THESIS

EVALUATING THE EFFECTIVENESS OF VARYING DOSES OF SUPPLEMENTAL TRYPTOPHAN AS A CALMATIVE IN HORSES

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ABSTRACT

EVALUATING THE EFFECTIVENESS OF VARYING DOSES OF SUPPLEMENTAL TRYPTOPHAN AS A CALMATIVE IN HORSES

Tryptophan (Trp), the amino acid precursor to serotonin, is a common ingredient in many commercial equine calming supplements. However, there is little scientific research to support the efficacy of tryptophan at modifying horse behavior. The objective of this study was to examine how various doses of tryptophan supplementation impacted reactive behavior and physiological stress measurements in the horse. Eleven horses (9 geldings, 2 mares) were given four treatments—0 mg Trp/kg bodyweight (CON), 20 mg Trp/kg bodyweight (LOW), 40 mg Trp/kg bodyweight (MED), and 60 mg Trp/kg bodyweight (HIGH)—in a randomized crossover design. Each treatment lasted three days. On Days 1 and 3 of each treatment, horses underwent a behavior test to measure startle response. Heart rate measurements and the speed at which the horses fled from startling stimuli were recorded. In addition, serum glucose, lactate, and cortisol levels were analyzed both immediately before the startle test and again 15 minutes after the test. Significant sedative effects were seen at LOW Day 1 on heart rate increase during the startle test ($P = 0.05$) and on change in serum lactate levels ($P = 0.03$). At MED Day 1, sedative effects were seen on change in serum cortisol levels ($P = 0.01$). Some excitatory effects were seen at MED Day 3 on the time for heart rate to return to baseline after the startle test ($P = 0.03$). No significant effects were seen at HIGH Day 1 or Day 3. A subset of blood samples was analyzed for serum free Trp and the ratio of Trp to other large neutral amino acids, which verified treatment effect.

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CHAPTER I: LITERATURE REVIEW

INTRODUCTION

This review will begin by covering some of the biochemical properties and pathways of tryptophan. We will discuss the physiology of the blood-brain barrier and how tryptophan is transported into the central nervous system. From here, we will delve into serotonin biosynthesis and metabolism in the brain. We'll cover some of the physiology and effects this neurotransmitter has on the body, particularly on mood. Finally, we'll review the research on supplemental tryptophan in humans and animals, focusing primarily on the horse. The purpose of this literature review is to provide a general understanding of the biochemistry, neurophysiology, and research that creates the platform which the experiment described in Chapter II is based upon.

TRYPTOPHAN

L-Tryptophan (Trp; L-α-aminoindole-3-propionic acid) is the least abundant amino acid found in tissues and food, occurring in approximately 1.4% of protein (Fernstrom and Wurtman, 1974; Voet and Voet, 1995). Tryptophan was first isolated from casein in 1902 (Yao et al., 2011). It is a nonpolar compound with an indole group and is the largest essential amino acid, with a molar mass of 204.225 g/mol. The carboxyl group on tryptophan has a pK_a value of 2.46 and the amine group has a pK_a of 9.41. (Voet and Voet, 1995) Only plants and microorganisms have the enzymes necessary to synthesize tryptophan, making it one of the essential amino acids for monogastric animals and young ruminants, before weaning (Yao et al., 2011).

The reactions in tryptophan synthesis are shown in Figures 1.1 and 1.2. Aromatic amino acid synthesis begins with the same steps: phosphoenolpyruvate (a glycolytic pathway intermediate) and erythrose-4-phosphate (a pentose phosphate pathway intermediate) combine and undergo seven enzyme-catalyzed reactions. The resulting compound, chorismate, can be utilized

to form tryptophan, tyrosine, or phenylalanine. In the biosynthesis of tryptophan, four more enzyme-catalyzed reactions take place to yield indole-3-glycerol phosphate. The last two steps require tryptophan synthase. The alpha unit of this enzyme first cleaves the compound into indole and glyceraldehyde-3-phosphate. The indole is then channeled through a tunnel in the enzyme to the beta subunit, where it is joined with serine to form tryptophan. The channeling step of tryptophan synthase is unique and important because it prevents the nonpolar indole intermediate from escaping the cell via diffusion and from being degraded (Voet and Voet, 1995).

Understanding the pathways of tryptophan degradation (shown in Figure 1.3) and their products is important in identifying how tryptophan is being metabolized and what it is being used for in the body. There are a few notable products of tryptophan degradation. Kynurenine is one such intermediate in tryptophan degradation. It is further degraded in several steps to yield alanine, which may go on to yield pyruvate. One of these steps is catalyzed by kynureninase, an enzyme dependent upon pyridoxal 5'-phosphate (PLP), the active form of vitamin B6. This is notable because, while many reactions involving amino acids are PLP-dependent, the cofactor usually cleaves different bonds than the ones broken in this reaction. PLP is also necessary in tryptophan biosynthesis and it will be an important cofactor in reactions to be discussed later. Quinolinate, which is an NAD^+ and $NADP^+$ precursor in the liver and kidneys, is another tryptophan metabolite. An alternative degradation pathway concludes with reactions identical to those seen in the breakdown of lysine; acetoacetate is the final metabolite (Voet and Voet, 1995).

