EFFECTS OF PITUITARY PARS INTERMEDIA DYSFUNCTION AND PRASCEND® TREATMENT ON ENDOCRINE AND IMMUNE FUNCTION IN SENIOR HORSES

DISSERTATION

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ABSTRACT OF DISSERTATION

EFFECTS OF PITUITARY PARS INTERMEDIA DYSFUNCTION AND PRASCEND® TREATMENT ON ENDOCRINE AND IMMUNE FUNCTION IN SENIOR HORSES

Pituitary pars intermedia dysfunction (PPID) is one of the most common endocrine diseases affecting senior horses. PPID causes abnormally high concentrations of adrenocorticotropic hormone (ACTH) in the plasma and a very distinct, long, shaggy haircoat (hypertrichosis). At present, the recommended treatment for PPID is daily oral administration of pergolide mesylate. Due to the increased ACTH levels associated with PPID, it is commonly thought that these horses are immunosuppressed and at increased risk of opportunistic infections, although current research in this area is sparse. Additionally, it is not well-understood how treatment with Prascend® (pergolide tablets) affects endocrine measures other than ACTH and if it also impacts the immune response.

To better understand how PPID influences endocrine and immune function in the horse, Non-PPID horses (n=10), untreated PPID horses (n=9), and PRASCEND-treated PPID horses (n=9) were followed over 15 months. Endocrine measures assessed included basal ACTH, ACTH responses to thyrotropin-releasing hormone (TRH) stimulation tests, basal insulin, insulin responses to oral sugar tests (OST), total cortisol, and free cortisol. Systemic immune function measures included basal and stimulated whole blood and peripheral blood mononuclear cell (PBMCs) cytokine and receptor expression, plasma myeloperoxidase levels, and complete blood counts. Localized immune function measures within the lung included cytokine and receptor expression after stimulation of cells obtained via bronchoalveolar lavage (BAL), myeloperoxidase levels in BAL fluid, and BAL fluid cytology. We hypothesized that PPID would affect immune function, but that any alterations would be corrected by treatment with PRASCEND.

Results for the endocrine analyses showed that basal ACTH was reduced in the PRASCEND-treated horses to the levels of the Non-PPID horses, but ACTH in response to TRH stimulation was only reduced in the PRASCEND-treated horses at non-fall timepoints. PPID did not affect basal insulin, insulin responses to OSTs, total cortisol, or free cortisol, and PRASCEND treatment did not appear to have an impact on these measures either. These results suggest that PPID and hyperinsulinemia/insulin dysregulation are distinct endocrine conditions, and that the excess ACTH in horses with PPID is inactive, as it is unable to stimulate a normal cortisol response.

In the immune function analyses, PPID horses had decreased expression of interferon gamma (IFNγ) from PBMCs stimulated with *Rhodococcus equi* and *Escherichia coli* and increased transforming growth factor beta (TGFβ) expression from the *E. coli*-stimulated PBMCs. TGFβ was also increased in PPID horses in the unstimulated whole blood samples. These results suggest that PPID horses are unable to mount an appropriate Th1 response, and that the regulatory subset of T-lymphocytes may be contributing to this decreased Th1 response. Results for the localized immune function analyses may indicate altered Th2 responses within the lung of PPID horses, although these results were severely limited by the sample size available for analyses. PRASCEND did not appear to affect immune function as measured in this study.

In summary, PRASCEND successfully reduces basal ACTH in PPID horses and remains the best choice for veterinarians in monitoring dosage and response to PRASCEND treatment. Insulin, total cortisol, and free cortisol were not affected by PPID status or PRASCEND treatment in this study. Immune function was altered in horses with PPID, and it is likely that these horses are indeed at increased risk of opportunistic infection. PRASCEND treatment did not correct the differences in immune function in this study. Additional research is needed to further understand which mechanisms are driving the alterations in immune function for horses with PPID.

KEYWORDS: equine, PPID, pituitary, pergolide, immune, endocrine

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To my husband, Bob, who has listened to me talk about the contents of this dissertation so often that he won't need to read it but will anyway. Thank you for your love and unwavering support.

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CHAPTER 1. LITERATURE REVIEW

1.1 Pituitary pars intermedia dysfunction and its effects on endocrine function

The population of geriatric horses is steadily rising with advances in veterinary medicine and a shift in the role of the horse from that of a work animal to that of a companion animal. The most recent results from the National Animal Health Monitoring System survey in 2015 indicated that almost half of the U.S. equine operations surveyed (44.6%) had at least one horse over twenty years of age [1]. Horses are generally considered to be old or geriatric when they are fifteen years of age or older [2]. Pituitary pars intermedia dysfunction (PPID) is a common disease of aging horses. Several studies have investigated the prevalence of PPID, with the most reliable data indicating a prevalence of about 20% in horses over fifteen years old, with a range in prevalence reported as approximately 10-22% in most of the epidemiological analyses conducted to date [2-6].

The most frequent clinical sign observed in PPID horses is the development of an abnormal haircoat, termed hypertrichosis, which is long, can be with or without curl, and often exhibits absent or delayed shedding [2,5,7-10]. This abnormal haircoat can be seen over the entire body or may be regional [7-9]. Additional clinical signs associated with PPID include polyuria, polydipsia, hyperhidrosis, muscle wasting, weight loss, and possibly changes in reproductive and immune function as well [5,7-12].

Pituitary pars intermedia dysfunction is caused by the loss of dopaminergic inhibition of the melanotropes of the pars intermedia of the pituitary [5,7,13]. In a healthy horse, adrenocorticotropic hormone (ACTH) is produced by the pars distalis of the pituitary, whereas in horses with PPID, ACTH is also aberrantly produced by the pars intermedia of the pituitary [5,7].

In both the pars distalis and pars intermedia, the pre-prohormone, pro-opiomelanocortin (POMC) is produced and cleaved by prohormone convertase I (PC-I) into ACTH and several other peptide hormones [5,7,13]. A second enzyme is present only in the pars intermedia, prohormone convertase II (PC-II), which further cleaves ACTH into alpha-melanocyte-stimulating hormone (α-MSH) and corticotropin-like intermediate peptide (CLIP); in a healthy horse, essentially all ACTH produced by the pars intermedia is cleaved into these products [5,7,13]. Without proper inhibition of the melanotropes of the pars intermedia by dopamine, as is the case in PPID horses, POMC production and PC-I activity increases, thereby increasing ACTH production [5,7]. However, PC-II activity does not increase to the same extent and is no longer sufficient to cleave the greater influx of ACTH into α-MSH and CLIP [5,7]. Therefore, this ACTH is released, increasing the plasma ACTH concentrations in horses with PPID [5,7-9,13].

In horses, seasonal variation in endocrine function is well-described. ACTH increases within the fall time period (mid-July to mid-November) for the United States in both healthy and PPID horses, although this seasonal rise is exaggerated in horses with PPID [5,7-9,14,15]. This change has been shown to be driven by seasonal changes in dopamine, but horses with PPID have been also been shown to have lower dopamine concentrations than Non-PPID horses in three of the four seasons [16,17]. If the seasonal changes in ACTH in a horse's geographic region are not considered when screening for PPID, false diagnosis of PPID is possible within this normal seasonal ACTH rise [15].

A normal hypothalamus-pituitary-adrenal axis response to increased ACTH concentrations is to also increase cortisol concentrations [18]. In response, both ACTH and cortisol can also control corticotropin-releasing hormone (CRH), POMC, and ACTH

release through negative feedback [18]. However, the pars intermedia of horses does not contain receptors for these hormones, so ACTH production from the pars intermedia is unable to be shut down through these normal pathways [5].

Due to the normal response of increasing cortisol as a result of increasing ACTH, cortisol has also been thought to be impacted by PPID. This, along with comparisons of PPID in horses to Cushing's disease in other species, has generally been the basis behind which PPID horses were assumed to be immunosuppressed [5,18-20]. However, Cushing's Disease in other species, such as humans and dogs, affects different areas of the pituitary than in horses, thus preventing comparisons from being made between PPID in horses and Cushing's Disease in other species [10]. Research on the impacts of ACTH on cortisol in horses with PPID has been limited mainly to investigations of total cortisol concentrations and often produces conflicting results. One study that examined total cortisol over twenty-four hours in each of the four seasons found that horses with PPID only had higher total cortisol levels at the summer timepoint (June) when compared to normal horses [16]. This same study found that PPID did not impact the ability of the horse to follow normal responses to day-length and the photoperiod [16]. Conversely, other studies have found no changes in resting total cortisol in horses with PPID [21,22].

In general, total cortisol analyses are not thought to be reflective of cortisol's active effects, as most cortisol is bound to corticosteroid-binding globulin (CBG) and is thus prevented from diffusing through cell membranes and exerting its effects; however, hydrocortisone bound to CBG has been recently shown to alter the function of equine neutrophils *in vitro* [23,24]. In the two main studies that have examined the effects of PPID on free cortisol, one study found no difference in free cortisol, but found increased total

urinary cortisol in PPID horses, while the other found increased free cortisol fraction in PPID horses [22,25]. In addition, another study found only a weak correlation between cortisol and ACTH in Non-PPID horses ($r = 0.31$ and $r = 0.25$, depending on the season in which the horses were sampled) [26].

While a tendency to investigate the more systemic endocrine effects of PPID is often seen in the literature, the potential for more localized effects of these hormones should not be underestimated. To investigate whether local cortisol contributes to poor immune responses within the eyes of PPID horses, Hart et al. examined the cortisol concentrations in tears and found that PPID horses had significantly higher cortisol concentrations in their tears [27]. Localized cortisol effects were also seen in one study that examined suspensory ligament failure in PPID horses; they found that horses with PPID had significantly higher numbers of glucocorticoid receptors within the suspensory ligament compared to Non-PPID horses [28].

Hyperinsulinemia and/or insulin dysregulation, and thus laminitis, are also frequently thought to be related to PPID; however, it is often debated as to whether the two conditions are related or whether they are distinct and are both common conditions found in older horses [5,7,10,29-32]. Mastro et al. examined insulin sensitivity and its associated pathways in PPID horses from a research herd; they found no differences in insulin sensitivity between the PPID and age-matched Non-PPID horses, which supports the conclusion that hyperinsulinemia and/or insulin dysregulation and PPID are distinct, rather than related, conditions [33]. While literature regarding the impact of PPID on insulin may be inconsistent, it is well-accepted that insulin is not an appropriate tool for diagnosing

PPID, and that veterinarians should monitor older horses for hyperinsulinemia and/or insulin dysregulation, regardless of a horse's PPID status [10,14,29,32,33].

1.2 Diagnosing pituitary pars intermedia dysfunction in horses

Given the role of ACTH in the pathogenesis of PPID, it follows that the current recommendations for the diagnosis of PPID in horses are focused on the measurement of ACTH, either basal or in response to a thyrotropin-releasing hormone (TRH) stimulation test [5,7-9]. In horses already displaying signs of hypertrichosis, a basal ACTH above the seasonal reference range is indicative of PPID [5,7-10]. However, detection of PPID before more severe clinical signs are apparent is possible by measuring the ACTH response to a TRH stimulation test [5,7-9,34]. Veterinarians can perform this test at the farm or facility where a horse is typically located, as it does not require specialized equipment. First, a blood sample is collected to measure basal ACTH concentrations (T0) [5,7-9,34]. Then, TRH is administered intravenously, and a second blood sample is collected ten minutes later (T10) for ACTH analysis [5,7-9]. The administration of TRH stimulates ACTH production because TRH receptors are present in the pars intermedia of horses; in PPID horses, exaggerated responses to the TRH stimulation test are seen [5,14,35].

After comparing the ACTH results to the seasonal reference ranges for the local region, a diagnosis of Non-PPID or PPID can be made [5,7-9]. In cases where the results of the TRH stimulation test are ambiguous, it is recommended that the test be repeated in several months (during a different season) [5,7,8]. Obtaining TRH has sometimes proven difficult, but it is currently available in ready-to-use compounded form or in research-grade powder form, which can be then be mixed with sterile 0.9% NaCl in aseptic fashion [5,7].

The dexamethasone-suppression test (DST) was also historically used to diagnose PPID but has fallen out of favor for several reasons [5,34,36]. To perform a DST, a baseline cortisol sample was taken in the evening, followed by intramuscular dexamethasone administration at a dose of 40 μg per kilogram of body weight, and a follow-up cortisol sample was taken between fifteen and nineteen hours later [5]. The reasoning behind this test is the thought that PPID impacts the hypothalamus-pituitary-adrenal axis responses of PPID horses, and that their high ACTH production by the pars intermedia stimulates cortisol increases that are not susceptible to negative feedback because the pars intermedia does not have glucocorticoid receptors [5,10]. As discussed in the previous section, the impact of PPID on cortisol is not clear and even in healthy Non-PPID horses, cortisol appears to be only weakly linked, if at all, to increased ACTH levels [5,26,34]. Given that hyperinsulinemia and laminitis occur in some horses with PPID, many veterinarians and owners are hesitant to administer dexamethasone, particularly when basal ACTH or the ACTH response to a TRH-stimulation test do not carry the same risks [10,21,37,38]. Additionally, the DST is not reliable in detecting PPID in the preclinical stages [5,7,34]. Since basal ACTH concentrations are sufficient for diagnosis in the more advanced presentations of PPID, the administration of the DST is rather unnecessary [5]. Further, the DST generally requires multiple visits from a veterinarian because of the need for the evening and morning cortisol samples. For all of these reasons, the TRH stimulation test is the recommended diagnostic test for PPID at this time [5,7-9].

It has been noted that feeding can impact ACTH levels in the horse [39]. Therefore, when it is desired to compare multiple results over time, feeding status should be consistently maintained (i.e. always fed or always fasting) [39]. Given the current

recommendations to also monitor insulin in geriatric horses, it is often more cost-efficient for owners and easier for veterinarians to sample in the same visit for both ACTH and insulin in a fasted horse [7-9,36].

1.3 Treating pituitary pars intermedia dysfunction in horses

Given the involvement of dopamine in the pathogenesis of PPID, it follows that the majority of treatment options for PPID have included the use of dopamine agonists [5,7-10]. Currently, the only FDA-approved treatment for PPID is daily oral administration of Prascend® (pergolide tablets) (Boehringer-Ingelheim Animal Health; Duluth, GA), a dopamine agonist [7-9,36]. Suggested starting doses are 0.002 milligrams (mg) per kilogram (kg) of body weight, but this can be increased up to 0.004 mg/kg of body weight if ACTH is not controlled [7]. PRASCEND is generally given orally once per day, but recent work suggests that twice per day administration may produce more stable responses [40]. It is currently recommended that ACTH concentrations be re-checked regularly to monitor the response to treatment with PRASCEND and to ensure dose adequacy [5,7-9,36]. Additionally, present recommendations indicate that the method of testing used to originally diagnose PPID in a horse should continue to be used to monitor dose adequacy and response to treatment in that horse. [8,9].

Aside from its proven ability to reduce ACTH concentrations in PPID horses, the impact of PRASCEND on other aspects of endocrine function is largely unknown at this time [40,41]. One study found that PRASCEND did not appear to affect glucose or insulin; nevertheless, PRASCEND and other dopamine agonists are frequently given to Non-PPID horses with hyperinsulinemia/insulin dysregulation in an attempt to lower insulin concentrations [42,43]. On the reproductive side of endocrine function, a single case report

noted that PRASCEND treatment did not appear to impact fertility in a stallion [11]. Other dopamine agonists, such as bromocriptine and cabergoline, have also been used in horses with PPID, but at this time, their efficacy and usefulness remain unclear [44].

Outside of the dopamine agonists, one drug sometimes used for the treatment of PPID is cyproheptadine, a serotonin antagonist [5,10]. Cyproheptadine appears to be able to reduce ACTH concentrations, but is not nearly as effective as PRASCEND, which explains its infrequent use [5,10,36,45,46]. It is sometimes used in addition to PRASCEND for horses that are not controlled on PRASCEND alone [5,46].

While PRASCEND is currently the only FDA-approved treatment for horses with PPID, it is unclear whether PRASCEND influences immune function in these horses.

1.4 Effects of pituitary pars intermedia dysfunction on immune function

The impact of PPID on immune function in horses is currently not well-described, and research in this area is comprised mainly of case reports, with few experimentally controlled studies. Despite this, PPID is frequently noted anecdotally to cause immunosuppression in horses [5,7-9,47-49]. Clinically, it appears that PPID horses may be at greater risk of infection caused by opportunistic bacteria and fungi due to the numerous reports of infection involving their respiratory and integumentary systems [5,7-9,47,49].

Several studies by McFarlane et al. have examined immune responses in PPID horses [48,50]. In one study, cytokine expression from total leukocytes and TNFα production from peripheral blood mononuclear cells (PBMCs) were analyzed. PPID horses had IFNγ and IL-6 expression that was similar to Non-PPID adult (younger) horses, but lower than age-matched Non-PPID horses [50]. Additionally, PPID horses had elevated IL-8 expression

compared to Non-PPID adult horses, but it was similar to the expression in the age-matched Non-PPID horses in this study [50]. For the PBMC stimulations in this same study, there were no differences between the groups in the unstimulated samples, but PPID horses had greater TNFα production from lipopolysaccharide (LPS)-stimulated PBMCs than both the age-matched and adult Non-PPID horses [50]. Release of TNFα is a normal response to LPS, and this is consistent with studies in horses and other species; however, it appears to have been more exaggerated in PPID horses in the described study [50-53].

Based on the differences in IL-8 described in the study above, McFarlane et al. further investigated neutrophil function in PPID horses [48]. This study revealed that neutrophils in horses with PPID had decreased oxidative burst, but normal phagocytosis, compared to Non-PPID horses [48]. Additionally, in response to IL-8, neutrophils from PPID horses showed similar chemotaxis to younger Non-PPID horses but decreased from age-matched Non-PPID horses [48]. Neutrophil adhesion was also decreased in PPID horses compared to Non-PPID horses when they were unstimulated or in the presence of mild stimuli, but was not different with maximal stimulation by phorbal 12-myristate 13-acetate (PMA) [48]. However, in both of the studies described above, the samples were collected over a period of sixty to ninety days, which may complicate the analysis [48,50].

In addition to the above studies that focused mainly on white blood cells (WBC) in PPID horses, some work has also been done in the area of parasitic load and response to anthelmintic treatment in PPID horses. One study found differences in fecal egg counts before and after ivermectin treatment with PPID horses having higher fecal egg counts than Non-PPID horses [54]. A second study in this area focused on horses diagnosed with PPID based only on basal ACTH levels, with follow-up testing in the PPID horses approximately

seven months later [55]. The authors found no difference between the PPID horses and Non-PPID horses in this study and did not find a difference in the PPID horses that were untreated or treated with pergolide [55]. However, the authors cautioned that they used horses that were pre-clinical or less severe cases of PPID in this study [55]. This can be seen in their given ranges for basal ACTH, in which some of the horses were very close to the study's Non-PPID cut-off values of less than 35pg/mL [55]. Given the work in the more severe cases of PPID which indicated a difference in fecal egg counts before and after anthelmintic treatment compared to Non-PPID horses, it appears likely that PPID has some level of effect on parasitic load in horses.

As mentioned in the section above on endocrine function in PPID horses, Hart et al. also investigated whether or not cortisol concentrations in the tears of horses may contribute to poor immune responses in the eye; they found that PPID horses had significantly higher cortisol concentrations in their tears, which may also indicate localized alterations in immune function in PPID horses [27].

1.5 Effects of pituitary pars intermedia dysfunction on the lung

Little is currently known regarding the impact of PPID on the lung of horses. It is often mentioned that these horses have higher rates of asthma or heaves, but to the author's knowledge, there is no literature at this time that supports this conclusion [46]. In one study that compared pathological findings in horses with PPID to both younger and age-matched Non-PPID horses, the PPID horses were found to have significantly higher rates of bronchiolitis and bronchointerstitial inflammation than either Non-PPID group [56]. In this same study, lungs were scored based on severity, and PPID horses also had significantly higher lung scores than both the younger and age-matched Non-PPID horses [56].

1.6 An overview of opportunistic infection

Across many species, opportunistic infections are frequently described in individuals with both primary and secondary immune dysfunction or suppression [19,57]. These infections are often caused by pathogens that are commonly found in the external environment and on or within the host, but do not generally cause disease in a healthy host [58]. Due to their exposure to the external environment, the respiratory and integumentary systems are often affected by opportunistic infection. Indeed, in adult horses, most pneumonia cases occur in conjunction with some degree of breakdown in immune function, whether due to underlying chronic disease, secondary infection after viral disease, or administration of immunosuppressive drugs, such as corticosteroids [58-60]. Additionally, pleuropneumonia is one of the most common causes of sepsis in adult horses [61].

Escherichia coli is one of the most commonly isolated bacteria from equine pneumonia cases [58,60,62-64]. *E. coli* is a ubiquitous gram-negative bacterium that is frequently encountered by adult horses [60]. Generally, commensal *E. coli* does not cause harm; however, breakdowns in immune function can permit infection to occur [60].

While *Rhodococcus equi* infections in horses are typically seen in foals, particularly in the first several months of life, *R. equi* infections have also been reported in adult horses and are generally accepted to be opportunistic pathogens in these cases [59,60,65-67]. However, *R. equi* infection is still not common in adults despite the abundant presence of this gram-positive bacterium in soil and its ability to thrive in manure [59,60,65-68]. Additionally, *R. equi* infection has often been found in immunocompromised humans, such as HIV-positive individuals and transplant recipients on immunosuppressive medications, and tends to have high mortality rates in these cases [69-72]. *R. equi* infections in immunocompetent humans

have also been described, but there does appear to be an association with age in these cases [69,73,74].

As in humans and foals, adult and aged horses can develop localized *R. equi* lesions within the lung, but also bacteremia, with *R. equi* spreading diffusely throughout the body [59,66,67,75,76]. Especially in foals, extrapulmonary lesions are quite common, demonstrating that the potential for an individual to develop bacteremia should not be underestimated [74,76-80]. Among the reported *R. equi* infections in adult horses, some cases note an immunocompromised host, while others do not [59,60,65-67]. The onset of *R. equi* infection is often insidious in adult horses, potentially due to low awareness, which is problematic in both human and veterinary medicine [65-67,71,81]. Pneumonia is the most common initial diagnosis, but *R. equi* is often not seen in these cultures, which can lead to delayed treatment and poor prognosis [66,67,70]. It has been hypothesized that the tendency for *R. equi* to not be identified in culture could be due to its slower growth habits (approximately forty-eight hours) compared to other commonly isolated species from equine pneumonia cases; therefore, the growth of other isolates may obscure *R. equi* since cultures from pneumonia cases in horses are frequently positive for multiple species of bacteria and/or fungi [59,60,66,70].

It is well-documented that the susceptibility of foals to *R. equi* infection is caused by their reduced expression of IFNγ and a reduced Th1 response compared to adult horses [82]. The Th1 response is crucial to clear an infection caused by *R. equi*, due to the intracellular nature of the bacterium and its ability to persist in the macrophages of the lung [82]. An appropriate Th1 response in young horses is generally developed by approximately six months of age, at which point the risk of *R. equi* infection drops substantially [82]. Additionally, the presence of the virulence-associated protein A (VapA) in *R. equi* is

known to prompt a strong immune response, but *R. equi* expression of VapA also appears to contribute to the ability of the bacteria to evade the host immune response [82]. In summary, both *R. equi* and *E. coli* infections in adult horses are generally considered opportunistic, meaning that a breakdown in the host immune response often precedes infection, particularly of the respiratory tract, by these bacteria.

Currently, studies of PPID in horses are rather limited. In addition, the results of the available works have often yielded conflicting results, making it difficult for clinicians and researchers alike to determine how to best care for horses with PPID. Further, it is not well-understood whether PPID affects the immune responses of horses and whether these horses should be considered immunosuppressed. Therefore, the use of horses housed in a research setting proves ideal and necessary for examining the effects of PPID and PRASCEND treatment on endocrine and immune function in horses.

1.7 Hypotheses and Specific Aims

The focus of the work in this dissertation was to gain further understanding of how endocrine function affects the immune response of horses with PPID and how treatment with a dopamine agonist, PRASCEND, affects these measures. Our hypotheses were that altered endocrine function in PPID horses would cause changes in immune function, both systemically and locally within the lung, when compared to age-matched Non-PPID horses, and that PRASCEND treatment would normalize the measured endocrine and immune responses to levels similar to the Non-PPID horses.

Specific Aim 1: To gain further understanding of the complexities of endocrine function in PPID horses and to investigate the endocrine effects of PRASCEND treatment (Chapter 2)

Specific Aim 2: To investigate the effects of PPID and PRASCEND treatment on systemic immune function and *in vitro* immune responses to stimuli (Chapter 3)

Specific Aim 3: To examine the effects of PPID and PRASCEND treatment on localized immune function and *in vitro* immune responses to stimuli within the lung (Chapter 4)

CHAPTER 2. EFFECTS OF PITUITARY PARS INTERMEDIA DYSFUNCTION AND PRASCEND® TREATMENT ON ENDOCRINE FUNCTION IN HORSES

The material in this chapter has been submitted in consideration for publication and is currently under review. The author of this dissertation is the primary author on this paper. Co-authors on this paper provided laboratory analyses, advice on study design or statistical methods, and reviewed the manuscript prior to submission.

2.1 Abstract

Background: It remains unclear how Pituitary Pars Intermedia Dysfunction (PPID) and treatment with Prascend® (pergolide tablets) affect endocrine function in horses.

Hypothesis/Objectives: Our hypothesis was that untreated (PPID Control) horses would have altered endocrine function compared to Non-PPID and PRASCEND-treated (PPID Treatment) horses as measured by ACTH, insulin, total cortisol, and free cortisol.

Animals: 28 horses from a research herd (10 Non-PPID, 9 PPID Control, and 9 PPID Treatment) horses.

Methods: Blood was collected regularly over approximately 15 months. PRASCEND treatment was initiated after Day 0 collections. Results were analyzed using a linear mixed model (SAS v9.4), with significance set at $P<.05$.

Results: Significant group ($P<.01$) and group by time ($P<.001$) effects were observed in basal ACTH such that PPID Treatment horses differed from Non-PPID horses only at Day 0 (Least squares mean/standard error: 244.44/54.5861 (PPID Treatment-Day 0) versus 54.62/51.7849 (Non-PPID-Day 0) (pg/mL); $P=.012$). PPID Treatment horses had

significantly lower ACTH change scores in response to TRH stimulation tests than PPID Control horses at non-fall timepoints only, Mid-late February 2018 (least squares mean/standard error: 441.72/84.99 versus 733.12/82.8638 (pg/mL); *P*=.016) and Early April 2018 (least squares mean/standard error: 272.84/87.4494 versus 563.57/82.7348 (pg/mL); *P*=.017). No significant group or group by time effects were seen in insulin, total cortisol, or free cortisol; however, significant time effects were seen in these measures (*P*<.0001).

Conclusions and clinical importance: Basal ACTH remains the best choice for determining successful response to PRASCEND treatment. Neither PPID nor PRASCEND appear to influence insulin, total cortisol, and free cortisol.

2.2 Introduction

Pituitary Pars Intermedia Dysfunction (PPID) is a common endocrine disease affecting older horses. Horses with PPID have increased plasma concentrations of adrenocorticotropic hormone (ACTH) and often develop distinct hypertrichosis, with difficulty shedding their coats appropriately [5,9]. Clinical signs often associated with PPID include abnormal sweating, polyuria, polydipsia, frequent infections, laminitis, and loss of muscle mass [5,9]. Currently, the only FDA-approved treatment for PPID is daily oral administration of Prascend® (pergolide tablets) (Boehringer-Ingelheim Animal Health, Inc., Duluth, GA), a dopamine agonist [36].

Due to increased ACTH concentrations, it is frequently thought that other endocrine changes may exist in horses with PPID, such as changes in insulin, total cortisol, and free cortisol. Presently, it is not well understood if and how PPID may affect these endocrine

measures, since many current studies have come to contradictory conclusions [8-10,14,22,25,27,42,83,84]. Additionally, it is unclear what impact PRASCEND treatment, which is known to decrease plasma ACTH levels, has on other aspects of endocrine function and on ACTH responses to thyrotropin-releasing hormone (TRH) stimulation tests [41,42,45,85]. Current literature is also rather mixed in whether PPID and hyperinsulinemia are separate or related endocrine diseases, and few long-term studies have been conducted to determine whether PRASCEND treatment improves insulin responses [86].

Challenges for studies seeking to further understand PPID include often needing to use client-owned horses, with samples collected and stored over a range of times, and horses often treated on different schedules based on owner availability. Given the known seasonal changes in ACTH, along with other hormones, this can be problematic when interpreting results . Thus, the objective of this study was to characterize the effects of long-term PRASCEND treatment with PPID on multiple endocrine parameters in university-owned horses under consistent treatment, seasonal, and environmental conditions. We hypothesized that untreated PPID horses would have altered endocrine function compared to the Non-PPID and PRASCEND-treated PPID horses.

2.3 Materials and Methods

2.3.1 Animal Selection and Study Design

Horses were initially selected for potential inclusion in the study from a university-owned research herd based on prior screenings and clinical history (absence or presence of hypertrichosis and/or absence or presence of history of hypertrichosis/difficulty shedding

and/or results of multiple TRH stimulation tests). PPID status was confirmed using ACTH responses to TRH stimulation tests approximately two weeks prior to Day 0 of the study (Early September 2017) and basal ACTH values on Day 0. An oral sugar test (OST) was also performed prior to the study, approximately one week after the TRH stimulation test, to assess hyperinsulinemia or insulin dysregulation status [87]. Horses that did not screen as either Non-PPID or PPID for both the TRH stimulation test and basal ACTH screenings were excluded from the study. As these initial screenings were obtained during the fall season in the USA (late August and early September), a basal ACTH value of ≥100pg/mL and ACTH 10 minutes post-TRH injection (T10) value of ≥400pg/mL were used for selection into the PPID group [8,9].

Thirty-one horses were originally selected to proceed through the study. However, three horses assigned to the PRASCEND-treated group had ACTH values that failed to become controlled and remained uncontrolled throughout the study; these horses were excluded from all analyses and are not discussed further. Non-PPID horses (n=10) were blocked into one group. An attempt was made to randomly assign the PPID horses into the untreated and treated groups, however, due to also attempting to block for insulin status, true randomization was not possible. PPID Control horses (n=9) remained untreated for the duration of the study, and PPID Treatment horses (n=9) began receiving PRASCEND within approximately 24 hours of their completion of all Day 0 sample collections. Horses were started at the lowest label dose (2 μg/kg) to the nearest half-tablet for their body weight, based on the manufacturer's label recommendations. Basal ACTH was checked weekly for the first 4 weeks, and the dose was increased each week until basal ACTH levels

were reduced to controlled (Non-PPID) values or until the maximum label dose (4 μg/kg) to the nearest half-tablet was reached.

Throughout the approximately 15-month study, six horses were euthanized due to non-study related health issues. The study ended with 9, 7, and 6 horses in the Non-PPID, PPID Control, and PPID Treatment groups, respectively. At the start of the study, there was no statistical difference in age between the groups (P =.17) (Table 2.1). Age ranges were 20-29 years for Non-PPID horses, 21-31 years for PPID Control horses, and 19-29 years for PPID Treatment horses. Only mares and geldings were included (Table 2.2). Breed representations, either confirmed or to the best of the researchers' knowledge, included Thoroughbred (n=14), American Quarter Horse (n=4), Standardbred (n=1), Standardbred cross (n=1), Mustang (n=1), Paint (n=1), Arabian (n=1), Tennessee Walking Horse (n=1), Appaloosa (n=1), and unknown or mixed breed (n=3) horses (Table 2.2). Breakdowns of sex and breed by group are included in Table 2.2. All horses were cared for and sampled with approval of the University of Kentucky's Institutional Animal Use and Care Committee (IACUC) (#2014-1225 and #2018-3004).

2.3.2 Sampling

On sampling days, horses were grain fasted (if part of their normal ration) until all collections were completed. Blood samples were collected via jugular venipuncture within a four-hour window during morning hours (approximately 08:00 to 12:00), for measurement of ACTH and insulin at fourteen timepoints and for measurement of total cortisol and free cortisol at ten of these timepoints. At each timepoint, all blood samples (not including the TRH stimulation tests or OSTs) were collected over a period of 1-4 days, but within the same time window above (08:00 to 12:00); when multiple days were needed

to complete all of the collections, a mixture of horses from the three groups were screened each day. Appendix 2 contains a sample timeline. TRH stimulation tests and OSTs were performed at six timepoints. Body weight, body condition score (BCS), cresty neck score (CNS), and hypertrichosis score were evaluated in each horse at 10, 7, and 8 timepoints, respectively.

For the TRH stimulation tests, a blood sample (T0) was collected, 1mg. of TRH was administered intravenously, and a second blood sample was collected 10 minutes later (T10) [8,9]. The TRH (Sigma Aldrich; St. Louis, MO) was prepared aseptically by dissolving 1 mg. of TRH in 1 mL of sterile saline (0.9% NaCl).

For OSTs, a blood sample (T0) was collected, Karo® light corn syrup (ACH Food Companies Inc, Cordova, TN) was administered orally, and a second blood sample was collected 60 minutes later (T60); the corn syrup dose used was 0.15 mL of corn syrup per kilogram of body weight with an extra 5 mL included for wastage during administration [87]. Horses were weighed for accurate dosing of corn syrup using a portable agricultural scale within one week prior to the test.

Regular assessment of BCS [88], CNS[89], and hypertrichosis score using a previously published method by Schott et al. [90] was completed by three individuals, and the average of the three scores was used. The hypertrichosis score range was 0 (normal) to 3 (severe coat changes) [90].

ACTH, insulin, total cortisol concentration, estimated free cortisol concentration, and free cortisol fraction (%) were analyzed. For ACTH analysis, blood was collected into tubes containing EDTA and kept on ice until processed within 6 hours. For insulin, total

cortisol, and free cortisol concentration analyses, blood was collected into tubes with no additive and allowed to clot. Plasma and serum were separated by centrifugation at 800g for 10 minutes, frozen at -20ºC, and sent to Cornell University's Animal Health Diagnostic Center (Ithaca, NY) for analysis by previously validated chemiluminescent assays (Immulite, Diagnostics Product Corporation; Los Angeles, CA) for ACTH and total cortisol and previously validated radioimmunoassay (EMD Millipore; Billerica, MA) for insulin [22,85]. Free cortisol concentration and free cortisol fraction were determined using the previously published radioactive ligand-binding ultrafiltration assay [91].

2.3.3 Statistical Methods

Age, basal ACTH, and basal insulin at Day 0 were summarized with descriptive statistics (PROC UNIVARIATE, SAS v9.4). Distributional assumptions were evaluated using graphical and numerical summaries for the presence of gross normality violations. The primary analyses were the longitudinal analysis of all measures over time; linear mixed models included main effects for group and time. The primary comparisons were the PPID Treatment to PPID Control groups and the PPID to Non-PPID groups, when the PPID groups were combined. To determine if groups changed differently over time, an F-test for the interaction of group and time was used. Graphical summaries and model fit were used to determine the specifications of the linear mixed model (PROC MIXED, SAS v9.4). To account for repeated measures, an ARMA (1,1) variance-covariance structure was used for ACTH, ACTH change scores in response to TRH stimulation tests, hypertrichosis scores, insulin change scores in response to OSTs, total cortisol, and estimated free cortisol concentration. A TOEP variance-covariance structure was used for BCS, CNS, and body weight. A TOEPH variance-covariance structure was used for basal insulin and free

cortisol fraction (%). All analyses were conducted using SAS v9.4 (Cary, NC), and a two-sided significance level of .05 was used for all statistical tests. Graphs were created using GraphPad Prism v.8.0.2 (San Diego, CA).

Results for PPID Control and PPID Treatment horses were analyzed first. If significant group by time differences were observed, these groups remained separate for comparison to Non-PPID horses. If no significant group by time differences were observed, the PPID Control and PPID Treatment groups were combined before comparison to Non-PPID horses. If no significant group or group by time differences were observed between the combined PPID group and Non-PPID group, all groups were combined for analysis of time effects only.

If starting differences ($P<.1$) were observed between PPID Control and PPID Treatment horses in the initial analyses, starting values were included as a covariate within the model to account for potential confounding. If starting differences ($P<.1$) were observed between PPID and Non-PPID horses, analyses were performed without starting values as a covariate within the model, in order to analyze group effects, and then with starting values as a covariate, in order to analyze group by time effects; results for each of the methods are presented if applicable.

For dynamic testing (TRH stimulation test and OST), the differences between the T0 sample and the second timepoint sample (T10 for TRH stimulation test and T60 for OST) were calculated; this is often referred to as the delta ACTH or delta insulin. In addition, T0 values were included in the model as a covariate to account for potential differences in disease severity. Thus, the dynamic testing results are further reported as ACTH or insulin change scores.

Pearson correlation coefficients were examined for basal ACTH, basal insulin, total cortisol, free cortisol fraction (%), and estimated free cortisol concentration (PROC CORR, SAS v.9.4).

2.4 Results

Basal ACTH

Significant group (P=.032), time (P<.0001), and group by time effects (P=.032) were seen in ACTH between the PPID Control and PPID Treatment groups. Therefore, the groups were kept separate for comparison to Non-PPID horses. Initial analyses of Non-PPID to PPID Control and PPID Treatment horses showed significant group (P<.01), time (P<.0001), and group by time (P<.001) effects (Table 2.3; Figure 2.1A). Between PPID Treatment horses and Non-PPID horses, ACTH only differed at Day 0 (Early September 2017) (P=.012). ACTH was significantly higher in PPID Control horses compared to Non-PPID horses at eight time points, but was not significantly higher at the Early September 2017 (P=.059), Early February 2018 (P=.068), Early April 2018 (P=.17), Mid-June 2018 (P=.16), Early August 2018 (P=.23), and Early September 2018 (P=.12) timepoints. ACTH in Non-PPID horses did not differ significantly from their starting ACTH values (Early September 2017) at any timepoint. Due to starting differences between PPID Control and Non-PPID horses and between PPID Treatment horses and Non-PPID horses, the analyses were run again with starting ACTH values (Early September 2017) included as a covariate in the model; significant group (P<.001), time (P<.0001), and group by time (P<.001) effects remained. The commonly seen rise in ACTH in the fall season was exaggerated in PPID Control horses compared to the Non-PPID and PPID Treatment groups.

ACTH change score in response to TRH stimulation test

Significant time (*P*<.0001) and group by time (*P*=.027) effects, but not a group effect alone), were seen in ACTH change scores between the PPID Control and PPID Treatment groups. PPID Treatment horses had significantly lower change scores in ACTH in response to the TRH stimulation test than PPID Control horses at only the Mid-late February 2018 timepoint (*P*=.047). ACTH change scores in PPID Treatment horses were comparable to PPID Control horses at the Early April 2018 timepoint (*P*=.05) and all other timepoints (*P*-value range: .24 - .86) Due to the group by time differences, the PPID Control and PPID Treatment horses were kept separate for comparison to Non-PPID horses.

Significant group (*P*<.0001), time (*P*<.0001), and group by time (*P*=.0005) effects were seen in ACTH change scores in response to the TRH stimulation test in the PPID Control, PPID Treatment, and Non-PPID comparison (Table 2.4; Figure 2.1B). Non-PPID horses had significantly lower ACTH change scores at all time points compared to both PPID Control and PPID Treatment horses (*P*<.01). PPID Treatment horses had significantly lower ACTH change scores than PPID Control horses at both non-fall timepoints (Mid-late February 2018 (*P*=.016) and Early April 2018 (*P*=.017)) but did not differ at the other timepoints (*P*-value range: .16 - .82). Significant time effects were seen in all groups between the non-fall timepoints (Mid-late February 2018 and Early April 2018) and the other timepoints within the fall season (*P*<.01).

Hypertrichosis scores

Significant time (P<.001) and group by time (P=.034) effects were seen in hypertrichosis scores between the PPID Control and PPID Treatment groups. However, PPID Control and PPID Treatment horses did not differ at any timepoint; the significant group by time effects appear to have been driven by the fact that PPID Treatment horses started out slightly lower than PPID Controls, although not significantly different, and ended the study with slightly higher, but not significantly different, scores than PPID Controls. Given the significant group by time effect, the PPID Control and PPID Treatment groups were kept separate for comparisons with the Non-PPID group.

Initial analysis of hypertrichosis scores for the Non-PPID, PPID Control, and PPID Treatment horses showed significant group (P<.0001), time (P <.0001), and group by time (P =.023) effects (Table 2.5; Figure 2.2). Both PPID groups had significantly higher hypertrichosis scores than Non-PPID horses at all timepoints but did not differ significantly from each other at any timepoint. Due to starting differences between the groups, the analysis was run again with starting hypertrichosis scores as a covariate. The time and group by time effects remained significant, suggesting that the groups did in fact change over time based on their group status, despite starting differences. However, this still appeared to be attributed to the fact that the PPID Treatment horses started lower and ended higher than PPID Control horses, even though the PPID groups were not statistically different at these timepoints.

Insulin

No significant group or group by time effects were seen in insulin between any of the groups. Significant time effects were observed in the PPID Control to PPID Treatment ($P<.0001$), Non-PPID to all PPID ($P<.0001$), and all groups combined ($P<.0001$) comparisons. Table 2.6 and Figure 2.3A display results for the Non-PPID to all PPID comparison.

Insulin change score in response to OST

No significant group, time, or group by time effects were seen in insulin change scores in response to an oral sugar test between any of the groups. Table 7 and Figure 3B display results for the Non-PPID to all PPID comparison.

Body Weight

No significant group or group by time effects were seen in body weight, measured in pounds, between the PPID Control and PPID Treatment horses; a significant time effect ($P<.0001$) was seen. After combining these groups for comparison to Non-PPID horses, significant group ($P=.0033$) and time ($P<.0001$) effects, but not a significant group by time effect, were observed with PPID horses weighing significantly less than Non-PPID horses at all timepoints ($P<.01$) (Table 2.8; Figure 2.4A). Due to significant starting differences between Non-PPID and PPID horses, the analysis was run again with starting weight as a covariate in the model to determine if significant group by time changes were present. This subsequent analysis revealed no significant group by time effects; therefore, these groups did not change over time as a result of their group status.

BCS & CNS

No significant group or group by time effects were seen in BCS between any of the groups. Significant time effects in BCS were observed when all PPID horses were compared to Non-PPID horses ($P<.001$) and when all groups were combined ($P=.0044$). Table 9 and Figure 4B display results for the Non-PPID to all PPID comparison.

No significant group, time, or group by time effects were seen in CNS between any of the groups. Table 2.10 and Figure 2.4C display results for the Non-PPID to all PPID comparison.

Total Cortisol

No significant group or group by time effects were seen in total cortisol concentration between any of the groups. Significant time effects were observed in the PPID Control to PPID Treatment ($P=.006$), Non-PPID to all PPID ($P<.0001$), and all groups combined ($P<.0001$) comparisons. Table 2.11 and Figure 2.5A display results for the Non-PPID to all PPID comparison.

Free Cortisol

No significant group or group by time effects were seen in estimated free cortisol concentration or free cortisol fraction (%) between any of the groups, but significant time effects were observed in all comparisons for both measures ($P<.0001$). Tables 2.12 and 2.13 and Figures 2.5B and 2.5C display results for the Non-PPID to all PPID comparison for estimated free cortisol concentration and free cortisol fraction (%), respectively.

Correlations

No significant correlations were seen between ACTH and insulin, ACTH and total cortisol, and ACTH and estimated free cortisol concentration or free cortisol fraction (%). Similarly, no significant correlations were observed between insulin and total cortisol and insulin and estimated free cortisol concentration or free cortisol fraction (%). Statistically significant correlations were seen between estimated free cortisol concentration and both total cortisol concentration and free cortisol fraction (%) (r=.77 and r=.62; P<.0001).

2.5 Discussion

The goal of this study was to evaluate endocrine function (ACTH, basal insulin, total and free cortisol) across seasons in horses with and without PPID and the extent to which PRASCEND treatment affects these measures in horses with PPID. The lack of change in total and free cortisol observed in this study provides evidence that excess ACTH generated in horses with PPID may be inactive, which has previously been hypothesized, and that PRASCEND treatment successfully reduces ACTH to the level of Non-PPID horses regardless of season [92-94]. Additionally, the results suggest that PPID and hyperinsulinemia and/or insulin dysregulation are likely distinct diseases, and that PRASCEND treatment does not impact insulin, total cortisol, or free cortisol.

