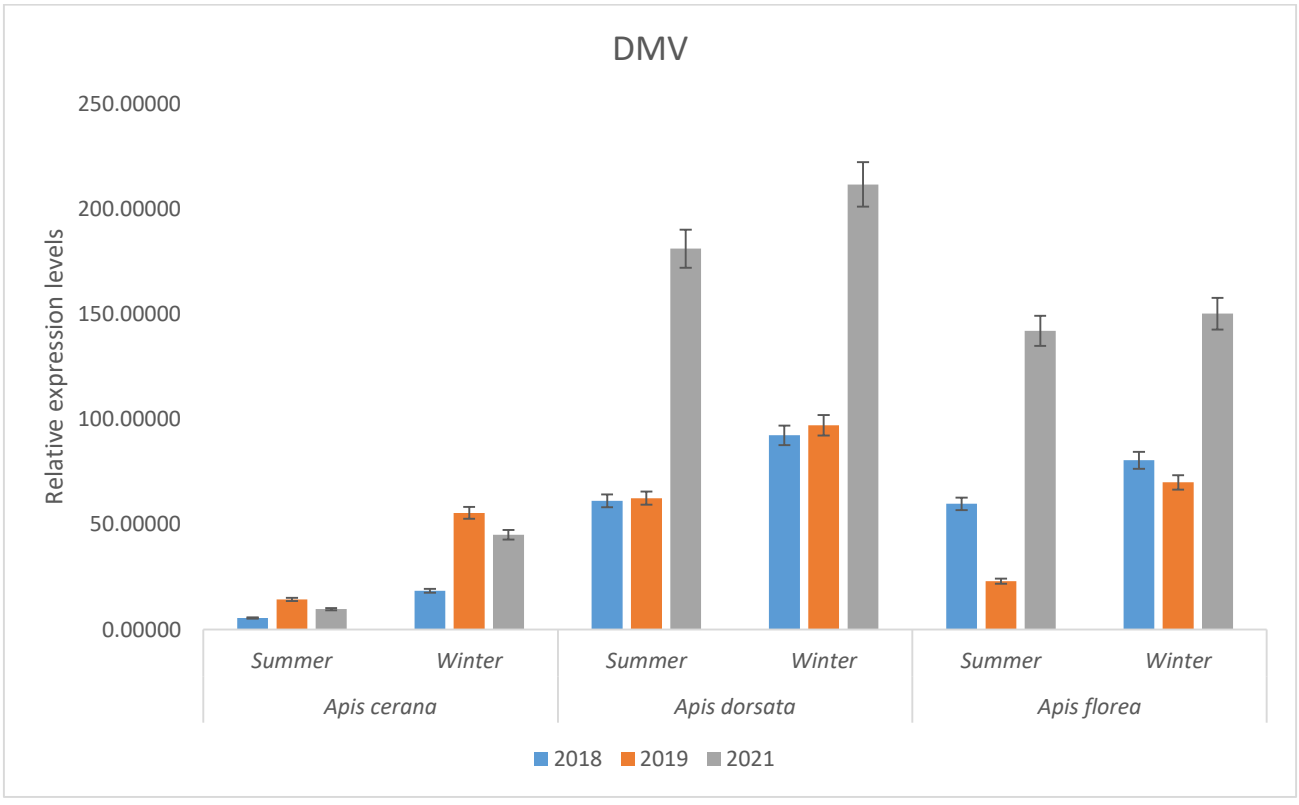

Figure 7: Relative expression levels of DMV gene member across the seasons and among the species. All the values are average of triplicates. Values are expressed as mean ± SD.