THE BLOOD-BRAIN BARRIER

While water, carbon dioxide, oxygen, and lipid-soluble compounds pass through plasma membranes and equate between the blood and brain easily, the blood-brain barrier is almost impermeable to larger compounds like plasma proteins. However, there are some areas of the

brain—parts of the hypothalamus, pineal gland, and medulla—where compounds diffuse more easily (Guyton and Hall, 2000). Sites of the blood-brain barrier include the arachnoid membrane, the blood vessels in the subarachnoid space, and the choroid plexus. The arachnoid villus is shown in Figure 1.4. When pressure of the cerebrospinal fluid exceeds that of the sinus blood, the arachnoid villi will open, allowing the bulk movement of metabolic and waste molecules from the CSF into venous sinus blood (Rapoport, 1976). Unlike other capillaries in the body, those found in the brain are connected by tight junctions and, as shown in Figure 1.5, glial cells cover about 85% of the vessel surface (Rapoport, 1976). This serves as a second site of the blood-brain barrier. The choroid plexus is the third site of the blood-brain barrier and is shown in Figure 1.6. The choroid plexus secretes cerebrospinal fluid and is located in the lateral, third, and fourth ventricles (Rapoport, 1976).

Tryptophan, like some other amino acids, binds non-covalently to serum albumin (Yao et al., 2011). Actually, most (80-90%) of circulating tryptophan is bound to albumin (Bosch et al., 2007). Several studies provide evidence that increasing free tryptophan relates to a greater ability of the amino acid to pass the blood brain barrier (Bosch et al., 2007; Davis et al., 2000; Farris et al., 1998; Fernstrom and Wurtman, 1972b; Grimmett and Sillence, 2005). Free tryptophan competes with other large neutral amino acids (LNAA)—including tyrosine, phenylalanine, leucine, isoleucine, and valine—for the same transporter to cross the blood-brain barrier (Fernstrom, 2013). This facilitated transporter is called the L-system. It has little dependence upon pH and no sodium dependence. LNAA, including tryptophan, are transported down their concentration gradients via the L-system (Rapoport, 1976). The concentrations of the amino acids found in plasma are normally much lower than the K_m values and saturation capacity of the transport system (Rapoport, 1976). The facilitated diffusion of tryptophan into the brain is a linear

function of concentration up to many times plasma levels (Rapoport, 1976). Once inside the brain, tryptophan can be used to synthesize serotonin, a biogenic amine with many functions, some of which will be discussed in the following sections.

"An excessive plasma concentration of one of a competing set of amino acids reduces brain uptake of others of the set, thereby modifying synthesis of protein, myelin, and neurotransmitters, and altering cellular respiration and replication" (Rapoport, 1976). A lot of what is known about competition between amino acids for entry into the brain is known because of aminoacidurias, diseases in which an individual lacks critical enzymes necessary in amino acid metabolism. Phenylketonuria, for example, occurs in people with a deficiency of phenylalanine hydroxylase, an enzyme necessary to convert phenylalanine to tyrosine. As a result, phenylalanine and its metabolic byproducts build up in the blood and have neurotoxic effects. All babies born in the United States are now tested for phenylketonuria at birth; if treated with a diet low in phenylalanine and high in the essential amino acids that compete with phenylalanine, detrimental effects such as mental retardation can be prevented. Understanding the etiology of this disease provided researchers with an understanding of the competitive inhibition between amino acids with similar structural and chemical properties for transport across the blood-brain barrier. High blood phenylalanine impairs serotonin synthesis by interfering with tryptophan transport into the brain. Serum serotonin is decreased in phenylketonuric patients and brain serotonin is decreased in phenylalanine-loaded animals. (Rapoport, 1976)

SEROTONIN

Location

Serotonin is an indoleamine found throughout the body and functions in several different systems. Most (80-95%) of serotonin in the body is located within the gastrointestinal tract,

specifically in enterochromaffin cells and in enteric neurons (Kim and Camilleri, 2000). Here, serotonin plays a crucial role in gut secretion, motility, and sensation (Yao et al., 2011). Serotonin is also an important constituent of platelets and contributes to blood coagulation (Kim and Camilleri, 2000). Although serotonin is a hormonally active substance in the blood, it does not have a direct effect on the brain because of the blood-brain barrier (Voet and Voet, 1995). Instead, precursors are transported across the barrier and serotonin is synthesized in the central nervous system, where it influences cognition, sleep, mood, and appetite. Serotonin is also a factor in some neurological conditions including depression, anxiety disorders, schizophrenia, and eating disorders (Yao et al., 2011). Scientists credit the multiple roles of serotonin as a hormone and a neurotransmitter to evolutionary opportunism rather than to related physiological significance (Voet and Voet, 1995).

The cell bodies of serotonergic neurons are located in the dorsal raphe nucleus of the midbrain. However, these neurons have projections extending to many regions of the brain and have different influences on mood and cognition, shown in Figure 1.7.