To our knowledge, this is the only long-term PRASCEND administration study that followed Non-PPID, PPID Control, and PPID Treatment horses from the beginning of one fall season until the end of the subsequent fall season, and that used non-client owned horses that were all managed in consistent fashion on the same farm. It has been reported

extensively that horses experience a seasonal fall rise in ACTH [15,83,95,96]. In this study, PRASCEND successfully reduced ACTH levels, so that despite statistically significant starting ACTH differences in Early September 2017, Non-PPID and PPID Treatment horses did not differ in ACTH values after six weeks of treatment (by Mid-October 2017). Therefore, despite treatment starting near the beginning of the seasonal fall ACTH rise, PRASCEND treatment was able to control ACTH within the fall seasonal rise (by Mid-October 2017). As seen in the results, the fall rise in ACTH is evident in both Non-PPID and PPID horses but is very exaggerated in the PPID Control horses. The results discussed earlier showed that ACTH in Non-PPID horses did not differ significantly at any point in the study from their starting ACTH values, further supporting the conclusion that the seasonal fall rise in ACTH is exaggerated in horses with PPID. This data demonstrates that the exaggerated fall ACTH rise in PPID horses can be corrected with PRASCEND treatment, in contrast to previous work [41].

The differences in ACTH change scores in response to the TRH stimulation test between PPID Control and PPID Treatment horses suggest that PRASCEND treatment may improve responses to the TRH stimulation at non-fall timepoints but does not reduce ACTH change scores to the level of a Non-PPID horse. Additionally, the ACTH change scores in response to TRH stimulation tests demonstrate clear temporal differences in both Non-PPID and PPID horses between non-fall and fall timepoints. Currently, veterinarians are encouraged to monitor basal ACTH to evaluate responses to PRASCEND dose and treatment [9]. The results of this study also suggest that monitoring of basal ACTH appears most appropriate for the assessment of responses to PRASCEND treatment.

While the ability to see differences between PPID and Non-PPID hypertrichosis scores was clear, the significant differences seen between PPID Control and PPID Treatment horses are difficult to interpret. Horses were scored at each timepoint, which might have led to high levels of inter-timepoint variation, potentially masking differences. Additionally, the scoring scale may not have been detailed enough to determine differences within the PPID groups but was sensitive enough to detect Non-PPID to PPID differences. For management purposes, some animals had to be body-clipped. While scorers were notified if a horse was body-clipped, this may also have impacted results by preventing differentiation between higher scores (i.e. difficult to determine whether a horse would have been 2 or 3 on the scoring scale before body-clipping). Anecdotally, improvement in hypertrichosis is commonly reported with PRASCEND treatment; therefore, the authors present these results to show that haircoat differences indeed existed between the Non-PPID and PPID horses but are unable to make conclusions on the efficacy of PRASCEND at improving hypertrichosis in horses with PPID.

The similarities in insulin and insulin change scores in response to OSTs between all three groups, along with the lack of correlation seen between ACTH and insulin in this study, support the idea that insulin dysregulation and PPID are distinct endocrine diseases or conditions, rather than PPID horses being pre-disposed to hyperinsulinemia and/or insulin dysregulation. Additionally, in this study, PRASCEND treatment did not influence basal insulin or insulin change scores in response to OSTs. However, the authors caution that given the small sample sizes, it would be beneficial to confirm these results in additional investigations.

The lack of changes in BCS and CNS demonstrate that these measures were consistent throughout the study and were not confounding variables in the analysis of basal insulin values or insulin change scores in response to OSTs; this also verifies that PRASCEND treatment did not lead to changes in BCS or CNS. The significant difference in weight between PPID and Non-PPID horses is consistent with common clinical signs in horses with PPID [9], and the lack of weight change over time shows that body weight was also not influencing changes in insulin values.

Due to increased ACTH concentrations in horses with PPID, it has frequently been assumed that cortisol levels are affected downstream, following normal hypothalamus-pituitary-adrenal (HPA) axis physiology. A recent publication examined free cortisol in horses with PPID and found no difference in free cortisol but found altered total urinary cortisol [25]. An additional study also previously examined the effects of PPID on total and free cortisol and found no differences in total cortisol, but significantly higher free cortisol fraction in PPID horses compared to age and season-matched control animals [22]. To our knowledge, no studies have evaluated the effects of PRASCEND on total or free cortisol. The study described in this manuscript expands upon previous works by confirming PPID status in all subjects via the currently recommended dynamic TRH stimulation testing and by sampling the same animals over time, at the same timepoints, to account for seasonal and individual animal differences [8,9]. The results from this study both support and contradict previously published work, as they support the hypothesis that total and free cortisol are not affected by PPID. Additionally, total and free cortisol do not appear to be affected by PRASCEND treatment. Significant time effects in both total and free cortisol suggest that normal temporal changes are present in both Non-PPID and PPID horses,

making it crucial that further work in this area include control animals for each timepoint and avoid comparisons between samples collected at separate points in time.

Furthermore, the lack of association between cortisol and ACTH supports earlier work hypothesizing that excess ACTH generated as a result of PPID may be inactive or incapable of prompting normal HPA axis responses [92-94]. This would explain why increased free and total cortisol is not seen in the plasma of PPID horses, despite their increased ACTH concentrations, and why PRASCEND treatment can reduce ACTH without impacting total or free cortisol. Since cortisol levels did not correlate with ACTH or change in response to PPID status or with PRASCEND treatment, it is likely that the excess ACTH is inactive, potentially due to its aberrant production in the pars intermedia of the pituitary [5,92,94]. However, this also raises additional questions on how and where the excess ACTH seen in horses with PPID is contributing to the disease process.

In summary, neither PPID nor treatment with PRASCEND influenced insulin, total cortisol, or free cortisol in the horses in this study. PRASCEND treatment successfully reduced basal ACTH but was limited in its ability to reduce ACTH change scores to TRH stimulation tests. Therefore, we suggest that current recommendations that veterinarians monitor basal ACTH when assessing responses to PRASCEND treatment are appropriate.

Table 2.1 Age, basal ACTH, and basal insulin at Day 0 (Early September 2017). PPID-pituitary pars intermedia dysfunction.

Group	Number in group at Day 0	Mean (Age in years)	Median (Age in years)	Standard Deviation (Age in years)	Mean/ Standard Deviation (Basal ACTH at Day 0)	Mean/ Standard Deviation (Basal Insulin at Day 0)
Non-PPID	10	23.600	23.000	2.590	54.62/ 24.628	27.562/ 16.458
PPID Control	9	26.555	28.000	3.711	197.333/ 78.876	28.521/ 10.826
PPID Treatment	9	24.444	24.000	3.844	244.444/ 112.424	50.863/ 21.477

Table 2.2. Sex and breed representation at Day 0 (Early September 2017).
PPID-pituitary pars intermedia dysfunction.

Group	Number of geldings	Number of mares	Breeds included:
Non-PPID	2	8	Quarter Horse (n=1) Thoroughbred (n=6) Paint (n=1) Arabian (n=1) unknown/mixed breed (n=1)
PPID Control	4	5	Quarter Horse (n=2) Thoroughbred (n=4) Standardbred (n=1) Tennessee Walking Horse (n=1) unknown/mixed breed (n=1)
PPID Treatment (PRASCEND)	2	7	Quarter Horse (n=1) Thoroughbred (n=4) Standardbred cross (n=1) Mustang (n=1) Appaloosa (n=1) unknown/mixed breed (n=1)

Table 2.3. Basal ACTH (pg/mL): Least squares mean and standard error values.

At Day 0 (Early September 2017), there were 10, 9, and 9 horses in the Non-PPID, PPID Control, and PPID Treatment groups, respectively. By the end of the study, there were 9, 7, and 6 horses in the Non-PPID, PPID Control, and PPID Treatment groups, respectively. * denotes a significant difference ($P<.05$) between Non-PPID and PPID Control. $^{\mu}$ denotes a significant difference ($P<.05$) between Non-PPID and PPID Treatment. $^{\#}$ denotes a significant difference ($P<.05$) between PPID Treatment and PPID Control groups. PPID-pituitary pars intermedia dysfunction; ACTH-adrenocorticotropic hormone.

Group	Time	Least Squares Mean	Standard Error	Group differences
Non-PPID	Early September 2017	54.62	51.7849	
Non-PPID	Mid-October 2017	43.55	51.7849	
Non-PPID	Early November 2017	33.39	51.7849	
Non-PPID	Early February 2018	22.51	51.7849	
Non-PPID	Early April 2018	19.2652	53.4461	
Non-PPID	Mid-June 2018	24.7312	53.4875	
Non-PPID	Early August 2018	50.0875	53.963	
Non-PPID	Mid-late August 2018	38.8408	53.5399	
Non-PPID	Early September 2018	56.2866	53.7659	
Non-PPID	Mid-September 2018	58.3969	53.7204	
Non-PPID	Early October 2018	92.6605	53.9811	
Non-PPID	Mid-October 2018	45.0534	53.9503	
Non-PPID	Late October 2018	34.3335	53.582	
Non-PPID	Mid-November 2018	27.3273	53.9257	
PPID Control	Early September 2017	197.33	54.5861	
PPID Control	Mid-October 2017	268.51	54.5861	*
PPID Control	Early November 2017	227.31	54.5861	*
PPID Control	Early February 2018	160.64	54.5861	
PPID Control	Early April 2018	124.36	54.5861	
PPID Control	Mid-June 2018	136.96	58.9986	
PPID Control	Early August 2018	146.44	60.207	
PPID Control	Mid-late August 2018	317.55	59.1067	*
PPID Control	Early September 2018	179.98	59.3118	
PPID Control	Mid-September 2018	273.37	58.0757	*
PPID Control	Early October 2018	429.49	59.8308	*

PPID Control	Mid-October 2018	366.04	60.234	*
PPID Control	Late October 2018	223.96	59.1864	*
PPID Control	Mid-November 2018	284.14	60.2105	*
PPID Treatment	Early September 2017	244.44	54.5861	‡
PPID Treatment	Mid-October 2017	62.9556	54.5861	#
PPID Treatment	Early November 2017	50.5	54.5861	#
PPID Treatment	Early February 2018	50.5553	57.0457	
PPID Treatment	Early April 2018	50.9431	58.9962	
PPID Treatment	Mid-June 2018	54.6641	62.294	
PPID Treatment	Early August 2018	83.4889	64.2716	
PPID Treatment	Mid-late August 2018	87.7006	62.3966	#
PPID Treatment	Early September 2018	98.4636	63.1949	
PPID Treatment	Mid-September 2018	100.65	62.5643	#
PPID Treatment	Early October 2018	97.4923	64.0234	#
PPID Treatment	Mid-October 2018	61.8212	64.1921	#
PPID Treatment	Late October 2018	68.6374	62.8891	
PPID Treatment	Mid-November 2018	64.7805	64.2513	#

Table 2.4. ACTH (pg/mL) change score (in response to thyrotropin-releasing hormone (TRH) stimulation test): Least squares mean and standard error values.

At Day 0 (Early September 2017), there were 10, 9, and 9 horses in the Non-PPID, PPID Control, and PPID Treatment groups, respectively. By the end of the study, there were 9, 7, and 6 horses in the Non-PPID, PPID Control, and PPID Treatment groups, respectively. * denotes a significant difference ($P<.05$) between Non-PPID and PPID Control. µ denotes a significant difference ($P<.05$) between Non-PPID and PPID Treatment. # denotes a significant difference ($P<.05$) between PPID Treatment and PPID Control groups. PPID-pituitary pars intermedia dysfunction; ACTH-adrenocorticotropic hormone.

Group	Time	Least Squares Mean	Standard Error	Group differences
Non-PPID	Late August 2017	165.1	78.7565	
Non-PPID	Early-mid November 2017	186.91	79.0622	
Non-PPID	Mid-late February 2018	-25.0414	80.8563	
Non-PPID	Early April 2018	-46.0584	80.9273	
Non-PPID	Early-mid September 2018	174.41	80.4203	
Non-PPID	Mid-October 2018	215.97	80.6503	
PPID Control	Late August 2017	925	88.4257	*
PPID Control	Early-mid November 2017	921.01	82.8611	*
PPID Control	Mid-late February 2018	733.12	82.8638	*
PPID Control	Early April 2018	563.57	82.7348	*
PPID Control	Early-mid September 2018	912.76	87.6444	*
PPID Control	Mid-October 2018	919.43	88.3016	*
PPID Treatment	Late August 2017	951.51	85.4795	µ#
PPID Treatment	Early-mid November 2017	755.41	82.9373	µ#
PPID Treatment	Mid-late February 2018	441.72	84.9901	µ#&
PPID Treatment	Early April 2018	272.84	87.4494	µ#&
PPID Treatment	Early-mid September 2018	871.7	89.9972	µ#
PPID Treatment	Mid-October 2018	813.06	90.0619	µ#

Table 2.5. Hypertrichosis score: Least squares mean and standard error values.

At Day 0 (Early September 2017), there were 10, 9, and 9 horses in the Non-PPID, PPID Control, and PPID Treatment groups, respectively. By the end of the study, there were 9, 7, and 6 horses in the Non-PPID, PPID Control, and PPID Treatment groups, respectively. * denotes a significant difference ($P<.05$) between Non-PPID and PPID Control. μ denotes a significant difference ($P<.05$) between Non-PPID and PPID Treatment. # denotes a significant difference ($P<.05$) between PPID Treatment and PPID Control groups. PPID-pituitary pars intermedia dysfunction.

Group	Time	Least Squares Mean	Standard Error	Group differences
Non-PPID	Early October 2017	0.05	0.2101	
Non-PPID	Early-mid November 2017	0.0167	0.2101	
Non-PPID	Mid-February 2018	0.3667	0.2101	
Non-PPID	Early April 2018	0.5281	0.2132	
Non-PPID	Late May 2018	0.2685	0.2132	
Non-PPID	Late June 2018	0.2779	0.2132	
Non-PPID	Early August 2018	0.09293	0.2132	
Non-PPID	Mid-October 2018	0.2501	0.2132	
PPID Control	Early October 2017	1.2222	0.2215	*
PPID Control	Early-mid November 2017	1.0184	0.2215	*
PPID Control	Mid-February 2018	1.7593	0.2215	*
PPID Control	Early April 2018	1.5	0.2215	*
PPID Control	Late May 2018	1.1845	0.2293	*
PPID Control	Late June 2018	1.5181	0.2292	*
PPID Control	Early August 2018	1.3993	0.2292	*
PPID Control	Mid-October 2018	1.2793	0.2294	*
PPID Treatment	Early October 2017	1.7963	0.2215	μ#
PPID Treatment	Early-mid November 2017	1.6111	0.2215	μ#
PPID Treatment	Mid-February 2018	1.8891	0.2255	μ#
PPID Treatment	Early April 2018	1.9223	0.2301	μ#
PPID Treatment	Late May 2018	1.5896	0.2301	μ#
PPID Treatment	Late June 2018	1.438	0.2355	μ#
PPID Treatment	Early August 2018	1.3398	0.2355	μ#
PPID Treatment	Mid-October 2018	1.7856	0.2356	μ#

Table 2.6. Basal insulin (uIU/mL): Least squares mean and standard error values.

At Day 0 (Early September 2017), there were 10 horses in the Non-PPID group and 18 horses in the PPID group. By the end of the study, there were 9 horses in the Non-PPID group and 13 horses in the PPID group. PPID-pituitary pars intermedia dysfunction.

Group	Time	Least Squares Mean	Standard Error
Non-PPID	Early September 2017	27.562	6.1047
Non-PPID	Mid-October 2017	21.668	5.148
Non-PPID	Early November 2017	18.555	3.1995
Non-PPID	Early February 2018	22.686	6.1181
Non-PPID	Early April 2018	18.09	12.3466
Non-PPID	Mid-June 2018	26.1476	21.129
Non-PPID	Early August 2018	16.0345	8.518
Non-PPID	Mid-late August 2018	13.7838	3.9131
Non-PPID	Early September 2018	14.738	2.7366
Non-PPID	Mid-September 2018	13.7832	4.1685
Non-PPID	Early October 2018	19.3905	3.7686
Non-PPID	Mid-October 2018	16.7949	5.1729
Non-PPID	Late October 2018	11.8217	4.7314
Non-PPID	Mid-November 2018	15.1288	2.1761
PPID	Early September 2017	39.6922	4.5502
PPID	Mid-October 2017	22.0261	3.8371
PPID	Early November 2017	16.1183	2.3848
PPID	Early February 2018	28.0769	4.6561
PPID	Early April 2018	32.6302	9.2488
PPID	Mid-June 2018	48.3645	17.0531
PPID	Early August 2018	28.2703	6.912
PPID	Mid-late August 2018	20.602	3.1713
PPID	Early September 2018	19.2748	2.2252
PPID	Mid-September 2018	20.4193	3.2397
PPID	Early October 2018	24.0439	3.0508
PPID	Mid-October 2018	24.8148	4.1992
PPID	Late October 2018	23.9773	3.7735
PPID	Mid-November 2018	19.2884	1.7476

Table 2.7. Insulin (uIU/mL) change score (in response to oral sugar test (OST)): Least squares mean and standard error values.

At Day 0 (Early September 2017), there were 10 horses in the Non-PPID group and 18 horses in the PPID group. By the end of the study, there were 9 horses in the Non-PPID group and 13 horses in the PPID group. PPID-pituitary pars intermedia dysfunction.

Group	Time	Least Squares Mean	Standard Error
Non-PPID	Late August 2017	16.7213	5.6309
Non-PPID	Early-mid November 2017	24.0003	5.6245
Non-PPID	Mid-late February 2018	24.2892	5.6205
Non-PPID	Early April 2018	18.9912	5.9202
Non-PPID	Early-mid September 2018	24.7916	5.9506
Non-PPID	Mid-October 2018	23.127	5.9764
PPID	Late August 2017	19.1173	4.2651
PPID	Early-mid November 2017	22.9834	4.2059
PPID	Mid-late February 2018	23.291	4.312
PPID	Early April 2018	20.1876	4.5514
PPID	Early-mid September 2018	29.0158	4.9258
PPID	Mid-October 2018	28.1987	4.9191

Table 2.8. Body weight (lbs): Least squares mean and standard error values.

At Day 0 (Early September 2017), there were 10 horses in the Non-PPID group and 18 horses in the PPID group. By the end of the study, there were 9 horses in the Non-PPID group and 13 horses in the PPID group. PPID-pituitary pars intermedia dysfunction; lbs-pounds.

Group	Time	Least Squares Mean	Standard Error	Group differences
Non-PPID	Early September 2017	1283.1	40.8431	
Non-PPID	Mid-October 2017	1280.1	40.8431	
Non-PPID	Early November 2017	1257.5	40.8431	
Non-PPID	Early-mid February 2018	1254.3	40.8431	
Non-PPID	Early April 2018	1227.51	40.957	
Non-PPID	Late May 2018	1264.4	41.0115	
Non-PPID	Late June 2018	1268.83	41.0544	
Non-PPID	Early August 2018	1234.07	41.1056	
Non-PPID	Early September 2018	1201.54	41.0723	
Non-PPID	Mid-October 2018	1204.78	41.092	
PPID	Early September 2017	1125.28	30.4427	*
PPID	Mid-October 2017	1108.78	30.4427	*
PPID	Early November 2017	1080.39	30.4427	*
PPID	Early-mid February 2018	1101.1	30.5136	*
PPID	Early April 2018	1077.51	30.5596	*
PPID	Late May 2018	1095.19	30.765	*
PPID	Late June 2018	1089.8	30.9197	*
PPID	Early August 2018	1088.04	31.0069	*
PPID	Early September 2018	1059.21	30.9735	*
PPID	Mid-October 2018	1058.22	31.0245	*

Table 2.9. Body Condition Score: Least squares mean and standard error values.

At Day 0 (Early September 2017), there were 10 horses in the Non-PPID group and 18 horses in the PPID group. By the end of the study, there were 9 horses in the Non-PPID group and 13 horses in the PPID group. PPID-pituitary pars intermedia dysfunction.

Group	Time	Least Squares Mean	Standard Error
Non-PPID	Early-mid September 2017	5.4867	0.2276
Non-PPID	Early-mid November 2017	5.3567	0.2276
Non-PPID	Mid-February 2018	5.0602	0.2276
Non-PPID	Early April 2018	5.1739	0.2304
Non-PPID	Late May 2018	5.5591	0.2319
Non-PPID	Early August 2018	5.07	0.2322
Non-PPID	Mid-October 2018	4.9013	0.2315
PPID	Early-mid September 2017	5.063	0.1696
PPID	Early-mid November 2017	4.9298	0.1696
PPID	Mid-February 2018	4.9528	0.1714
PPID	Early April 2018	4.9623	0.1726
PPID	Late May 2018	4.9954	0.178
PPID	Early August 2018	4.9454	0.1804
PPID	Mid-October 2018	4.7687	0.1792

Table 2.10. Cresty Neck Score: Least squares mean and standard error values.

At Day 0 (Early September 2017), there were 10 horses in the Non-PPID group and 18 horses in the PPID group. By the end of the study, there were 9 horses in the Non-PPID group and 13 horses in the PPID group. PPID-pituitary pars intermedia dysfunction.

Group	Time	Least Squares Mean	Standard Error
Non-PPID	Early-mid September 2017	1.35	0.23
Non-PPID	Early-mid November 2017	1.2	0.23
Non-PPID	Mid-February 2018	1.1332	0.23
Non-PPID	Early April 2018	1.1502	0.2328
Non-PPID	Late May 2018	1.3776	0.2346
Non-PPID	Early August 2018	1.1727	0.2345
Non-PPID	Mid-October 2018	1.2824	0.2335
PPID	Early-mid September 2017	1.4815	0.1715
PPID	Early-mid November 2017	1.3888	0.1715
PPID	Mid-February 2018	1.3602	0.1729
PPID	Early April 2018	1.2656	0.1741
PPID	Late May 2018	1.4461	0.18
PPID	Early August 2018	1.4305	0.182
PPID	Mid-October 2018	1.5726	0.1801

Table 2.11. Total Cortisol (ug/dL): Least squares mean and standard error values.

At Day 0 (Early September 2017), there were 10 horses in the Non-PPID group and 18 horses in the PPID group. By the end of the study, there were 9 horses in the Non-PPID group and 13 horses in the PPID group. PPID-pituitary pars intermedia dysfunction.

Group	Time	Least Squares Mean	Standard Error
Non-PPID	Early September 2017	3.857	0.4415
Non-PPID	Mid-October 2017	4.453	0.4415
Non-PPID	Early November 2017	3.877	0.4415
Non-PPID	Early February 2018	4.13	0.4415
Non-PPID	Early April 2018	3.8458	0.4526
Non-PPID	Mid-June 2018	5.0622	0.4525
Non-PPID	Mid-September 2018	3.6702	0.4529
Non-PPID	Early October 2018	3.6504	0.4524
Non-PPID	Mid-October 2018	4.6622	0.4528
Non-PPID	Late October 2018	3.4502	0.4524
PPID	Early September 2017	4.2078	0.329
PPID	Mid-October 2017	3.9567	0.329
PPID	Early November 2017	3.6839	0.329
PPID	Early February 2018	3.6883	0.3337
PPID	Early April 2018	4.5759	0.3386
PPID	Mid-June 2018	4.7303	0.3565
PPID	Mid-September 2018	4.1113	0.3575
PPID	Early October 2018	3.8601	0.3561
PPID	Mid-October 2018	4.4507	0.3571
PPID	Late October 2018	4.0983	0.3563

Table 2.12. Estimated Free Cortisol Concentration (ug/dL): Least squares mean and standard error values.

At Day 0 (Early September 2017), there were 10 horses in the Non-PPID group and 18 horses in the PPID group. By the end of the study, there were 9 horses in the Non-PPID group and 13 horses in the PPID group. PPID-pituitary pars intermedia dysfunction.

Group	Time	Least Squares Mean	Standard Error
Non-PPID	Early September 2017	0.2144	0.03755
Non-PPID	Mid-October 2017	0.2382	0.03755
Non-PPID	Early November 2017	0.217	0.03755
Non-PPID	Early February 2018	0.2744	0.03755
Non-PPID	Early April 2018	0.2115	0.03905
Non-PPID	Mid-June 2018	0.3463	0.03905
Non-PPID	Mid-September 2018	0.2799	0.03907
Non-PPID	Early October 2018	0.2827	0.03904
Non-PPID	Mid-October 2018	0.3093	0.03906
Non-PPID	Late October 2018	0.2577	0.03904
PPID	Early September 2017	0.2544	0.02799
PPID	Mid-October 2017	0.2389	0.02799
PPID	Early November 2017	0.1964	0.02799
PPID	Early February 2018	0.2165	0.0286
PPID	Early April 2018	0.2985	0.02927
PPID	Mid-June 2018	0.3886	0.03166
PPID	Mid-September 2018	0.325	0.03172
PPID	Early October 2018	0.3229	0.03164
PPID	Mid-October 2018	0.3315	0.03169
PPID	Late October 2018	0.3291	0.03165

Table 2.13. Free Cortisol Fraction (%): Least squares mean and standard error values. At Day 0 (Early September 2017), there were 10 horses in the Non-PPID group and 18 horses in the PPID group. By the end of the study, there were 9 horses in the Non-PPID group and 13 horses in the PPID group. PPID-pituitary pars intermedia dysfunction.

Group	Time	Least Squares Mean	Standard Error
Non-PPID	Early September 2017	5.5754	0.6839
Non-PPID	Mid-October 2017	5.349	1.0727
Non-PPID	Early November 2017	5.4958	0.4062
Non-PPID	Early February 2018	6.4789	0.6326
Non-PPID	Early April 2018	5.3631	0.5613
Non-PPID	Mid-June 2018	6.9072	0.3034
Non-PPID	Mid-September 2018	7.5914	0.3131
Non-PPID	Early October 2018	7.7452	0.6856
Non-PPID	Mid-October 2018	6.7057	0.3545
Non-PPID	Late October 2018	7.4663	0.3258
PPID	Early September 2017	6.1802	0.5097
PPID	Mid-October 2017	6.3086	0.7995
PPID	Early November 2017	5.4835	0.3027
PPID	Early February 2018	5.9078	0.4846
PPID	Early April 2018	6.0496	0.421
PPID	Mid-June 2018	8.0187	0.2508
PPID	Mid-September 2018	7.8824	0.256
PPID	Early October 2018	8.4763	0.5669
PPID	Mid-October 2018	7.3311	0.2933
PPID	Late October 2018	7.7702	0.2688

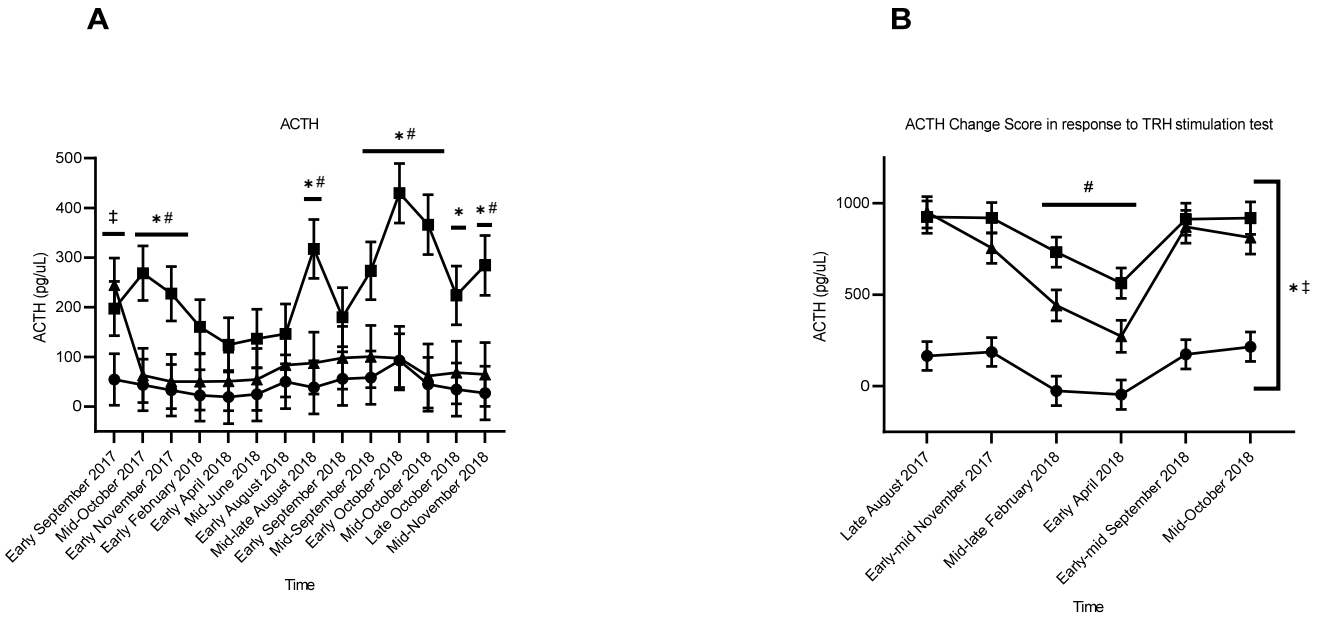

Figure 2.1. (A) Basal ACTH and (B) ACTH change scores (TRH stimulation test)

Mean +/- SEM. Non-PPID horses are represented by lines with closed circles. PPID Treatment (PRASCEND) horses are represented by lines with closed triangles. PPID Control horses are represented by lines with closed squares. At Day 0 (Early September 2017), there were 10, 9, and 9 horses in the Non-PPID, PPID Control, and PPID Treatment groups, respectively. By the end of the study, there were 9, 7, and 6 horses in the Non-PPID, PPID Control, and PPID Treatment groups, respectively. * denotes a significant difference (P<.05) between Non-PPID and PPID Control. ‡ denotes a significant difference (P<.05) between Non-PPID and PPID Treatment. # denotes a significant difference (P<.05) between PPID Treatment and PPID Control groups. ACTH-adrenocorticotropic hormone; TRH-thyrotropin-releasing hormone; PPID-pituitary pars intermedia dysfunction.

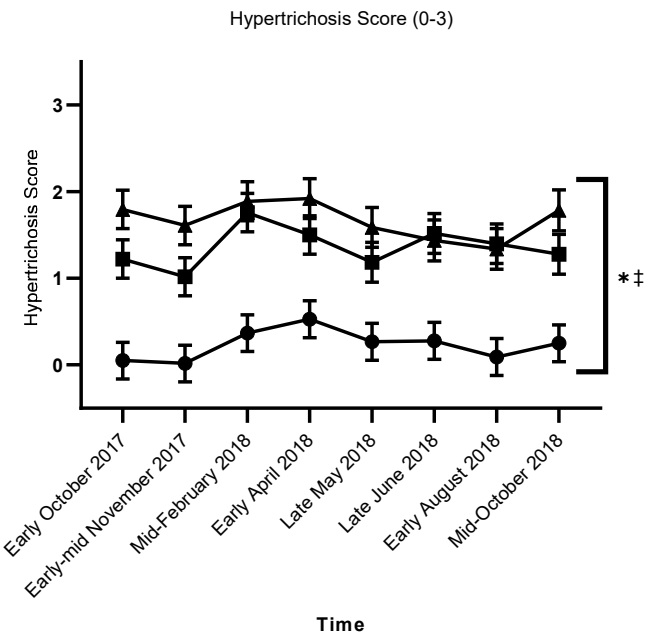

Figure 2.2. Hypertrichosis scores

(Mean +/- SEM). Non-PPID horses are represented by lines with closed circles. PPID Treatment (PRASCEND) horses are represented by lines with closed triangles. PPID Control horses are represented by lines with closed squares. At Day 0 (Early September 2017), there were 10, 9, and 9 horses in the Non-PPID, PPID Control, and PPID Treatment groups, respectively. By the end of the study, there were 9, 7, and 6 horses in the Non-PPID, PPID Control, and PPID Treatment groups, respectively. * denotes a significant difference ($P<.05$) between Non-PPID and PPID Control. ‡ denotes a significant difference ($P<.05$) between Non-PPID and PPID Treatment. PPID-pituitary pars intermedia dysfunction.

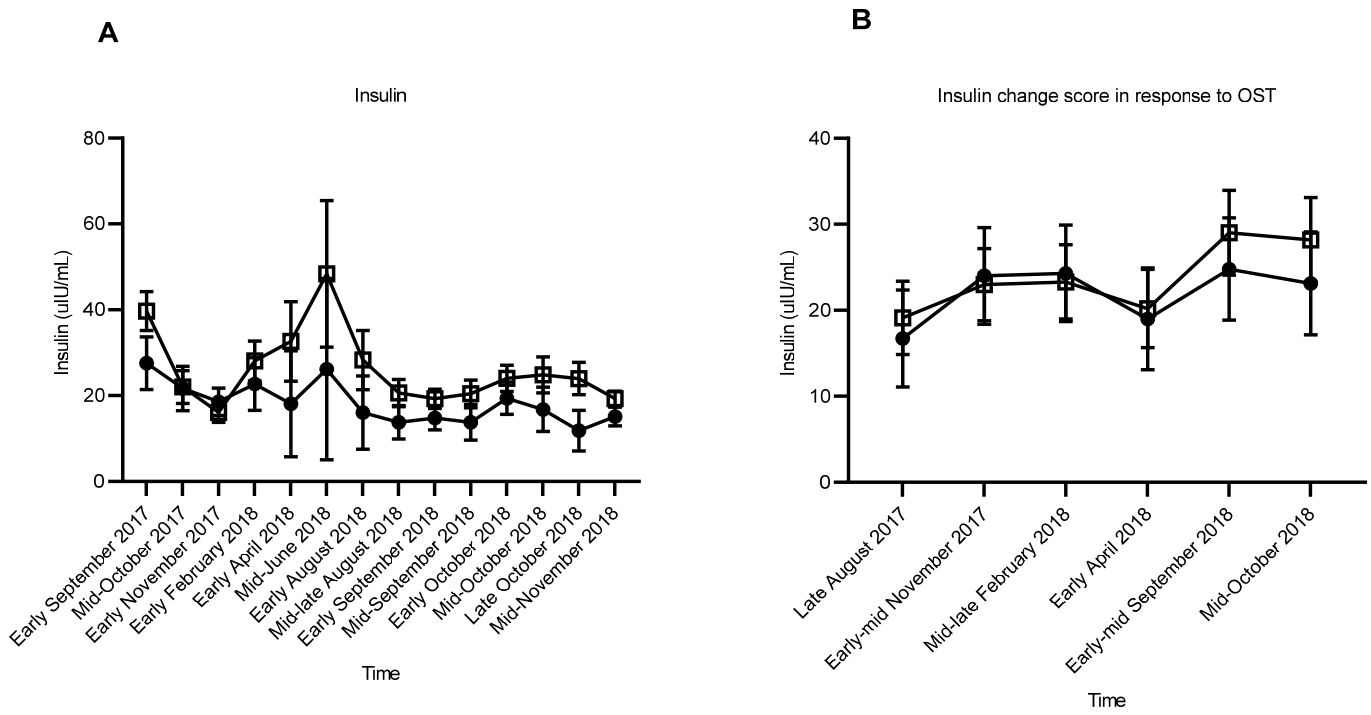

Figure 2.3 (A) Basal Insulin and (B) Insulin change scores (OST)

Mean +/- SEM. Non-PPID horses are represented by lines with closed circles. PPID horses are represented by lines with open squares. At Day 0 (Early September 2017), there were 10 horses in the Non-PPID group and 18 horses in the PPID group. By the end of the study, there were 9 horses in the Non-PPID group and 13 horses in the PPID group. OST- oral sugar test; PPID- pituitary pars intermedia dysfunction.

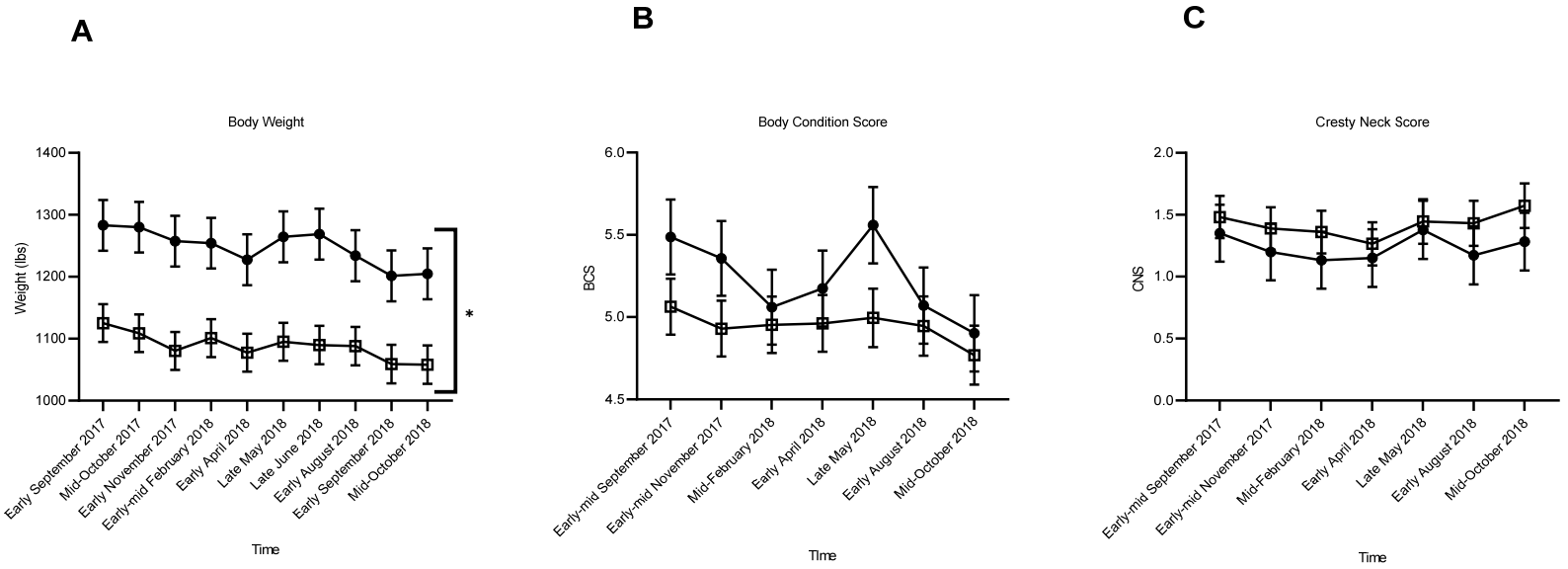

Figure 2.4. (A) Body Weight, (B) body condition score, and (C) cresty neck score

Mean +/- SEM. Non-PPID horses are represented by lines with closed circles. PPID horses are represented by lines with open squares. At Day 0 (Early September 2017), there were 10 horses in the Non-PPID group and 18 horses in the PPID group. By the end of the study, there were 9 horses in the Non-PPID group and 13 horses in the PPID group. * denotes a significant difference (P<.01) between Non-PPID and PPID groups. PPID-pituitary pars intermedia dysfunction

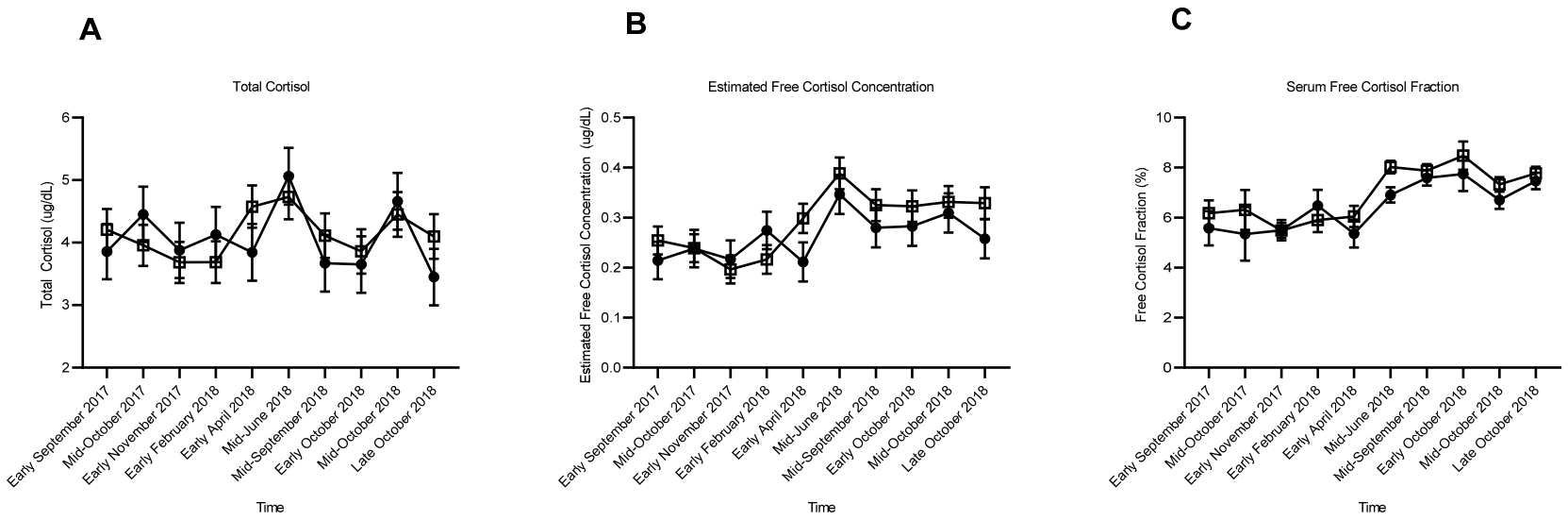

Figure 2.5. (A) Total Cortisol, (B) Estimated Free Cortisol Concentration, (C) Serum Free Cortisol Fraction

Mean +/- SEM. Non-PPID horses are represented by lines with closed circles. PPID horses are represented by lines with open squares. At Day 0 (Early September 2017), there were 10 horses in the Non-PPID group and 18 horses in the PPID group. By the end of the study, there were 9 horses in the Non-PPID group and 13 horses in the PPID group. PPID-pituitary pars intermedia dysfunction.

CHAPTER 3. EFFECTS OF PITUITARY PARS INTERMEDIA DYSFUNCTION AND PRASCEND® TREATMENT ON SYSTEMIC IMMUNE FUNCTION IN HORSES

3.1 Abstract

Background: It remains unclear how Pituitary Pars Intermedia Dysfunction (PPID) and treatment with Prascend® (pergolide tablets) affect systemic immune function in horses.

Animals: 28 horses from a research herd (10 Non-PPID, 9 untreated PPID (PPID Control), and 9 PRASCEND-treated PPID horses (PPID Treatment)) were used.

Hypotheses: Our hypotheses were that PPID horses would have altered immune function compared to Non-PPID horses, and that after treatment with PRASCEND, immune function in PPID Treatment horses would normalize to that of the Non-PPID horses.

Methods: Horses were sampled over approximately fifteen months for analysis of complete blood counts, plasma myeloperoxidase, basal whole blood cytokine and receptor gene expression, *in vitro* whole blood stimulations for cytokine and receptor gene expression and *in vitro* stimulated peripheral blood mononuclear cell (PBMC) stimulations for cytokine and receptor gene expression. For all *in vitro* stimulations, heat-inactivated *Rhodococcus equi*, heat-inactivated *Escherichia coli*, and phorbal 12-myristate 13-acetate (PMA)/ionomycin were used. PRASCEND treatment was initiated after Day 0 collections. Results were analyzed using a linear mixed model (SAS v9.4), with significance set at $P<.05$.

Results: PPID horses had decreased white blood cell (WBC) counts, decreased absolute lymphocyte counts, and decreased red blood cell counts compared to Non-PPID horses. In whole blood (Tempus™) samples, PPID horses had increased expression of IL-8 at the

Early September 2017, Early April 2018, and Mid-October 2018 timepoints. In the whole blood stimulations, a decrease in IL-8 expression to *R. equi* stimulation was also seen at the Early November 2017 timepoint, and a decrease in IL-6 expression to *R. equi* stimulation was observed at the Early September 2017 and Early November 2017 timepoints in PPID horses. Compared to Non-PPID horses, PPID horses had lower TLR4 expression in unstimulated whole blood at the Mid-October 2017 timepoint, higher TLR4 expression in *R. equi*-stimulated whole blood at the Mid-October 2017 and Early November 2017 timepoints, and greater TGFβ expression in unstimulated whole blood at the Early September 2017 timepoint. PPID horses displayed decreased IFNγ production from PBMCs in response to stimulation with *R. equi* and *E. coli*. Additionally, in response to *E. coli* stimulation, PPID horses displayed increased TGFβ expression. Compared to PPID Treatment horses, PPID Control horses had greater IL-13 expression in unstimulated whole blood at the Mid-October 2017 timepoint only. PPID Treatment horses also had greater IL-17α expression from PBMCs in response to *R. equi* than both PPID Control and Non-PPID horses at the Early February 2018 timepoint only.