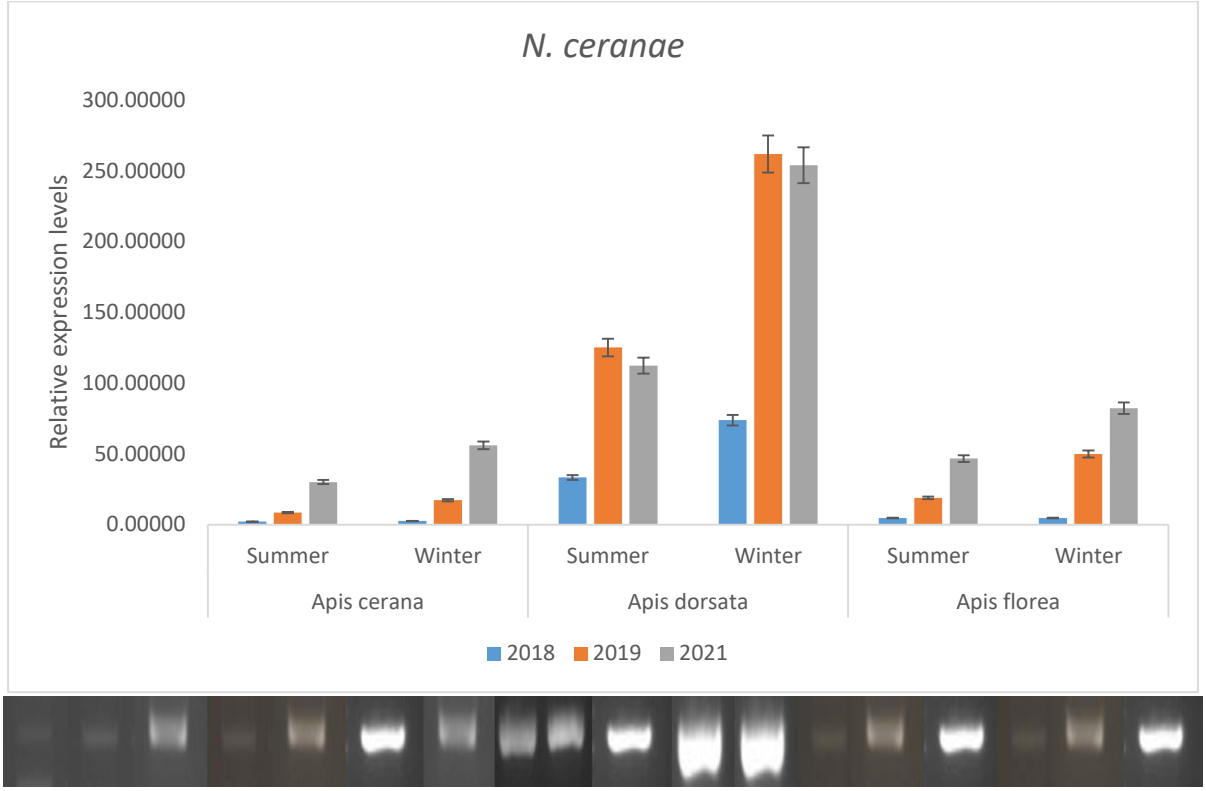

Figure 8: Relative expression levels of *N. ceranae* gene member across the seasons and among the species. All the values are average of triplicates. Values are expressed as mean ± SD. 1.2% agarose gel with the expressed gene members can be seen in the below image.

CONCLUSION

Feral bees offer a great and valuable model to investigate the potential negative consequences of domestication and other human interventions. This could aid us in interpreting the host–pathogen interactions. Undoubtedly, a host manages its gene member's expression in timely manner and of course when in need. On infestation, a host stimulates its immune system to over express some of the genes which could help them to cope up with the infection. We found the gene members like hymenopaectin, ago2, PGRP-LC, PGRP-s2 and defensin to be strongly upregulated with high loads of parasite. In our study, we found higher loads of parasite infection in winter season than the summer season, and a correlation could be seen between the season load of infection and immune gene member's upregulation.

Feral colonies were obviously more susceptible to pathogen infection due to low maintenance of pesticides and treatment. As such these feral bees have their own way of handling these infections. Here, we quantified the pathogen levels along immune gene expression and confirmed the positive correlation between the components. Our study also strongly suggests to use these gene members as indicators for estimating the overall bee health. This could surely help us breeding efforts and focus more towards increasing these traits in selected honey bee stocks, this could decrease the overall colony losses for the beekeeping industry. This study could also pave ways to understand the role of feralization on pathogen dynamics and Eco immunology among the managed species.

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