There are at least thirteen different G protein-coupled receptors that mediate serotonin activity as a neurotransmitter (Hannon and Hoyer, 2008). Some receptors are specific to certain areas of the brain and on the serotonergic neurons themselves. Based on structure, transduction properties, and mode of operation, these receptors are divided into seven types (5-HT_1 through 5-HT_7) (Hannon and Hoyer, 2008). Corr (2006) describes these receptors in more detail: the two main presynaptic receptors are 5-HT_{1A}, which slows neuronal firing, and 5-HT_{1D}, which detects 5-HT in the synapse. When 5-HT_{1D} is occupied, release of 5-HT is inhibited. Postsynaptic receptors include 5-HT_{1A}, 5-HT_{1D}, 5-HT_{2A}, 5-HT_{2C}, 5-HT_3, and 4-HT_4 (Corr, 2006). Serotonergic neurons also contain norepinephrine receptors that modulate 5-HT release (Corr, 2006). In addition,

serotonergic neurons in the dorsal raphe nucleus can be inhibited by GABAergic interneurons from the prefrontal cortex (Robbins, 2005). These examples demonstrate the interconnectedness of neural pathways. Although a lot of research in psychology and pharmacology is devoted to further understanding the complex functions and mechanisms of modification of serotonin receptors, drug effects are oftentimes non-specific and influence more than one transmitter system (Mench and Shea-Moore, 1995). Hannon and Hoyer provide a more detailed review of 5-HT receptors (2008).

Metabolism

Once tryptophan crosses the blood-brain barrier, brain serotonin biosynthesis occurs in a two-step process, shown in Figure 1.8. First, tryptophan is hydroxylated into 5-hydroxytryptophan (5-HTP) by tryptophan hydroxylase. This is the rate-limiting step. Oxygen and tetrahydrobiopterin also form dihydrobiopterin in this first step. Next, 5-HTP decarboxylase, a PLP-dependent enzyme, cleaves the carboxyl group from 5-HTP to produce 5-hydroxytryptamine (5-HT), also known as serotonin (Voet and Voet, 1995). Serotonin biosynthesis rates are about 20 times higher in neuron cell bodies than in terminals (Boadle-Biber, 1993).

In the central nervous system, serotonin is inactivated primarily by reuptake by serotonergic neurons. Highly selective sodium- and chloride-dependent membrane transporters recycle the neurotransmitter back to terminal buttons (Corr, 2006). A lot of research has been dedicated to understanding the serotonin transporter gene (5-HTT), which is involved in serotonin transmission, early brain development, adult neurogenesis, and plasticity (Corr, 2006). Serotonin can also be destroyed by monoamine oxidase and aldehyde dehydrogenase, yielding 5-hydroxyindole acetic acid (5-HIAA) (Kim and Camilleri, 2000). Melatonin is another potential metabolite of serotonin. Melatonin is a hormone primarily produced in the pineal gland but some biosynthesis also occurs in the retina and gastrointestinal tract (Esteban et al., 2004). Melatonin

synthesis increases in the evening and is critical in the maintenance of the circadian clock (Piccione et al., 2005).

Because the hydroxylation of tryptophan is the rate-limiting step in serotonin biosynthesis, an increase in brain tryptophan can potentially double serotonin synthesis (Bosch et al., 2007). Esteban and others (2004) showed that, under normal conditions, the rate-limiting enzyme tryptophan hydroxylase is far from being saturated by its substrate. The availability of cofactors such as magnesium, vitamin B_3, and vitamin B_6 may also play a role in the hydroxylation of tryptophan (Alberghina et al., 2010b).

Two-thirds of the tryptophan available for serotonin biosynthesis comes from intracellular degradation. Diet serves as the secondary source but only 1-2% of dietary tryptophan is converted to serotonin (Yao et al., 2011). While these proportions may seem low, the rate of serotonin synthesis has displayed more sensitivity to its dietary precursor than any other any other neurotransmitter (Fernstrom, 2013). Further evidence suggests that the amount of tryptophan available to the brain may influence serotonin biosynthesis. Studies have shown an increase in the main metabolite of serotonin, 5-hydroxyindoleacetic acid (5-HIAA), as a result of large doses of tryptophan (Fernstrom and Wurtman, 1974). Increasing dietary tryptophan stimulates an increase in brain serotonin synthesis in several species (Adeola and Ball, 1992; Laycock and Ball, 1990; Leathwood, 1987; Shea et al., 1990). Conversely, tryptophan depletion can be achieved by providing a tryptophan-free diet. Because tryptophan depletion creates a significant reduction in brain serotonin synthesis and release, it is a valuable tool used to study the brain serotonergic system and pharmacology effecting that system (Bell et al., 2001; Kantak et al., 1980).

In human medicine, drugs that increase serotonergic activity are used to treat a range of psychological and neurological conditions. These compounds act on various serotonin receptors

to stimulate more neurotransmitter release and/or to inhibit reuptake of the neurotransmitter from the synaptic cleft. Other drugs inhibit monoamine oxidase activity, reducing the rate of serotonin degeneration. Additional treatments directly or indirectly effect dopamine, norepinephrine, and GABA pathways.

The serotonin syndrome is a consequence of excess serotonergic agonism in the central nervous system and has been seen across species, including humans, monkeys, rabbits, mice, and rats (Boyer and Shannon, 2005; Gillman, 1999). Serotonin syndrome has been associated with the use of MAOIs, tricyclic antidepressants, SSRIs, opiate analgesics, anti-migraine drugs, herbal products, and other drugs (Boyer and Shannon, 2005). The severity of the condition ranges from barely perceptible to lethal and presents a spectrum of clinical findings in people. Symptoms include: tremor, hyperreflexia, spontaneous muscle spasms, muscle rigidity, hyperthermia, agitation, sweating, shivering, diarrhea, incoordination, and delirium (Boyer and Shannon, 2005). The combination of serotonergic drugs has induced the rapid onset (minutes to hours) and progression of serotonin syndrome (Gillman, 1999). A single dose of an SSRI has also been observed to cause serotonin syndrome (Boyer and Shannon, 2005).