Conclusions: Overall, the most striking and temporally consistent results were the decreases in absolute lymphocyte and red blood counts, as well as the decreased IFNγ production from PBMCs in PPID horses after *in vitro* stimulation to *R. equi* and *E. coli* and the increased TGFβ production from PBMCs in PPID horses after *E. coli* stimulation compared to Non-PPID horses. Treatment with PRASCEND did not seem to influence immune function in a clinically relevant way.

3.2 Introduction

Due to their increased plasma ACTH concentrations, PPID horses are often thought to be immunosuppressed compared to Non-PPID horses [7,48]. The normal hypothalamus-pituitary-adrenal axis responses to increased ACTH concentrations dictate that cortisol, which is known to impact immune function in many species, should also be increased [18,19]. Therefore, while it is commonly stated that horses with PPID have altered immune responses and clinically appear to have more frequent infections than Non-PPID horses, research in this area is currently rather sparse. In the available published research, some differences in immune function in PPID horses have been described; however, the mechanisms behind any potential differences in the immune function of PPID horses are unclear. Additionally, while daily treatment with Prascend® (pergolide tablets) (Boehringer-Ingelheim Animal Health; Duluth, GA) is currently the preferred treatment option for PPID, it is not known whether it influences immune function.

In animals with decreased immune function, opportunistic infections are often noted, and can result in severe disease. Infection with *R. equi*, a gram-positive bacterium, and *E. coli*, a gram-negative bacterium, are generally considered opportunistic infections in adult horses, and thus were selected for *in vitro* stimulations in this work to aid in the evaluation of the immune response in PPID horses [59,65-67]. Our hypothesis was that PPID horses would have altered immune function, both basally and in response to *in vitro* stimuli, compared to Non-PPID horses, and that PRASCEND treatment would normalize the immune function in PPID Treatment horses. To analyze this, complete blood counts, basal whole blood cytokine and receptor expression, and peripheral blood mononuclear cell (PBMC) and whole blood responses to *in vitro* stimulation with heat-inactivated *R. equi*, heat-

inactivated *E. coli*, and phorbal 12-myristate 13-acetate (PMA)/ionomycin were measured. Additionally, based on published work by McFarlane et al. indicating that PPID horses had decreased oxidative burst in neutrophils, basal plasma myeloperoxidase (MPO), a marker of neutrophil degranulation, was measured [48].

3.3 Materials and Methods

3.3.1 Animal Selection and Study Design

Horses were initially selected for potential inclusion in the study from a university-owned research herd based on prior screenings and clinical history (absence or presence of hypertrichosis and/or absence or presence of history of hypertrichosis/difficulty shedding and/or results of multiple TRH stimulation tests). PPID status was confirmed using ACTH responses to TRH stimulation tests approximately two weeks prior to Day 0 of the study (Early September 2017) and basal ACTH values on Day 0. An oral sugar test (OST) was also performed prior to the study, approximately one week after the TRH stimulation test, to assess hyperinsulinemia or insulin dysregulation status [87]. Horses that did not screen as either Non-PPID or PPID for both the TRH stimulation test and basal ACTH screenings were excluded from the study. As these initial screenings were obtained during the fall season in the USA (late August and early September), a basal ACTH value of ≥100pg/mL and ACTH 10 minutes post-TRH injection (T10) value of ≥400pg/mL were used for selection into the PPID group [8,9].

Thirty-one horses were originally selected to proceed through the study. However, three horses assigned to the PRASCEND-treated group had ACTH values that failed to

become controlled and remained uncontrolled throughout the study; these horses were excluded from all analyses and are not discussed further. Non-PPID horses (n=10) were all assigned into one group. An attempt was made to randomly assign the PPID horses into the untreated and treated groups, however, due to also attempting to block for insulin status, true randomization was not possible. PPID Control horses (n=9) remained untreated for the duration of the study, and PPID Treatment horses (n=9) began receiving PRASCEND within approximately 24 hours of their completion of all Day 0 sample collections. Horses were started at the lowest label dose (2 μg/kg) to the nearest half-tablet for their body weight, based on the manufacturer's label recommendations. Basal ACTH was checked weekly for the first 4 weeks, and the dose was increased each week until basal ACTH levels were reduced to controlled (Non-PPID) values or until the maximum label dose (4 μg/kg) to the nearest half-tablet was reached.

Throughout the approximately 15-month study, six horses were euthanized due to non-study related health issues. The study ended with 9, 7, and 6 horses in the Non-PPID, PPID Control, and PPID Treatment groups, respectively. At the start of the study, there was no statistical difference in age between the groups (*P*=.1747) (Table 2.1). Age ranges were 20-29 years for Non-PPID horses, 21-31 years for PPID Control horses, and 19-29 years for PPID Treatment horses. Only mares and geldings were included (Table 2.2). Breed representations, either confirmed or to the best of the researchers' knowledge, included Thoroughbred (n=14), American Quarter Horse (n=4), Standardbred (n=1), Standardbred cross (n=1), Mustang (n=1), Paint (n=1), Arabian (n=1), Tennessee Walking Horse (n=1), Appaloosa (n=1), and unknown or mixed breed (n=3) horses (Table 2.2). Breakdowns of sex and breed by group are included in Table 2.2. All horses were cared for

and sampled with approval of the University of Kentucky's Institutional Animal Use and Care Committee (IACUC) (#2014-1225 and #2018-3004).

3.3.2 Sampling

On sampling days, horses were grain fasted (if part of their normal ration) until all collections were completed. At six main timepoints (Early September 2017, Mid-October 2017, Early November 2017, Early February 2018, Early April 2018, and Mid-October 2018), resting blood samples were collected aseptically via jugular venipuncture within a four-hour window during morning hours (approximately 08:00 to 12:00) for systemic immune measures. This allowed for sampling and analyses for short PRASCEND treatment time periods (6 weeks and 8 weeks), and longer PRASCEND treatment time periods, as well as analyses of PPID effects within different seasons. Summer samples were not collected to avoid sampling during the seasonal fall rise in ACTH. At each timepoint, all blood samples were collected over a period of 1-4 days, but within the same time window above (08:00 to 12:00); when multiple days were needed to complete all of the collections, a mixture of horses from the three groups were screened each day. The systemic immune function measures investigated at the six main time points include complete blood counts (CBCs), plasma myeloperoxidase (MPO) analysis, and cytokine/receptor gene expression analysis of unstimulated whole blood and stimulated peripheral blood mononuclear cells (PBMCs). Stimulated whole blood cytokine and gene expression analyses were performed at the first three timepoints only (Early September 2017, Mid-October 2017, Early November 2017). Rectal temperature, pulse, and respiration were also recorded at the six main timepoints. Appendix 2 contains a sample timeline.

Complete blood counts:

Complete blood counts and fibrinogen analyses were performed by the laboratory at Rood and Riddle Equine Hospital (Lexington, Kentucky) using a Beckman Coulter AcT diff and refractometer, respectively.

Plasma myeloperoxidase (MPO):

Myeloperoxidase is considered a marker for neutrophil degranulation. For plasma MPO analysis, blood was collected into tubes containing EDTA and placed on ice. Within six hours of collection, plasma was separated by centrifugation at 800g for 10 minutes and frozen at -20ºC until used for a previously-validated equine MPO enzyme-linked immunosorbent assay (ELISA) [97]. Plasma was diluted 1:100 in phosphate-buffered saline (PBS) (Gibco™; Life Technologies) for the assay and absorbance was read at 450nm, as recommended by the manufacturer.

*Whole blood (*Tempus™*) cytokine and receptor gene expression*

Whole blood was collected directly from each horse into Tempus™ Blood RNA tubes. This allowed for analysis of circulating cytokine and receptor gene expression with immediate RNA stabilization as opposed to the other stimulations, which were either processed and frozen prior to stimulation (PBMC stimulations) or had longer incubation times after blood collection (whole blood stimulations). The blood tubes were stored at room temperature until approximately 24 hours after collection, at which point they were frozen at -20ºC until further processed. RNA was isolated using the iPrep PureLink system and stored at -80ºC until reverse transcribed (described below). Gene expression of IFNγ,

IL-12α, IL-13, IL-17α, IL-1β, IL-6, IL-8, TGFβ, TNFα, TLR2, and TLR4 was then measured using RT-PCR (described below).

PBMC (unstimulated and stimulated) cytokine and receptor gene expression

Blood was collected into sodium heparin tubes for isolation of PBMCs using a Ficoll® (GE Healthcare) density gradient. After isolation, the cells were washed twice with phosphate-buffered saline (PBS) and frozen in freeze media (50% HyClone™ RPMI 1640 media (GE Healthcare), 40% fetal bovine serum (Sigma Aldrich; St. Louis, MO), and 10% dimethyl sulfoxide (DMSO) (Sigma Aldrich)) at -80°C , and transferred to liquid nitrogen after at least 24 hours in the -80°C until they were needed for further analyses.

After thawing the PBMCs and resuspending in complete RPMI (96.4% HyClone™ RPMI 1640 media (GE Healthcare), 2.5% fetal equine serum, 1% penicillin, streptomycin, L-Glutamine solution (Sigma Aldrich), and 0.1% 2-mercaptoethanol (Gibco™)), the cells were counted using a Vi-CELL counter (Beckman Coulter; Indianapolis, Indiana), and plated at $4x10^6$ cells per well into a total of four wells of a cell culture plate. For the six main sampling timepoints, each horse had four cell stimulations. One well remained unstimulated, while one well was stimulated with heat-inactivated *Rhodococcus equi* 103^+ (10^7 colony-forming units (CFU) in 10 µL of complete RPMI) for 24 hours, one well was stimulated with heat-inactivated *Escherichia coli* (ATCC #35218) (10^7 CFU in 10 µL of complete RPMI) for 24 hours, and one well was stimulated with 10 µL phorbal 12-myristate 13-acetate (PMA)/ionomycin for the last four hours of the incubation. A dose response pilot study was conducted in advance to determine the best dose for each of the bacteria used in the stimulations that would elicit a sub-maximal response. The cells were incubated at 37°C with 5% CO_2 after plating and bacterial stimulations. Brefeldin A was

added to all wells after 20 hours of incubation. After 24 hours, approximately half of the cells were removed, and TRIzol™ (Invitrogen™) was added. These samples were stored at -80°C until RNA was isolated via phenol-chloroform extraction. After RNA isolation, the samples were placed back in the -80°C freezer until reverse transcribed (described below). Gene expression of IFNγ, IL-1β, IL-6, IL-8, IL-10, IL-17α, IL-12α, IL-13, TGFβ, TNFα, and TLR4 was then measured using RT-PCR (described below).

Stimulated whole blood cytokine and receptor gene expression

At the first three timepoints only, (Early September 2017, Mid-October 2017, Early November 2017), whole blood was collected into several 3 mL sodium heparin tubes. For each horse, one tube remained unstimulated (nothing added), one was stimulated with heat-inactivated *R. equi* suspended in PBS (10^6 CFU per mL of blood), one was stimulated with heat-inactivated *E. coli* suspended in PBS (10^7 CFU per mL of blood), and one was stimulated with PMA/ionomycin (10 μL per mL of blood). A dose response pilot study was conducted in advance to determine the best dose for each of the bacteria used in the stimulations that would elicit a sub-maximal response. After addition of stimuli, the blood tubes were incubated at 37°C with 5% CO_2 for 24 hours. At the end of the 24-hour stimulations, all of the blood tubes were transferred to Tempus™ Blood RNA tubes. The blood tubes were stored at room temperature for approximately 24 hours after collection, at which point they were frozen at -20°C until further processed. RNA was isolated using the manufacturer's protocol for Tempus™ Blood RNA extraction kits (Qiagen) and the iPrep PureLink system; samples were then stored at -80°C until reverse transcribed (described below) [98]. Gene expression of IFNγ, IL-12α, IL-13, IL-17α, IL-1β, IL-6, IL-8, TGFβ, TNFα, TLR2, and TLR4 was then measured using RT-PCR (described below).

Reverse transcription

Prior to reverse transcription, RNA was quantified via Epoch microplate spectrophotometer (Biotek; Winooski, Vermont). Master mix reagents (Promega; Madison, Wisconsin) were used to transcribe the RNA; after addition of the reagents, samples were placed into a thermocycler (Applied Biosystems; Foster City, California) for a cycle of 15 minutes at 42ºC, followed by a cycle of 5 minutes at 95ºC [98-102]. After completion of RNA reverse transcription to cDNA, the samples were stored -80ºC until used for RT-PCR (described below).

RT-PCR

For the desired cytokine analyses described in each subsection and the endogenous control gene, β-gus, equine-specific intron spanning primers and probes (Applied Biosystems), nuclease-free water (Qiagen), TaqMan™ (Applied Biosystems), and the desired cDNA samples were loaded onto plates in duplicate via a robotic pipetting machine (Eppendorf) [98-102]. RT-PCR was then performed using the 7900HT Fast RT-PCR System (Applied Biosystems) with the first cycle of 10 minutes at 95ºC, and an additional 40 cycles of 15 seconds at 95ºC and 60 seconds at 60ºC [98-102]. After determination of cycle threshold values using linear regression analysis (LinRegPCR version 2018; Heart Failure Research Center, Amsterdam University Medical Centers), relative quantity (RQ) values were calculated using the $2^{-\Delta\Delta Ct}$ method [98-103]. The calibrators for the PBMC and whole blood stimulations were the average ΔCT of the Day 0 (Early September 2017) media/unstimulated values for all of the Non-PPID horses. For the whole blood analyses using Tempus™ Blood RNA tubes, the calibrator was the average ΔCT of the Day 0 (Early

September 2017) samples for all of the Non-PPID horses. All RQs were natural log transformed prior to statistical analyses.

Rhodococcus equi culture and heat-inactivation

A *Rhodococcus equi* 103^{+} culture was kindly supplied by the lab of Dr. David Horohov, University of Kentucky. The stock solution was streaked onto plates containing tryptic soy agar (VWR®) using a sterile inoculating loop (VWR® International). After approximately 48 hours of growth at 37°C, one colony was selected and used to inoculate a tube of tryptic soy broth (Corning®). After sealing the tube and brief vortexing, a sterile inoculating loop was used to inoculate additional broth tubes. These broth tubes were then incubated at 37°C for approximately 48 hours with caps in aerobic growth positioning. The main broth tube was then aliquoted and frozen for future use. The additional broth tubes were used to establish a growth curve and to determine the ideal time to heat-inactivate the bacteria. Based on the growth curve results, the bacteria were then pulled in the exponential growth period (after approximately 40 hours of growth) for all further work. Heat inactivation time was selected so that the minimum heat and time necessary for inactivation was achieved, in order to minimize any changes to the bacteria. Inoculated broth tubes were placed into a water bath at 60°C for various times and then streaked onto tryptic soy agar plates to confirm inactivation. The plates were checked at 48 hours after plating to determine if growth was present. The minimum time needed for heat-inactivation of *R. equi* was 50 minutes at 60°C.

Once the growth curve and heat inactivation time were determined, the final batches were prepared in similar fashion to the above descriptions. The stock solution was streaked onto tryptic soy agar plates and after approximately 48 hours of growth, a colony was

selected to inoculate a broth tube. After sealing the tube and briefly vortexing it, a sterile inoculating loop was used to inoculate multiple broth tubes, which were then incubated at 37°C. After approximately 40 hours, the broth tubes were removed from the incubator, and the volume of broth was measured as the tubes were combined into a sterile glass bottle. After a brief vortex, 100µL of broth was removed and serially diluted five times before plating 25µL in duplicate. The number of colonies was then counted on these two plates at approximately 48 hours of growth and averaged; this count was used to determine the colony-forming units (CFU) per milliliter. Then, the broth was aliquoted into new sterile tubes, and heat-inactivated for 50 minutes at 60°C. After heat inactivation, the broth was combined again into a sterile glass bottle, 25µL was plated in duplicate, and the final volume of broth was measured, so that accurate counts could be obtained. The final heat-inactivated broth was then frozen at -20°C. When needed for stimulations, the broth was thawed, centrifuged at 3,400g for 10 minutes, and the pellet resuspended in either saline (whole-blood stimulations) or complete RPMI (PBMC stimulations). The final broth was positive for *R. equi* and VapA expression on Real Time PCR, performed by the University of Kentucky Veterinary Diagnostic Lab (Lexington, Kentucky).

Escherichia coli culture and heat-inactivation

Escherichia coli (ATCC #35218) was obtained from VWR (Microbiologics, Inc.; St. Cloud, Minnesota) in KWIK-STIK™ vials. The KWIK-STIK™ vial was crushed, and the sterile swab was streaked onto plates containing Difco™ nutrient agar (VWR®; BD Biosciences). After approximately 24 hours of growth at 37°C, one colony was selected and used to inoculate a tube of Difco™ nutrient broth (VWR®, BD Biosciences). After sealing the tube and brief vortexing, a sterile inoculating loop was used to inoculate

additional broth tubes. These broth tubes were then incubated at 37°C for approximately 24 hours with caps in aerobic growth positioning. The main broth tube was then aliquoted and frozen for future use. The additional broth tubes were used to establish a growth curve and to determine the ideal time to heat-inactivate the bacteria. Based on the growth curve results, the bacteria were then pulled in the exponential growth period (after approximately 24 hours of growth) for all further work. Heat inactivation time was selected so that the minimum heat and time necessary for inactivation was achieved, in order to minimize any changes to the bacteria. Inoculated broth tubes were placed into a water bath at 60°C for various times and then streaked onto tryptic soy agar plates to confirm inactivation. The plates were checked at 24 hours after plating to determine if growth was present. The minimum time needed for heat-inactivation of *E. coli* was 7.5 minutes at 60°C.

Once the growth curve and heat inactivation time were determined, the final batches were prepared in similar fashion to the above descriptions. The stock solution was streaked onto Difco™ nutrient agar plates and after approximately 24 hours of growth, a colony was selected to inoculate a broth tube. After sealing the tube and briefly vortexing it, a sterile inoculating loop was used to inoculate multiple broth tubes, which were then incubated at 37°C. After approximately 24 hours, the broth tubes were removed from the incubator, and the volume of broth was measured as the tubes were combined into a sterile glass bottle. After a brief vortex, 100µL of broth was removed and serially diluted six times before plating 25µL in duplicate. The number of colonies was then counted on these two plates at 24 hours of growth and averaged; this count was used to determine the CFU/mL. Then, the broth was aliquoted into new sterile tubes, and heat-inactivated for 7.5 minutes at 60°C. After heat inactivation, the broth was combined again into a sterile glass bottle, 25µL was

plated in duplicate, and the final volume of broth was measured, so that accurate counts could be obtained. The final heat-inactivated broth was then frozen at -20°C. When needed for stimulations, the broth was thawed, centrifuged at 3,400g for 10 minutes, and the pellet resuspended in either saline (whole-blood stimulations) or complete RPMI (PBMC stimulations).

3.3.3 Statistical Methods

Distributional assumptions were evaluated using graphical and numerical summaries for the presence of gross normality violations. The primary analyses were the longitudinal analysis of all measures over time; linear mixed models included main effects for group and time. The primary comparisons were the PPID Treatment to PPID Control groups and the PPID to Non-PPID groups, when the PPID groups were combined. To determine if groups changed differently over time, an F-test for the interaction of group and time was used. Graphical summaries and model fit were used to determine the specifications of the linear mixed model (PROC MIXED, SAS v9.4). To account for repeated measures, an UN, ARMA(1,1), AR(1), TOEP, or TOEPH variance-covariance structure was used for each measure, depending on the best model fit based on information criteria (BIC, AIC). All analyses were conducted using SAS v9.4 (Cary, North Carolina), and a two-sided significance level of .05 was used for all statistical tests. Graphs were created using GraphPad Prism v.8.0.2 (San Diego, California).

Results for PPID Control and PPID Treatment horses were analyzed first. If significant group by time differences were observed, these groups remained separate for comparison to Non-PPID horses. If no significant group by time differences were observed,

the PPID Control and PPID Treatment groups were combined before comparison to Non-PPID horses. If no significant group or group by time differences were observed between the combined PPID group and Non-PPID group, all groups were combined for analysis of time effects only.

If starting differences ($P<.1$) were observed between PPID Control and PPID Treatment horses in the initial analyses, starting values were included as a covariate within the model to address the potential for confounding. If starting differences ($P<.1$) were observed between PPID and Non-PPID horses, analyses were performed without starting values as a covariate within the model, in order to analyze group effects, and then with starting values as a covariate, in order to analyze group by time effects; results for each of the methods are presented if applicable.

For the stimulated samples (whole blood stimulations and PBMC stimulations), the media/unstimulated values for each cytokine/receptor were included within the model as a covariate to account for changes based on where the individual animals started. Additionally, for the RT-PCR and plasma MPO results, outliers were removed prior to statistical analyses if they were determined to be more than five times lower or higher than the median absolute deviation (MAD) from the median for each cytokine and receptor and each stimulation, as well as the plasma MPO (Tables 3.1-3.3).

3.4 Results

Body Temperature:

Due to having no degrees of freedom in the PPID Control to PPID Treatment analysis for the variance-covariance structure with the best information criteria, the UN variance-covariance structure was used. No group, time, or group by time effects were found in the PPID Control to PPID Treatment comparison. After combining the PPID Control and PPID Treatment groups for comparison to the Non-PPID group, significant group (P=.043) and time (P=.0249) effects, but not a significant group by time effect alone, were observed (Figure 3.1). In this analysis, the PPID horses had significantly lower body temperature than the Non-PPID horses at the Mid-October 2018 timepoint only (P=.0278).

Pulse:

Due to having no degrees of freedom in the PPID Control to PPID Treatment analysis for the variance-covariance structure with the best information criteria, the UN variance-covariance structure was used. No significant group or group by time effects were found in the PPID Control to PPID Treatment comparison, but a significant time effect was observed (P=.0445).

After combining the PPID Control and PPID Treatment groups for comparison to the Non-PPID group, significant group (P=.0014) and time (P=.0098) effects, but not a significant group by time effect alone, were observed. In this analysis, the PPID horses had significantly lower heart rates than the Non-PPID horses at the Early September 2017 (P=.0009), Early February 2018 (P=.0005), and Mid-October 2018 (P=.021) timepoints (Figure 3.2A). However, due to starting differences between the two groups, the analysis

was run again with starting values as a covariate (Figure 3.2B). In this subsequent analysis, no significant group, time, or group by time effects were observed; therefore, the groups did not change over time as a result of their group status.

Respiration:

Due to having no degrees of freedom in the PPID Control to PPID Treatment analysis for the variance-covariance structure with the best information criteria, the UN variance-covariance structure was used. No significant group, time, or group by time effects were found in the PPID Control to PPID Treatment comparison; however, due to starting differences between the two groups, the analysis was run again with starting values as a covariate. In this subsequent analysis, no significant time or group by time effects were observed, so the groups did not change over time as a result of their group status. The group effect could not be determined in this comparison due to having no degrees of freedom.

After combining the PPID Control and PPID Treatment groups for comparison to the Non-PPID group, significant group (P=.0336) and time (P=.0289) effects, but not a significant group by time effect alone, were observed (Figure 3.3). In this analysis, the PPID horses had significantly higher respiration rates than the Non-PPID horses at the Early November 2017 (P=.038) and Mid-October 2018 (P=.0017) timepoints only.

Complete blood counts:

White blood cell (WBC), absolute segmented neutrophil, absolute banded neutrophil, absolute monocyte, absolute lymphocyte, absolute eosinophil, absolute basophil, and red blood counts (RBC) were measured and described below. Fibrinogen,

hemoglobin (Hgb), packed cell volume (PCV), and total protein were also measured and described below.

White blood cell (WBC) count

No significant group or group by time effects were seen in white blood cell (WBC) counts between the PPID Control and PPID Treatment groups; a significant time effect (*P*=.0046) was observed in this comparison. After combining the PPID Control and PPID Treatment groups for comparison to Non-PPID horses, a significant group by time effect (*P*=.041), but not a group or time effect alone, was observed (Figure 3.4). PPID horses had significantly lower WBC counts than Non-PPID horses at the Early February 2018 and Early August 2018 timepoints only.

Absolute segmented neutrophil count, absolute monocyte count, hemoglobin, and packed cell volume

No significant group or group by time effects were seen in absolute segmented neutrophil counts, absolute monocyte counts, hemoglobin, or packed cell volume between any of the groups. Significant time effects in absolute segmented neutrophil counts were observed in the PPID Control to PPID Treatment (*P*=.0099) and all groups combined (*P*=.0395) comparisons. Significant time effects in absolute monocyte counts were observed in the PPID Control to PPID Treatment (*P*=.027), Non-PPID to PPID (*P*=.0002), and all groups combined (*P*<.0001) comparisons. Significant time effects in hemoglobin were observed in the PPID Control to PPID Treatment (*P*=.0022), Non-PPID to PPID (*P*=.0011), and all groups combined (*P*=.0006) comparisons. Significant time effects in

packed cell volume were observed in the PPID Control to PPID Treatment (P<.0001), Non-PPID to PPID (P<.0001), and all groups combined (P<.0001) comparisons.

Absolute banded neutrophil count

No significant group, time, or group by time effects were seen in absolute banded neutrophil counts between the PPID Control and PPID Treatment groups. After combining the PPID Control and PPID Treatment groups for comparison to Non-PPID horses, significant time (P=.0039) and group by time (P=.0112) effects, but not a group effect alone, were observed. This appeared to be driven by the Non-PPID horses having higher counts than PPID horses at the Early April 2018 timepoint only, and the PPID horses having higher counts than the Non-PPID horses at the Early February 2018 timepoint. This group by time effect may not represent a biologically significant result as the small sample sizes may have allowed a few high results to influence the group by time effect and also because the groups do not appear to be visually distinct from one another in graphical representation.

Additionally, none of the horsed exhibited a clinically significant left-shift at any point in the study, defined as greater than 300/μL banded neutrophils [104].

Absolute lymphocyte count

No significant group or group by time effects were seen in absolute lymphocyte counts between the PPID Control and PPID Treatment horses; a significant time effect (P=.0284) was observed in this comparison. After combining these groups for comparison to Non-PPID horses, significant group (P=.028) and time (P=.0044) effects, but not a group by time effect, were observed with PPID horses generally having lower absolute

lymphocyte counts than Non-PPID horses (Figure 3.5A). Due to starting differences between the Non-PPID and PPID horses, the analysis was run again with starting values as covariate in the model to determine if significant group by time changes were present (Figure 3.5B). This subsequent analysis revealed no significant group by time effects; therefore, these groups did not change over time as a result of their group status.

Absolute basophil count

A significant group effect (*P*=.062), but no time or group by time effect, was seen in absolute basophil counts between the PPID Control and PPID Treatment groups. After combining the PPID Control and PPID Treatment groups for comparison to Non-PPID horses, no significant group or group by time effects were seen. Significant time effects in absolute basophil counts were observed in the Non-PPID to PPID (*P*=.0164) and all groups combined (*P*=.0233) comparisons.

Absolute eosinophil count

No significant group or group by time effects were seen in absolute eosinophil counts between the PPID Control and PPID Treatment horses. Due to starting differences between the PPID Control and PPID Treatment horses, the analysis was run again with starting values as a covariate in the model to determine if significant group by time changes were present. This subsequent analysis revealed no significant group by time effects; therefore, these groups did not change over time as a result of their group status. After combining these groups for comparison to Non-PPID horses, no significant group or group by time effects were observed. Significant time effects in absolute eosinophil counts were

observed in the PPID Control to PPID Treatment (P=.0015), Non-PPID to PPID (P<.0001), and all groups combined (P<.0001) comparisons.

Fibrinogen

A significant group effect (P=.0303), but no time or group by time effect, was seen in fibrinogen between the PPID Control and PPID Treatment groups. After combining the PPID Control and PPID Treatment groups for comparison to Non-PPID horses, no significant group or group by time effects were found. No significant time effects were observed between any of the groups.

Total Protein

No significant group or group by time effects were seen in total protein between the PPID Control and PPID Treatment horses. Due to starting differences between the PPID Control and PPID Treatment horses, the analysis was run again with starting values as a covariate in the model to determine if significant group by time changes were present. This subsequent analysis revealed no significant group by time effects; therefore, these groups did not change over time as a result of their group status. After combining these groups for comparison to Non-PPID horses, no significant group or group by time effects were observed. Significant time effects in total protein were observed in the PPID Control to PPID Treatment (P<.0001), Non-PPID to PPID (P<.0001), and all groups combined (P<.0001) comparisons.

Red blood cell count

No significant group or group by time effects were seen in red blood cell counts between the PPID Control and PPID Treatment horses; a significant time effect (P<.0001)

was observed in this comparison. After combining these groups for comparison to Non-PPID horses, significant group (P=.0203) and time (P<.0001) effects, but not a group by time effect, were observed with PPID horses generally having lower red blood cell counts than Non-PPID horses (Figure 3.6A). Due to starting differences between the Non-PPID and PPID horses, the analysis was run again with starting values as a covariate in the model to determine if significant group by time changes were present (Figure 3.6B). This subsequent analysis revealed no significant group by time effects; therefore, these groups did not change over time as a result of their group status.

Summary of significant CBC results:

PPID horses had significantly lower WBC counts than Non-PPID horses at the Early February 2018 and Early August 2018 timepoints only. PPID horses generally had lower absolute lymphocyte counts and red blood counts than Non-PPID horses. Significant time effects for the Non-PPID to PPID comparisons are shown in Figures 3.7 and 3.8.

Plasma myeloperoxidase:

No significant group or group by time effects were found in plasma myeloperoxidase concentrations between any of the groups. Significant time effects were observed in the PPID Control and PPID Treatment (P<.0001), Non-PPID to PPID (P<.0001), and all horses combined comparisons (P<.0001). Figure 3.9 displays the Non-PPID to PPID comparison.

Whole blood (Tempus™) cytokine/receptor gene expression:

No significant group or group by time effects were seen in IL-12α, IL-1β, IL-6, TGFβ, or TNFα between any of the groups. Significant time effects were seen in IL-12α expression for the PPID Control to PPID Treatment comparison (P=.0006), Non-PPID to PPID comparison (P<.0001), and when all groups were combined (P<.0001). Significant time effects were seen in IL-1β expression for the PPID Control to PPID Treatment comparison (P=.001), Non-PPID to PPID comparison (P<.0001), and when all groups were combined (P<.0001). Significant time effects were seen in IL-6 expression for the PPID Control to PPID Treatment comparison (P<.0001), Non-PPID to PPID comparison (P<.0001), and when all groups were combined (P<.0001). Significant time effects were seen in TGFβ expression for the PPID Control to PPID Treatment comparison (P<.0001), Non-PPID to PPID comparison (P<.0001), and when all groups were combined (P<.0001). Significant time effects were seen in TNFα expression for the Non-PPID to PPID comparison (P=.0172) and when all groups were combined (P=.0165).

Significant group by time effects were seen in IL-13 expression between the PPID Control and PPID Treatment groups (P=.0025). Therefore, the groups were kept separate for comparison to Non-PPID horses. In the Non-PPID to PPID Control to PPID Treatment comparison, there were no significant group or group by time differences between any of the groups. Therefore, all of the groups were combined for analysis of time effects. Significant time effects were seen in the PPID Control to PPID Treatment (P<.0001), Non-PPID to PPID Control to PPID Treatment (P<.0001), and all groups combined (P<.0001) comparisons.

No significant group or group by time effects were seen in IL-17α or TLR4 between any of the groups. Due to starting differences between the PPID and Non-PPID groups, these analyses were run again with starting values as a covariate in the model; the group and group by time effects remained insignificant. Significant time effects in IL-17α expression were observed in the PPID to Non-PPID comparison (P=.0007) and when all groups were combined (P=.0014). Significant time effects in TLR4 expression were observed in the PPID Control to PPID Treatment (P<.0001), Non-PPID to PPID (P<.0001), and all groups combined (P<.0001) comparisons.

No significant group or group by time effects were seen in IFNγ or TLR2 between any of the groups. Due to starting differences between the PPID Control and PPID Treatment groups, these analyses were run again with starting values as covariates in the model; the group and group by time effects remained insignificant. Significant time effects in IFNγ expression were observed in the PPID Control to PPID Treatment (P=.0036), Non-PPID to PPID (P=.0014), and all groups combined (P=.0004) comparisons. Significant time effects in TLR2 expression were observed in only the Non-PPID to PPID (P=.0149), and all groups combined (P=.0204) comparisons.

No significant group or group by time effects were seen in IL-8 between the PPID Control and PPID Treatment horses. After combining these groups for comparison to Non-PPID horses, a significant group (P=.0102) effect was seen with PPID horses having greater IL-8 expression than Non-PPID horses (Figure 3.10). Due to starting differences between Non-PPID and PPID horses, the analysis was run again with starting values as a covariate in the model to determine if significant group by time changes were present. This subsequent analysis revealed no significant group by time effects; therefore, these groups

did not change in their IL-8 response over time. Significant time effects were observed in the PPID Control to PPID Treatment (P=.0002) and Non-PPID to PPID (P<.0001) comparisons.

Summary of significant whole blood (Tempus™) cytokine and receptor expression results:

Aside from time effects in many of the measured cytokines and receptors, the only significant group or group by time difference was in the comparison of Non-PPID to all PPID horses for IL-8 expression. In this analysis, PPID horses had greater IL-8 expression in whole blood compared to Non-PPID horses. Significant time effects for the Non-PPID to PPID comparisons are shown in Figures 3.11 and 3.12.

PBMC (unstimulated/stimulated) cytokine and receptor gene expression

PBMC stimulation- Media:

No significant group, time, or group by time effects were seen in TLR4, TNFα, TGFβ, IL-6, IL-13, and IFNγ between any of the groups. Due to starting differences between the PPID Control and PPID Treatment groups in the TLR4 analysis, the analysis was performed again with starting values as a covariate in the model; the group, time, and group by time effects remained insignificant.

No significant group or group by time effects were seen in IL-1β, IL-8, IL-10, IL-12α, and IL-17α between any of the groups. Significant time effects were seen in IL-1β expression for the Non-PPID to PPID comparison (P=.03) and when all groups were combined (P=.033). Significant time effects were seen in IL-8 expression for the Non-PPID to PPID comparison (P=.034) and when all groups were combined (P=.024).

Significant time effects were seen in IL-10 and IL-12α expression for all comparisons (P<.01). Significant time effects were observed in IL-17α expression in the Non-PPID to PPID comparison (P=.045), but not when all groups were combined.

Summary of PBMC stimulation- Media:

Aside from time effects, there were no differences between PPID Treatment and PPID Control horses and between PPID and Non-PPID horses in the unstimulated samples.

PBMC stimulation- R. equi:

No significant group, time, or group by time effects were seen in TGFβ, TNFα, IL-12α, and IL-6 between any of the groups.

No significant group or group by time effects were seen in TLR4 between any of the groups. Significant time effects in TLR4 were observed when PPID Control and PPID Treatment horses were compared (P=.021), but not in the Non-PPID to PPID comparison or when all groups were combined.

No significant group, time, or group by time effects were seen in IL-1β between the PPID Control and PPID Treatment horses or in the PPID to Non-PPID comparison. Due to starting differences between the PPID and Non-PPID groups, this analysis was run again with starting values as a covariate in the model; the group, time, and group by time effects remained insignificant. No time effects were observed when all groups were combined.

No significant group, time, or group by time effects were seen in IL-8 between the PPID Control and PPID Treatment horses. Significant time (P=.02) and group by time effects (P=.042) were seen in IL-8 expression between the Non-PPID and PPID groups of horses. Due to starting differences between the PPID and Non-PPID groups, this analysis

was run again with starting values as a covariate in the model; significant time (P=.018) and group by time effects (P=.044) remained. Non-PPID and PPID horses differed at only one timepoint, Early September 2017, which appeared to drive the significant group by time effect seen. This suggests that the result is likely not biologically relevant.

No significant group, time, or group by time effects were seen in IL-13 between the PPID Control and PPID Treatment horses. Significant time (P=.018) and group by time (P<.01) effects were seen in IL-13 expression between the Non-PPID and PPID groups of horses. Due to starting differences between the PPID and Non-PPID groups, this analysis was run again with starting values as a covariate in the model; the significant time effect was no longer present, but the significant group by time effect remained (P=.047). Non-PPID and PPID horses differed at only one timepoint, Early September 2017, which appeared to drive the significant group by time effect seen. This suggests that the result is likely not biologically relevant.

No significant group, time, or group by time effects were seen in IFNγ between the PPID Control and PPID Treatment horses. Due to starting differences between these groups, this analysis was run again with starting values as a covariate in the model; the group, time, and group by time effects remained insignificant. After combining these groups for comparison to Non-PPID horses, a significant group effect (P<.01), but not a time or group by time effect, was seen with PPID horses having less IFNγ expression than Non-PPID horses in response to *R. equi* stimulation (Figure 3.13B). Due to significant starting differences between Non-PPID and PPID horses, the analysis was run again with starting values as a covariate in the model to determine if significant group by time changes

were present (Figure 3.13C). This subsequent analysis revealed no significant group by time effects; therefore, these groups did not change over time.

A significant group by time effect ($P<.01$), but not a group or time effect, was seen in IL-17α expression between the PPID Control and PPID Treatment groups. Therefore, the groups were kept separate for comparison to Non-PPID horses. Initial analyses of Non-PPID to PPID Control and PPID Treatment horses showed a significant group by time ($P<.001$) effect, but not a group or time effect (Figure 3.14B). The only point at which significant differences existed between the three groups was at the Early February 2018 timepoint, where the PPID Treatment horses had significantly higher expression of IL-17α in response to *R. equi* stimulation than both PPID Control and Non-PPID horses. Given that this was the only significant difference present, it appears that the significant group by time effects are being driven by the differences at this timepoint only. Therefore, it is unlikely that this result is biologically significant.

Significant group effects were seen in IL-10 expression between the PPID Control and PPID Treatment groups ($P=.039$). Due to starting differences between these groups, this analysis was run again with starting values as a covariate in the model; the group effect was no longer significant. Significant time effects were seen in the analysis with starting values as a covariate in the model. After combining the PPID Control and PPID Treatment groups for comparison to Non-PPID horses, no significant group or group by time effects were seen. Significant time effects in IL-10 expression in response to *R. equi* stimulation were observed when all PPID horses were compared to Non-PPID horses and when all groups were combined ($P<.01$).

Summary of PBMC stimulation- R. equi:

PPID horses had decreased IFNγ expression compared to Non-PPID horses in response to stimulation with *R. equi*. Significant time effects were also seen in some of the responses to *in vitro R. equi* stimulation.

PBMC stimulation- E. coli:

No significant group, time, or group by time effects were seen in IL-12α, IL-6, and TNFα, between any of the groups.

No significant group or group by time effects were seen in IL-13, IL-8, or TLR4 between any of the groups. Significant time effects were seen in IL-13 expression for the Non-PPID to PPID comparison (P=.007) and when all groups were combined (P=.006). Significant time effects were seen in IL-8 expression for the PPID Control to PPID Treatment comparison (P=.011), Non-PPID to PPID comparison (P<.0001), and when all groups were combined (P<.0001). Significant time effects were seen in TLR4 expression for the PPID Control to PPID Treatment comparison (P=.03), Non-PPID to PPID comparison (P<.0001), and when all groups were combined (P<.0001).

No significant group or group by time effects were seen in IL-1β between any of the groups. Due to starting differences between Non-PPID and PPID horses, the analysis was run again with starting values as a covariate in the model to determine if significant group by time changes were present. This subsequent analysis revealed no significant group or group by time effects. Significant time effects were seen in IL-1β expression for the Non-PPID to PPID comparison (P<.0001) and when all groups were combined (P<.001).

No significant group or group by time effects were seen in IL-10 between any of the groups. Due to starting differences between the PPID Control and PPID Treatment groups, this analysis was run again with starting values as a covariate in the model; the group and group by time effects remained insignificant. Significant time effects in IL-10 expression were observed in all comparisons ($P<.0001$).

No significant group, time, or group by time effects were seen in IFNγ between the PPID Control and PPID Treatment horses. After combining these groups for comparison to Non-PPID horses, a significant group effect, but not a time or group by time effect, was seen with PPID horses having less IFNγ expression than Non-PPID horses in response to *E. coli* stimulation ($P<.01$) (Figure 3.15B). Due to starting differences between Non-PPID and PPID horses, the analysis was run again with starting values as a covariate in the model to determine if significant group by time changes were present (Figure 3.15C). This subsequent analysis revealed no significant group by time effects; therefore, the groups did not change in their IFNγ response over time as a result of their group status.

A significant group by time ($P=.0497$) effect, but not a group effect, were seen in IL-17α expression between the PPID Control and PPID Treatment groups. Therefore, the groups were kept separate for comparison to Non-PPID horses. In the Non-PPID to PPID Control to PPID Treatment comparison, there were no significant group or group by time differences between any of the groups. There were no significant differences between the PPID Control and PPID Treatment horses in either comparison; the significant group by time effect in the PPID Control to PPID Treatment comparison appears to be driven by a single timepoint (Mid-October 2018), where the groups differed slightly, although were not statistically different ($P=.075$). Significant time effects were seen in PPID Control to

PPID Treatment (*P*=.04), Non-PPID to PPID Control to PPID Treatment (*P*=.013), and all groups combined (*P*=.008) comparisons.

No significant group, time, or group by time effects were seen in TGFβ between the PPID Control and PPID Treatment horses. After combining these groups for comparison to Non-PPID horses, a significant group effect (*P*=.04) was seen with PPID horses having greater TGFβ expression than Non-PPID horses in response to *E. coli* stimulation (Figure 3.16B). No significant time or group by time effects were seen in the Non-PPID to PPID comparison.

Summary of PBMC stimulation- E. coli:

PPID horses had decreased IFNγ expression and increased TGFβ expression compared to Non-PPID horses in response to stimulation with *E. coli*. Time effects were often seen in responses to *E. coli* stimulation.

PBMC stimulation- PMA/ionomycin:

No significant group or group by time effects were seen in IFNγ, IL-6, IL-8, TNFα, TGFβ, or TLR4 between any of the groups. Significant time effects were seen in IFNγ expression for the PPID Control to PPID Treatment comparison (*P*=.04), Non-PPID to PPID comparison (*P*=.003), and when all groups were combined (*P*=.004). Significant time effects were seen in IL-6 expression for the PPID Control to PPID Treatment comparison (*P*=.0035), Non-PPID to PPID comparison (*P*<.0001), and when all groups were combined (*P*<.0001). Significant time effects were seen in IL-8 expression for the Non-PPID to PPID comparison (*P*=.0055) and when all groups were combined (*P*=.0083). Significant time effects were seen in TNFα expression for the PPID Control to PPID Treatment comparison

(*P*=.025), Non-PPID to PPID comparison (*P*=.029), and when all groups were combined (*P*=.024). Significant time effects were seen in TGFβ expression only for the Non-PPID to PPID comparison (*P*=.018). Significant time effects were seen in TLR4 expression for the Non-PPID to PPID comparison (*P*<.0001) and when all groups were combined (*P*=.0002).

No significant group, time, or group by time effects were seen in IL-10 and IL-13 between any of the groups. Due to significant starting differences between PPID Control and PPID Treatment horses, these analyses were run again with starting values as a covariate in the model; the results remained the same with no significant group, time, or group by time effects observed.

No significant group, time, or group by time effects were seen in IL-17α between the PPID Control and PPID Treatment groups. Due to starting differences between the PPID Control and PPID Treatment groups, this analysis was run again with starting values as a covariate in the model; the group, time, and group by time effects remained insignificant. No significant group or group by time effects were seen in IL-17α for the Non-PPID to PPID comparison or when all groups were combined, but significant time effects in IL-17α expression were observed in the Non-PPID to PPID comparison (*P*=.027) and when all groups were combined (*P*=.032).

No time or group by time effects were seen in IL-12α between the PPID Control and PPID Treatment horses, but significant group effects were seen (*P*=.032). Due to significant starting differences between PPID Control and PPID Treatment horses, the analysis was run again with starting values as a covariate in the model; this analysis revealed no significant group, time, or group by time effects. Since there were no significant group by time differences, these groups were combined for the comparison to

Non-PPID horses. No significant group, time, or group by time differences were seen in the Non-PPID to PPID comparison or when all groups were combined.

Significant group (P=.034) and time effects (P=.015), but not group by time effects, were seen in IL-1β expression between the PPID Control and PPID Treatment horses. Since there were no significant group by time differences, these groups were combined for the comparison to Non-PPID horses. No significant group or group by time differences were seen in the Non-PPID to PPID comparison or when all groups were combined. Significant time effects in IL-1β expression were observed in the Non-PPID to PPID comparison (P=.005) and when all groups were combined (P=.0033).