In the past, serotonin syndrome has been misdiagnosed as neuroleptic malignant syndrome, which presents with many of the same symptoms as serotonin syndrome but is caused by dopamine antagonists instead of serotonergic drugs (Sternbach, 1991). There are only a few distinguishing symptoms between the two conditions; namely that patients presenting with serotonin syndrome typically have hyperactive gut sounds and dilated pupils (Boyer and Shannon, 2005). Nonetheless, patient medication history is extremely important in accurate diagnosis and treatment of serotonin syndrome. In mild to moderate cases, supportive care and cessation of proserotonergic agents is effective at alleviating symptoms associated with the syndrome (Boyer and Shannon, 2005).

Sedation, paralysis, intubation, and treatment with 5-HT_2 blockers may be necessary in life-threatening cases (Boyer and Shannon, 2005; Gillman, 1999). Many cases of serotonin syndrome can be resolved within 24 hours; however, symptoms may persist in patients taking longer acting drugs (Boyer and Shannon, 2005).

FACTORS IMPACTING RATE OF SEROTONIN SYNTHESIS

The rate of brain serotonin synthesis depends on a number of things, including age, sex, breed, social status, level of arousal, and other individual characteristics. External factors such as diet and exercise can also impact the rate of serotonin synthesis.

Age

Some researches have reported that the permeability of the blood-brain barrier decreases with age (Grimmett and Sillence, 2005). In accordance with this line of thought, higher plasma tryptophan and serotonin levels are seen in foals than in adult horses (Ferlazzo et al., 2012). However, one study showed a positive correlation between age and plasma tryptophan throughout the first year in a foal's life (Alberghina et al., 2014). These data suggest that throughout a horse's lifetime, the permeability of the blood-brain barrier stops increasing, stabilizes, and eventually decreases. Farabollini et al. (1988) found that neonatal rats given serotonin antagonists were less anxious and responded more to environmental and social cues as adults. However, brain serotonin levels and turnover are similar in control and animals treated neonatally, suggesting that early effects may change receptor sensitivity (Farabollini et al., 1988).

Breed

While there is some evidence supporting the effect of breed and genetics on tryptophan availability/serotonin biosynthesis, their mechanisms remain unclear. One study noted that Arabian-type horses have higher plasma tryptophan than Anglo-Arabians (Alberghina et al.,

2014). In another study, Bagshaw et al. (1994) found that resting serum serotonin is lower in Arabian mares than in Standardbred mares. However, serum tryptophan concentrations were not significantly different between the two breeds. A separate group of researchers saw lower blood serotonin in Arabian mares compared to Swedish Warmblood mares, even though all horses were fed the same diets and housed in the same conditions (as cited in Grimmett and Sillence, 2005). It is unclear whether breed differences relate to differing absorption, transport, metabolism, and/or excretion of tryptophan. Momozawa and others (2006) investigated polymorphisms in the equine serotonin transporter gene, which controls serotonin reuptake from the synaptic gap. However, of the haplotypes identified, none were associated with anxiety scores determined through caretaker questionnaire.

Sex

Research evaluating gender differences in serotonin biosynthesis in the human brain report conflicting results (as cited in Kim and Camilleri, 2000). However, research in animals tends to suggest that females are more sensitive to dietary changes in tryptophan than males. Dickson and Curzon (1986) reported that female rats that were fed tryptophan were more likely to exhibit side effects liked to serotonin syndrome than their male counterparts. Rouvinen et al. (1999) supplemented silver foxes with tryptophan over the course of several months. While females showed reduced fear and increased exploratory behavior, the same response was not seen in males. Henry and others found that female swine have less hypothalamic serotonin and are more sensitive to dietary changes in amino acid ratio than males (Henry et al., 1992; Henry et al., 1996). Not only do female and male (neutered and intact) pigs have different concentrations of serotonin and 5-HT metabolites in the brain, but the areas that seem to have the most serotonergic activity vary (Henry

et al., 1996). These studies provide evidence that androgens inhibit serotonergic function in male rats and boars.

Other researchers have noted that female rats are more vulnerable than males in models of depression and show less sensitivity to serotonin receptor agonists (Kennett et al., 1986). In addition, female rats seem less sensitive than males to neonatal manipulation with serotonin receptor agonists or antagonists; possibly because of interaction between the developing serotonin system and testosterone (Albonetti et al., 1994).

Social status

Some studies show that subordinate animals have higher levels of serotonin, yet may be less sensitive to tryptophan supplementation than their dominant counterparts (Mench and Shea-Moore, 1995; Raleigh, 1987). A different study showed that dominant males have twice as much blood serotonin than submissive males (Mench and Shea-Moore, 1995). These researchers hypothesized that the dominant animals were metabolizing more dietary tryptophan to serotonin peripherally instead of in the central nervous system.