Summary of PBMC stimulation- PMA/ionomycin:

Aside from some time effects, no differences were observed in PMA/ionomycin-stimulated samples.

Summary of overall PBMC stimulation results:

PPID does not appear to affect the cytokine and receptor gene expression of unstimulated or PMA/ionomycin-stimulated PBMCs. However, PPID horses showed decreased *in vitro* expression of IFNγ in response to both *R. equi* and *E. coli* compared to Non-PPID horses. Additionally, PPID horses had greater *in vitro* TGFβ expression in response to *E. coli* than Non-PPID horses. Oral administration of PRASCEND does not appear to influence the *in vitro* immune responses of peripheral blood mononuclear cells. Significant time effects for the Non-PPID to PPID or PPID Control to PPID Treatment to Non-PPID comparisons are shown in Figures 3.17-3.21.

Stimulated whole blood cytokine and receptor gene expression

Whole blood stimulation- unstimulated:

No significant group, time, or group by time effects were seen in IL-1β and TLR2 between any of the groups.

No significant group or group by time effects were seen in IFNγ or TNFα between any of the groups. Significant time effects in IFNγ expression were observed in the PPID Control to PPID Treatment (P=.0086), Non-PPID to PPID (P<.0001), and all groups combined (P<.0001) comparisons. Significant time effects in TNFα expression were observed in the PPID Control to PPID Treatment (P=.0056), Non-PPID to PPID (P<.0001), and all groups combined (P<.0001) comparisons.

No significant group or group by time effects were seen in IL-12α, IL-17α, IL-6, or IL-8 between any of the groups. Due to starting differences between the Non-PPID and PPID groups in the expression of these four cytokines, the analyses for these comparisons were run again with starting values as covariates in the model; the group and group by time effects remained insignificant. Significant time effects in IL-12α expression were observed in the PPID Control to PPID Treatment (P=.0263), Non-PPID to PPID (P<.0001), and all groups combined (P<.0001) comparisons. Significant time effects in IL-6 expression were observed in the PPID Control to PPID Treatment (P=.004), Non-PPID to PPID (P<.0001), and all groups combined (P<.0001) comparisons. Significant time effects in IL-8 expression were observed in the PPID Control to PPID Treatment (P=.0018), Non-PPID to PPID (P=.0002), and all groups combined (P<.0001) comparisons. No time effects were seen for IL-17α in any of the comparisons.

No significant group or group by time effects were seen in TLR4 between the PPID Control and PPID Treatment groups, but a significant time effect was seen ($P<.0001$). After combining these groups for comparison to Non-PPID horses, significant group ($P=.036$), time ($P<.0001$), and group by time ($P=.0319$) effects were seen with PPID horses having greater TLR4 expression than Non-PPID horses (Figure 3.22A). Due to starting differences between Non-PPID and PPID horses, the analysis was run again with starting values as a covariate to determine if truly significant group by time changes were present (Figure 3.22B). This subsequent analysis revealed no significant group by time effects; therefore, these groups did not actually change in their TLR4 expression over time based on their group status. The PPID horses differed from the Non-PPID horses at only one of the three timepoints (Early September 2017); it is likely that the significant group difference observed is driven by the large starting difference between the two groups, especially given that there are only three timepoints in this data set.

No significant group or group by time effects were seen in TGFβ between the PPID Control and PPID Treatment horses. After combining these groups for comparison to Non-PPID horses, a significant group effect ($P=.031$), but not a group by time effect, was seen with PPID horses having greater TGFβ expression than Non-PPID (Figure 3.23A). Due to starting differences between the Non-PPID and PPID groups, the analysis for this comparison was run again with starting values as a covariate; the group by time effects remained insignificant; therefore, these groups did not change in their TGFβ expression over time based on their group status (Figure 3.23B). Significant time effects were observed in the PPID Control to PPID Treatment ($P=.0002$) and Non-PPID to PPID ($P<.0001$) comparisons.

Significant time (P<.0001) and group by time (P=.0012) effects were observed in IL-13 expression between the PPID Control and PPID Treatment groups. Therefore, the groups were kept separate for comparison to Non-PPID horses. In the Non-PPID to PPID Control and PPID Treatment comparison, significant time (P<.0001) and group by time effects (P=.0035) were also seen (Figure 3.24A). Due to starting differences between the Non-PPID and PPID groups, the analysis for this comparison was run again with starting values as a covariate; the significant group by time effect remained (P=.0001) (Figure 3.24B). However, the only point at which a significant difference existed between the three groups after including starting values as a covariate was between the PPID Control and PPID Treatment groups and between the Non-PPID and PPID Treatment horses at the Mid-October timepoint only. At this timepoint, PPID Treatment horses had significantly lower expression of IL-13 than both PPID Control and Non-PPID horses. Given that this was the only significant difference present, it appears that the significant group by time effects are being driven by the differences at this timepoint only. These results are difficult to draw any conclusions from, because graphically, the groups appear quite similar. It is possible that this result is not biologically significant but is just statistically significant due to the few samples and timepoints for this data set.

Summary of whole blood stimulation- unstimulated:

PPID horses had significantly higher expression of TGFβ in whole blood than Non-PPID horses. Aside from time effects, the other significant differences (TLR4 and IL-13) appeared to be driven by single timepoint differences within a group.

Whole blood stimulation- R. equi:

No significant group or group by time effects were seen in IFNγ, IL-12α, IL-13, IL-17α, IL-1β, TLR2, TLR4, or TNFα between any of the groups. Significant time effects in IFNγ expression were observed in the PPID Control to PPID Treatment (*P*=.0004), Non-PPID to PPID (*P*<.0001), and all groups combined (*P*<.0001) comparisons. Significant time effects in IL-12α expression were observed in the PPID Control to PPID Treatment (*P*<.0001), Non-PPID to PPID (*P*<.0001), and all groups combined (*P*<.0001) comparisons. Significant time effects in IL-13 expression were observed in the PPID Control to PPID Treatment (*P*=.0033), Non-PPID to PPID (*P*=.0001), and all groups combined (*P*=.0001) comparisons. Significant time effects in IL-17α expression were observed in the PPID Control to PPID Treatment (*P*<.0001), Non-PPID to PPID (*P*<.0001), and all groups combined (*P*<.0001) comparisons. Significant time effects in IL-1β expression were observed in the PPID Control to PPID Treatment (*P*<.0001), Non-PPID to PPID (*P*<.0001), and all groups combined (*P*<.0001) comparisons. Significant time effects in TLR2 expression were observed in the Non-PPID to PPID (*P*=.0026) and all groups combined (*P*=.0076) comparisons. Significant time effects in TLR4 expression were observed in the PPID Control to PPID Treatment (*P*=.0047), Non-PPID to PPID (*P*=.0013), and all groups combined (*P*=.0005) comparisons. Significant time effects in TNFα expression were observed in the PPID Control to PPID Treatment (*P*=.0083) and all groups combined (*P*=.0248) comparisons.

Significant group, but not time or group by time, effects were seen in TGFβ expression between the PPID Control and PPID Treatment groups (*P*=.0396). Therefore, the groups were kept separate for comparison to Non-PPID horses. In the Non-PPID to

PPID Control to PPID Treatment comparison, there were no significant group, time, or group by time differences between any of the groups. Therefore, all of the groups were combined for analysis of time effects. No significant time effects were seen in any of the comparisons. The initial significant group effect seen between the PPID Control and PPID Treatment appears to have been driven by a significant difference between these two groups at only one timepoint, Mid-October 2017; given the small sample size and few timepoints in the data set, this likely led to the statistically significant difference, but not a biologically significant difference.

No significant group or group by time effects were seen in IL-6 between the PPID Control and PPID Treatment horses. After combining these groups for comparison to Non-PPID horses, a significant group effect (P=.0172), but not a group by time effect, was seen with PPID horses having less IL-6 expression than Non-PPID horses (Figure 3.25C). Due to starting differences between the Non-PPID and PPID groups, the analysis for this comparison was run again with starting values as a covariate; the group by time effects remained insignificant; therefore, these groups did not change in their IL-6 expression in response to *R. equi* over time based on their group status (Figure 3.25D). Significant time effects were observed in the PPID Control to PPID Treatment (P=.0001) and Non-PPID to PPID (P<.0001) comparisons.

No significant group or group by time effects were seen in IL-8 between the PPID Control and PPID Treatment horses. After combining these groups for comparison to Non-PPID horses, a significant group effect was seen with PPID horses having less IL-8 expression than Non-PPID horses in response to *R. equi* (P=.034) (Figure 3.26C).

Significant time effects in IL-8 expression were also observed in the PPID Control to PPID Treatment (P<.0001) and Non-PPID to PPID (P<.0001) comparisons.

Summary of whole blood stimulation- R. equi:

PPID horses had less IL-6 and IL-8 expression than Non-PPID horses in response to *in vitro R. equi* stimulation of whole blood. Time effects were also observed in whole blood responses to *R. equi* stimulation.

Whole blood stimulation- E. coli:

No significant group, time, or group by time effects were seen in IFNγ, IL-12α, IL-13, TGFβ, or TNFα between any of the groups.

No significant group or group by time effects were seen in IL-17α, IL-1β, IL-8, TLR2 between any of the groups. Significant time effects in IL-17α expression were observed in the Non-PPID to PPID (P=.0038) comparison and the all groups combined (P=.0095) comparison. A significant time effect in IL-1β expression was observed in the all groups combined (P=.0317) comparison. Significant time effects in IL-8 expression were observed in the PPID Control to PPID Treatment (P=.0061), Non-PPID to PPID (P=.0005), and all groups combined (P=.0003) comparisons. Significant time effects in TLR2 expression were observed in the PPID Control to PPID Treatment (P=.0374), Non-PPID to PPID (P=.0296), and all groups combined (P=.0266) comparisons.

Significant group (P=.0327) and group by time (P=.0314) effects were seen in IL-6 expression between the PPID Control and PPID Treatment groups. Due to initial starting

differences, the analysis was run again with starting values as a covariate in the model; the group by time effect remained significant (*P*=.045). Therefore, the groups were kept separate for comparison to Non-PPID horses. In the initial Non-PPID to PPID Control to PPID Treatment comparison, there were no significant group, time, or group by time differences between any of the groups. However, starting differences were present between the PPID Control and PPID Treatment horses, so the analysis was run again with starting values as a covariate in the model. The group, time, and group by time effects remained insignificant. Therefore, all of the groups were combined for analysis of time effects. No significant time effects were seen in any of the comparisons.

No significant group, time, or group by time effects were seen in TLR4 between the PPID Control and PPID Treatment horses. After combining these groups for comparison to Non-PPID horses, a significant group effect (*P*=.0044), but not a time or group by time effect, was seen with PPID horses having greater TLR4 expression than Non-PPID horses in response to *E. coli* at both the Mid-October 2017 and Early November 2017 timepoints (Figure 3.27C).

Summary of whole blood stimulation- E. coli:

PPID horses had greater TLR4 expression than Non-PPID horses in response to *E. coli* at both the Mid-October 2017 and Early November 2017 timepoints

Whole blood stimulation- PMA/ionomycin:

No significant group, time, or group by time effects were found in IL-12α and IL-13 between any of the groups.

No significant group or group by time effects were seen in IFNγ, IL-1β, IL-6, TGFβ, or TNFα between any of the groups. Significant time effects in IFNγ expression were observed in the PPID Control to PPID Treatment (*P*=.0005), Non-PPID to PPID (*P*=.0023), and all groups combined (*P*=.0031) comparisons. Significant time effects in IL-1β expression were observed in the Non-PPID to PPID (*P*=.0197) and all groups combined (*P*=.0194) comparisons. Significant time effects in IL-6 expression were observed in the PPID Control to PPID Treatment (*P*=.0275), Non-PPID to PPID (*P*=.0256), and all groups combined (*P*=.0276) comparisons. Significant time effects in TGFβ expression were observed in the PPID Control to PPID Treatment (*P*=.0162), Non-PPID to PPID (*P*=.0058), and all groups combined (*P*=.0064) comparisons. Significant time effects in TNFα expression were observed in the Non-PPID to PPID (*P*=.0177) and all groups combined (*P*=.0318) comparisons.

A significant group by time effect (*P*=.0235) was seen in IL-17α expression between the PPID Control and PPID Treatment groups. Therefore, the groups were kept separate for comparison to Non-PPID horses. In the Non-PPID to PPID Control to PPID Treatment comparison, there were no significant group or group by time differences between any of the groups. Therefore, all of the groups were combined for analysis of time effects. Significant time effects in IL-17α expression were observed in the PPID Control to PPID Treatment (*P*=.0002), Non-PPID to PPID Control to PPID Treatment (*P*=.0046), and all groups combined (*P*=.0011) comparisons.

No significant group or group by time effects were seen in TLR2 between PPID Control and PPID Treatment horses. Due to starting differences between these groups, this analysis was run again with starting values as a covariate in the model; the group, time,

and group by time effects remained insignificant. After combining the PPID Control and PPID Treatment groups for comparison to Non-PPID horses, no significant group or group by time effects were seen. Significant time effects in TLR2 expression were observed in the Non-PPID to PPID (*P*=.0179) and all groups combined (*P*=.0306) comparisons.

A significant group effect (*P*=.036), but no group by time effect, was seen in TLR4 expression between the PPID Control and PPID Treatment groups. Due to starting differences between these groups, this analysis was run again with starting values as a covariate in the model; the group effect was no longer significant and the group by time effect remained insignificant. After combining the PPID Control and PPID Treatment groups for comparison to Non-PPID horses, no significant group or group by time effects were seen. No significant time effects were observed between any of the groups.

A significant group effect (*P*=.0401), but no group by time effect, was seen in IL-8 expression between the PPID Control and PPID Treatment groups. Due to not having a significant group by time effect and having no starting differences, these groups were combined for comparison to the Non-PPID horses; no significant group or group by time differences were observed. Therefore, all of the groups were combined for analysis of time effects. No significant time effects were seen in any of the comparisons.

Summary of whole blood stimulation- PMA/ionomycin:

Aside from time effects, no differences were found.

Summary of overall whole blood stimulation results:

PPID horses had significantly higher expression of TGFβ in unstimulated whole blood than Non-PPID horses. PPID horses had less IL-6 and IL-8 expression than Non-PPID horses in response to *R. equi* stimulation of whole blood. PPID horses had greater TLR4 expression than Non-PPID horses in response to *E. coli* stimulation at both the Mid-October 2017 and Early November 2017 timepoints. Time effects were also often observed in the cytokine and receptor gene expression measured in unstimulated whole blood and in whole blood responses to *in vitro R. equi*, *E. coli*, and PMA/ionomycin stimulation.

3.5 Discussion

The goal of this study was to investigate systemic immune function in horses with PPID across seasons and to evaluate the extent to which PRASCEND treatment affects these measures. Immune function was measured using complete blood counts, plasma MPO concentrations, and gene expression of cytokines and receptors important for immune function in whole blood (Tempus™ Blood RNA tubes) and *R. equi*, *E. coli*, and PMA/ionomycin-stimulated whole blood and isolated PBMCs. Overall, the most striking and consistently significant results were related to the complete blood counts and PBMC stimulations.

Decreased WBC and absolute lymphocyte counts were observed in the PPID horses compared to the Non-PPID horses and were not impacted by PRASCEND treatment; this is consistent with previously documented findings of decreased WBC counts in PPID horses [10,50]. In theory, this alone is sufficient to place PPID horses at greater risk of infection. The inclusion of age-matched Non-PPID controls rules out the possibility that

these findings are related to normal decreases in the lymphocyte populations due to age-related immunosenescence [105]. This work also confirms that lymphocytes from PPID horses do not respond differently to *in vitro* PMA/ionomycin stimulation than those from age-matched Non-PPID horses; differences in lymphocyte responses to PMA/ionomycin in aged horses compared to younger, adult horses have been reported [101]. However, for the bacterial stimulations, which can be considered more biologically relevant than PMA/ionomycin stimulations, significant differences in the PBMC responses were apparent between PPID and Non-PPID horses.

For the PBMC stimulations with heat-inactivated *R. equi* and heat-inactivated *E. coli*, each horse's PBMCs were counted and plated at 4 million cells per well. This allowed us to evaluate the activity of these cells without confounding due to the potential differences in a horse's baseline WBC and absolute lymphocyte counts. Even after this normalization in the number of cells that were stimulated *in vitro*, PPID horses consistently demonstrated significantly decreased IFNγ production in response to *in vitro R. equi* and *E. coli* stimulation. PPID horses also displayed significantly increased TGFβ expression in response to *in vitro E. coli* stimulation compared to Non-PPID horses. PRASCEND treatment did not impact these responses.

In foals with *R. equi* infection, it is widely accepted that a decreased ability to enact a Th1 response, combined with reduced IFNγ expression, allows *R. equi* to establish infection within the lung [66,82,106]. It is not uncommon for foals to develop bacteremia in conjunction with *R. equi* pneumonia, and this has also been demonstrated in adult horses [59,66,67]. Decreased WBC counts have also been reported in foals that went on to later develop *R. equi* pneumonia [107]. This supports the conclusion that insufficiencies in WBC

counts and decreased IFNγ expression may predispose adult horses with PPID to infection with *R. equi* and indicates that the response of PPID horses to *R. equi* bears resemblance to that of the foal.

Of note, the bacteria used in this study were heat-inactivated; this method was selected to ensure that all samples could be processed in a short period of time, with minimal changes to the bacterial cultures. The ability to grow a single batch of bacteria (both *R. equi* and *E. coli*), obtain these bacteria during the logarithmic growth phase, and perform all stimulations at once was ideal for comparing the results with minimal confounding variables. Additionally, the added benefit of using dead bacteria was that the bacteria could not replicate during the incubation time (ensuring each well was exposed to the same dose for the entire incubation time) and could not infect the cells. The measured cytokine responses are truly the measurement of the initial immune responses to these stimuli, since these bacteria, particularly *R. equi*, were not permitted to establish infection.

In the *E. coli* stimulation, PPID horses not only had decreased IFNγ expression compared to Non-PPID horses, but also had increased TGFβ expression. This decreased IFNγ expression is consistent with the PBMC responses to *R. equi*; however, the increased TGFβ difference was not observed in the *R. equi* stimulation. In the unstimulated whole blood samples, a significant increase in TGFβ expression was also seen in PPID horses compared to Non-PPID horses.

In contrast to work by McFarlane and Holbrook, where lipopolysaccharide (LPS)-stimulated PBMCs from PPID horses produced significantly greater TNFα than adult and age-matched Non-PPID horses, no differences in TNFα were observed in our analyses [50]. This may be a consequence of the LPS model in general. In human work, the LPS model,

which is reflective of endotoxemia , does not achieve adequate similarities to actual sepsis cases [108,109]. Nevertheless, LPS stimulations can prove valuable when it is desired to only view the pathways associated with LPS activation of PBMCs. The contrast in findings between the work of McFarlane and Holbrook and the current study may be due to the use of LPS versus intact, but inactivated, bacteria. However, these differences might also be due to the single timepoint of analysis in the McFarlane and Holbrook study, which may have been influenced by the significant temporal changes in the immune response as described in our findings from the present study; the timeframe in which samples were collected was not described in the study by McFarlane and Holbrook [50]. Overall, the findings from the currently presented study suggest that PPID horses consistently are unable to mount a successful Th1 response to opportunistic bacteria, but also hint at differences within the T_{reg} subpopulation of lymphocytes.

In general, the immune response to gram-negative and gram-positive bacteria is initially characterized by IFNγ release, as well as IL-12 for gram-negative bacteria [110]. This was observed in the *R. equi* and *E. coli*-stimulated samples of both the Non-PPID and PPID horses compared to their unstimulated samples, although with the PPID horses responding with significantly lower IFNγ release than Non-PPID horses in both stimulations. The most appropriate immune response to both the *R. equi* and *E. coli* stimulations for such a short incubation time (24 hours) would have been the activation of the Th1 response. An appropriate Th1 response is generally characterized by increased levels of IFNγ, produced by monocytes/macrophages depending on location, which then encourages Th1 differentiation of CD4 T-cells [111]. These differentiated Th1 CD4 T-cells also produce IFNγ, which further stimulates Th1 differentiation of CD4 T-cells [111]. Additionally, this IFNγ

production can inhibit differentiation of CD4 T-cells into Th2 and Th17 subsets [111]. The Th2 subset is most appropriate for extracellular infection, such as helminths, while the Th17 subset is important for neutrophil recruitment [111]. The T_{reg} subset of CD4 T-cells is stimulated to develop by TGFβ, and these cells continue to produce TGFβ to further promote the development of more T_{reg} cells [111]. This TGFβ production also shuts down differentiation of CD4 T-cells into the Th1 and Th2 lineage [111]. T_{reg} cells essentially serve as a check for the immune response, to ensure that an immune response is "turned off" when no longer required.

Given the results of this study, it appears that PPID horses mount inadequate Th1 responses to *R. equi* and *E. coli* stimulation. These findings also suggest that T_{reg} cells may be shutting down the Th1 response with TGFβ expression in response to *E. coli* stimulation. While this study is one of the larger and more extensive studies on immune function in horses with PPID, the sample sizes are still small, so it is possible that a difference in TGFβ may have existed in response to *R. equi* stimulation between the Non-PPID and PPID horses, but there was not enough power to detect it.

While the role of lymphocytes in the immune response is crucial, the innate immune response is also important to prevent infection. In foals with *R. equi* infection, inadequate neutrophil function also appears to play a role in the ability of *R. equi* to establish infection [82,107]. Neutrophil function in horses with PPID has been previously examined and reported; in one study, neutrophils of horses with PPID exhibited significantly lower oxidative burst, but no difference in phagocytosis, compared to Non-PPID horses [48]. In a separate study, PPID horses and age-matched Non-PPID horses were found to have increased IL-8 expression compared to Non-PPID adult horses [50]. In the work currently presented, no

differences in IL-8 expression from unstimulated or stimulated PBMCs were observed. However, in the currently presented study, when whole blood was collected directly into Tempus™ Blood RNA tubes, which immediately stabilize any RNA, expression of IL-8 was found to be increased in PPID horses compared to our age-matched Non-PPID horses. This suggests that PPID horses may have altered neutrophil activation and/or recruitment at a basal level. Due to the other published work that demonstrated decreased oxidative burst, plasma myeloperoxidase, a marker of neutrophil degranulation, was examined in this study [48]. No differences were observed in plasma MPO in the present work, which contradicts the previous work that demonstrated differences in oxidative burst of neutrophils of PPID horses.

In the remaining significant results, including body temperature, respiration, pulse, IL-17α expression in the *R. equi* PBMC stimulation, as well as several of the unstimulated and stimulated whole blood samples, the significant differences appeared to be driven mainly by single timepoint differences, as well as differences in starting values. These differences were not consistent over time, unlike the results discussed at length above. In the whole blood unstimulated and stimulated samples, this was likely due to the decreased number of sampling timepoints, as these samples were only available for the first three timepoints of the study. The temperature differences, where PPID horses tended to have lower body temperature than Non-PPID horses, and the respiration differences, where PPID horses had higher respiration rates than Non-PPID horses, may also indicate an issue with temperature regulation in horses with PPID. Strong time effects were frequently seen in complete blood count results, plasma MPO results, and in both stimulated and unstimulated PBMC and whole blood cytokine and receptor expression, and in whole blood

(Tempus™ Blood RNA tubes) cytokine and receptor expression. With these significant time effects, having at least six timepoints of data appeared to minimize the effects of the wide variation in the immune response over time and provided more consistent, and likely more relevant, findings related to PPID status and PRASCEND treatment effects.

Overall, the results from this study suggest that horses with PPID are likely at increased risk for opportunistic infection and may have a reduced ability to recover from bacteremia due to their decreased WBC counts, decreased expression of IFNγ to both *R. equi* and *E. coli* stimulations, increased expression of TGFβ in PBMC responses to *E. coli*, and increased IL-8 expression in whole blood (Tempus™ Blood RNA tubes). Indeed, these results suggest that PPID can cause inadequate immune responses to *R. equi* that are quite similar to those seen in foals under six months of age, which may offer an explanation as to why some adult horses appear susceptible to *R. equi* infection. The lack of difference in the PMA/ionomycin-stimulated samples demonstrates that in the presence of a strong stimuli, PPID horses do not respond differently than Non-PPID horses. This further supports the conclusion that PPID horses are likely at higher risk of opportunistic infection rather than infection from pathogens that also affect healthy hosts. In addition, the many significant time effects demonstrate that immune responses in horses, regardless of PPID status, are quite variable over time; this stresses the importance of careful study design and the need for adequate control groups when investigating the equine immune response.

Table 3.1. Number of outliers removed from whole blood (Tempus™ Blood RNA tubes) cytokine and receptor gene expression sample analyses

Cytokine/Receptor	**# of whole blood (Tempus™) outliers removed**
IFNγ	3
IL-12α	1
IL-13	4
IL-17α	0
IL-1β	4
IL-6	1
IL-8	3
TGFβ	2
TLR2	2
TLR4	1
TNFα	1

Table 3.2. Number of outliers removed from peripheral blood mononuclear cell (PBMC) (unstimulated/stimulated) cytokine and receptor gene expression sample analyses

Cytokine/Receptor	**# of media outliers removed**	**# of *R. equi* outliers removed**	**# of *E. coli* outliers removed**	**# of PMA outliers removed**
IFNγ	4	1	2	0
IL-10	2	2	2	0
IL-17α	0	0	4	0
IL-12α	0	0	0	0
IL-13	2	0	0	0
IL-6	0	4	4	3
IL-8	1	2	3	0
TLR4	2	1	4	2
TGFβ	2	0	0	0
TNFα	1	0	0	1
IL-1β	1	2	2	0

Table 3.3. Number of outliers removed from whole blood (unstimulated/stimulated) cytokine and receptor gene expression sample analyses

Cytokine/Receptor	**# of unstimulated outliers removed**	**# of *R. equi* outliers removed**	**# of *E. coli* outliers removed**	**# of PMA outliers removed**
IFNγ	0	1	1	0
IL-12α	0	1	2	0
IL-13	0	0	0	0
IL-17α	0	2	0	0
IL-1β	1	0	2	0
IL-6	0	0	0	0
IL-8	0	0	3	1
TGFβ	0	0	0	0
TLR2	0	1	0	1
TLR4	0	0	0	0
TNFα	0	0	1	1

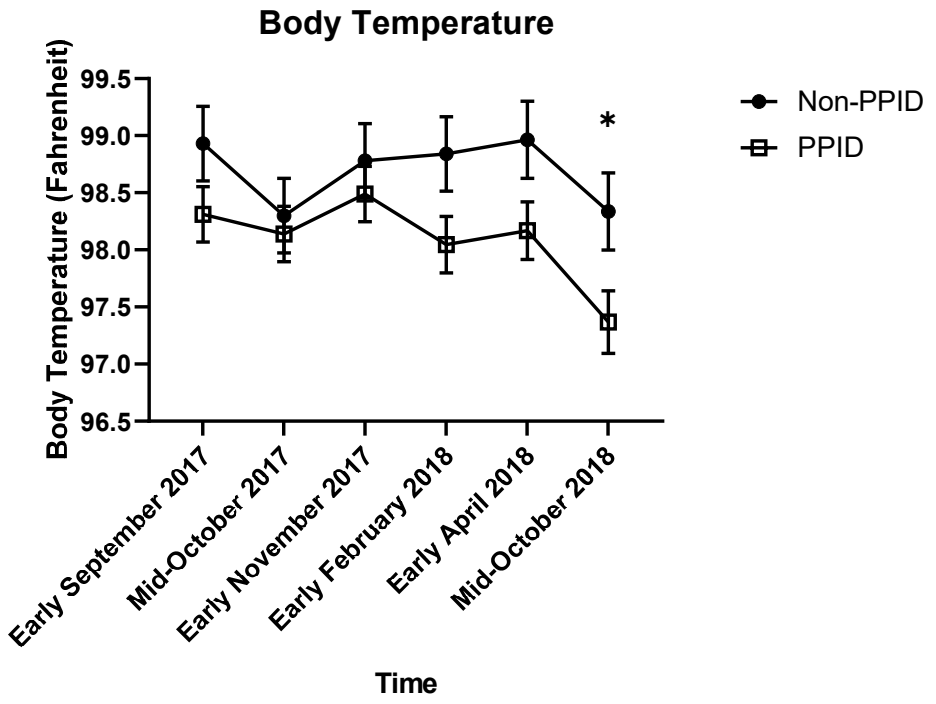

Figure 3.1. Body Temperature

Mean +/- SEM. Non-PPID horses are represented by lines with closed circles. PPID horses are represented by lines with open squares. At Day 0 (Early September 2017), there were 10 horses in the Non-PPID group and 18 horses in the PPID group. By the end of the study, there were 9 horses in the Non-PPID group and 13 horses in the PPID group. * denotes a significant difference ($P<.05$) between Non-PPID and PPID groups. PPID-pituitary pars intermedia dysfunction.

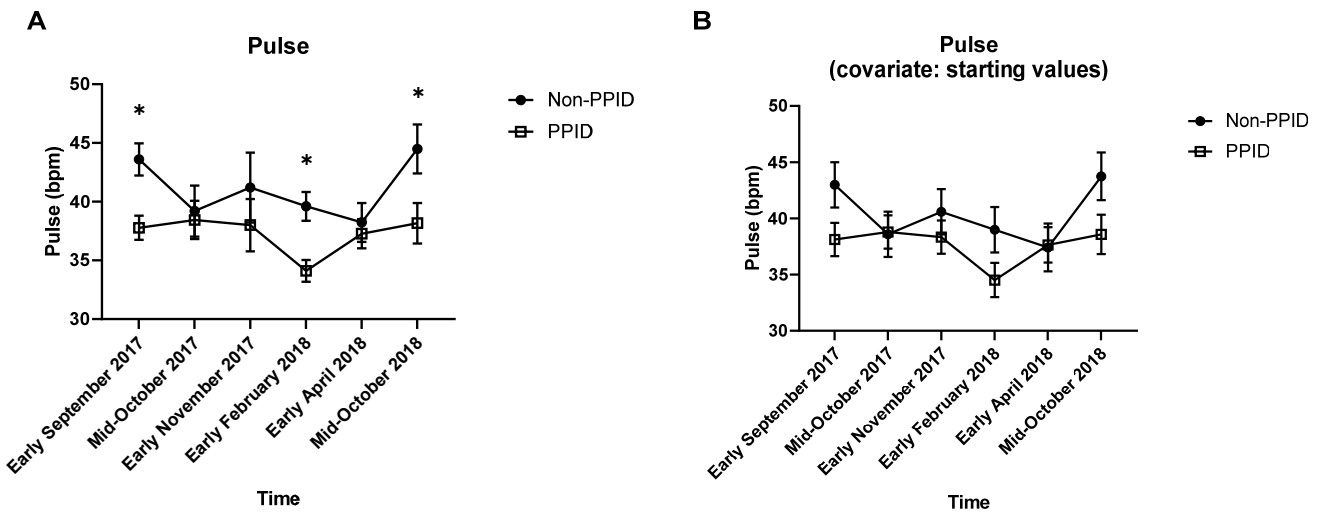

Figure 3.2. (A) Pulse (B) Pulse (starting values as a covariate in the model)

Mean +/- SEM. Non-PPID horses are represented by lines with closed circles. PPID horses are represented by lines with open squares. At Day 0 (Early September 2017), there were 10 horses in the Non-PPID group and 18 horses in the PPID group. By the end of the study, there were 9 horses in the Non-PPID group and 13 horses in the PPID group. * denotes a significant difference ($P<.05$) between Non-PPID and PPID groups. PPID-pituitary pars intermedia dysfunction.

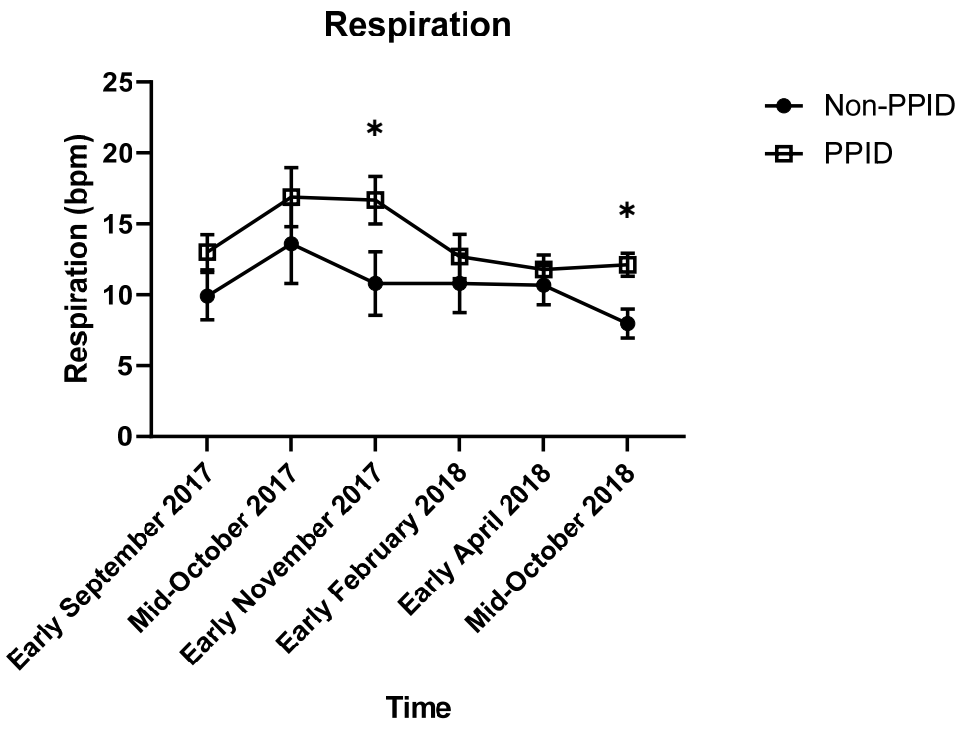

Figure 3.3. Respiration

Mean +/- SEM. Non-PPID horses are represented by lines with closed circles. PPID horses are represented by lines with open squares. At Day 0 (Early September 2017), there were 10 horses in the Non-PPID group and 18 horses in the PPID group. By the end of the study, there were 9 horses in the Non-PPID group and 13 horses in the PPID group. * denotes a significant difference ($P<.05$) between Non-PPID and PPID groups. PPID-pituitary pars intermedia dysfunction.

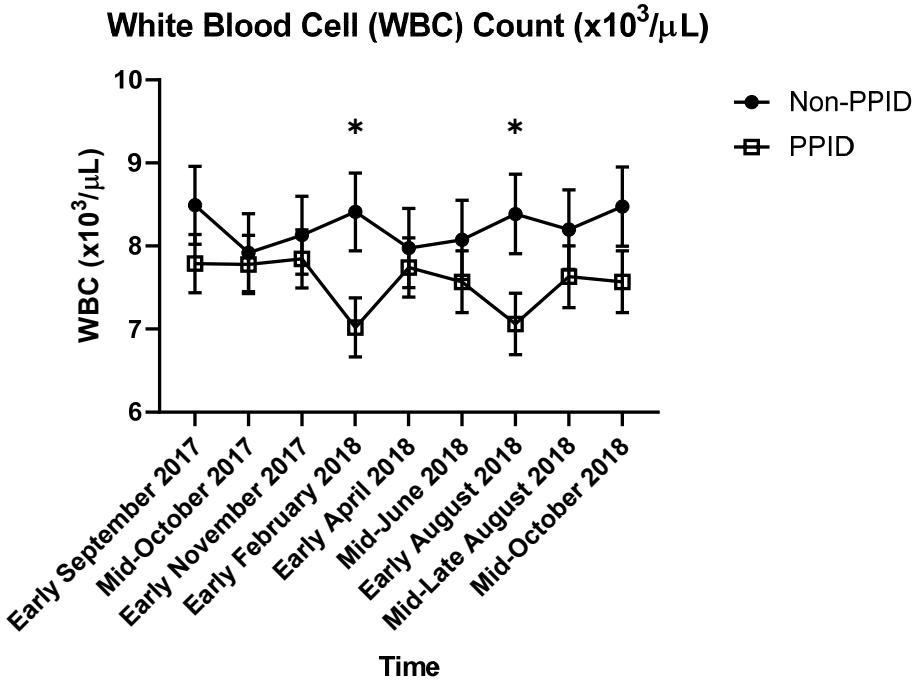

Figure 3.4. White Blood Cell count (x10^3/μl)

Mean +/- SEM. Non-PPID horses are represented by lines with closed circles. PPID horses are represented by lines with open squares. At Day 0 (Early September 2017), there were 10 horses in the Non-PPID group and 18 horses in the PPID group. By the end of the study, there were 9 horses in the Non-PPID group and 13 horses in the PPID group. * denotes a significant difference (P<.05) between Non-PPID and PPID groups. PPID-pituitary pars intermedia dysfunction.

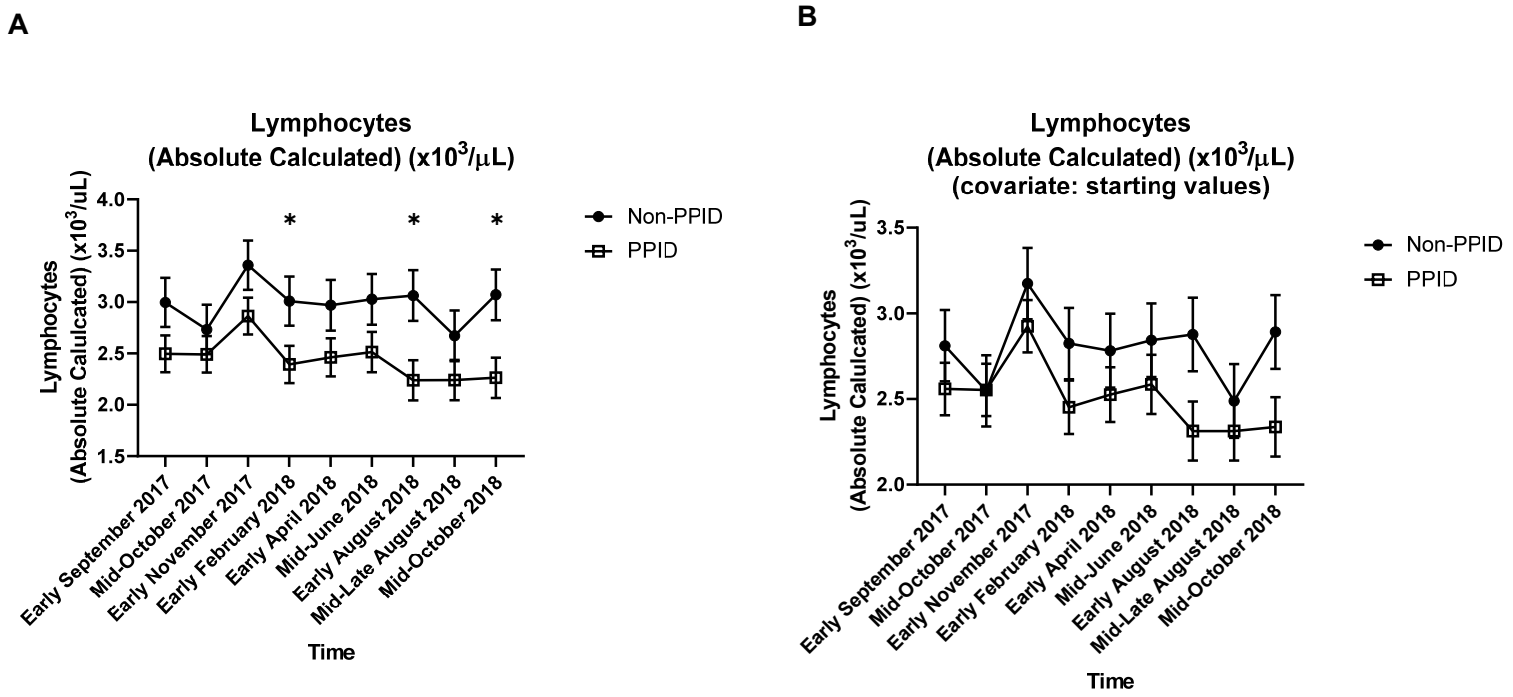

Figure 3.5. (A) Absolute lymphocyte counts (B) Absolute lymphocyte counts (starting values as a covariate in the model)

Mean +/- SEM. Non-PPID horses are represented by lines with closed circles. PPID horses are represented by lines with open squares. At Day 0 (Early September 2017), there were 10 horses in the Non-PPID group and 18 horses in the PPID group. By the end of the study, there were 9 horses in the Non-PPID group and 13 horses in the PPID group. * denotes a significant difference (P<.05) between Non-PPID and PPID groups. PPID-pituitary pars intermedia dysfunction.

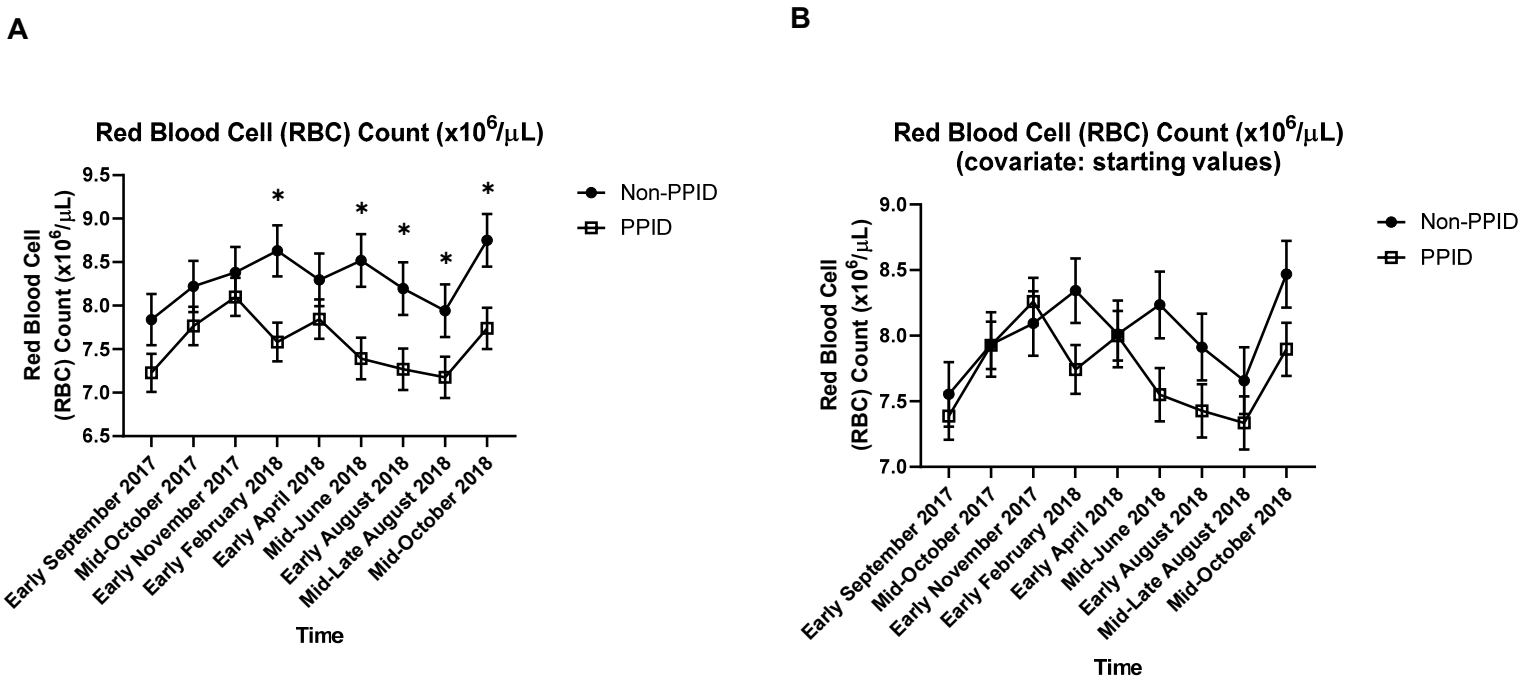

Figure 3.6. (A) Red blood cell counts (B) Red blood cell counts (starting values as covariate in the model)

Mean +/- SEM. Non-PPID horses are represented by lines with closed circles. PPID horses are represented by lines with open squares. At Day 0 (Early September 2017), there were 10 horses in the Non-PPID group and 18 horses in the PPID group. By the end of the study, there were 9 horses in the Non-PPID group and 13 horses in the PPID group. * denotes a significant difference ($P<.05$) between Non-PPID and PPID groups. PPID-pituitary pars intermedia dysfunction.

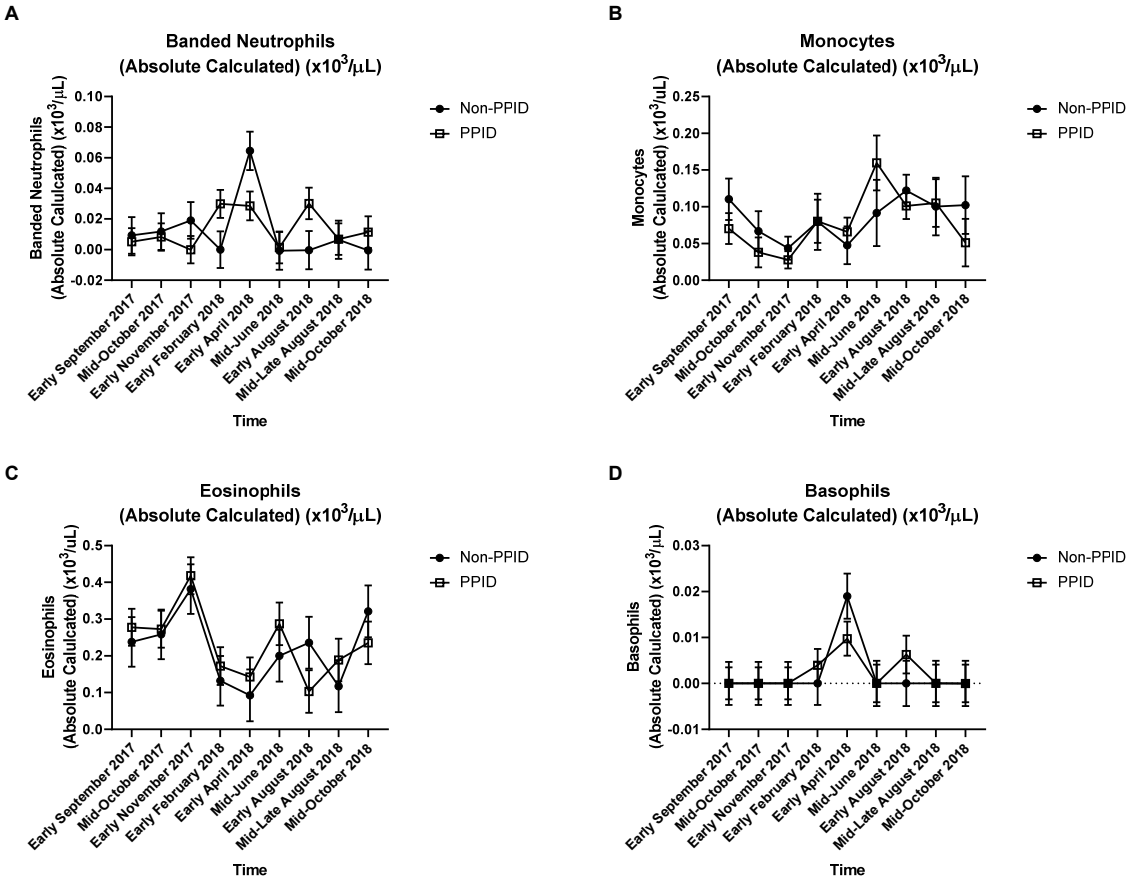

Figure 3.7. Significant time effects for (A) absolute banded neutrophils, (B) absolute monocytes, (C) absolute eosinophils, and (D) absolute basophils.

Mean +/- SEM. Non-PPID horses are represented by lines with closed circles. PPID horses are represented by lines with open squares. At Day 0 (Early September 2017), there were 10 horses in the Non-PPID group and 18 horses in the PPID group. By the end of the study, there were 9 horses in the Non-PPID group and 13 horses in the PPID group. These graphs show only the significant time effects ($P<.05$) for the respective analyses. PPID-pituitary pars intermedia dysfunction.

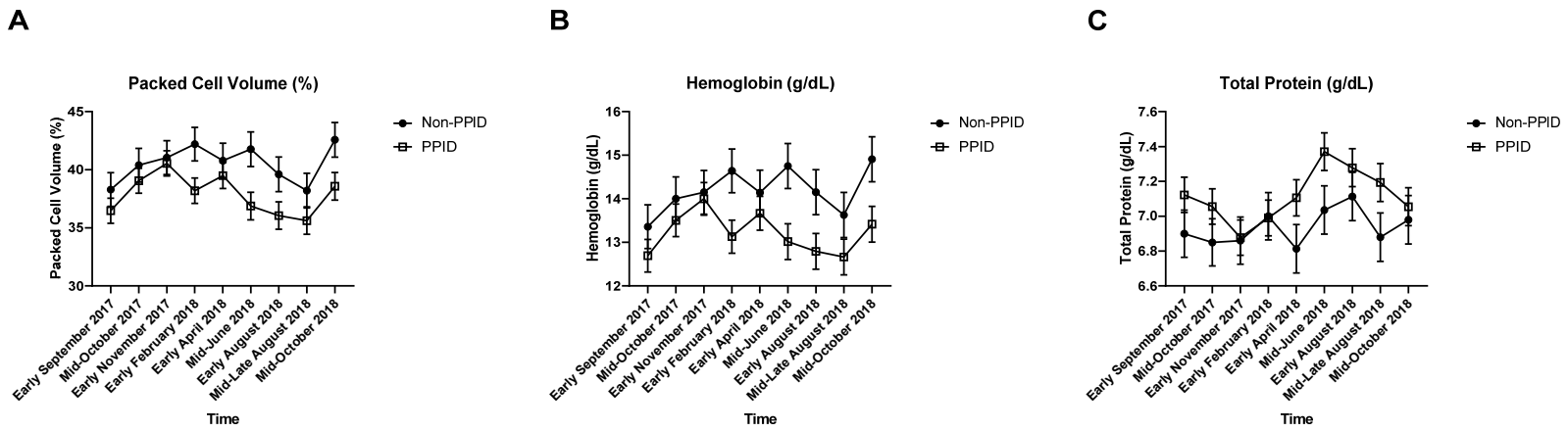

Figure 3.8. Significant time effects for (A) packed cell volume, (B) hemoglobin, and (C) total protein

Mean +/- SEM. Non-PPID horses are represented by lines with closed circles. PPID horses are represented by lines with open squares. At Day 0 (Early September 2017), there were 10 horses in the Non-PPID group and 18 horses in the PPID group. By the end of the study, there were 9 horses in the Non-PPID group and 13 horses in the PPID group. These graphs show only the significant time effects (P<.05) for the respective analyses. PPID-pituitary pars intermedia dysfunction.

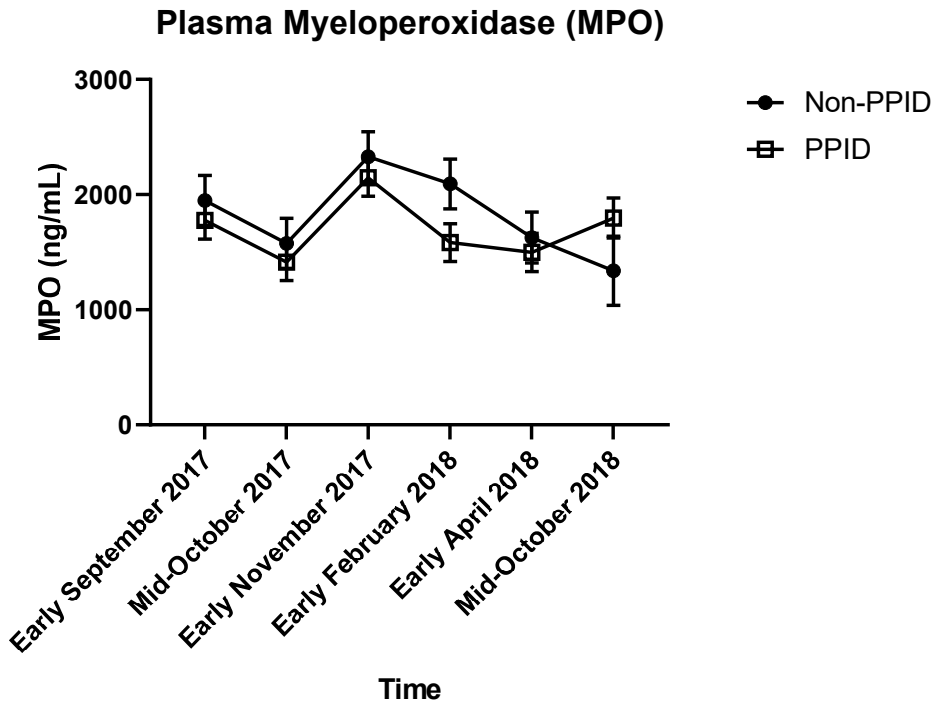

Figure 3.9. Plasma myeloperoxidase concentration

Mean +/- SEM. Non-PPID horses are represented by lines with closed circles. PPID horses are represented by lines with open squares. At Day 0 (Early September 2017), there were 10 horses in the Non-PPID group and 18 horses in the PPID group. By the end of the study, there were 9 horses in the Non-PPID group and 13 horses in the PPID group. PPID-pituitary pars intermedia dysfunction.

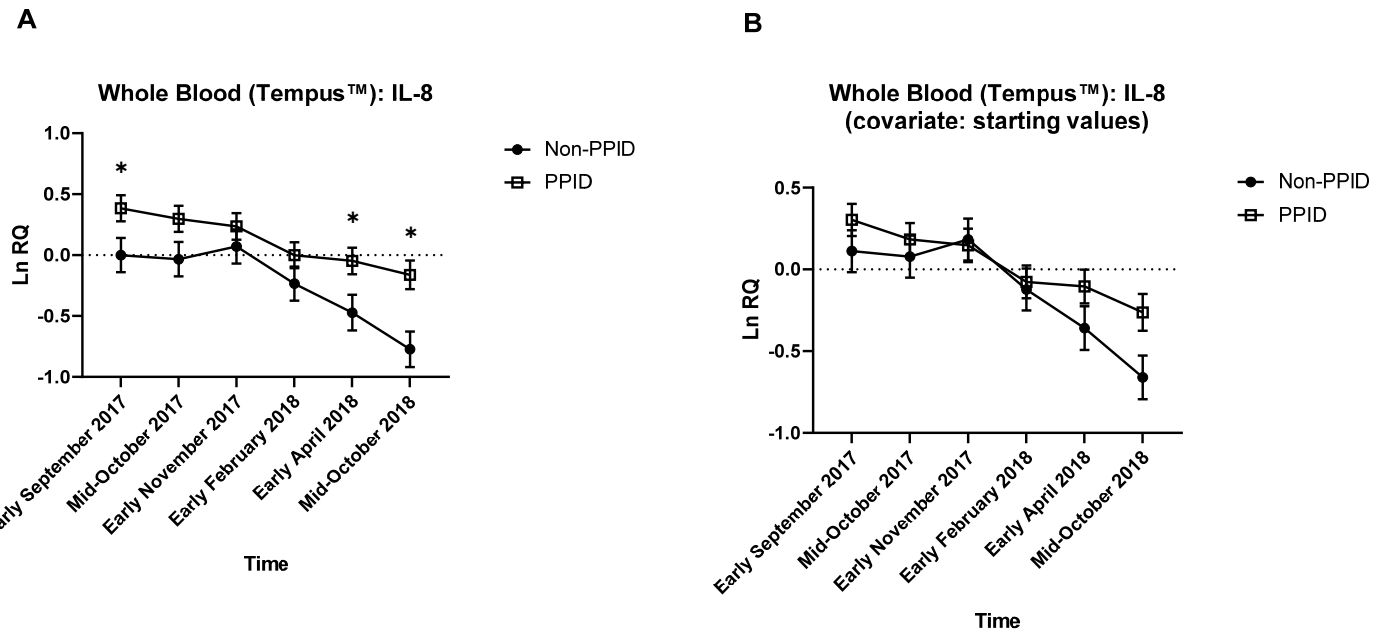

Figure 3.10. (A) Whole blood (Tempus™) IL-8 expression (B) Whole blood (Tempus™) IL-8 expression (starting values as covariate in the model)

Mean +/- SEM. RQs are natural log-transformed (Ln). Non-PPID horses are represented by lines with closed circles. PPID horses are represented by lines with open squares. At Day 0 (Early September 2017), there were 10 horses in the Non-PPID group and 18 horses in the PPID group. By the end of the study, there were 9 horses in the Non-PPID group and 13 horses in the PPID group. * denotes a significant difference ($P<.05$) between Non-PPID and PPID groups. PPID-pituitary pars intermedia dysfunction; IL-interleukin; RQ-relative quantity.

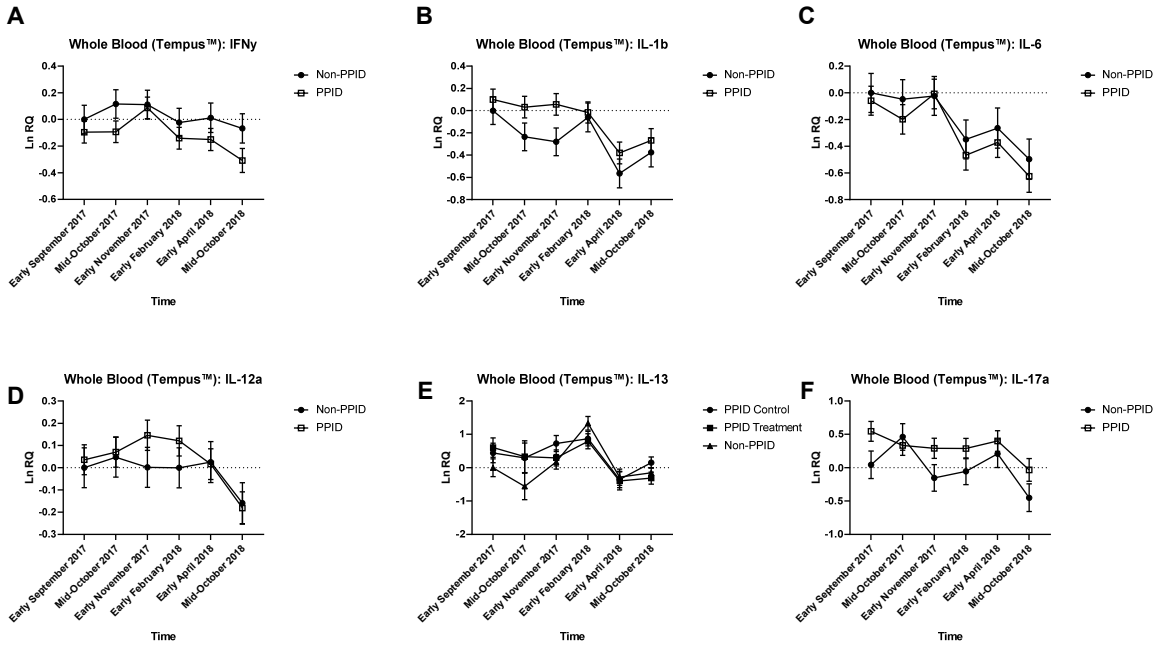

Figure 3.11. Significant time effects in whole blood (Tempus™) cytokine expression for (A) IFNγ, (B) IL-1β, (C) IL-6, (D) IL-12α, (E) IL-13, and (F) IL-17α

Mean +/- SEM. (A-D, F) Non-PPID horses are represented by lines with closed circles. PPID horses are represented by lines with open squares. At Day 0 (Early September 2017), there were 10 horses in the Non-PPID group and 18 horses in the PPID group. By the end of the study, there were 9 horses in the Non-PPID group and 13 horses in the PPID group. (E) Non-PPID horses are represented by lines with closed circles. PPID Treatment horses are represented by lines with closed triangles. PPID Control horses are represented by lines with closed squares. At Day 0 (Early September 2017), there were 10, 9, and 9 horses in the Non-PPID, PPID Control, and PPID Treatment groups, respectively. By the end of the study, there were 9, 7, and 6 horses in the Non-PPID, PPID Control, and PPID

Treatment groups, respectively. These graphs show only the significant time effects (P<.05) for the respective analyses. PPID-pituitary pars intermedia dysfunction; IFNγ-interferon gamma; IL-interleukin; RQ-relative quantity.

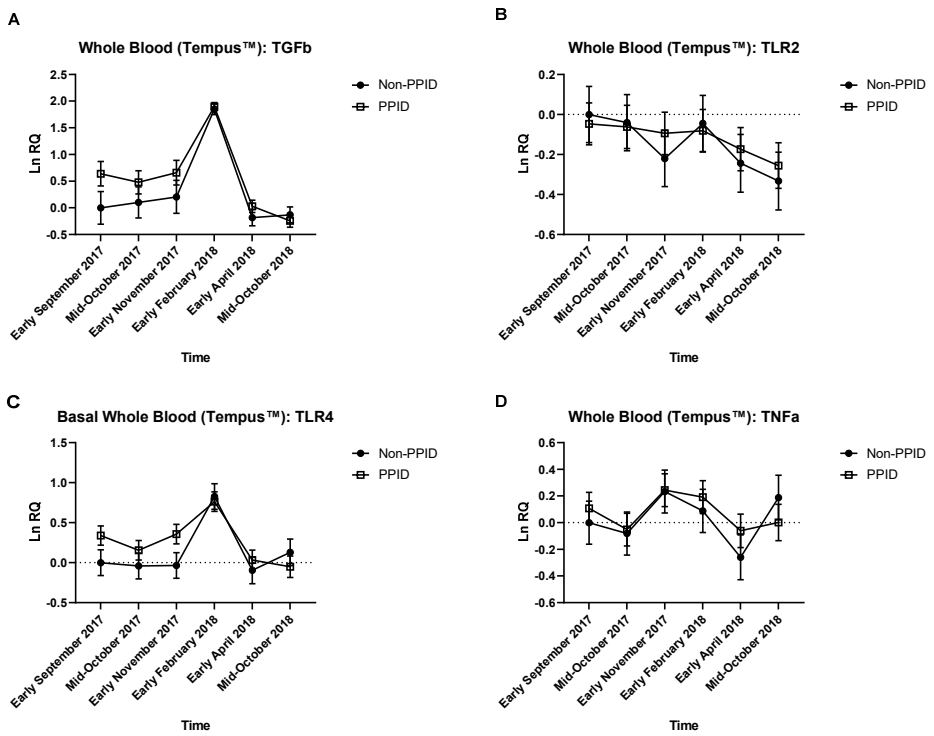

Figure 3.12. Significant time effects in whole blood (Tempus™) cytokine and receptor expression for (A) TGFβ, (B) TLR2, (C) TLR4, (D) TNFα

Mean +/- SEM. Non-PPID horses are represented by lines with closed circles. PPID horses are represented by lines with open squares. At Day 0 (Early September 2017), there were 10 horses in the Non-PPID group and 18 horses in the PPID group. By the end of the study, there were 9 horses in the Non-PPID group and 13 horses in the PPID group. These graphs show only the significant time effects (P<.05) for the respective analyses. PPID-pituitary pars intermedia dysfunction.; TGFβ-transforming growth factor beta; TLR-toll-like receptor; TNFα-tumor necrosis factor alpha; RQ-relative quantity.

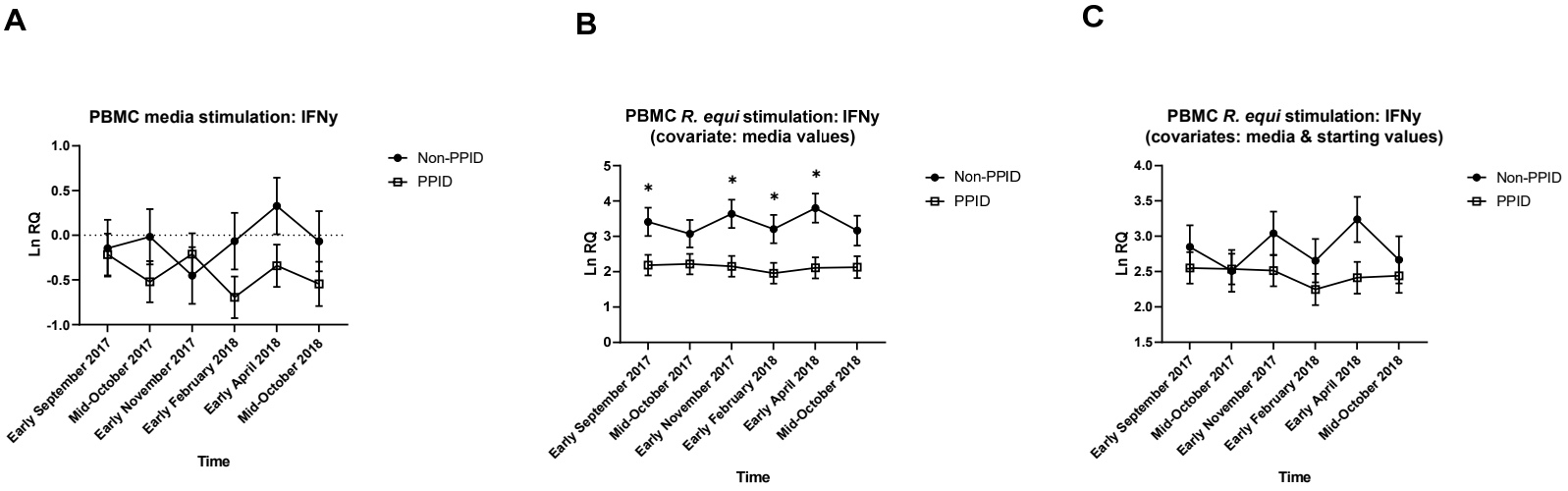

Figure 3.13. (A) Unstimulated peripheral blood mononuclear cell (PBMC) IFNγ expression (B) PBMC IFNγ expression after heat-inactivated *R. equi*-stimulation and with media as a covariate in the model. (C) PBMC IFNγ expression after heat-inactivated *R. equi*-stimulation and with media and starting values as a covariate in the model

Mean +/- SEM. RQs are natural log-transformed (Ln). Non-PPID horses are represented by lines with closed circles. PPID horses are represented by lines with open squares. At Day 0 (Early September 2017), there were 10 horses in the Non-PPID group and 18 horses in the PPID group. By the end of the study, there were 9 horses in the Non-PPID group and 13 horses in the PPID group. * denotes a significant difference ($P<.05$) between Non-PPID and PPID groups. PPID-pituitary pars intermedia dysfunction; IFNγ-interferon gamma; *R. equi-Rhodococcus equi*; RQ-relative quantity.

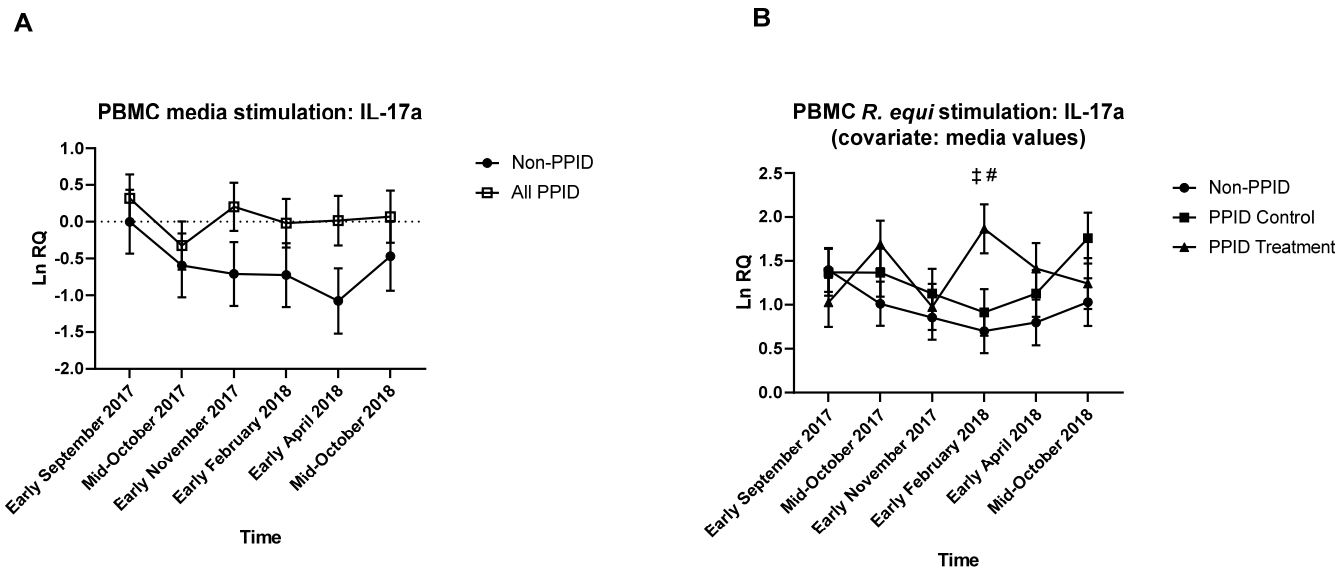

Figure 3.14. (A) Unstimulated peripheral blood mononuclear cell (PBMC) IL-17α expression (B) PBMC IL-17α expression after heat-inactivated *R. equi*-stimulation and with media as a covariate in the model

Mean +/- SEM. RQs are natural log-transformed (Ln). (A) Non-PPID horses are represented by lines with closed circles. PPID horses are represented by lines with open squares. At Day 0 (Early September 2017), there were 10 horses in the Non-PPID group and 18 horses in the PPID group. By the end of the study, there were 9 horses in the Non-PPID group and 13 horses in the PPID group. (B) Non-PPID horses are represented by lines with closed circles. PPID Treatment horses are represented by lines with closed triangles. PPID Control horses are represented by lines with closed squares. At Day 0 (Early September 2017), there were 10, 9, and 9 horses in the Non-PPID, PPID Control, and PPID Treatment groups, respectively. By the end of the study, there were 9, 7, and 6 horses in the Non-PPID, PPID Control, and PPID Treatment groups, respectively. $^{\mu}$ denotes a significant difference ($P<.05$) between Non-PPID and PPID Treatment. $^{\#}$ denotes a significant difference ($P<.05$) between PPID Treatment and PPID Control groups. PPID-pituitary pars intermedia dysfunction; IL-interleukin; *R. equi-Rhodococcus equi*; RQ- relative quantity

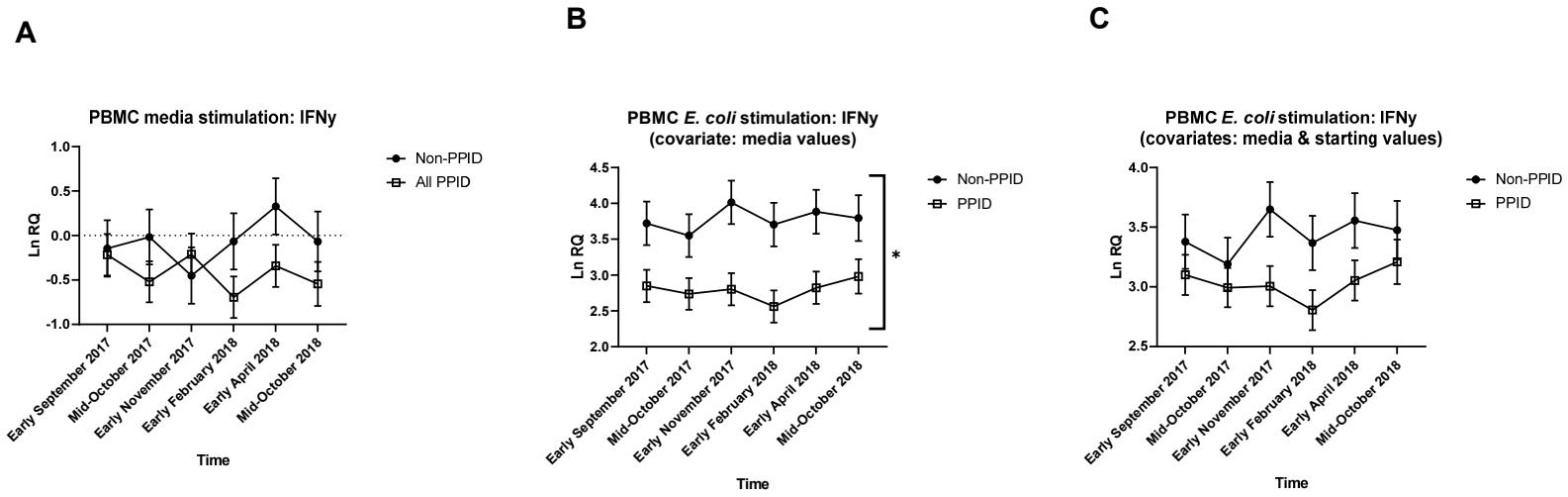

Figure 3.15. (A) Unstimulated peripheral blood mononuclear cell (PBMC) IFNγ expression (B) PBMC IFNγ expression after heat-inactivated *E. coli*-stimulation and with media as a covariate in the model. (C) PBMC IFNγ expression after heat-inactivated *E. coli*-stimulation and with media and starting values as a covariate in the model

Mean +/- SEM. RQs are natural log-transformed (Ln). Non-PPID horses are represented by lines with closed circles. PPID horses are represented by lines with open squares. At Day 0 (Early September 2017), there were 10 horses in the Non-PPID group and 18 horses in the PPID group. By the end of the study, there were 9 horses in the Non-PPID group and 13 horses in the PPID group. * denotes a significant difference ($P<.05$) between Non-PPID and PPID groups. PPID-pituitary pars intermedia dysfunction; IFNγ-interferon gamma; *E. coli-Escherichia coli*; RQ-relative quantity.

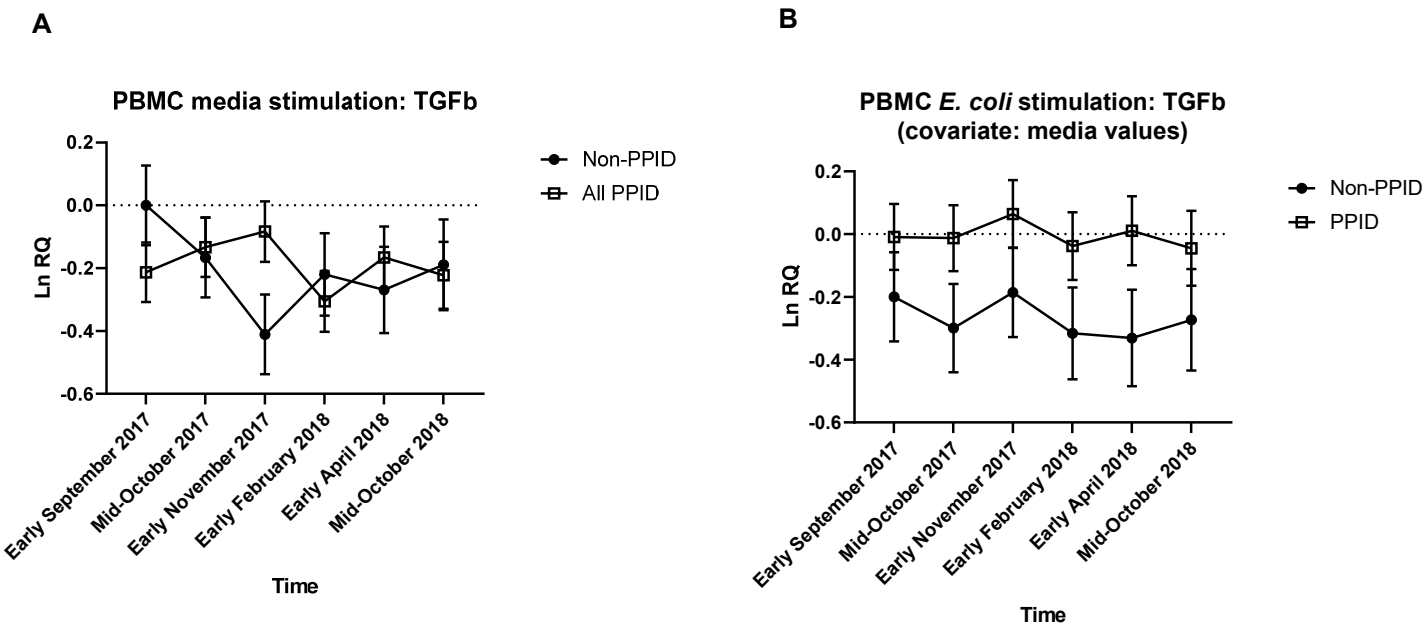

Figure 3.16. (A) Unstimulated peripheral blood mononuclear cell (PBMC) TGFβ expression (B) PBMC TGFβ expression after heat-inactivated *E. coli*-stimulation and with media as a covariate in the model

Mean +/- SEM. RQs are natural log-transformed (Ln). Non-PPID horses are represented by lines with closed circles. PPID horses are represented by lines with open squares. At Day 0 (Early September 2017), there were 10 horses in the Non-PPID group and 18 horses in the PPID group. By the end of the study, there were 9 horses in the Non-PPID group and 13 horses in the PPID group. PPID-pituitary pars intermedia dysfunction; TGFβ-transforming growth factor beta; *E. coli*-*Escherichia coli*; RQ-relative quantity.

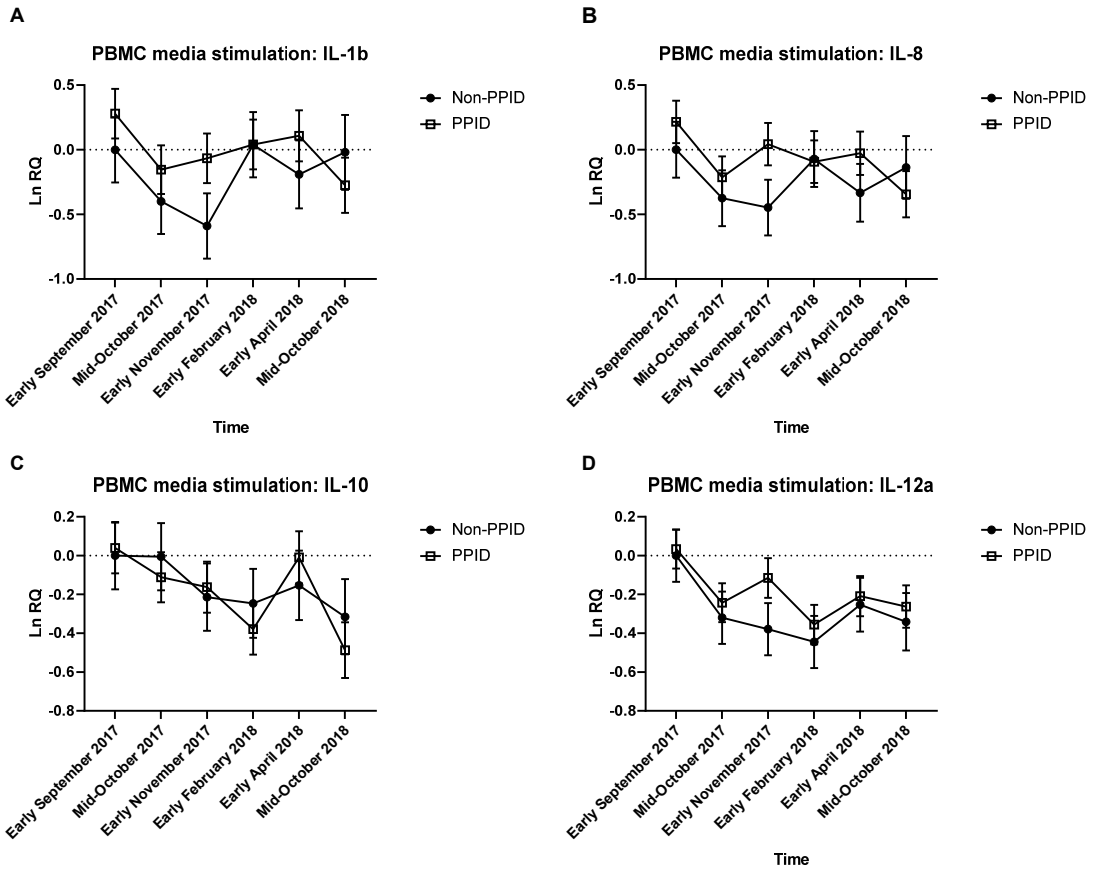

Figure 3.17. Significant time effects in unstimulated peripheral blood mononuclear cell (PBMC) cytokine expression for (A) IL-1β, (B) IL-8, (C) IL-10, (D) IL-12α

Mean +/- SEM. Non-PPID horses are represented by lines with closed circles. PPID horses are represented by lines with open squares. At Day 0 (Early September 2017), there were 10 horses in the Non-PPID group and 18 horses in the PPID group. By the end of the study, there were 9 horses in the Non-PPID group and 13 horses in the PPID group. These graphs show only the significant time effects (P<.05) for the respective analyses. PPID-pituitary pars intermedia dysfunction; IL-interleukin; RQ-relative quantity

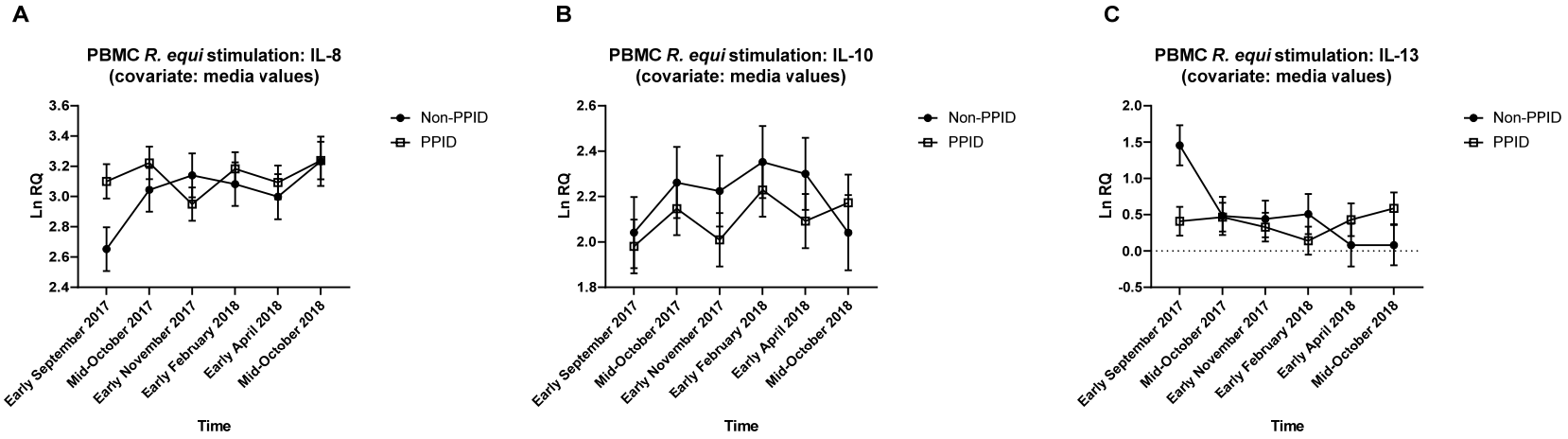

Figure 3.18. Significant time effects in *R. equi*-stimulated peripheral blood mononuclear cell (PBMC) cytokine expression with media as a covariate in the model for (A) IL-8, (B) IL-10, (C) IL-13.

Mean +/- SEM. Non-PPID horses are represented by lines with closed circles. PPID horses are represented by lines with open squares. At Day 0 (Early September 2017), there were 10 horses in the Non-PPID group and 18 horses in the PPID group. By the end of the study, there were 9 horses in the Non-PPID group and 13 horses in the PPID group. These graphs show only the significant time effects ($P<.05$) for the respective analyses. PPID-pituitary pars intermedia dysfunction; *R. equi-Rhodococcus equi*; IL-interleukin; RQ-relative quantity.

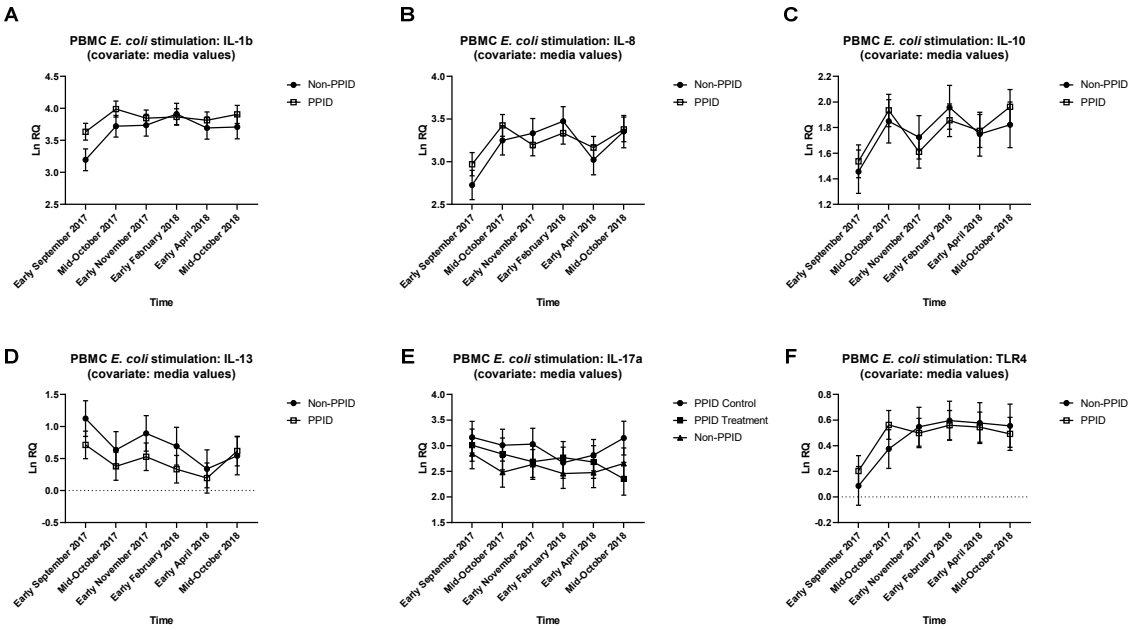

Figure 3.19. Significant time effects in *E. coli*-stimulated peripheral blood mononuclear cell (PBMC) cytokine expression with media as a covariate in the model for (A) IL-1β, (B) IL-8, (C) IL-10, (D) IL-13, (E) IL-17α, and (F) TLR4.

Mean +/- SEM. (A-D, F) Non-PPID horses are represented by lines with closed circles. PPID horses are represented by lines with open squares. At Day 0 (Early September 2017), there were 10 horses in the Non-PPID group and 18 horses in the PPID group. By the end of the study, there were 9 horses in the Non-PPID group and 13 horses in the PPID group. (E) Non-PPID horses are represented by lines with closed circles. PPID Treatment horses are represented by lines with closed triangles. PPID Control horses are represented by lines with closed squares. At Day 0 (Early September 2017), there were 10, 9, and 9 horses in the Non-PPID, PPID Control, and PPID Treatment groups, respectively. By the end of the study, there were 9, 7, and 6 horses in the Non-PPID, PPID Control, and PPID Treatment groups, respectively. These graphs show only the significant time effects ($P<.05$) for the respective analyses. PPID-pituitary pars intermedia dysfunction; *E. coli-Escherichia coli*; IL-interleukin; TLR-toll-like receptor; RQ-relative quantity.