Level of arousal

Several researchers have provided evidence that serotonin synthesis and precursor sensitivity are greater at higher states of arousal. This is probably because more neurotransmitter is released when the neurons fire more frequently (Young, 1991). A study by Trulson (1979) provides support for this hypothesis, showing dorsal raphe activity in cats increases with state of arousal. In addition, Chamberlain et al. (1987) concluded that altered tryptophan levels in the diet influence aggression in vervet monkeys more reliably at higher levels of arousal. Firing of action potentials may enhance tryptophan uptake into 5-HT neurons via a carrier specific to these neurons and/or tryptophan hydroxylase activity may be enhanced as a result of neuronal firing. In an *in*

vitro experiment, an increase in serotonin synthesis was only observed in the presence of electrical field stimulation, even though available tryptophan was the same in stimulated and unstimulated tissues. However, *in vivo* studies consistently show that administration of exogenous tryptophan enhances 5-HT formation; this may be reflective of the fact that 5-HT neurons fire tonically *in vivo* (as cited in Boadle-Biber, 1993). Additionally, Schaechter and Wurtman (1990) provide evidence that elevating tryptophan levels in the rat hypothalamus proportionally increases 5-HT levels and release; these data support the hypothesis that serotonin release is proportionate to intracellular serotonin levels.

Other Individual Characteristics

There is some evidence in the literature that tryptophan supplementation is most effective in individuals who exhibit dysregulation of behaviors that may be under control of the serotonergic system. For example, Bell and others (2001) performed a study in humans which showed that healthy individuals experienced some mood lowering, memory impairment, and an increase in aggression due to tryptophan depletion. However, patients who had been treated for depression or panic disorder and responded well to antidepressants (particularly serotonergic agents), experienced a relapse. Interestingly, patients with untreated depression experienced no worsening in mood symptoms. These findings suggest that individual serotonergic systems may vary in sensitivity even when factors such as age and sex are held constant. Further support is provided Weld and others (1998), who saw changes in serotonin turnover (evidenced by concentrations of 5-HIAA in cerebral spinal fluid) as a result of tryptophan supplementation only in animals who displayed self-injurious behavior.

Diet

We have already discussed how the amount of tryptophan in the diet has an impact on serotonin biosynthesis. As shown in Figure 1.9, the profile of other nutrients in the diet can influence the amount free tryptophan available for transport transport into the central nervous system and for serotonin synthesis.

The release of insulin triggers the uptake of free amino acids into peripheral tissues such as muscle (not the brain). However, because most tryptophan is bound to albumin, the rate of absorption into peripheral tissues is slower. As a result, the ratio Trp:LNAA increases, favoring tryptophan uptake by the brain and, furthermore, serotonin synthesis (Bellisle et al., 1998). Fernstrom and Wurtman (1972b) conducted a study in which insulin was administered to fasted rats. Surprisingly, this increased total plasma tryptophan up to 40% while also decreasing the concentration of other LNAA in the plasma. Two hours after receiving the insulin, brain tryptophan levels were elevated by 36% and brain serotonin levels were elevated by 28%. Research by Noble and others (2007) provides further supporting evidence in the role of insulin in serotonin biosynthesis by showing that plasma tryptophan follows the glycemic response after meal-feeding Thoroughbred horses. Additionally, the Trp:LNAA ratio peaked after horses were fed a "starch and sugar" meal but stayed relatively constant in horses fed a "fat and fiber" meal (Wilson et al., 2007). There is some discrepancy in the literature, however; Alberghina and others (2010b) found that horses kept on a high fiber diet had higher plasma serotonin and tryptophan levels than horses fed a high starch diet. The conflicting results of this study may be because blood samples were taken three and six hours after feeding—at least an hour after the spikes in insulin, serotonin, and tryptophan were reported in previous studies. Also, Kim and Camilleri (2000) claim that plasma measurements of serotonin are often inaccurate, since serotonin is easily released with the agitation

and lysis of platelets. Wurtman (2011) draws the association that "carbohydrate cravers" may be unknowingly attempting to increase serotonin synthesis using this mechanism in order to compensate for disorders linked with low serotonin.

High fat diets (or lipolysis) may also increase the amount of free tryptophan available to the central nervous system, as non-esterified fatty acids displace tryptophan from its binding site on albumin (Bosch et al., 2007). High protein diets increase plasma tryptophan levels but, because of the corresponding increase in competing LNAA, brain tryptophan and serotonin do not increase. Removing the competing amino acids from the diet has been shown to increase tryptophan and serotonin (Fernstrom and Wurtman, 1972a).

Exercise

There is evidence that exercise increases free tryptophan, the tryptophan to LNAA ratio, and brain serotonin (as cited in Bruschetta et al., 2013; Farris et al., 1998). Exercise may increase free Trp:LNAA by releasing free fatty acids, which displace tryptophan from its binding site on albumin (Grimmett and Sillence, 2005). Additionally, multiple studies have looked at the effects of tryptophan and/or serotonin in central fatigue during exercise but the results are conflicting and range from a negative correlation between serotonin and exercise endurance to the absence of an effect between tryptophan supplementation and time until exercise fatigue (Alberghina et al., 2010a; Bruschetta et al., 2013; Farris et al., 1998; Piccione et al., 2005; Vervuert et al., 2005). Some researchers believe that exercise releases peripheral serotonin from platelets, which is transported in the plasma to neurons or vascular endothelial cells (Alberghina et al., 2010a; Bruschetta et al., 2013). Whether it be through serotonin release from platelets, decreased motivation, or increased survival methods, the mechanisms by which serotonin impacts exercise endurance have yet to be elucidated.