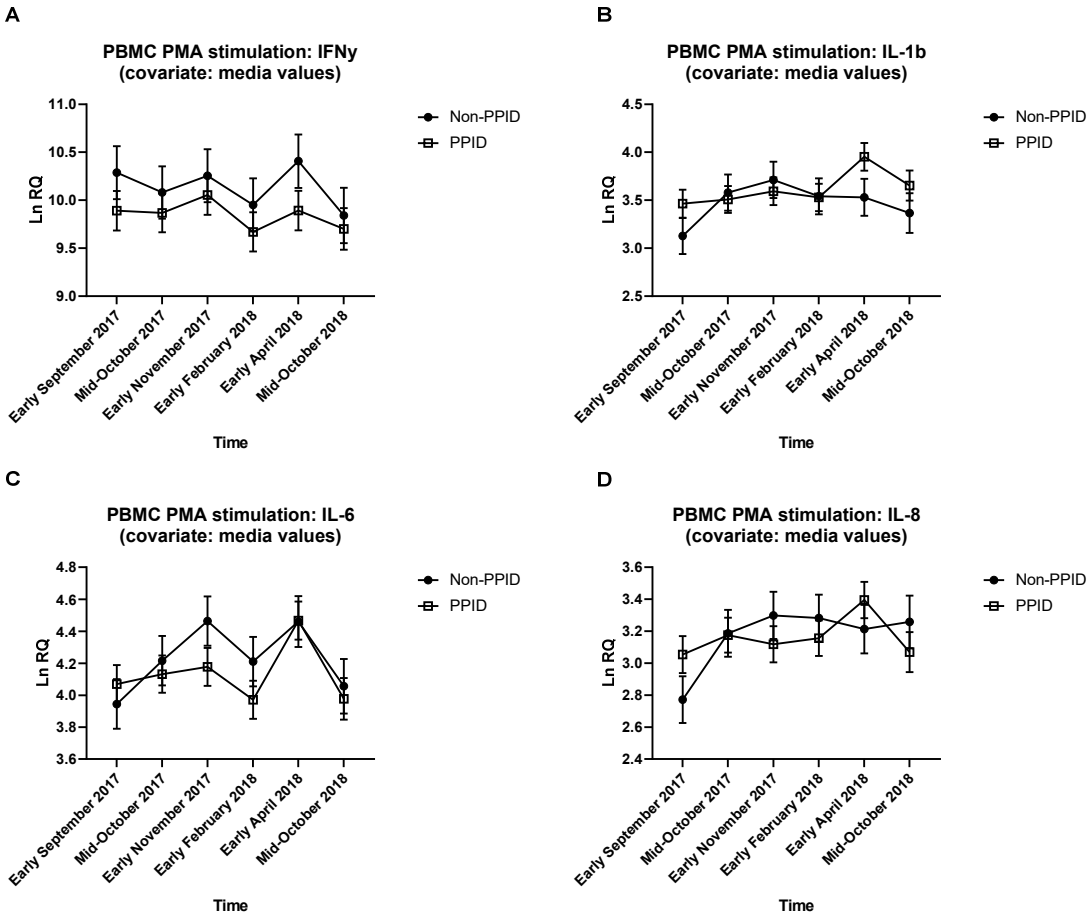

Figure 3.20. Significant time effects in PMA/ionomycin-stimulated peripheral blood mononuclear cell (PBMC) cytokine expression for (A) IFNγ, (B) IL-1β, (C) IL-6, and (D) IL-8

Mean +/- SEM. Non-PPID horses are represented by lines with closed circles. PPID horses are represented by lines with open squares. At Day 0 (Early September 2017), there were 10 horses in the Non-PPID group and 18 horses in the PPID group. By the end of the study, there were 9 horses in the Non-PPID group and 13 horses in the PPID group. These graphs show only the significant time effects (P<.05) for the respective analyses. PPID-pituitary pars intermedia dysfunction; IFNγ-interferon gamma; IL-interleukin; RQ-relative quantity.

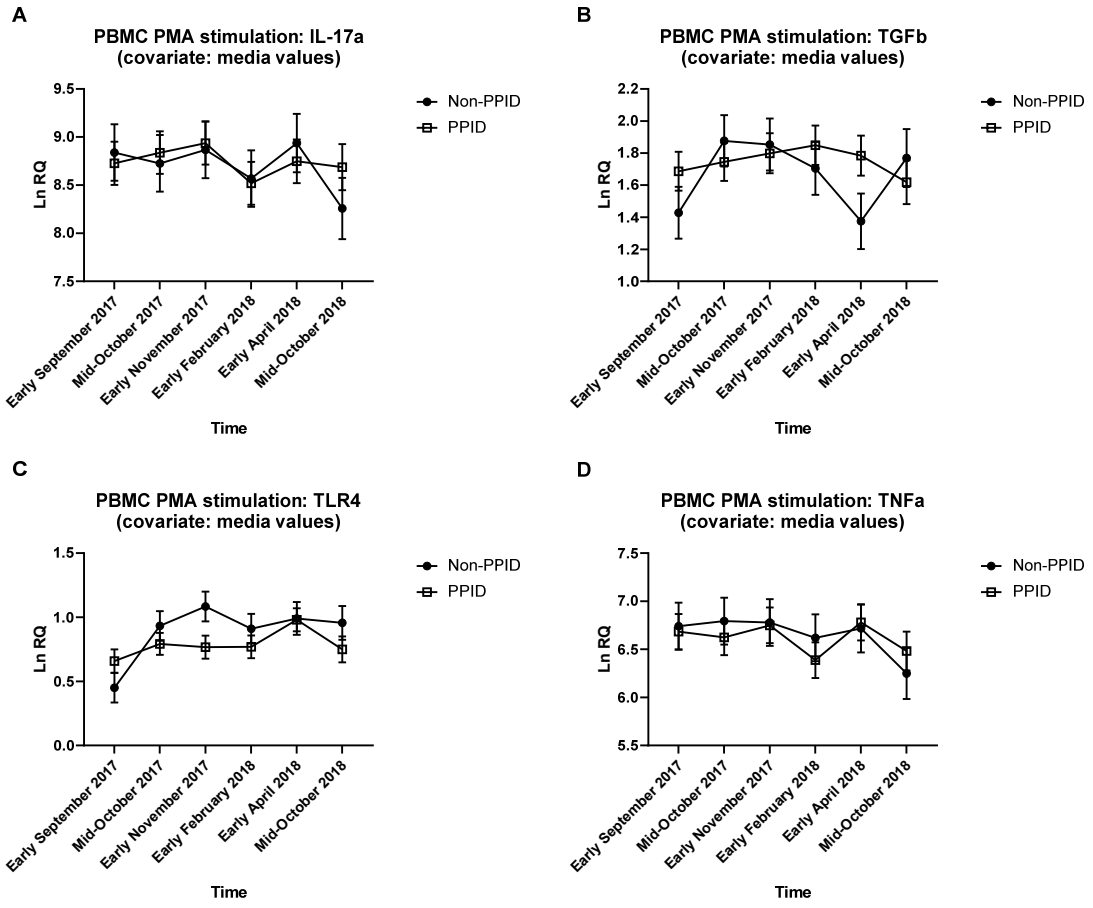

Figure 3.21. Significant time effects in PMA/ionomycin-stimulated peripheral blood mononuclear cell (PBMC) cytokine expression for (A) IL-17α, (B) TGFβ, (C) TLR4, and (D) TNFα

Mean +/- SEM. Non-PPID horses are represented by lines with closed circles. PPID horses are represented by lines with open squares. At Day 0 (Early September 2017), there were 10 horses in the Non-PPID group and 18 horses in the PPID group. By the end of the study, there were 9 horses in the Non-PPID group and 13 horses in the PPID group. These graphs show only the significant time effects ($P<.05$) for the respective analyses. PPID-pituitary pars intermedia dysfunction; IL-interleukin; TGFβ-transforming growth factor beta; TLR-toll-like receptor; TNFα-tumor necrosis factor alpha; RQ-relative quantity

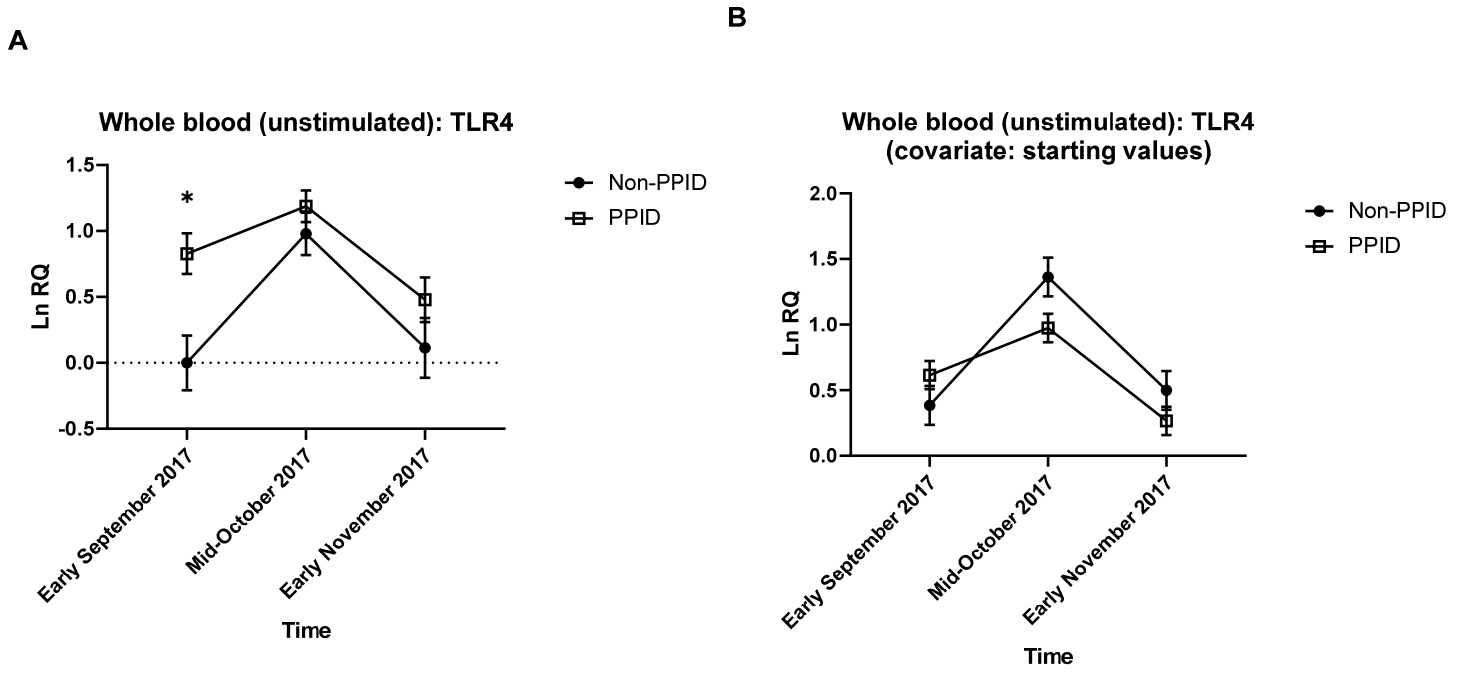

Figure 3.22. (A) Unstimulated whole blood TLR4 expression (B) Unstimulated whole blood TLR4 expression with starting values as a covariate in the model

Mean +/- SEM. RQs are natural log-transformed (Ln). Non-PPID horses are represented by lines with closed circles. PPID horses are represented by lines with open squares. At Day 0 (Early September 2017), there were 10 horses in the Non-PPID group and 18 horses in the PPID group. By the end of the study, there were 9 horses in the Non-PPID group and 13 horses in the PPID group. * denotes a significant difference ($P<.05$) between Non-PPID and PPID groups. PPID-pituitary pars intermedia dysfunction; TLR4-toll-like receptor 4; RQ-relative quantity.

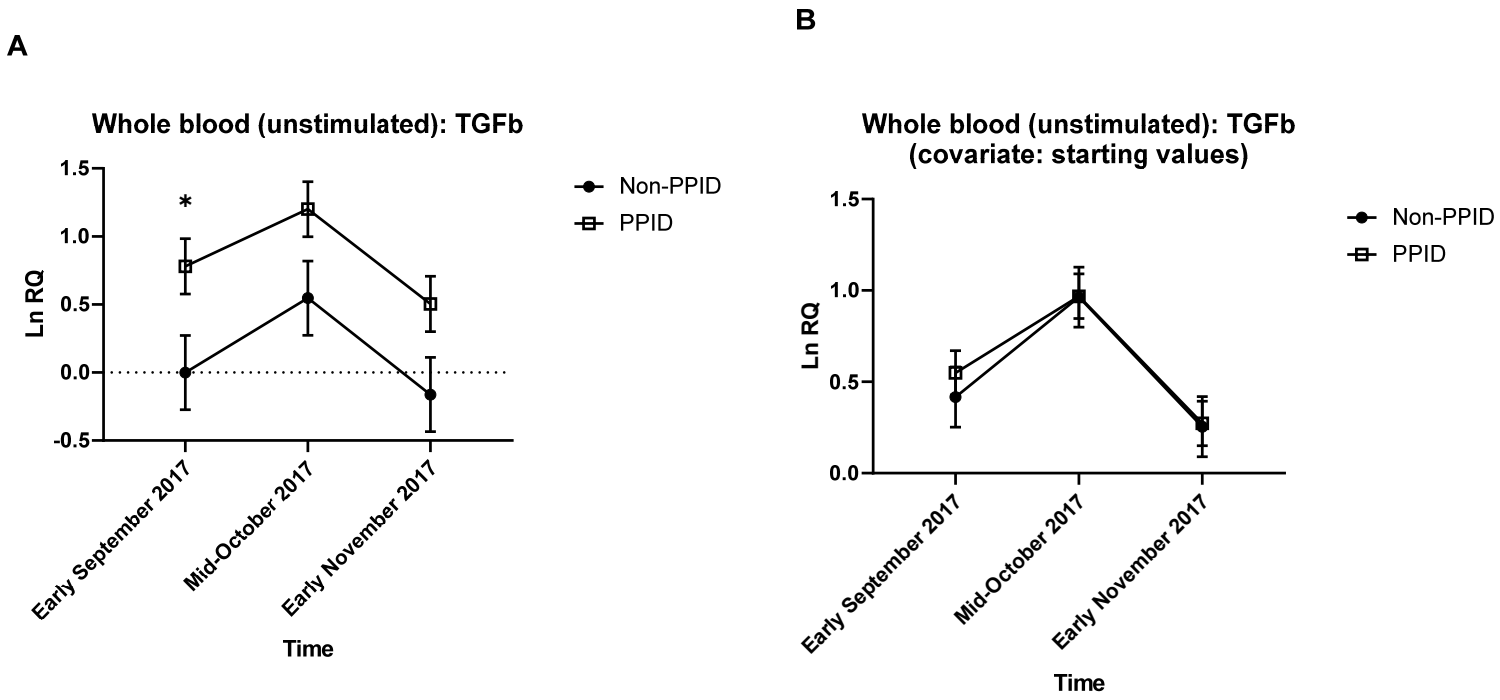

Figure 3.23. (A) Unstimulated whole blood TGFβ expression (B) Unstimulated whole blood TGFβ expression with starting values as a covariate in the model

Mean +/- SEM. RQs are natural log-transformed (Ln). Non-PPID horses are represented by lines with closed circles. PPID horses are represented by lines with open squares. At Day 0 (Early September 2017), there were 10 horses in the Non-PPID group and 18 horses in the PPID group. By the end of the study, there were 9 horses in the Non-PPID group and 13 horses in the PPID group. * denotes a significant difference ($P<.05$) between Non-PPID and PPID groups. PPID-pituitary pars intermedia dysfunction; TGFβ-transforming growth factor beta; RQ-relative quantity.

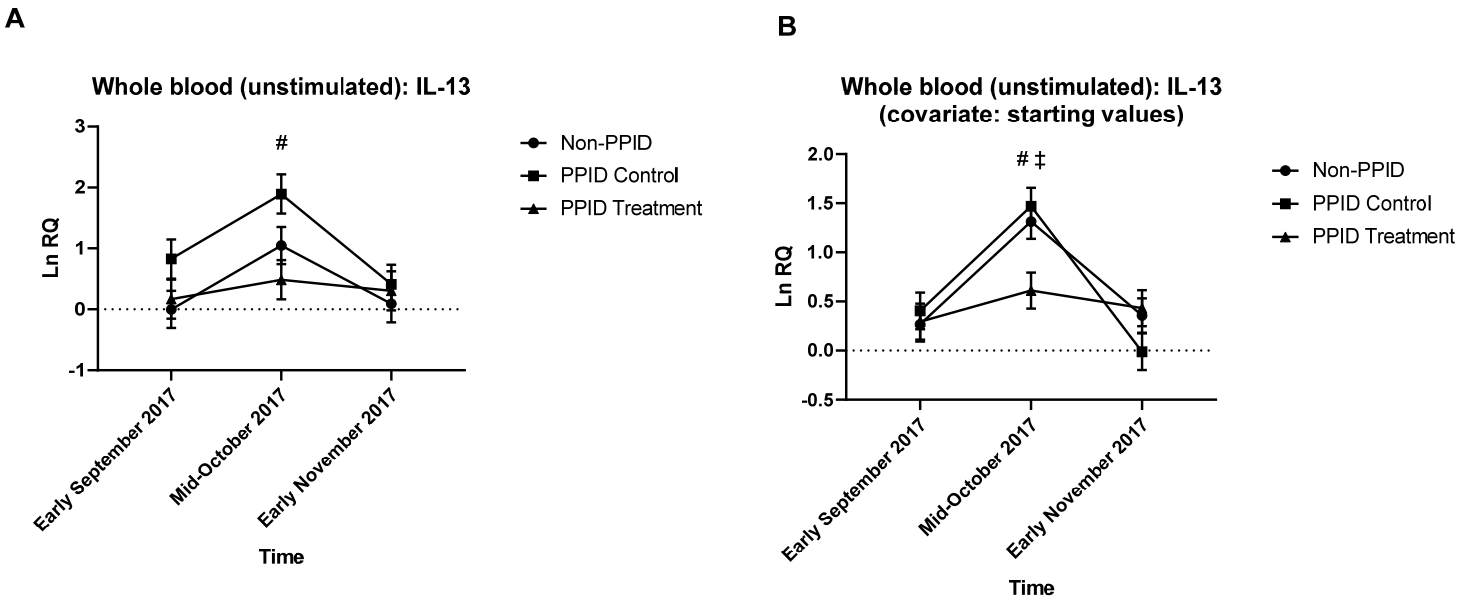

Figure 3.24. (A) Unstimulated whole blood IL-13 expression (B) Unstimulated whole blood IL-13 expression with starting values as a covariate in the model

Mean +/- SEM. RQs are natural log-transformed (Ln). Non-PPID horses are represented by lines with closed circles. PPID Treatment horses are represented by lines with closed triangles. PPID Control horses are represented by lines with closed squares. At Day 0 (Early September 2017), there were 10, 9, and 9 horses in the Non-PPID, PPID Control, and PPID Treatment groups, respectively. By the end of the study, there were 9, 7, and 6 horses in the Non-PPID, PPID Control, and PPID Treatment groups, respectively. ‡ denotes a significant difference (P<.05) between Non-PPID and PPID Treatment. # denotes a significant difference (P<.05) between PPID Treatment and PPID Control groups. PPID-pituitary pars intermedia dysfunction; IL-interleukin; RQ-relative quantity.

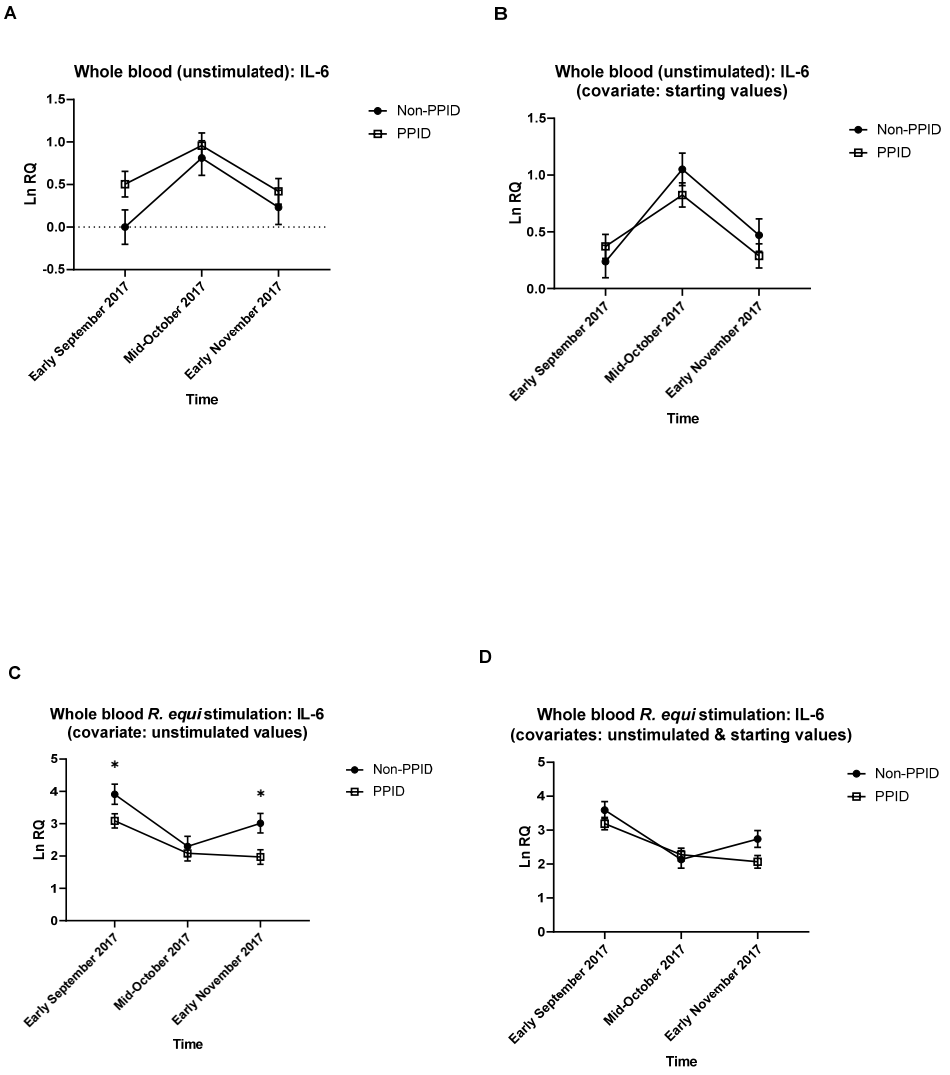

Figure 3.25. (A) Unstimulated whole blood IL-6 expression (B) Unstimulated whole blood IL-6 expression with starting values as a covariate in the model. (C) Heat-inactivated *R. equi*-stimulated whole blood IL-6 expression (D) Heat-inactivated *R. equi*-stimulated whole blood IL-6 expression with starting values as a covariate in the model

Mean +/- SEM. RQs are natural log-transformed (Ln). Non-PPID horses are represented by lines with closed circles. PPID horses are represented by lines with open squares. At Day 0 (Early September 2017), there were 10 horses in the Non-PPID group and 18 horses in the PPID group. By the end of the study, there were 9 horses in the Non-PPID group and 13 horses in the PPID group. * denotes a significant difference ($P<.05$) between Non-PPID and PPID groups. PPID-pituitary pars intermedia dysfunction; IL-interleukin; *R. equi*-*Rhodococcus equi*; RQ- relative quantity.

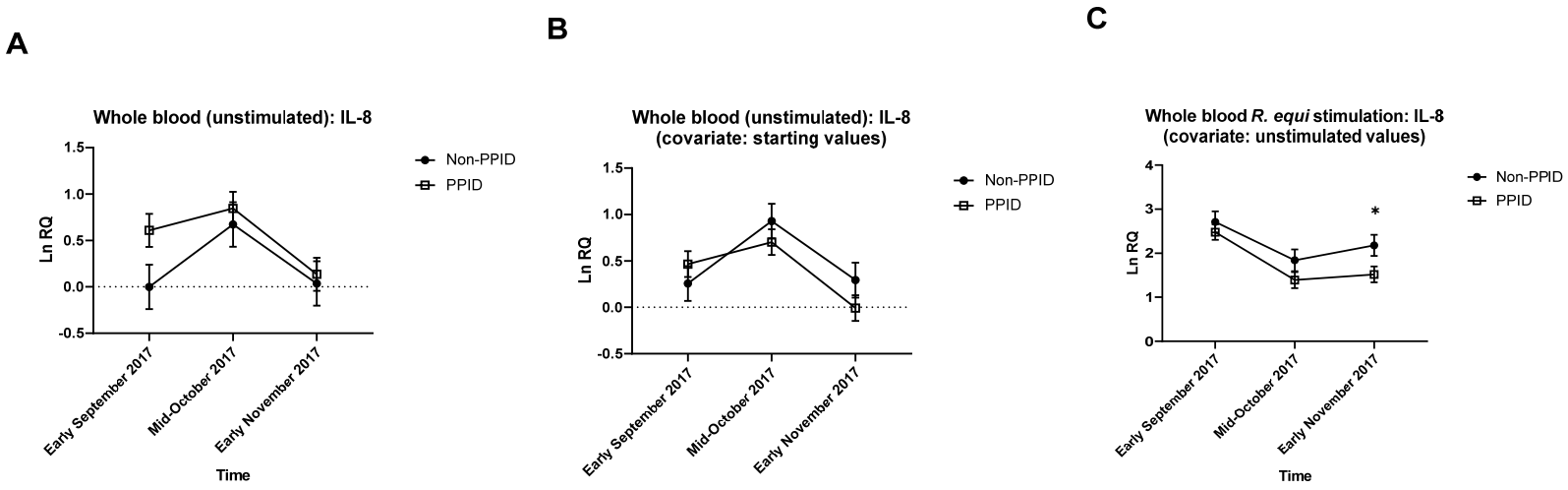

Figure 3.26. (A) Unstimulated whole blood IL-8 expression (B) Unstimulated whole blood IL-8 expression with starting values as a covariate in the model. (C) Heat-inactivated *R. equi*-stimulated whole blood IL-8 expression

Mean +/- SEM. RQs are natural log-transformed (Ln). Non-PPID horses are represented by lines with closed circles. PPID horses are represented by lines with open squares. At Day 0 (Early September 2017), there were 10 horses in the Non-PPID group and 18 horses in the PPID group. By the end of the study, there were 9 horses in the Non-PPID group and 13 horses in the PPID group. * denotes a significant difference ($P<.05$) between Non-PPID and PPID groups. PPID-pituitary pars intermedia dysfunction; IL-interleukin; *R. equi-Rhodococcus equi*; RQ- relative quantity.

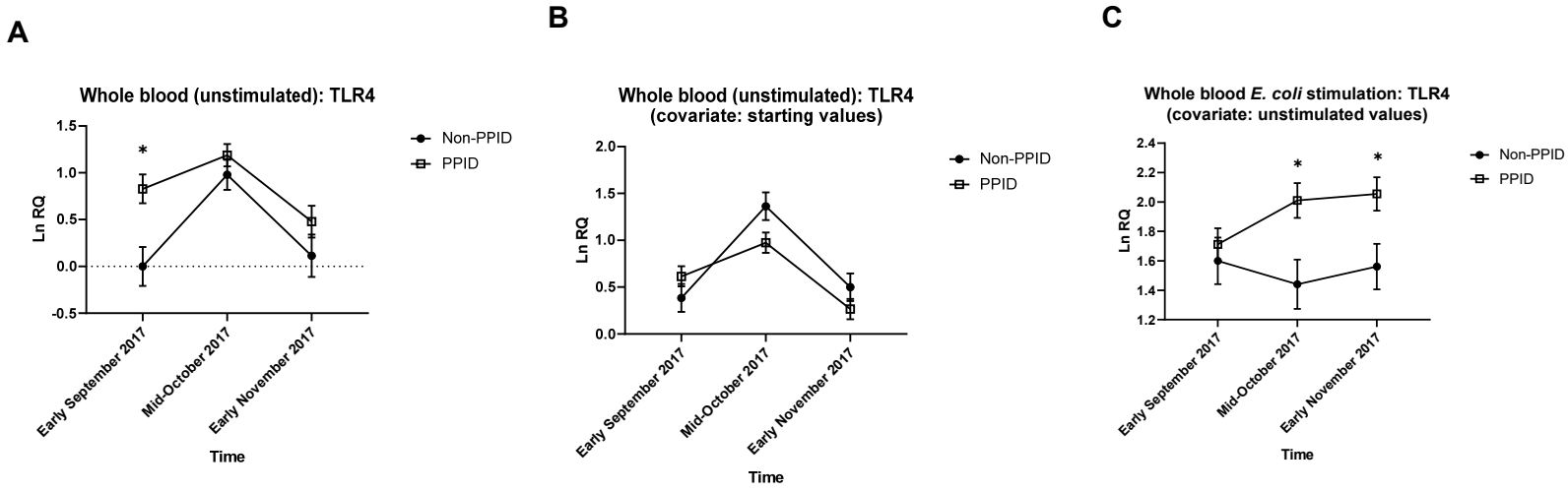

Figure 3.27. (A) Unstimulated whole blood TLR4 expression (B) Unstimulated whole blood TLR4 expression with starting values as a covariate in the model. (C) Heat-inactivated *E. coli*-stimulated whole blood TLR4 expression

Mean +/- SEM. RQs are natural log-transformed (Ln). Non-PPID horses are represented by lines with closed circles. PPID horses are represented by lines with open squares. At Day 0 (Early September 2017), there were 10 horses in the Non-PPID group and 18 horses in the PPID group. By the end of the study, there were 9 horses in the Non-PPID group and 13 horses in the PPID group. * denotes a significant difference ($P<.05$) between Non-PPID and PPID groups. PPID-pituitary pars intermedia dysfunction; TLR-toll-like receptor; *E. coli-Escherichia coli*; RQ-relative quantity.

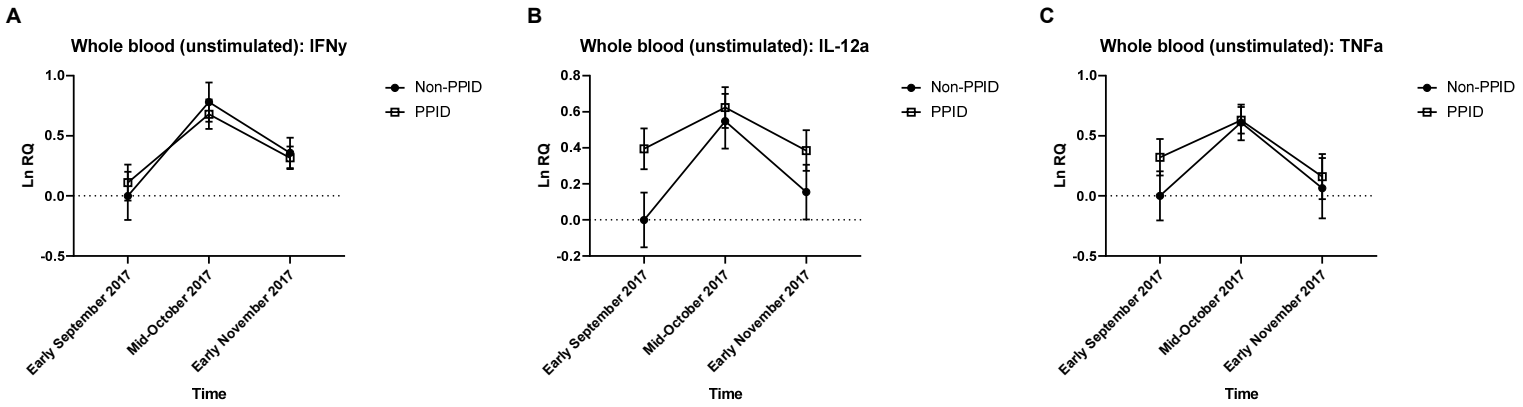

Figure 3.28. Significant time effects in unstimulated whole blood cytokine expression for (A) IFNγ, (B) IL-12α, and (C) TNFα

Mean +/- SEM. RQs are natural log-transformed (Ln). Non-PPID horses are represented by lines with closed circles. PPID horses are represented by lines with open squares. At Day 0 (Early September 2017), there were 10 horses in the Non-PPID group and 18 horses in the PPID group. By the end of the study, there were 9 horses in the Non-PPID group and 13 horses in the PPID group. These graphs show only the significant time effects ($P<.05$) for the respective analyses. PPID-pituitary pars intermedia dysfunction; IFNγ-interferon gamma; IL-interleukin, TNFα-tumor necrosis factor alpha; RQ-relative quantity.

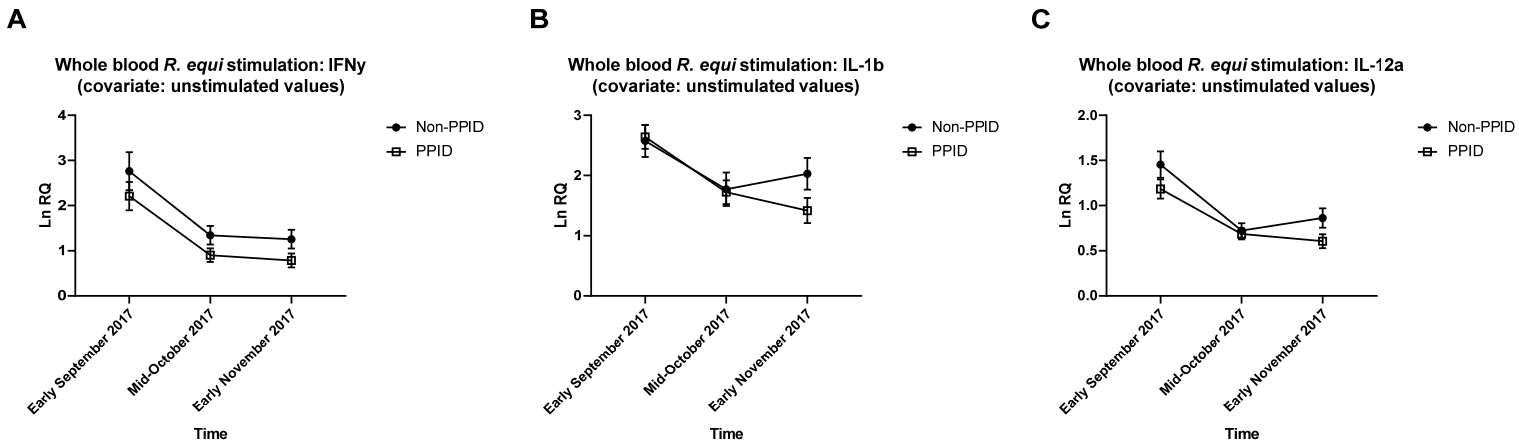

Figure 3.29. Significant time effects in *R-equi*-stimulated whole blood cytokine expression of (A) IFNγ, (B) IL-1β, and (C) IL-12α

Mean +/- SEM. RQs are natural log-transformed (Ln). Non-PPID horses are represented by lines with closed circles. PPID horses are represented by lines with open squares. At Day 0 (Early September 2017), there were 10 horses in the Non-PPID group and 18 horses in the PPID group. By the end of the study, there were 9 horses in the Non-PPID group and 13 horses in the PPID group. These graphs show only the significant time effects ($P<.05$) for the respective analyses. PPID-pituitary pars intermedia dysfunction; IFNγ-interferon gamma; IL-interleukin; *R. equi-Rhodococcus equi*; RQ-relative quantity.

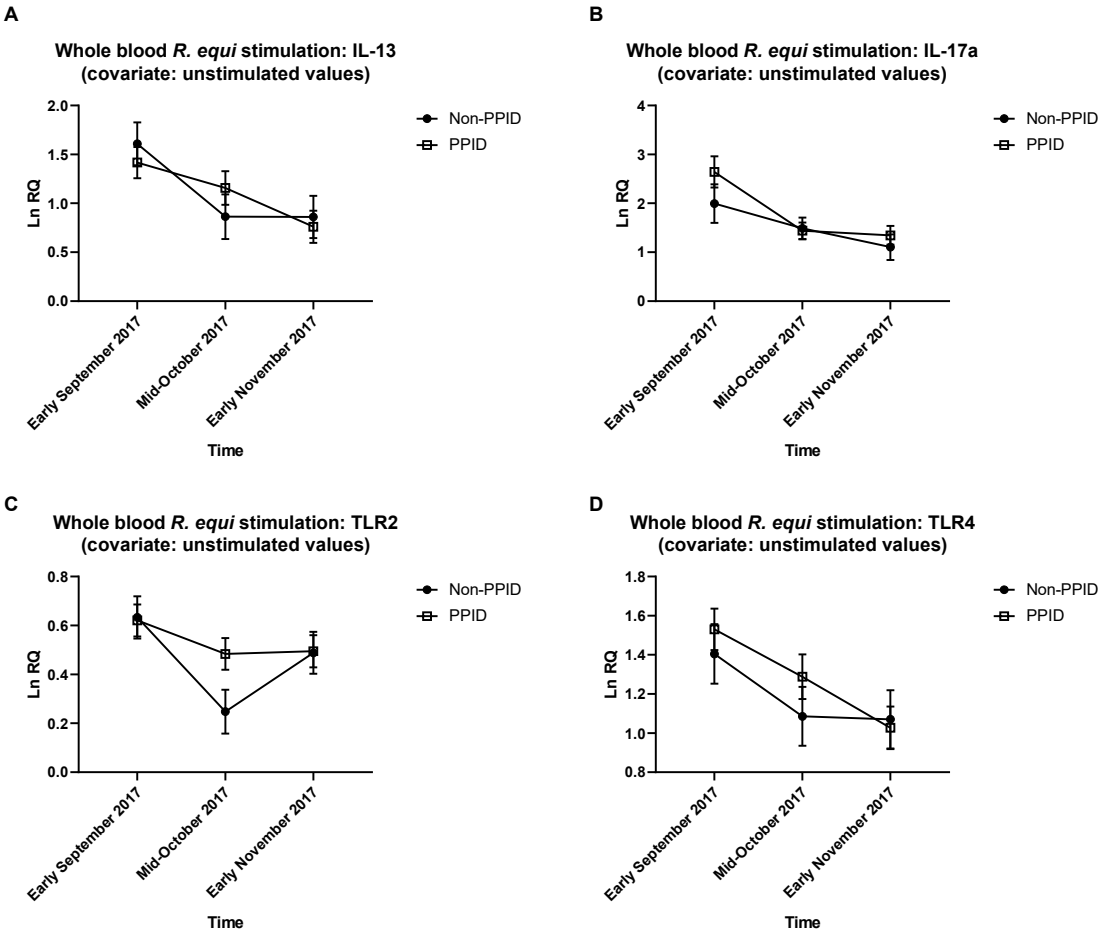

Figure 3.30. Significant time effects in *R-equi*-stimulated whole blood cytokine expression of (A) IL-13, (B) IL-17α, (C) TLR2, and (D) TLR4

Mean +/- SEM. Non-PPID horses are represented by lines with closed circles. PPID horses are represented by lines with open squares. At Day 0 (Early September 2017), there were 10 horses in the Non-PPID group and 18 horses in the PPID group. By the end of the study, there were 9 horses in the Non-PPID group and 13 horses in the PPID group. These graphs show only the significant time effects (P<.05) for the respective analyses. PPID-pituitary pars intermedia dysfunction; IL-interleukin; TLR-toll-like receptor; *R. equi-Rhodococcus equi;* RQ-relative quantity.

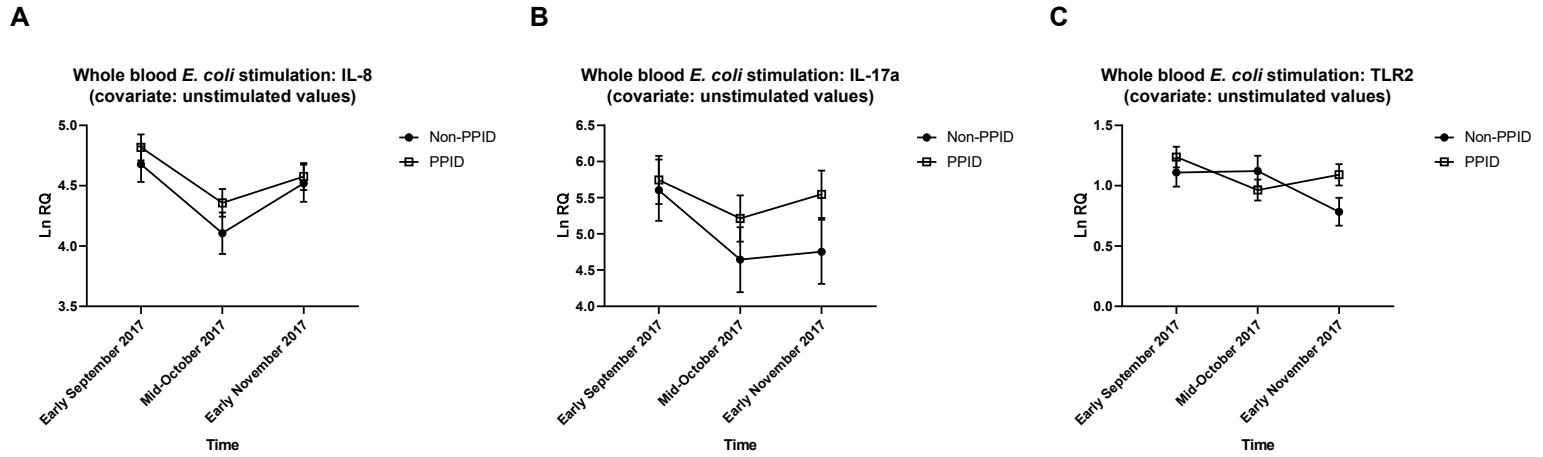

Figure 3.31. Significant time effects in *E. coli*-stimulated whole blood cytokine expression of (A) IL-8, (B) IL-17α, and (C) TLR2

Mean +/- SEM. RQs are natural log-transformed (Ln). Non-PPID horses are represented by lines with closed circles. PPID horses are represented by lines with open squares. At Day 0 (Early September 2017), there were 10 horses in the Non-PPID group and 18 horses in the PPID group. By the end of the study, there were 9 horses in the Non-PPID group and 13 horses in the PPID group. These graphs show only the significant time effects (P<.05) for the respective analyses. PPID-pituitary pars intermedia dysfunction; *E. coli-Escherichia coli*; IL-interleukin, TLR-toll-like receptor; RQ-relative quantity.

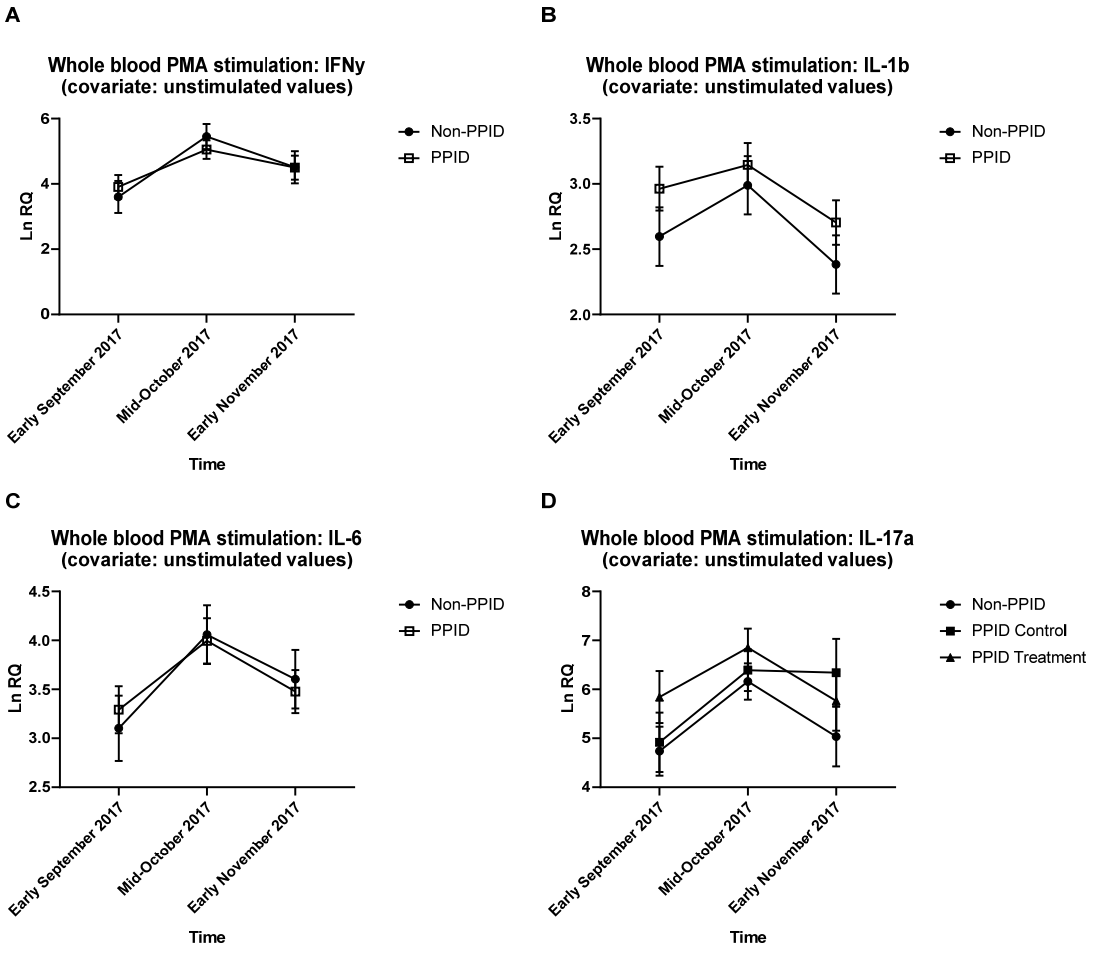

Figure 3.32. Significant time effects in PMA/ionomycin-stimulated whole blood cytokine expression of (A) IFNγ, (B) IL-1β, (C) IL-6, and (D) IL-17α

Mean +/- SEM. (A-C) Non-PPID horses are represented by lines with closed circles. PPID horses are represented by lines with open squares. At Day 0 (Early September 2017), there were 10 horses in the Non-PPID group and 18 horses in the PPID group. By the end of the study, there were 9 horses in the Non-PPID group and 13 horses in the PPID group. (D) Non-PPID horses are represented by lines with closed circles. PPID Treatment horses are represented by lines with closed triangles. PPID Control horses are represented by lines with closed squares. At Day 0 (Early September 2017), there were 10, 9, and 9 horses in the Non-PPID, PPID Control, and PPID Treatment groups, respectively. By the end of the study, there were 9, 7, and 6 horses in the Non-PPID, PPID Control, and PPID Treatment groups, respectively. These graphs show only the significant time effects (P<.05) for the respective analyses. PPID-pituitary pars intermedia dysfunction; IFNγ-interferon gamma; IL-interleukin; RQ-relative quantity.