TRYPTOPHAN SUPPLEMENTATION IN HORSES

Research in cattle, poultry, swine, dogs, foxes, humans, mice, fish, and monkeys have shown sedative effects, including decreases in aggression, fear, stress, depression, stereotypic behavior, and/or overall activity level, as a result of various doses of tryptophan (as cited in Grimmett and Sillence, 2005). For a review of these studies, see Table 1.1. However, other than slight effects seen in one study, no research has been able to show a behavioral effect in horses. This suggests that there may be a potential difference in tryptophan doses required to achieve specific behavioral effects in horses.

Bagshaw et al. (1994) conducted the first study assessing the behavioral effects of tryptophan supplementation in horses. However, the doses given to horses were less than 1% of the average amount of tryptophan administered using the commercial supplements available today. These researchers actually found that tryptophan supplementation corresponded with a significant increase in activity both when horses were isolated and when they had visual contact with other horses. However, treatment horses did show some significant decreases in heart rate throughout the behavioral tests and stereotypic behavior in one horse was reduced.

Grimmett and Sillence published a review in 2005 summarizing the current research and evaluating areas of future research in the use of tryptophan as a calmative in horses. Since this time, there has been very little research in supplementing tryptophan to equines. Malmkvist and Christensen (2007) supplemented young horses with a commercial tryptophan product but found no significant differences between treatment and control groups with respect to mean heart rate or behavioral observations taken during a voluntary approach test and handling test. Another study by Noble et al. (2008) looked at the effects of supplementing horses with a commercial dose of L-tryptophan on plasma tryptophan levels and response to approaching a novel object/person. Results

showed that plasma tryptophan levels increased three-fold as a result of supplementation. The ratio of tryptophan to other large neutral amino acids also increased. However, no significant behavioral effects were noted.

Paradis et al. (1991) evaluated toxicity effects of tryptophan supplementation in horses, either via oral administration or intravenous infusion. They found that plasma tryptophan concentrations peak after dosing and return to pre-dosing levels within 48 hours. These researchers also demonstrated that orally administered tryptophan can be metabolized into 3-methylindole or, more commonly, indole. Both of these compounds may have toxic effects, causing hemolytic anemia and/or respiratory distress. These consequences were seen in one of the four ponies receiving 350 mg tryptophan/kg bodyweight and in three of the four ponies receiving 600 mg tryptophan/kg bodyweight.

It is clear from the evidence, or lack thereof, provided by previous research that the safety and efficacy of using tryptophan as a calmative in horses warrants further investigation. However, supplement companies continue to market products containing tryptophan to horse owners, insuring that the ingredient is safe and effective for use in equines. For a review of these products, see Table 1.2. Horse owners find these supplements appealing because they do not require veterinary oversight yet promise a safer, more relaxed, and easy-to-ride horse.

TABLE 1.1: Review of research supplementing tryptophan to various species

Author	Species	Treatments	Supplement Schedule	Results
Young et al., 1986	Human males	~129 mg/kg	1x	increase self-reported depression symptoms
Liebermann et al., 1986	Humans	50 mg/kg	1x	sedative effect
Nakanishi et al., 1997	Calves	160 mg/kg	7d	calves supplemented with tryptophan showed increased lying time and decreased exploratory behavior in the two-weeks post weaning; feed utilization was not different between treatment and control calves.
Laycock & Ball, 1990	Chickens	5000 mg/kg	6d	hysteria episodes decreased; feed consumption increased, egg laying increased; plasma Trp increased
Shea et al., 1990	Male broiler breeders	control, 0.38%, 0.75%, 1.5%	4 - 20 weeks of age	Birds fed supplemental tryptophan showed less aggressive pecking; low levels (0.38%) were as effective as high levels (1.5%)
DeNapoli et al., 2000	Dogs	Low protein, low Trp; low protein, high Trp; high protein, low Trp; high protein, high Trp	daily, 7d	No differences in fearfulness between diets; dogs on high protein diet supplemented with Trp showed less dominance aggression than dogs feed a high protein diet without Trp; dogs on low protein diet with supplemented Trp showed less territorial aggression than dogs fed a low protein diet without supplemental Trp
Paradis et al., 1991	Horses	350 mg/kg	1x, feed withheld for 24hrs prior to admin	Four ponies developed hemoglobinuric nephrosis and bronchiolar degeneration; one pony also developed hemolysis, hemoglobinuria, and increased respiratory rate
Paradis et al., 1991	Horses	100 mg/kg IV	1x, feed withheld for 24hrs prior to admin	Ponies did not show any pathology due to Trp supplementation