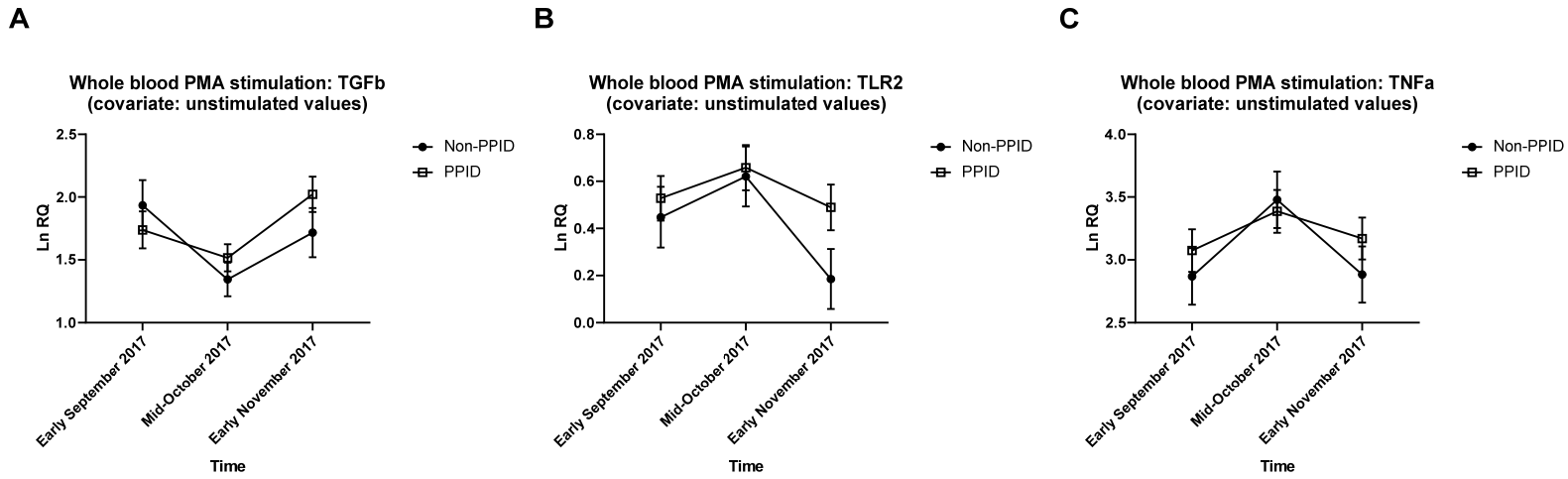

Figure 3.33. Significant time effects in PMA/ionomycin-stimulated whole blood cytokine expression of (A) TGFβ, (B) TLR2, and (C) TNFα

Mean +/- SEM. RQs are natural log-transformed (Ln). Non-PPID horses are represented by lines with closed circles. PPID horses are represented by lines with open squares. At Day 0 (Early September 2017), there were 10 horses in the Non-PPID group and 18 horses in the PPID group. By the end of the study, there were 9 horses in the Non-PPID group and 13 horses in the PPID group. These graphs show only the significant time effects ($P<.05$) for the respective analyses. PPID-pituitary pars intermedia dysfunction; TGFβ-transforming growth factor beta, TLR-toll-like receptor; TNFα-tumor necrosis factor alpha RQ-relative quantity

CHAPTER 4. EFFECTS OF PITUITARY PARS INTERMEDIA DYSFUNCTION AND PRASCEND® TREATMENT ON LOCALIZED IMMUNE FUNCTION WITHIN THE LUNG OF HORSES

4.1 Abstract

Background: It remains unclear how Pituitary Pars Intermedia Dysfunction (PPID) and treatment with Prascend® (pergolide tablets) affect local immune function within the lung of horses.

Hypotheses: Our hypotheses were that PPID horses would have altered immune function within the lung compared to Non-PPID horses, and that treatment with PRASCEND would normalize immune function in PPID Treatment horses to that of the Non-PPID horses.

Animals: 28 horses from a research herd (10 Non-PPID, 9 untreated PPID (PPID Control), and 9 PRASCEND-treated PPID horses (PPID Treatment)) were used.

Methods: Horses were sampled three times over approximately fifteen months for analysis of bronchoalveolar lavage fluid (BALF) cytology, BALF myeloperoxidase, and bronchoalveolar lavage (BAL) cell cytokine and receptor gene expression in response to stimuli. Bronchoalveolar lavages (BAL) were performed at Day 0, 8 weeks, and 1-year-and-6-weeks to allow for evaluations of the short-term (8 weeks) and long-term (1-year-and-6-weeks) effects of PRASCEND treatment. For the *in vitro* stimulations, heat-inactivated *R. equi*, heat-inactivated *E. coli*, and phorbal 12-myristate 13-acetate (PMA)/ionomycin were used. PRASCEND treatment was initiated after Day 0 collections. Results were analyzed using a linear mixed model (SAS v9.4), with significance set at $P<.05$.

Results: PPID horses had a lower percentage of eosinophils in BALF than Non-PPID horses at the Early September 2017 timepoint only. In unstimulated BAL samples, PPID horses had lower IL-13 expression than Non-PPID horses at the Mid-October 2018 timepoint only. In *R. equi*-stimulated samples, PPID horses had lower IL-13 expression than Non-PPID horses at the Early November 2017 and Mid-October 2018 timepoints. The TGFβ analysis for *R. equi*-stimulated samples also showed significant group by time effects, but the Non-PPID and PPID horses were not different at any timepoint. For IL-12α and TLR2 expression in *R. equi*-stimulated samples, significant group by time effects were observed, but these appeared to be driven by starting differences. PPID horses had lower BALF myeloperoxidase concentrations than Non-PPID horses at the Mid-October 2018 timepoint only.

Conclusions: The identified significant effects may indicate altered Th2 responses within the lungs of PPID horses, but it is also possible that these results are not biologically significant or real differences as they were generally not temporally consistent. This was likely due to the small sample size and few timepoints. Often, significant results were only apparent at single timepoints and were likely heavily influenced by one or several values. Although it significantly reduced plasma ACTH concentrations, PRASCEND did not appear to affect immune function within the lung in this study.

4.2 Introduction

As a result of their increased plasma ACTH concentrations**,** horses with PPID are often thought to have reduced immune function compared to Non-PPID horses, which may predispose them to infection caused by opportunistic bacteria or fungi, particular within

the respiratory tract [7-9,48]. Generally, this is thought to occur because of the normal hypothalamus-pituitary-adrenal axis response to increased ACTH, which is to increase cortisol concentrations, thereby impacting immune function [18,19]. Unfortunately, the currently published literature on immune function in horses with PPID is rather sparse.

Furthermore, to the author's knowledge, there is currently no published research to investigate the immune function of the lung in horses with PPID. However, in individuals with compromised immune function, opportunistic infection is common within the respiratory tract due to its exposure to the external environment [58,59]. It has been noted that horses with PPID frequently experience sinusitis, bronchopneumonia, and other infections [7,47-49,58,59,112]. When available, cultures often show infection with multiple bacterial and fungal species considered opportunistic [58,59,66,70]. Additionally, one study that described pathological changes in PPID horses during post-mortem examinations showed increased findings of bronchiolitis and bronchointerstitial inflammation in PPID horses compared to both young and aged Non-PPID horses [56].

Due to the anecdotal evidence for increased susceptibility to respiratory infections in horses with PPID, as well as the pathological findings reported in the lungs of PPID horses, it followed that analysis of the immune function within the lung of the PPID horse was needed to determine if altered local immune responses are responsible for the potential increased susceptibility to infection. In order to analyze this, bacteria considered opportunistic in adult horses were used for *in vitro* stimulation of cells obtained via bronchoalveolar lavage (BAL). In general, most pneumonia cases in horses are caused by bacteria or fungi that are considered opportunistic [58,59]. Although frequently seen in foals under six months of age, *Rhodococcus equi* infection in adult horses is not very common,

and generally occurs when immune function is compromised [59,65-67,82]. This can occur secondary to chronic diseases or viral infection, as well as after administration of immunosuppressive drugs, such as corticosteroids [58,59]. *Escherichia coli* is commonly isolated from pneumonia cases in adult horses [58].

R. equi and *E. coli* were selected for the *in vitro* stimulations of BAL cells in order to analyze the immune response to opportunistic bacteria for adult horses that were both gram-positive and gram-negative, respectively. The hypotheses for this work was that PPID horses would have altered localized immune responses basally and in response to stimuli within the lung when compared to Non-PPID horses; additionally, we anticipated that treatment with Prascend® (pergolide tablets) (Boehringer-Ingelheim Animal Health; Duluth, GA) would normalize the local immune responses within the lung to those of the age-matched Non-PPID horses.

4.3 Materials and Methods

4.3.1 Animal Selection and Study Design

Horses were initially selected for potential inclusion in the study from a university-owned research herd based on prior screenings and clinical history (absence or presence of hypertrichosis and/or absence or presence of history of hypertrichosis/difficulty shedding and/or results of multiple TRH stimulation tests). PPID status was confirmed using ACTH responses to TRH stimulation tests approximately two weeks prior to Day 0 of the study (Early September 2017) and basal ACTH values on Day 0. An oral sugar test (OST) was also performed prior to the study, approximately one week after the TRH stimulation test,

to assess hyperinsulinemia or insulin dysregulation status [87]. Horses that did not screen as either Non-PPID or PPID for both the TRH stimulation test and basal ACTH screenings were excluded from the study. As these initial screenings were obtained during the fall season in the USA (late August and early September), a basal ACTH value ≥100pg/mL and ACTH 10 minutes post-TRH injection (T10) value ≥400pg/mL were used for selection into the PPID group [8,9].

Thirty-one horses were originally selected to proceed through the study. However, three horses assigned to the PRASCEND-treated group had ACTH values that failed to become controlled and remained uncontrolled throughout the study; these horses were excluded from all analyses and are not discussed further. Non-PPID horses (n=10) were all assigned to one group. An attempt was made to randomly assign the PPID horses into the untreated and treated groups, however, due to also attempting to block for insulin status, true randomization was not possible. PPID Control horses (n=9) remained untreated for the duration of the study, and PPID Treatment horses (n=9) began receiving PRASCEND within approximately 24 hours of their completion of all Day 0 sample collections. Horses were started at the lowest label dose (2 μg/kg) to the nearest half-tablet for their body weight, based on the manufacturer's label recommendations. Basal ACTH was checked weekly for the first 4 weeks, and the dose was increased each week until basal ACTH levels were reduced to controlled (Non-PPID) values or until the maximum label dose (4 μg/kg) to the nearest half-tablet was reached.

Throughout the approximately 15-month study, six horses were euthanized due to non-study related health issues. The study ended with 9, 7, and 6 horses in the Non-PPID, PPID Control, and PPID Treatment groups, respectively. At the start of the study, there

was no statistical difference in age between the groups (*P*=.1747) (Table 2.1). Age ranges were 20-29 years for Non-PPID horses, 21-31 years for PPID Control horses, and 19-29 years for PPID Treatment horses. Only mares and geldings were included (Table 2.2). Breed representations, either confirmed or to the best of the researchers' knowledge, included Thoroughbred (n=14), American Quarter Horse (n=4), Standardbred (n=1), Standardbred cross (n=1), Mustang (n=1), Paint (n=1), Arabian (n=1), Tennessee Walking Horse (n=1), Appaloosa (n=1), and unknown or mixed breed (n=3) horses (Table 2.2). Breakdowns of sex and breed by group are included in Table 2.2. All horses were cared for and sampled with approval of the University of Kentucky's Institutional Animal Use and Care Committee (IACUC) (#2014-1225 and #2018-3004).

4.3.2 Sampling

On sampling days, horses were grain fasted (if part of their normal ration) until all collections were completed and had recovered from sedation. At three timepoints (Early September 2017, Early November 2017, and Mid-October 2018), bronchoalveolar lavages were performed within a four-hour window (approximately 10:00 to 14:00) of the blood collections for endocrine and systemic immune function analyses, as described in Chapters 2 and 3. At each timepoint, all samples (not including the TRH stimulation tests or OSTs) were collected over a period of 1-4 days, but within the same time window above (10:00 to 14:00); when multiple days were needed to complete all of the collections, a mixture of horses from the three groups were screened each day. Blood and BAL collections for each horse were performed on the same day. The local immune function measures investigated at the three time points included BALF cytology, BALF myeloperoxidase (MPO) analysis, and cytokine and receptor gene expression analysis of unstimulated and stimulated

bronchoalveolar lavage cells. Rectal temperature, pulse, and respiration were also recorded at these three timepoints. Appendix 2 contains a sample timeline.

BALF cytology and BAL sample collections

Horses were sedated intravenously (dormosedan and torbugesic) prior to the start of the procedure. A sterile bronchoalveolar lavage tube was lubricated with sterile lube, inserted into the nasal passage, and guided until seated in a bronchiole. Approximately 60mL of sterile saline (0.9% NaCl) was infused into the lung and immediately removed. This was repeated five times for a total saline infusion of approximately 300mL, with total recovery of approximately 200mL of BALF, which was then kept on ice. Approximately 6mL of the recovered sample was placed into a blood tube with EDTA for cytological analysis. A sterile swab was used to make slides for each sample. BALF differential counts were performed by a board-certified pathologist at the University of Kentucky's Veterinary Diagnostic Laboratory (Lexington, KY).

BAL cell (unstimulated and stimulated) cytokine and receptor gene expression

The recovered BAL specimens were centrifuged at 400g for ten minutes to allow for isolation of the BAL cells. The supernatant was then collected and filtered through a 2-micron filter and frozen at -20°C. The remaining cells were washed twice with cold phosphate-buffered saline (PBS) and filtered through a sterile gauze square to remove any mucous [113]. The cells were then frozen in freeze media (50% HyClone™ RPMI 1640 media (GE Healthcare), 40% fetal bovine serum (Sigma Aldrich; St. Louis, MO), and 10% dimethyl sulfoxide (DMSO) (Sigma Aldrich)) at -80°C, and transferred to liquid nitrogen after at least 24 hours in the -80°C until they were needed for further analyses.

After thawing the BAL cells and resuspending in complete RPMI (96.4% HyClone™ RPMI 1640 media (GE Healthcare), 2.5% fetal equine serum, 1% penicillin, streptomycin, L-Glutamine solution (Sigma Aldrich), and 0.1% 2-mercaptoethanol (Gibco™)), the cells were counted using a Vi-CELL counter (Beckman Coulter; Indianapolis, Indiana), and plated at $4x10^6$ cells per well into a total of four wells of a cell culture plate. Due to the lower cell counts associated with BAL collections, not all horses had enough cells for all stimulations, therefore, the number of samples available for each stimulation and timepoint varied. If cell counts permitted, each horse had a total of four cell stimulations. One well remained unstimulated, one well was stimulated with heat-inactivated *Rhodococcus equi* 103^+ (10^7 CFU in 10 µL of complete RPMI) for 24 hours, one well was stimulated with heat-inactivated *Escherichia coli* (ATCC #35218) (10^7 CFU in 10 µL of complete RPMI) for 24 hours, and one well was stimulated with 10 µL phorbal 12-myristate 13-acetate (PMA)/ionomycin for the last four hours of incubation. A dose response pilot study was conducted in advance to determine the best dose for each of the bacteria used in the stimulations that would elicit a sub-maximal response. The cells were incubated at 37°C with 5% CO_2 after plating and bacterial stimulations. Brefeldin A was added to all wells after 20 hours of incubation. After 24 hours, approximately half of the cells were removed, and TRIzol™ (Invitrogen™) was added. These samples were stored at -80°C until RNA was isolated via phenol-chloroform extraction. After RNA isolation, the samples were placed back in the -80°C freezer until reverse transcribed (described below). Gene expression of IFNγ, IL-12α, IL-13, IL-17α, IL-1β, IL-6, IL-8, TGFβ, TNFα, TLR2, and TLR4 was then measured using RT-PCR (described below).

Reverse transcription

Prior to reverse transcription, RNA was quantified via Epoch microplate spectrophotometer (Biotek; Winooski, Vermont). Master mix reagents (Promega; Madison, Wisconsin) were used to transcribe the RNA; after addition of the reagents, samples were placed into a thermocycler (Applied Biosystems; Foster City, California) for a cycle of 15 minutes at 42ºC, followed by a cycle of 5 minutes at 95ºC [98-102]. After completion of RNA reverse transcription to cDNA, the samples were stored -80ºC until used for RT-PCR (described below).

RT-PCR

For the desired cytokine analyses and the endogenous control gene, β-gus, equine-specific intron spanning primers and probes (Applied Biosystems), nuclease-free water (Qiagen), TaqMan™ (Applied Biosystems), and the desired cDNA samples were loaded onto plates in duplicate via a robotic pipetting machine (Eppendorf) [98-102]. RT-PCR was then performed using the 7900HT Fast RT-PCR System (Applied Biosystems) with the first cycle of 10 minutes at 95ºC, and an additional 40 cycles of 15 seconds at 95ºC and 60 seconds at 60ºC [98-102]. After determination of cycle threshold values using linear regression analysis (LinRegPCR version 2018; Heart Failure Research Center, Amsterdam University Medical Centers), relative quantity (RQ) values were calculated using the $2^{-\Delta\Delta Ct}$ method [98-103]. The calibrator for the BAL stimulations was the average ΔCT of the Day 0 (Early September 2017) media values for all of the Non-PPID horses. All RQs were natural log transformed prior to statistical analyses.

Rhodococcus equi culture and heat-inactivation

A *Rhodococcus equi* 103^{+} culture was kindly supplied by the lab of Dr. David Horohov. The stock solution was streaked onto plates containing tryptic soy agar (VWR®) using a sterile inoculating loop (VWR® International). After approximately 48 hours of growth at 37°C, one colony was selected and used to inoculate a tube of tryptic soy broth (Corning®). After sealing the tube and briefly vortexing, a sterile inoculating loop was used to inoculate additional broth tubes. These broth tubes were then incubated at 37°C for approximately 48 hours with caps in aerobic growth positioning. The main broth tube was then aliquoted and frozen for future use. The additional broth tubes were used to establish a growth curve and to determine the ideal time to heat-inactivate the bacteria. Based on the growth curve results, the bacteria were then pulled in the exponential growth period (after approximately 40 hours of growth) for all further work. Heat inactivation time was selected so that the minimum heat and time necessary for inactivation was achieved, in order to minimize any changes to the bacteria. After approximately 40 hours of growth, the inoculated broth tubes were placed into a water bath at 60°C for various times and then streaked onto tryptic soy agar plates to confirm inactivation. The plates were checked at 48 hours after plating to determine if growth was present. The minimum time needed for heat-inactivation of *R. equi* was 50 minutes at 60°C.

Once the growth curve and heat inactivation time were determined, the final batches were prepared in similar fashion to the above descriptions. The stock solution was streaked onto tryptic soy agar plates and after approximately 48 hours of growth, a colony was selected to inoculate a broth tube. After sealing the tube and briefly vortexing it, a sterile inoculating loop was used to inoculate multiple broth tubes, which were then incubated at

37°C. After approximately 40 hours, the broth tubes were removed from the incubator, and the volume of broth was measured as the tubes were combined into a sterile glass bottle. After a brief vortex, 100µL of broth was removed and serially diluted five times before plating 25µL in duplicate. The number of colonies was then counted on these two plates at approximately 48 hours of growth and averaged; this count was used to determine the colony-forming units (CFU) per milliliter. Then, the broth was aliquoted into new sterile tubes, and heat-inactivated for 50 minutes at 60°C. After heat inactivation, the broth was combined again into a sterile glass bottle, 25µL was plated in duplicate to confirm inactivation, and the final volume of broth was measured, so that accurate counts could be obtained. The final heat-inactivated broth was then frozen at -20°C. When needed for stimulations, the broth was thawed, centrifuged at 3,400g for ten minutes, and the pellet resuspended in complete RPMI. The final broth was positive for *R. equi* and VapA expression on Real Time PCR, performed by the University of Kentucky Veterinary Diagnostic Lab (Lexington, Kentucky).

Escherichia coli culture and heat-inactivation

Escherichia coli (ATCC #35218) was obtained from VWR (Microbiologics, Inc.; St. Cloud, Minnesota) in KWIK-STIK™ vials. The KWIK-STIK™ vial was crushed, and the sterile swab was streaked onto plates containing Difco™ nutrient agar (VWR®; BD Biosciences). After approximately 24 hours of growth at 37°C, one colony was selected and used to inoculate a tube of Difco™ nutrient broth (VWR®, BD Biosciences). After sealing the tube and briefly vortexing, a sterile inoculating loop was used to inoculate additional broth tubes. These broth tubes were then incubated at 37°C for approximately 24 hours with caps in aerobic growth positioning. The main broth tube was then aliquoted

and frozen for future use. The additional broth tubes were used to establish a growth curve and to determine the ideal time to heat-inactivate the bacteria. Based on the growth curve results, the bacteria were then pulled in the exponential growth period (after approximately 24 hours of growth) for all further work. Heat inactivation time was selected so that the minimum heat and time necessary for inactivation was achieved, in order to minimize any changes to the bacteria. Bacteria were placed into a water bath at 60°C for various times and then streaked onto tryptic soy agar plates to confirm inactivation. The plates were checked at 24 hours after plating to determine if growth was present. The minimum time needed for heat-inactivation of *E. coli* was 7.5 minutes at 60°C.

Once the growth curve and heat inactivation time were determined, the final batches were prepared in similar fashion to the above descriptions. The stock solution was streaked onto Difco™ nutrient agar plates and after approximately 24 hours of growth, a colony was selected to inoculate a broth tube. After sealing the tube and briefly vortexing it, a sterile inoculating loop was used to inoculate multiple broth tubes, which were then incubated at 37°C. After approximately 24 hours, the broth tubes were removed from the incubator, and the volume of broth was measured as the tubes were combined into a sterile glass bottle. After a brief vortex, 100µL of broth was removed and serially diluted six times before plating 25µL in duplicate. The number of colonies was then counted on these two plates at approximately 24 hours of growth and averaged; this count was used to determine the CFU/mL. Then, the broth was aliquoted into new sterile tubes, and heat-inactivated for 7.5 minutes at 60°C. After heat inactivation, the broth was combined again into a sterile glass bottle, 25µL was plated in duplicate, and the final volume of broth was measured, so that accurate counts could be obtained. The final heat-inactivated broth was then frozen at -

20°C. When needed for stimulations, the broth was thawed, centrifuged at 3,400g for ten minutes, and the pellet resuspended in complete RPMI.

BALF myeloperoxidase:

For BALF MPO analysis, the filtered BALF obtained during the BAL cell isolation process was used for a previously-validated equine MPO ELISA [97]. Filtered BALF was diluted 1:50 in phosphate-buffered saline (PBS) (Gibco™; Life Technologies) for the assay and absorbance was read at 450nm, as recommended by the manufacturer [97,114].

4.3.3 Statistical Methods

Distributional assumptions were evaluated using graphical and numerical summaries for the presence of gross normality violations. The primary analyses were the longitudinal analysis of all measures over time; linear mixed models included main effects for group and time. The primary comparisons were the PPID Treatment to PPID Control groups and the PPID to Non-PPID groups, when the PPID groups were combined. To determine if groups changed differently over time, an F-test for the interaction of group and time was used. Graphical summaries and model fit were used to determine the specifications of the linear mixed model (PROC MIXED, SAS v9.4). To account for repeated measures, an UN, ARMA(1,1), AR(1), TOEP, or TOEPH variance-covariance structure was used for each measure, depending on the best model fit based on information criteria (BIC, AIC). All analyses were conducted using SAS v9.4 (Cary, North Carolina),

and a two-sided significance level of .05 was used for all statistical tests. Graphs were created using GraphPad Prism v.8.0.2 (San Diego, California).

Results for PPID Control and PPID Treatment horses were analyzed first. If significant group by time differences were observed, these groups remained separate for comparison to Non-PPID horses. If no significant group by time differences were observed, the PPID Control and PPID Treatment groups were combined before comparison to Non-PPID horses. If no significant group or group by time differences were observed between the combined PPID group and Non-PPID group, all groups were combined for analysis of time effects only.

If starting differences ($P<.1$) were observed between PPID Control and PPID Treatment horses in the initial analyses, starting values were included as a covariate within the model. If starting differences ($P<.1$) were observed between PPID and Non-PPID horses, analyses were performed without starting values as a covariate within the model, in order to analyze group effects, and then with starting values as a covariate, in order to analyze group by time effects; results for each of the methods are presented if applicable.

For the BAL stimulations, the media (unstimulated) values for each cytokine/receptor were included within the model as a covariate to account for changes based on where the individual animals started. Additionally, for the RT-PCR and BALF MPO results, outliers were removed prior to statistical analyses if they were determined to be more than five times lower or higher than the median absolute deviation (MAD) from the median for each cytokine and receptor and each stimulation (Table 4.1) or for MPO.

4.4 Results

BALF cytology

Percent mast cells in BALF:

No significant group, time, or group by time effects were seen in percent mast cells in BALF between any of the groups.

Percent macrophages, percent lymphocytes, and percent neutrophils in BALF:

No significant group or group by time effects were seen in percent macrophages, percent lymphocytes, or percent neutrophils in BALF between any of the groups. Significant time effects in percent macrophages in BALF were observed in the PPID Control to PPID Treatment ($P<.0001$), Non-PPID to PPID ($P<.0001$), and all groups combined ($P<.0001$) comparisons. Significant time effects in percent lymphocytes in BALF were observed in the PPID Control to PPID Treatment ($P<.0001$), Non-PPID to PPID ($P<.0001$), and all groups combined ($P<.0001$) comparisons. Significant time effects in percent neutrophils in BALF were observed in the Non-PPID to PPID ($P=.0082$), and all groups combined ($P=.0195$) comparisons.

Percent eosinophils in BALF:

No significant group, time, or group by time effects were seen in percent eosinophils in BALF between the PPID Control and PPID Treatment horses. After combining the PPID Control and PPID Treatment groups for comparison to Non-PPID horses, significant time ($P=.0311$) and group by time ($P=.0311$) effects, but not a group effect alone, were found (Figure 4.1A). In this analysis, the PPID horses had lower percentages of eosinophils in BALF at the Early September 2017 timepoint when

compared to Non-PPID horses. Due to starting differences between the Non-PPID and PPID horses, the analysis was run again with starting values as a covariate to determine if the significant group by time changes remained (Figure 4.1B). This subsequent analysis still revealed significant group by time effects; therefore, these groups did change over time as a result of their group status.

Summary of significant BALF cytology results:

Aside from time effects, the only significant difference observed was in the percentage of eosinophils in BALF, with PPID horses having a lower percentage of eosinophils in BALF than Non-PPID horses at the Early September 2017 timepoint only. It is likely that this effect was driven by the significant starting differences, which combined with the small sample sizes, may have led to this significant result, and may not be biologically relevant. Significant time effects for the Non-PPID to PPID comparisons are shown in Figure 4.2.

BAL (unstimulated/stimulated) cytokine and receptor gene expression

BAL stimulation- Media:

No significant group, time, or group by time effects were seen in IL-12α, IL-6, IL-8, or TLR2 between any of the groups.

No significant group or group by time effects were seen in TGFβ or TLR4 between any of the groups. Significant time effects in TGFβ expression were observed in the PPID Control to PPID Treatment (P=.0015), Non-PPID to PPID (P=.0008), and all groups

combined (*P*=.0004) comparisons. Significant time effects in TLR4 expression were observed in the PPID Control to PPID Treatment (*P*=.0087), Non-PPID to PPID (*P*=.002), and all groups combined (*P*=.0007) comparisons.

No significant group or group by time effects were seen in IFNγ, TNFα, or IL-1β between any of the groups. Due to starting differences between the PPID Control and PPID Treatment groups, these analyses were run again with starting values as a covariate in the model; the group and group by time effects remained insignificant. No significant time effects in IFNγ and IL-1β expression were observed in any of the comparisons. Significant time effects in TNFα expression were observed in the PPID Control to PPID Treatment (*P*=.0483) and all groups combined (*P*=.0385) comparisons.

A significant group effect (*P*=.0193), but not a group by time effect, was seen in IL-17α expression between the PPID Control and PPID Treatment groups. Due to starting differences between these groups, this analysis was run again with starting values as a covariate in the model; the group effect was no longer significant and the group by time effect remained insignificant. After combining the PPID Control and PPID Treatment groups for comparison to Non-PPID horses, no significant group or group by time effects were seen. No significant time effects were observed between any of the groups.

Results for IL-13 are reported very cautiously as there was very late and/or no amplification of IL-13 in many of the samples. Therefore, the number of samples available for interpretation is very small. In the PPID Control to PPID Treatment comparison, 19 of the 45 samples were missing. In the Non-PPID to PPID comparison, 33 of the 75 samples were missing. No significant group or group by time effects were seen in IL-13 between the PPID Control and PPID Treatment groups, but a significant time effect was seen

(*P*=.021). A significant group by time effect, but not a group or time effect alone, was seen in the Non-PPID to PPID comparison (*P*=.0002) (Figure 4.3). This appears to be driven by a significant difference between the groups at the Mid-October 2018 timepoint only; however, this must be interpreted cautiously as only 3 Non-PPID and 4 PPID samples were available for this timepoint.

Summary of BAL stimulation- Media:

There was a possible IL-13 difference with PPID horses having lower IL-13 expression than Non-PPID horses at the Mid-October 2018 timepoint only, but this is likely driven by a few timepoints and the small number of samples, given how many samples had no amplification for this analysis. Significant time effects were observed.

BAL stimulation- R. equi:

No significant group, time, or group by time effects were seen in IL-1β, TLR4, or TNFα between any of the groups.

A significant group effect (*P*=.0142), but not a group by time effect, was seen in IFNγ expression between the PPID Control and PPID Treatment groups. Due to starting differences between these groups, this analysis was run again with starting values as a covariate; the group effect remained significant and the group by time effect remained insignificant. After combining the PPID Control and PPID Treatment groups for comparison to Non-PPID horses, no significant group or group by time effects were seen. No significant time effects were observed between any of the groups.

A significant group effect (*P*=.0013), but no group by time effect, was seen in IL-6 expression between the PPID Control and PPID Treatment groups. Due to starting differences between these groups, this analysis was run again with starting values as a covariate; the group effect remained significant and the group by time effect remained insignificant. After combining the PPID Control and PPID Treatment groups for comparison to Non-PPID horses, no significant group or group by time effects were seen. No significant time effects were observed between any of the groups.

No significant group or group by time effects were seen in IL-8 expression between the PPID Control and PPID Treatment groups. Due to starting differences between these groups, this analysis was run again with starting values as a covariate; the group effect was then significant (*P*=.0193), but the group by time effect remained insignificant. After combining the PPID Control and PPID Treatment groups for comparison to Non-PPID horses, no significant group or group by time effects were seen. Significant time effects were observed in the all horses combined comparison only (*P*=.0488).

A significant group effect (*P*=.0331), but not a group by time effect, was seen in IL-17α expression between the PPID Control and PPID Treatment groups. After combining the PPID Control and PPID Treatment groups for comparison to Non-PPID horses, no significant group or group by time effects were seen. No significant time effects were observed between any of the groups.

No significant group, time, or group by time effects were seen in TGFβ between the PPID Control and PPID Treatment groups. After combining these groups for comparison to Non-PPID horses, a significant group by time effect (*P*=.048) was observed; no group or time effects were observed (Figure 4.4B). This appears to be a consequence of

the small sample size as the Non-PPID and PPID groups were not statistically different at any timepoint, therefore, this result may have been heavily influenced by one or a few individual values.

A significant group effect (P=.0383), but no group by time effect, was seen in TLR2 expression between the PPID Control and PPID Treatment groups. Due to starting differences between these groups, this analysis was run again with starting values as a covariate; the group effect was no longer significant, but the group by time effect was then significant (P=.0146) (Figure 4.5C). Due to the significant group by time effects, the PPID Control and PPID Treatment groups remained separate for comparison to the Non-PPID group. In the PPID Control to PPID Treatment to Non-PPID comparison, no significant group or group by time effects were observed (Figure 4.5B). Due to starting differences between the PPID Treatment and PPID Control groups only, this analysis was run again with starting values as a covariate; the group effect remained not significant, but the group by time effect (P=.0196) was then significant (Figure 4.5C). No significant time effects were observed in any of the comparisons. The statistically significant results reported here appear to be a consequence of the small sample size, which allowed one or a few values to influence the results.

Significant group (P<.0001) and group by time (P=.0178) effects were seen in IL-12α expression between the PPID Control and PPID Treatment groups. Due to starting differences between these groups, this analysis was run again with starting values as a covariate; the group effect (P=.0002) and group by time effect (P=.0144) remained significant; however, the starting differences were not able to be corrected by including them as a covariate. Due to the significant group by time effects, the PPID Control and

PPID Treatment groups remained separate for comparison to the Non-PPID group. In the PPID Control to PPID Treatment to Non-PPID comparison, significant group (P=.0047) and group by time (P=.0402) effects were observed (Figure 4.6B). Due to starting differences between the PPID Treatment and PPID Control groups in this comparison, the analysis was run again with starting values as a covariate in the model (Figure 4.6C). The group effect (P=.0013) and group by time effect (P=.0284) remained significant; however, the starting differences were not able to be corrected by including them as a covariate (Figure 4.6C). PPID Treatment horses did not differ from Non-PPID horses at any timepoint, and PPID Control horses differed from Non-PPID horses at only the Early November 2017 timepoint (Figure 4.6B). PPID Control and PPID Treatment horses differed at the Early September 2017 and Early November 2017 timepoint, but not at the Mid-October 2018 timepoint (Figure 4.6B). No significant time effects were observed in any of the comparisons. Graphically, the significant group by time effect appears to be driven by a single value at the Early November 2017 timepoint in the PPID Control group, which heavily influenced the analysis. Removal of this value in the PPID Control to PPID Treatment to Non-PPID analysis eliminated the significant group by time effect, but not the significant group effect. The group effect appears to be driven by significant starting differences that were not able to be corrected by including them in the model as a covariate.

Results for IL-13 are reported very cautiously as there was very late and/or no amplification of IL-13 in many of the samples. Therefore, the number of samples available for interpretation is very small. In the PPID Control to PPID Treatment comparison, 22 of the 41 samples were missing. In the Non-PPID to PPID comparison, 38 of the 67 samples were missing. No significant group, time, or group by time effects were seen in IL-13

between the PPID Control and PPID Treatment groups. Significant group (P=.01) and group by time effects (P=.0051), but not time effects, were seen in the Non-PPID to PPID comparison (Figure 4.7B). However, these results must be interpreted cautiously as few samples were available and results may be heavily influenced by only one or a few values.

Summary of BAL stimulation- R. equi:

There was a possible IL-13 difference with PPID horses having lower IL-13 expression than Non-PPID horses at the Early November 2017 and Mid-October 2018 timepoints, but this was likely driven by a few timepoints and the small number of samples, given how many samples had no amplification for this analysis. For TGFβ responses to *in vitro R. equi* stimulation, the significant group by time result appears to be a consequence of the small sample size as the Non-PPID and PPID groups were not statistically different at any timepoint, therefore, this result may have been heavily influenced by one or a few values. For IL-12α and TLR2 expression, significant effects were observed, but these appear driven by starting differences and were likely impacted by the small sample sizes and single timepoint differences.

BAL stimulation- E. coli:

No significant group, time, or group by time effects were seen in IL-17α, IL-1β, IL-6, IL-8, TGFβ, TLR4, or TNFα between any of the groups.

No significant group or group by time effects were seen in IL-13 expression between the PPID Control and PPID Treatment groups. After combining the PPID Control and PPID Treatment groups for comparison to Non-PPID horses, no significant group or

group by time effects were seen. Significant time effects were observed in the Non-PPID to PPID comparison (*P*=.0022) and the all horses combined comparison only (*P*=.0003).

No significant group or group by time effects were seen in TLR2 expression between the PPID Control and PPID Treatment groups. Due to starting differences between these groups, this analysis was run again with starting values as a covariate in the model; the group and group by time effects remained insignificant. After combining the PPID Control and PPID Treatment groups for comparison to Non-PPID horses, no significant group or group by time effects were seen. Significant time effects were observed in the PPID Control and PPID Treatment (*P*=.0488), Non-PPID to PPID (*P*=.004), and all horses combined comparisons (*P*=.0028).

Significant group by time (*P*=.005) effects were seen in IL-12α expression between the PPID Control and PPID Treatment groups; no significant group or time effects were seen in this comparison. Due to starting differences between these groups, this analysis was run again, with starting values as a covariate in the model; the results remained the same. Due to the significant group by time effects, the PPID Control and PPID Treatment groups remained separate for comparison to the Non-PPID group. In the PPID Control to PPID Treatment to Non-PPID comparison, no significant group, time, or group by time effects were observed. Starting differences between the groups were again present, so the analysis was run again with starting values as a covariate in the model; the results remained the same. Therefore, all horses were combined for analysis of time effects; no significant time effects were observed.

Significant group by time (*P*=.011) effects were seen in IFNγ expression between the PPID Control and PPID Treatment groups; no significant group or time effects were

seen in this comparison. Due to the significant group by time effects, the PPID Control and PPID Treatment groups remained separate for comparison to the Non-PPID group. In the PPID Control to PPID Treatment to Non-PPID comparison, no significant group, time, or group by time effects were observed. Therefore, all horses were combined for analysis of time effects; no significant time effects were observed.

Summary of BAL stimulation- E. coli:

Aside from significant time effects, there were no significant findings.

BAL stimulation- PMA/ionomycin:

No significant group, time, or group by time effects were seen in IL-13, TGFβ, TLR4, or TNFα between any of the groups.

No significant group or group by time effects were seen in TLR2 between any of the groups. Significant time effects in TLR2 expression were observed in the Non-PPID to PPID (P=.0245) and all horses combined comparisons (P=.0195).

No significant group or group by time effects were seen in IL-1β expression between the PPID Control and PPID Treatment groups. After combining the PPID Control and PPID Treatment groups for comparison to Non-PPID horses, no significant group or group by time effects were seen. Significant time effects were observed only in the PPID Control and PPID Treatment (P=.033) comparison.

Significant group by time (P=.016) effects were seen in IL-17α expression between the PPID Control and PPID Treatment groups; no significant group or time effects were

seen in this comparison. Due to starting differences between these groups, this analysis was run again with starting values as a covariate in the model; the results remained the same, except that the group by time effect was no longer significant. This indicates that the initial significant group by time effect was caused by the starting differences between the two groups and was not a truly significant effect. After combining the PPID Control and PPID Treatment groups for comparison to Non-PPID horses, no significant group, time, or group by time effects were seen. No significant time effects were observed in the all groups combined comparison.

Significant group by time (*P*=.0329) effects were seen in IL-8 expression between the PPID Control and PPID Treatment groups; no significant group or time effects were seen in this comparison. Due to starting differences between these groups, this analysis was run again with starting values as a covariate in the model; the results remained the same, except that the group by time effect was no longer significant. This indicates that the initial significant group by time effect was caused by the starting differences between the two groups and was not a truly significant effect. After combining the PPID Control and PPID Treatment groups for comparison to Non-PPID horses, no significant group, time, or group by time effects were seen. No significant time effects were observed in the all groups combined comparison.

Significant time (*P*=.0442) and group by time (*P*=.0003) effects were seen in IFNγ expression between the PPID Control and PPID Treatment groups; no significant group effects were seen in this comparison. Due to starting differences between these groups, this analysis was run again with starting values as a covariate in the model; the results remained the same. Due to the significant group by time effects, the PPID Control and PPID

Treatment groups remained separate for comparison to the Non-PPID group. In the PPID Control to PPID Treatment to Non-PPID comparison, no significant group, time, or group by time effects were observed. Due to starting differences between these groups, this analysis was run again with starting values as a covariate in the model; the group and group by time effects remained insignificant, but a significant time effect was observed (*P*=.0257). Therefore, all horses were combined for analysis of time effects; no significant time effects were observed in this comparison.

Significant group by time (*P*=.0485) effects were seen in IL-6 expression between the PPID Control and PPID Treatment groups; no significant group or time effects were seen in this comparison. Due to the significant group by time effects, the PPID Control and PPID Treatment groups remained separate for comparison to the Non-PPID group. In the PPID Control to PPID Treatment to Non-PPID comparison, significant group by time effects, but not group or time effects alone, were observed. Due to starting differences between these groups, this analysis was run again with starting values as a covariate in the model; the group by time effects were no longer significant, indicating that these groups did not change over time based on their group status. Since no significant group or group by time effects were observed in this comparison, all horses were combined for analysis of time effects; no significant time effects were observed.

Significant group (*P*=.0205) and group by time (*P*=.0012) effects were seen in IL-12α expression between the PPID Control and PPID Treatment groups; no significant time effects were seen in this comparison. Due to starting differences between these groups, this analysis was run again with starting values as a covariate in the model; the group by time effect remained significant, but the group effect alone did not. Due to the significant group

by time effects, the PPID Control and PPID Treatment groups remained separate for comparison to the Non-PPID group. In the PPID Control to PPID Treatment to Non-PPID comparison, no significant group, time, or group by time effects were observed. Due to starting differences between these groups, this analysis was run again with starting values as a covariate in the model; the results remained the same. Since no significant group or group by time effects were observed in this comparison, all horses were combined for analysis of time effects; no significant time effects were observed.

Summary of BAL stimulation- PMA/ionomycin:

Aside from significant time effects, there were no significant findings.

Summary of overall BAL stimulation results:

Some significant group and group by time effects were observed in the *R. equi*-stimulated samples; however, most of these effects appeared to have been influenced by the small sample sizes and were difficult to draw any conclusions from. Significant IL-13 effects were noted in the media and *R. equi*-stimulated samples, but there were many samples with late or no amplification during the RT-PCR for IL-13, so only about half of the samples were able to be used in the analysis. No significant findings were observed in the *E. coli* and PMA/ionomycin stimulations aside from time effects. Significant time effects were often observed; for the Non-PPID to PPID comparisons, the significant time effects are shown in Figure 4.8.

BALF myeloperoxidase:

No significant group or group by time effects were found in BALF myeloperoxidase concentrations between the PPID Control and PPID Treatment horses; a significant time effect (P=.0133) was observed in this comparison. After combining the PPID horses for comparison to the Non-PPID horses, significant group (P=.0322) and group by time (P=.0322) effects were observed with PPID horses having lower BALF myeloperoxidase concentrations than Non-PPID horses at the Mid-October 2018 timepoint only (Figure 4.9). A significant time effect was not observed in the PPID to Non-PPID comparison. It is possible that these significant effects are not biologically significant as they were only apparent at one timepoint (Mid-October 2018), and the data set for the BAL samples was small.

4.5 Discussion

The goal of this study was to examine localized immune function in horses with PPID and to evaluate the extent to which short-term (8 weeks) and long-term (1-year-and-6-weeks) PRASCEND treatment affects these measures. Localized immune function was measured using BALF cytology, BALF myeloperoxidase concentrations, and gene expression of cytokines and receptors important for immune function in heat-inactivated *R. equi*, heat-inactivated *E. coli*, and PMA/ionomycin stimulations of BAL cells. Overall, the findings suggested that alterations may exist in the Th2 response within the lung of PPID horses when compared to Non-PPID horses but are opposite to what would be expected in asthmatic lungs. However, it was difficult to draw conclusions as the sample sizes limited statistical power.

Low cell counts in the recovered BAL fluid limited the number of stimulations that could be done. In these cases, the unstimulated and *R. equi* and *E. coli* stimulations were prioritized over the PMA/ionomycin stimulation. Additionally, unlike in the peripheral blood mononuclear cell stimulations, which had six timepoints over the study, the BAL cells were only collected at three main timepoints (Early September 2017, Early November 2017, and Mid-October 2018). Due to frequently observed significant time effects associated with the local immune response of the lung, the fewer timepoints and fewer samples due to low cell counts made it more difficult to analyze any effects of PPID status or PRASCEND treatment that were temporally consistent as well as significant.

Several of the significant results that may warrant further investigation included the differences in the percentage of eosinophils in BALF with PPID horses having lower counts at the initial starting timepoint (Early September 2017), as well as the decreased IL-13 in the unstimulated BAL cells and BALF MPO at the Mid-October 2018 timepoint, and the decreased IL-13 in the *R. equi*-stimulated BAL cells at the Early November 2017 and Mid-October 2018 timepoints when comparing PPID horses to Non-PPID horses. Unfortunately, many of the IL-13 samples had no or late amplification on the RT-PCR results, which combined with the already small sample sizes, left the IL-13 analyses vulnerable to the influence of one or a few sample values. If indeed the cytology results for the percentage of eosinophils in BALF are real, this points towards an decrease in the Th2 immune response of the lung in PPID horses compared to Non-PPID horses, which also may have coincided with the decrease in neutrophil degranulation, based on the BALF MPO results. In the general, a Th1 immune response would have been most appropriate in response to the *R. equi* stimuli, given the intracellular nature of *R. equi* and its ability to

replicate within macrophages [82,106,111]. Overzealous eosinophil and Th2 responses in the lung are associated with asthma and are responsible for the aberrant allergic and asthmatic responses of the lung in both humans and horses, but these were not seen in the available results from this study [115-118].

Overall, it is difficult to draw conclusions regarding the immune response within the lung of PPID horses based on the results obtained in this study. However, the slight differences that were found may point towards an altered immune response in the lung of PPID horses that is worth further exploring, albeit with either a larger sample size or more frequent sampling. An altered Th2 response in the lung would be consistent with the higher rates of bronchiolitis and bronchointerstitial inflammation observed in the post-mortem evaluations of Glover et al. in PPID horses and the anecdotal evidence suggesting that PPID horses have higher risk of respiratory infections [56]. Despite significantly reducing plasma ACTH concentrations, PRASCEND treatment did not appear to affect immune function within the lungs of PPID horses as measured in this study. Additionally, the significant time effects seen in the BALF cytology results (percentages of neutrophils, lymphocytes, macrophages, and eosinophils) and in some of the BAL immune responses emphasize the importance of having appropriate control groups when investigating the immune response of the lung in horses regardless of PPID status.

Table 4.1. Number of outliers removed from bronchoalveolar lavage cell (BAL) (unstimulated/stimulated) cytokine and receptor gene expression sample analyses

Cytokine/Receptor	**# of media outliers removed**	**# of *R. equi* outliers removed**	**# of *E. coli* outliers removed**	**# of PMA outliers removed**
IFNγ	0	0	0	1
IL-12α	0	0	0	0
IL-13	0	0	1	0
IL-17α	1	0	1	0
IL-1β	0	1	0	0
IL-6	0	0	3	0
IL-8	0	0	0	0
TGFβ	0	0	0	0
TLR2	1	1	0	1
TLR4	1	1	1	1
TNFα	1	1	0	0

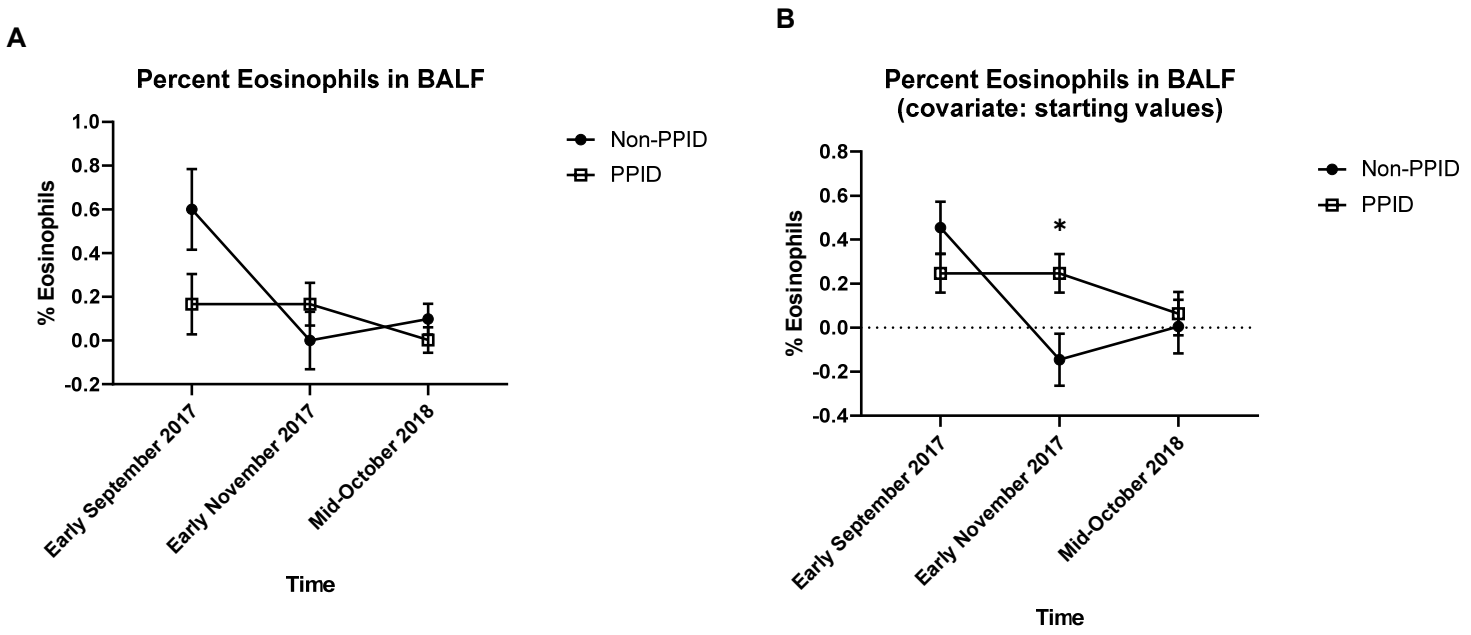

Figure 4.1. (A) Percent eosinophils in bronchoalveolar lavage fluid (BALF) cytology (B) Percent eosinophils in bronchoalveolar lavage fluid (BALF) cytology (starting values as a covariate in the model)

Mean +/- SEM. Non-PPID horses are represented by lines with closed circles. PPID horses are represented by lines with open squares. At Day 0 (Early September 2017), there were 10 horses in the Non-PPID group and 18 horses in the PPID group. By the end of the study, there were 9 horses in the Non-PPID group and 13 horses in the PPID group. * denotes a significant difference ($P<.05$) between Non-PPID and PPID groups. PPID-pituitary pars intermedia dysfunction.

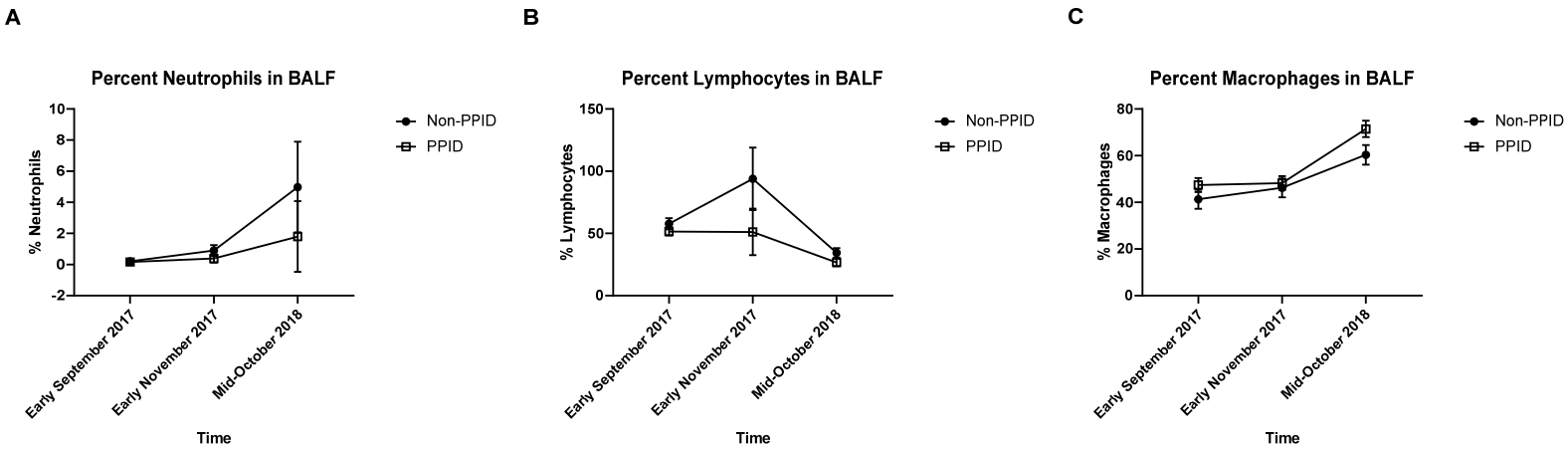

Figure 4.2. Significant time effects in bronchoalveolar lavage fluid (BALF) cytology for (A) percent neutrophils (B) percent lymphocytes, (C) percent macrophages

Mean +/- SEM. Non-PPID horses are represented by lines with closed circles. PPID horses are represented by lines with open squares. At Day 0 (Early September 2017), there were 10 horses in the Non-PPID group and 18 horses in the PPID group. By the end of the study, there were 9 horses in the Non-PPID group and 13 horses in the PPID group. These graphs show only the significant time effects ($P<.05$) for the respective analyses. PPID-pituitary pars intermedia dysfunction.

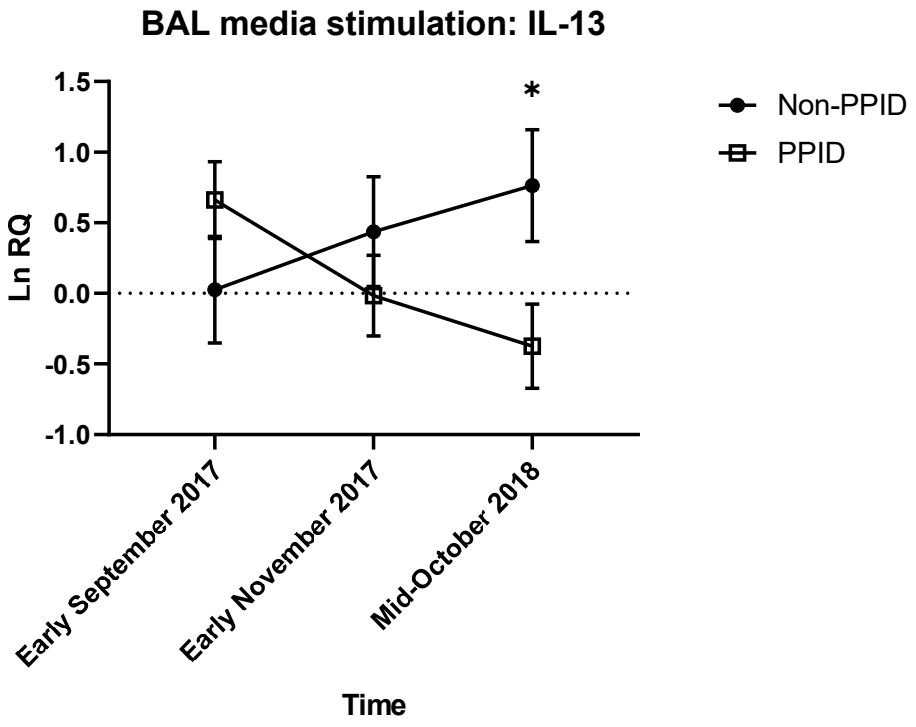

Figure 4.3. Unstimulated bronchoalveolar lavage (BAL) cell IL-13 expression

Mean +/- SEM. RQs are natural log-transformed (Ln). Non-PPID horses are represented by lines with closed circles. PPID horses are represented by lines with open squares. At Day 0 (Early September 2017), there were 10 horses in the Non-PPID group and 18 horses in the PPID group. By the end of the study, there were 9 horses in the Non-PPID group and 13 horses in the PPID group. * denotes a significant difference ($P<.05$) between Non-PPID and PPID groups. PPID-pituitary pars intermedia dysfunction; IL-interleukin; RQ-relative quantity.

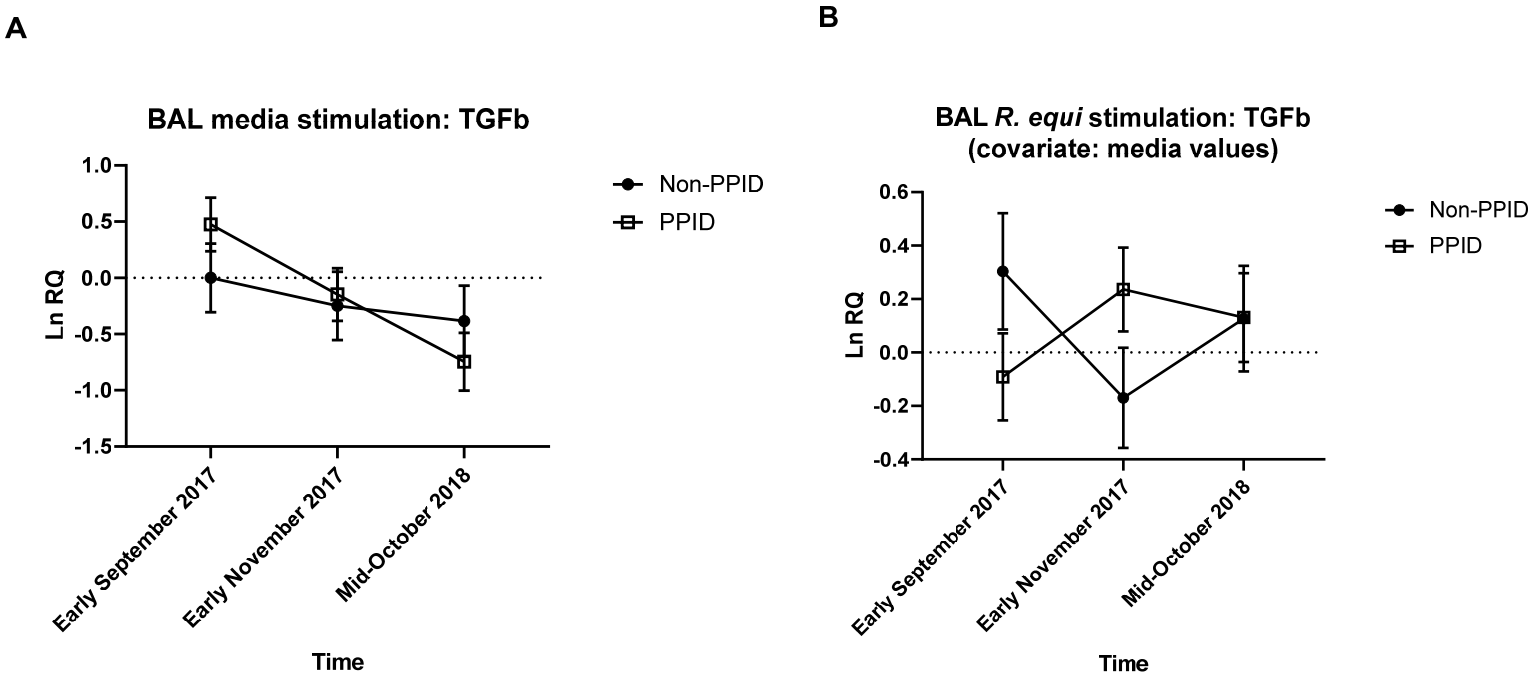

Figure 4.4. (A) Unstimulated bronchoalveolar lavage (BAL) cell TGFβ expression (B) Heat-inactivated *R. equi*-stimulated BAL cell TGFβ expression with media values as a covariate in the model

Mean +/- SEM. RQs are natural log-transformed (Ln). Non-PPID horses are represented by lines with closed circles. PPID horses are represented by lines with open squares. At Day 0 (Early September 2017), there were 10 horses in the Non-PPID group and 18 horses in the PPID group. By the end of the study, there were 9 horses in the Non-PPID group and 13 horses in the PPID group. PPID-pituitary pars intermedia dysfunction; TGFβ-transforming growth factor beta; *R. equi*-*Rhodococcus equi*; RQ-relative quantity.

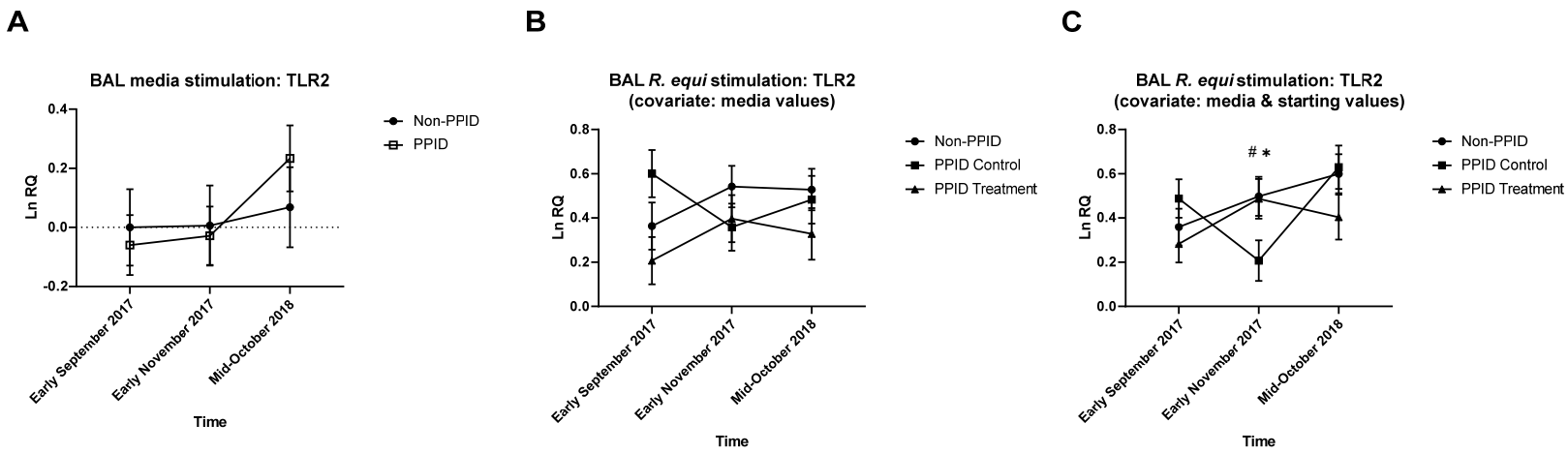

Figure 4.5. (A) Unstimulated bronchoalveolar lavage (BAL) cell TLR2 expression (B) Heat-inactivated *R. equi*-stimulated BAL cell TLR2 expression with media values as a covariate in the model. (C) Heat-inactivated *R. equi*-stimulated BAL cell TLR2 expression with media and starting values as covariates in the model

Mean +/- SEM. RQs are natural log-transformed (Ln). (A) Non-PPID horses are represented by lines with closed circles. PPID horses are represented by lines with open squares. At Day 0 (Early September 2017), there were 10 horses in the Non-PPID group and 18 horses in the PPID group. By the end of the study, there were 9 horses in the Non-PPID group and 13 horses in the PPID group. (B, C) Non-PPID horses are represented by lines with closed circles. PPID Treatment horses are represented by lines with closed triangles. PPID Control horses are represented by lines with closed squares. At Day 0 (Early September 2017), there were 10, 9, and 9 horses in the Non-PPID, PPID Control, and PPID Treatment groups, respectively. By the end of the study, there were 9, 7, and 6 horses in the Non-PPID, PPID Control, and PPID Treatment groups, respectively. * denotes a significant difference (P<.05) between Non-PPID and PPID Control. # denotes a significant difference (P<.05) between PPID Treatment and PPID Control groups. PPID-pituitary pars intermedia dysfunction; TLR2-toll-like receptor 2; *R. equi*-*Rhodococcus equi*; RQ-relative quantity.

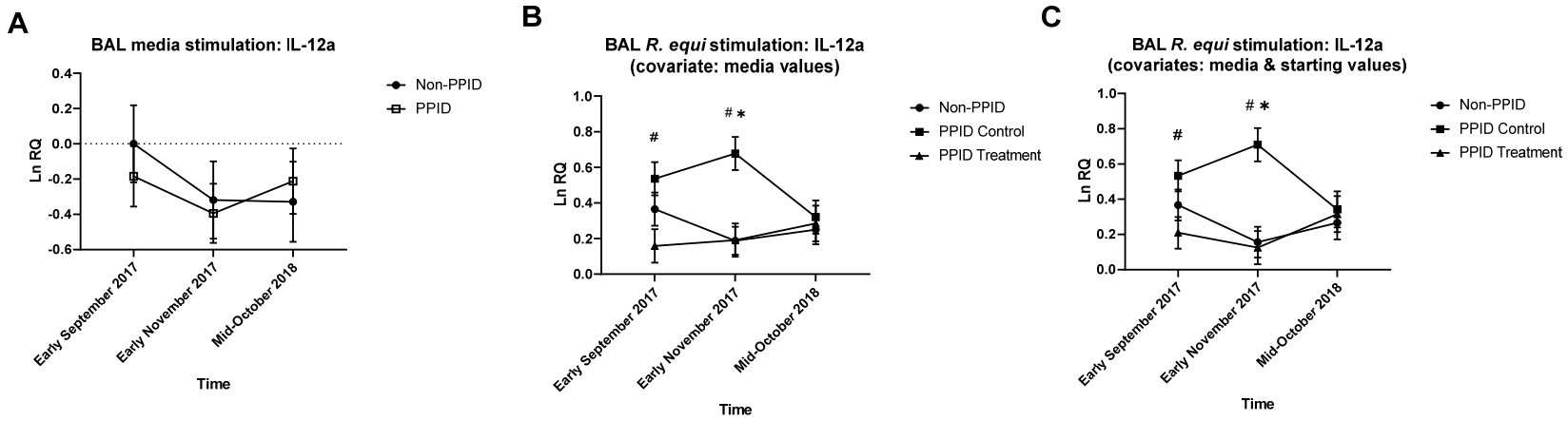

Figure 4.6. (A) Unstimulated bronchoalveolar lavage (BAL) cell IL-12α expression (B) Heat-inactivated *R. equi*-stimulated BAL cell IL-12α expression with media values as a covariate in the model. (C) Heat-inactivated *R. equi*-stimulated BAL cell IL-12α expression with media and starting values as covariates in the model

Mean +/- SEM. RQs are natural log-transformed (Ln). (A) Non-PPID horses are represented by lines with closed circles. PPID horses are represented by lines with open squares. At Day 0 (Early September 2017), there were 10 horses in the Non-PPID group and 18 horses in the PPID group. By the end of the study, there were 9 horses in the Non-PPID group and 13 horses in the PPID group. (B, C) Non-PPID horses are represented by lines with closed circles. PPID Treatment horses are represented by lines with closed triangles. PPID Control horses are represented by lines with closed squares. At Day 0 (Early September 2017), there were 10, 9, and 9 horses in the Non-PPID, PPID Control, and PPID Treatment groups, respectively. By the end of the study, there were 9, 7, and 6 horses in the Non-PPID, PPID Control, and PPID Treatment groups, respectively. * denotes a significant difference (P<.05) between Non-PPID and PPID Control. # denotes a significant difference (P<.05) between PPID Treatment and PPID Control groups. PPID-pituitary pars intermedia dysfunction; IL-interleukin; *R. equi-Rhodococcus equi*; RQ-relative quantity.

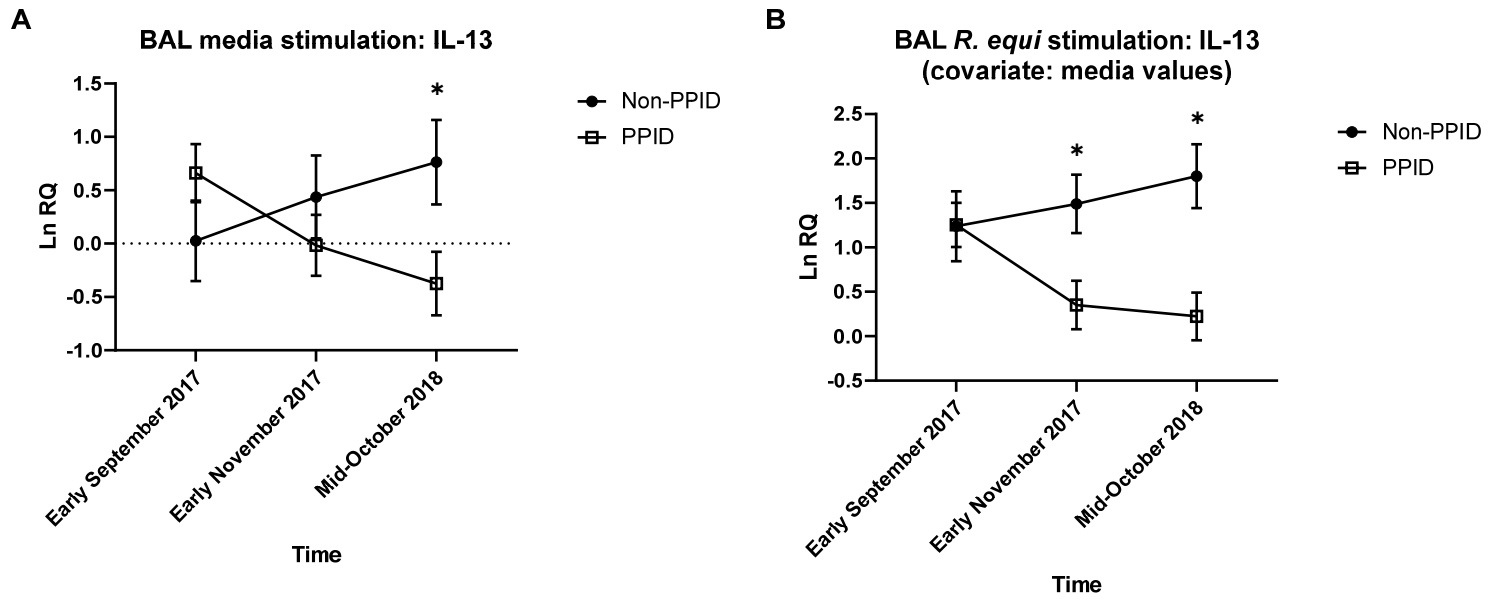

Figure 4.7. (A) Unstimulated bronchoalveolar lavage (BAL) cell IL-13 expression (B) Heat-inactivated *R. equi*-stimulated BAL cell IL-13 expression with media values as a covariate in the model

Mean +/- SEM. RQs are natural log-transformed (Ln). Non-PPID horses are represented by lines with closed circles. PPID horses are represented by lines with open squares. At Day 0 (Early September 2017), there were 10 horses in the Non-PPID group and 18 horses in the PPID group. By the end of the study, there were 9 horses in the Non-PPID group and 13 horses in the PPID group. * denotes a significant difference ($P<.05$) between Non-PPID and PPID groups. PPID-pituitary pars intermedia dysfunction; IL-interleukin; *R. equi-Rhodococcus equi*; RQ-relative quantity.

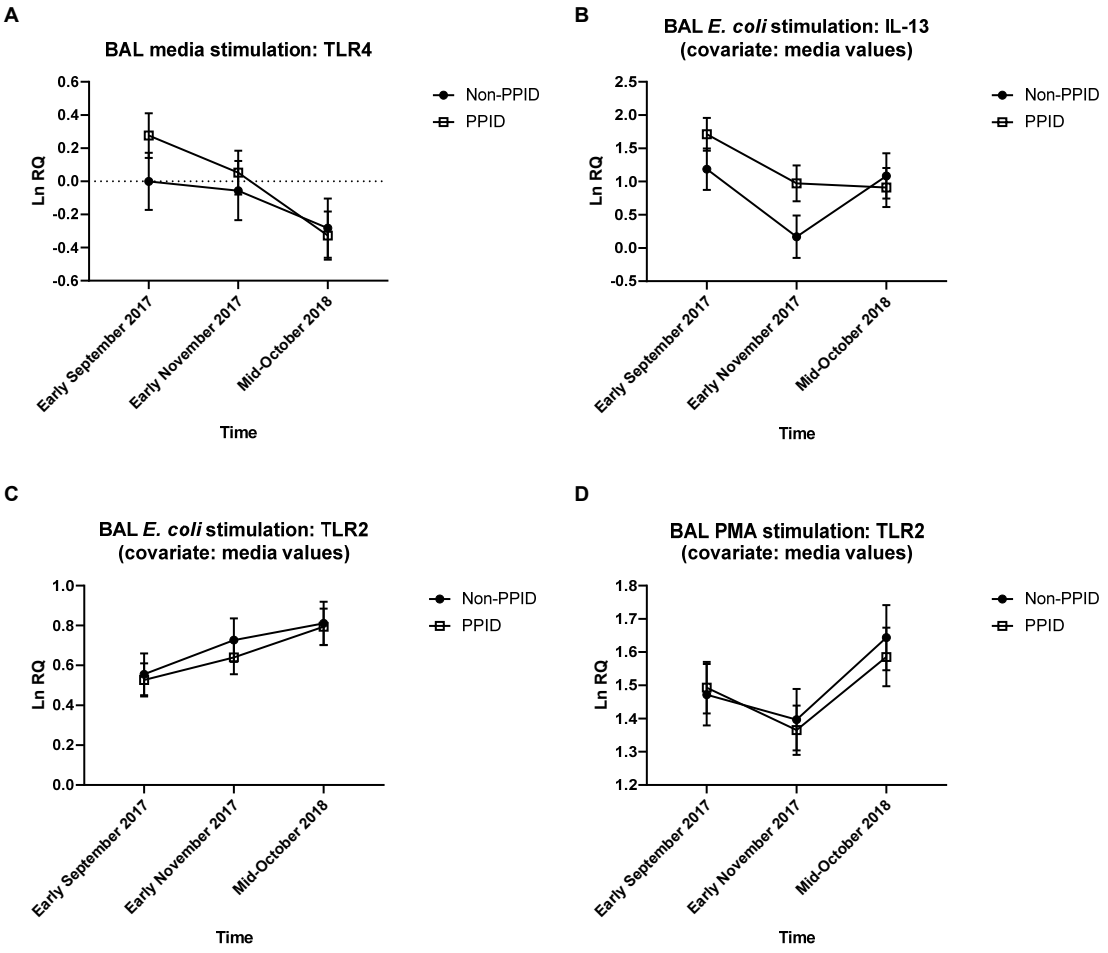

Figure 4.8. Significant time effects for bronchoalveolar lavage (BAL) cell cytokine expression for (A) TLR4 in unstimulated samples, (B) IL-13 in *E. coli*-stimulated samples, (C) TLR2 in *E. coli*-stimulated samples, and (D) TLR2 in PMA/ionomycin-stimulated samples

Mean +/- SEM. (A-C) Non-PPID horses are represented by lines with closed circles. PPID horses are represented by lines with open squares. At Day 0 (Early September 2017), there were 10 horses in the Non-PPID group and 18 horses in the PPID group. By the end of the study, there were 9 horses in the Non-PPID group and 13 horses in the PPID group. These graphs show only the significant time effects ($P<.05$) for the respective analyses. PPID-pituitary pars intermedia dysfunction; TLR-toll-like receptor; *E. coli-Escherichia coli*; IL-interleukin; RQ-relative quantity.

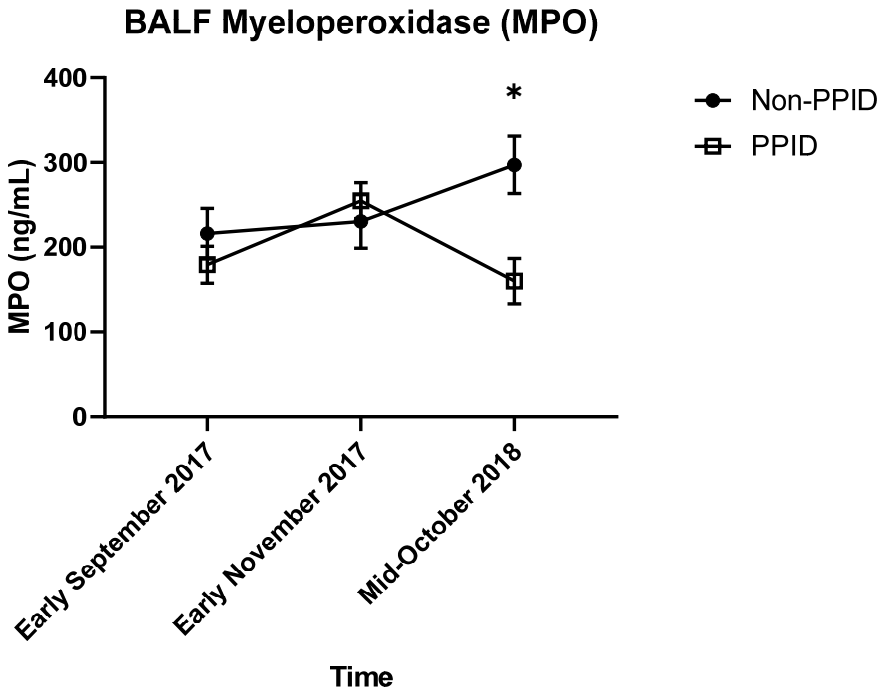

Figure 4.9. Myeloperoxidase concentrations in bronchoalveolar lavage fluid (BALF).

Mean +/- SEM. Non-PPID horses are represented by lines with closed circles. PPID horses are represented by lines with open squares. At Day 0 (Early September 2017), there were 10 horses in the Non-PPID group and 18 horses in the PPID group. By the end of the study, there were 9 horses in the Non-PPID group and 13 horses in the PPID group. * denotes a significant difference ($P<.05$) between Non-PPID and PPID groups. PPID-pituitary pars intermedia dysfunction.

CHAPTER 5. FINAL CONCLUSIONS AND FUTURE RESEARCH

In this study, PPID horses did not differ from age-matched Non-PPID horses in their serum insulin, total cortisol, or free cortisol concentrations. PPID horses had significantly higher resting plasma ACTH concentrations than Non-PPID horses at the beginning of the study. In the PPID Treatment horses, PRASCEND treatment successfully reduced ACTH to the level of a Non-PPID horses within only a few weeks of treatment, despite being in the midst of the seasonal fall rise in ACTH. However, PRASCEND treatment only appeared to influence the ACTH response to TRH stimulation tests at non-fall timepoints. Therefore, the authors conclude that current recommendations for veterinarians to assess resting ACTH concentrations for the purpose of monitoring responses to PRASCEND treatment to be appropriate, even if the original method to diagnose PPID was the TRH stimulation test. Other than significantly reducing ACTH in PPID horses, PRASCEND treatment did not appear to influence other endocrine measures in this study.

While laminitis and associated hyperinsulinemia are often thought to be more common in PPID horses, no differences were seen in basal insulin or in the insulin response to an oral sugar test between PPID and Non-PPID horses in this study. This supports the conclusion that hyperinsulinemia/insulin dysregulation and PPID are distinct endocrine conditions rather than related to one another, although they can both occur simultaneously in the same horse.

In contrast to what is expected in a normal hypothalamus-pituitary-adrenal axis response to increased ACTH concentrations, no differences were seen in total or free cortisol between PPID and Non-PPID horses. Therefore, it is reasonable to conclude that other published works are accurate in their suggestions that the ACTH produced from the

pars intermedia of horses is not capable of stimulating a downstream cortisol response and may not be bioavailable.

The results from this study indicate that PPID horses are likely at risk of opportunistic infection but do not appear to respond differently to strong stimuli, such as PMA/ionomycin. This conclusion is also consistent with work published by McFarlane et al. regarding neutrophils in horses with PPID; they found that neutrophils from horses with PPID responded similarly to Non-PPID horses in response to PMA, but differently when exposed to weaker stimuli [48]. In our work, PPID horses had decreased WBC and absolute lymphocyte counts compared to the Non-PPID horses. Further, PPID horses consistently demonstrated significantly decreased IFNγ production from PBMCs in response to *in vitro* stimulations with *R. equi* and *E. coli*. PPID horses also had consistent and significant increases in TGFβ expression from *E. coli*-stimulated PBMCs and in unstimulated whole blood samples compared to Non-PPID horses. Additionally, in whole blood (Tempus™ Blood RNA tubes) analyses, PPID horses had increased IL-8 expression compared to age-matched Non-PPID horses. In this study, PRASCEND treatment did not appear to affect these immune responses.

It was difficult to draw conclusions from the analyses of localized immune function within the lung due to small sample sizes combined with low BAL cell counts and poor or no amplification of IL-13 for RT-PCR results. Nevertheless, some of the significant results included a lower percentage of eosinophils in BALF in PPID horses compared to Non-PPID horses at the initial starting timepoint (Early September 2017), decreased IL-13 in unstimulated BAL cells and decreased BALF MPO at the Mid-October 2018 timepoint in

PPID horses, as well as decreased IL-13 in the *R. equi*-stimulated BAL cells of PPID horses at the Early November 2017 and Mid-October 2018 timepoints.

Overall, the systemic immune function results are suggestive of a reduced ability of PPID horses to generate a Th1 response *in vitro* to heat-inactivated *R. equi* and *E. coli*, but not to stronger stimuli, such as PMA/ionomycin. Additionally, the TGFβ results indicate that the T_{reg} subpopulation of lymphocytes may be contributing to this inability to evoke a Th1 response. Altogether, this would be consistent with a higher risk of opportunistic infection in PPID horses.

If indeed the differences in the percentage of eosinophils in BALF and MPO in BALF noted in the localized immune function analyses are real, the results point to possible alterations in the Th2 immune response within the lung of PPID horses. This would also be consistent with a higher risk of opportunistic infection in PPID horses, as an altered Th2 response in the lung may predispose a horse to infection, while a Th1 response would be more protective. However, due to the statistical difficulties in interpreting the BAL analyses, this conclusion is mostly speculative at this time.

Given that clear differences in the immune responses of PPID horses were found in this work despite finding no difference in free or total cortisol concentrations compared to Non-PPID horses, the question of what is causing the differences in immune function arises. In other species, ACTH has been shown to have immune-modulating capabilities [119-122]. Therefore, it is certainly possible that despite the ACTH from the pars intermedia appearing to be incapable of eliciting a normal downstream cortisol response, it may still be capable of exerting its immunomodulatory effects. Additionally, it is possible that the excess of other products generated when ACTH is cleaved in the pars intermedia, such as

α-MSH and CLIP, may be contributing to these immunological changes. Treatment with PRASCEND did not appear to influence the measured immune responses though, which would have been expected; this could be an indication that the statistical power was insufficient to detect these changes or that an entirely different mechanism is responsible for altering immune function in PPID horses.

Additional research is needed to elucidate the causes of immune dysfunction in horses with PPID. Ideally, work would continue to analyze individual factors of immune function in PPID horses to determine exactly where the breakdowns in function occur. Furthermore, determining what changes within the endocrine system are responsible for the alterations in immune function may help to develop additional treatment options.

APPENDICES

APPENDIX 1. LIST OF ABBREVIATIONS

ACTH: adrenocorticotropic hormone

α-MSH: alpha-melanocyte-stimulating hormone

BAL: bronchoalveolar lavage

BALF: bronchoalveolar lavage fluid

BCS: body condition score

β-Gus: beta-glucuronidase

CBC: complete blood count

CBG: cortisol-binding globulin

CD: cluster of differentiation

CLIP: corticotropin-like intermediate peptide

CFU: colony-forming units

CNS: cresty neck score

CRH: corticotropin-releasing hormone

DST: dexamethasone suppression test

DMSO: dimethyl sulfoxide

E. coli: *Escherichia coli*

EDTA: ethylenediaminetetraacetic acid

ELISA: enzyme-linked immunosorbent assay

HPA: hypothalamus-pituitary-adrenal

IFNγ: interferon gamma

IL: interleukin

kg: kilogram

Ln- natural-log

LPS: lipopolysaccharide

μg: microgram

μL: microliter

mg: milligram

MPO: myeloperoxidase

OST: oral sugar test

PBMC: peripheral blood mononuclear cell

PBS: phosphate-buffered saline

PC-I: prohormone convertase I

PC-II: prohormone convertase II

PMA: phorbal 12-myristate 13-acetate

POMC: pro-opiomelanocortin

PPID: Pituitary Pars Intermedia Dysfunction

RBC: red blood cell

R. equi: *Rhodococcus equi*

RNA: ribonucleic acid

RQ- relative quantity

RT-PCR: reverse-transcription polymerase chain reaction

TGFβ- transforming growth factor-beta

Th1: CD4 T-cell subset 1

Th2: CD4 T-cell subset 2

Th17: CD4 T-cell subset 17

TLR2: Toll-like receptor 2

TLR4: Toll-like receptor 4

TNFα- tumor necrosis factor alpha

T_{reg}: Regulatory T-cell

TRH: thyrotropin-releasing hormone

VapA: virulence-associated protein A

WBC: white blood cell

APPENDIX 2. SAMPLE TIMELINES

Endocrine function analyses

	Basal ACTH	Basal Insulin	Total Cortisol	Free Cortisol	TRH/OST Testing
Late August 2017					X
Early September 2017	X	X	X	X	
Mid-October 2017	X	X	X	X	
Early November 2017	X	X	X	X	
Early-mid November 2017					X
Early February 2018	X	X	X	X	
Mid-late February 2018					X
Early April 2018	X	X	X	X	X
Late May 2018					
Mid-June 2018	X	X	X	X	
Early August 2018	X	X			
Mid-late August 2018	X	X			
Early September 2018	X	X			
Early-mid September 2018					X
Mid-September 2018	X	X	X	X	
Early October 2018	X	X	X	X	
Mid-October 2018	X	X	X	X	X
Late October 2018	X	X	X	X	
Mid-November 2018	X	X			

Seasonal PPID & treatment responses

Short-term treatment responses

Long-term treatment responses

Systemic immune function analyses

	Complete Blood Counts (CBCs)	Plasma MPO	Whole blood (Tempus™)	PBMCs stimulations	Whole blood stimulations
Early September 2017	X	X	X	X	X
Mid-October 2017	X	X	X	X	X
Early November 2017	X	X	X	X	X
Early February 2018	X	X	X	X	
Early April 2018	X	X	X	X	
Mid-June 2018	X				
Early August 2018	X				
Mid-late August 2018	X				
Mid-October 2018	X	X	X	X	

Seasonal PPID & treatment responses

Short-term treatment responses

Long-term treatment responses

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Localized immune function analyses

		BALF MPO	BALF cytology	BAL cell stimulations	
PPID responses	Early September 2017	X	X	X	Short-term treatment responses
	Early November 2017	X	X	X	
	Mid-October 2018	X	X	X	Long-term treatment response

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October 2016-present	Gamma Sigma Delta Honor Society

Invited Publications

Miller AB, Loynachan AT, Adams AA. 2017. Causes of Mortality in Aged Horses in Kentucky. *Equine Disease Quarterly* 26(2): 3. Lexington, KY: Dept. of Veterinary Science, University of Kentucky.

Extension Publications:

Miller AB, Camargo, FC, Adams AA. 2018. Know the Facts: Equine Cushing's Disease or PPID. University of Kentucky, Lexington, KY.

Conference Proceedings:

Miller AB, Bush HM, Barker VD, Grubbs ST, Adams AA. 2019. Effects of Pituitary Pars Intermedia Dysfunction and Prascend® Treatment on Endocrine and Immune Function. 2019 American College of Veterinary Internal Medicine Forum, Phoenix, AZ.

Macon EL, Harris PA, Barker VD, Elzinga SE, **Miller AB**, Siard MH, Adams AA. 2018. Effects of season on morphometric measurements and insulin responses to the oral sugar test (OST) in control vs insulin dysregulated (ID) horses. American Association of Equine Practitioners' 64th Annual Convention, San Francisco, CA.

Miller AB, Loynachan AT, Adams AA. 2018. Evaluation of myeloperoxidase in the plasma and bronchoalveolar lavage fluid (BALF) of horses with pituitary pars intermedia dysfunction (PPID). 36th Veterinary Comparative Respiratory Society Symposium. Auburn University, Auburn, AL.