Paradis et al., 1991	Horses	600 mg/kg	1x, feed withheld for 24hrs prior to admin	Four out of five ponies showed restlessness, increased respiratory rate, hemolysis, and hemoglobinuria
Bagshaw et al., 1994	Horses	0, 0.05, & 0.1 mg/kg	1x, 2h prior to testing	Horses dosed with 0.1 mg/kg had higher rates of walking/sniffing and heart rate during isolation compared to horses dosed with 0 mg/kg; one horse showed a reduction in sterotypic head twisting after being dosed
Noble et al., 2008	Horses	12.5 mg/kg	1x	plasma Trp increased, with peak 1.5-2 hours post-dosing; supplement made no difference in behavior around novel object/person
Malmkvist & Christensen, 2007	Horses	12.9 mg/kg	1x, 2-3h prior to test	No difference in heart rate or novel stimulus approach test
Farris et al., 1998	Horses	100 mg/kg IV: glucose with Trp; no glucose with Trp; glucose without Trp; no glucose, no Trp	1x	Horses dosed with tryptophan immediately before exercise and either glucose or a placebo during exercise had a lower mean time to exhaustion than horses dosed with two rounds of placebos; these horses also had higher plasma prolactin.
O'Reilly, 2006 (Thesis)	Horses	3g 5x/day	21d	Tryptophan had no effect on cribbing behavior
Adeola & Ball, 1992	Pigs	0, 5, 10, 25 g Trp/kg diet	5d	doubled plasma Trp; pigs showing higher stress pre-slaughter had lower hypothalamic serotonin content; ; reduced PSE score; hypothalamic serotonin concentration increased with Trp supplementation but the increase peaks at day 5 and then decreases: researchers suspect an adaptive response
Shen, et al., 2015	Pigs	0.0% & 0.8%	12d	Piglets fed diets with 0.8% Trp had a higher average daily gain and feed efficiency and lower salivary cortisol concentrations after a stressful situation

Shen et al., 2015	Pigs	0.0%, 0.8%, & 0.7% (with the same amino acid ratios as 0.8%)	16d	Pigs fed diets supplemented with Trp had better feed efficiency
Koopmans et al., 2005	Pigs	diets with high and normal Trp:LNAA	7d and 12d	Pigs at the higher level of supplementation showed higher plasma Trp, lower basal plasma cortisol, lower basal plasma noradrenaline, but no difference in plasma adrenaline concentrations compared to controls; during social stress, pigs on the higher tryptophan diet showed less avoidance behavior, but similar amounts of physical activity and aggression; post-stress plasma cortisol, noradrenaline, and adrenaline were less in pigs provided supplement.
Peeters et al., 2004	Pigs	5 g/L in drinking water	3d	Pigs that received tryptophan spent significantly more time lying down during simulated transport
Li et al., 2006	Pigs	Control, 2x recommendation, 4x recommendation	7d, 3d, 3d	Trp-supplemented pigs spent more time laying, less time eating, and less time fighting
Poletto et al., 2014	Pigs (gestating sows)	~66 mg/kg	7d	Trp-supplemented sows showed less aggression and increased exploratory behavior when mixed with other sows
Martinez-Trejo et al., 2009	Weaned piglets	0.23%, 0.27%, 0.31%, 0.35%	3d before and after weaning (6d total)	Highest levels of tryptophan supplementation show less appendage biting and aggression than the two lowest
Hilakivi-Clarke et al., 1990	Male mice	50, 75, 100, 125, 200 mg/kg intraperitoneal	1x, 60 min prior to test	Antidepressant-like effects in Porsolt's Swim Test At 125 and 200 mg/kg injection, response was no different than control
Janczak et al., 2001	Mice	2.08 g/L in drinking water	dialy for 2 weeks before behavior test	Mice treated with tryptophan showed reduced exploratory behavior in resident-intruder test, number of fights, and time spent fighting

Schaechter & Wurtman, 1990	Rat Hypothalamic Slices	2 μM Tryptophan	superfused over 130 min	Superfusing hypothamalic slices with medium containing tryptophan increases total serotonin release by 115.0 ± 5.9%. Superfusing tryptophan in the medium also increases electrically-stimulated total serotonin release by 125.9 ± 3.9%. In slices with reduced tryptophan levels (due to leucine supplemented medium), total serotonin release decreased by 88.7 ± 2.0%.
Esteban et al., 2004	Rats	300 mg	5d	Rats that received tryptophan during the day (8:00), there was an increase in 5-HT and 5-HIAA in the brain; rats that received tryptophan at night (20:00), the 5-HT/5-HIAA did not change but there was a significant increase in circulating melatonin
Basic et al., 2013	Salmon	Control diet, 2x Trp, 3x Trp, 4x Trp	7d	Trp supplemented fish had lower basal levels of cortisol 1 and 10 days after supplementation stopped
Rouvinen et al., 1999	Silver Foxes	1.2 g/MJ ME	4 months	Supplementing Trp increased exploratory behavior in females; Trp did not have a significant effect on fur growth, fur quality, or weight gain
Raleigh, 1987	Vervet monkeys	10, 20, 40 mg/kg	6d	Monkeys showed dose-dependent increases in eating, and decreases in locomotion, vigilance, and aggression
Chamberlain et al., 1986	Vervet monkeys	diets: balanced, Trp-free diet, Trp-supplemented	1x; observed 5 hours after	Male and females on Trp-supplemented diet showed reduced competitive aggression; when males were given a mixture containing no Trp, spontaneous and competitive aggression increased
Weld et al., 1998	Rhesues monkeys (male)	100 mg/kg, twice per day	21d	In monkeys with a history of self-injurious behavior, the supplement reduced the behavior and increased serotonin turnover (measured by concentrations of 5-HIAA in CSF). Serotonin metabolism and behavior were not affected in monkeys with no history of self-injurious behavior.

TABLE 1.2: Summary of paste supplements containing tryptophan available to horse owners in the United States

Product	Dosing Directions	Dose (mg Trp/ kg BW)*	Other Ingredients
Divine Equine (Oralx Corp., Ogden, UT)	administer 4 hours before stressful event	3.4	valerian root, black cohash, passion flower, ginger root, hops, wood betony, cherry extract for flavoring, benzol alcohol .05%, sorbic acid as a preservative, xanthan gum
SmartCalm Ultra Paste (SmartPak Equine, Plymouth, MA)	administer 2-4 hours before stressful event	2	active: magnesium, taurine, inositol, thiamine, vitamin e inactive: ascorbyl palmitate, artificial flavor, citric acid, coconut oil, methylparaben, silicon dioxide, soy lecithin, vegetable oil (cold pressed), vitamin e supplement
B-Kalm (Farnam Companies, Inc., Phoenix, AZ)	administer 1.5-2 hours prior to exercise	20	inactive ingredients: dextrose, ethyl alcohol, ground limestone, potassium sorbate, sodium bentonite, sodium benzoate, sodium saccharin, thiamine hydrochloride with artificial flavor and color, water, and xanthan gum
Vision (VitaFlex Nutrition, Council Bluffs, IA)	administer 2 hours before competition/race/trailering	5	active: thiamine, inositol, riboflavin, magnesium, vitamin b6, valerian root extract inactive: artificial flavoring, corn starch, glucose, glycerin, maltodextrins, methylparaben, propylparaben, silicon dioxide, sorbic acid, sorbitol, sucrose, water
Tryptoplex (Oralx Corp., Ogden, UT)	administer 2 hours prior to event	3.4	water, magnesium amino acid hydrochloride, pyridoxine hydrochloride, ginger glycyrrhiza, juniper berries, cherry extract for flavoring, benzoic acid (a preservative) sorbic acid (a preservative) xanthan gum
Calmex-V (Med-Vet Pharmaceuticals, Eden Prarie, MN)	2-4 hours prior to trailering/riding when needed; may be given 24 and 48 hours prior to trailering/riding	1.5	active: valerian root, thiamine, taurine, inositol inactive: distilled water, glycerin, sodium propionate, xanthan gum
Easy Going (ProFormula Laboratories, Inc., Ft. Lauderdale, FL)	administer 3 hours prior to event	8.08	valerian root, passion flower, kava kava, ginger root, hops, wood betony, ethyl alcohol, aloe vera gel, .05% potassium sorbate as a persvative, acacia gum
Quietex II (Farnam Companies, Inc., Phoenix, AZ)	administer 2 hours before training or competition	5	active: thiamine, inositol, magnesium, vitamin b6, valerian root extract inactive: corn starch, glucose, glycerin, malt syrup, maltodextrins, methylparaben, propylparaben, silicon dioxide, sorbic acid, sorbitol, sucrose, water
EQUI+Calm (Equine Healthcare International, Aberdeen, NC)	administer once the night before and once the morning of performance; additional tubes can be administered at 6-12 hour intervals	not quantified	active: magnesium, melatonin, arginine, leucine, theanine, thiamine, phenylalanine, bismuth inactive: maple flavor, praline flavor, deionized water, vitamin c, glycerine, xanthan gum, methyl paraben, propyl paraben, acesulfame k sweetner
Perfect Prep EQ Extreme Paste (Perfect Products, LLC, Morrow, OH)	feed 90 minutes before increased stress; effects begin within 1 hour and last 6-8 hours	not quantified	active: magnesium, inositol, thiamine proprietary blend: soybean oil, magnesium amino acid chelate, thiamine mononitrate, soy lecithin, inositol, silica gel, vitamin e supplment, citric acid, natural and artificial flavors, polysorbate 80, ascorbyl palmitate, methylparaben, coconut oil

Perfect Prep EQ Supreme Paste (Perfect Products, LLC, Morrow, OH)	administer 90 minutes prior to activity; adjust for desired results; effects begin 1 hour after administration and can last up to 6 hours	not quantified	active: magnesium, inositol, thiamine, l tryptophan, vitamin "behave" (a proprietary blend of b vitamins) proprietary blend: soybean oil, magnesium amino acid chelate, thiamine mononitrate, soy lecithin, inositol, silica gel, vitamin e supplement, citric acid, natural & artificial flavors, pyrodoxine hcl, riboflavin, ascorbyl palmitate, methylparaben, coconut oil
Oxy-Calm (Meal and More, Inc., Morrice, MI)	feed 2 hours before event; can be given again 4 hours later	not quantified	vitamin e supplement, sugars, salt, flavorings, vegetable oil, tryptophan, thiamine mononitrate, guar gum, xanthan gum, wheat germ oil, dried active yeast, lactobacillus acidophilus fermentation product
At-Ease Megadose (Richdel, Inc., Carson City, NV)	administer one dose 1-3 hours before and one dose immediately before desired event	2	soy oil, magnesium oxide, corn starch, thiamine mononitrate, salt, dextrose, pyridoxine hydrochloride, methyl & propyl paraben (a preservative), silicon dioxide, artificial apple flavoring
Calming Oral Gel (Kaeco Group Inc., Savannah, MO)	administer 2-4 hours prior to competition, event, transporting, etc.	3.4	valerian root, black cohosh, passion flower, ginger root, hops, wood betony, apple flavor, benzyl alcohol .05%, sorbic acid as a preservative, xanthan gum
Formula Calm B (dac, Dover, Ohio)	give one to three times daily before and during events	0.5	magnesium sulfate, taurine, thiamine mononitrate, inositol, gylcerin, silica gel, soybean oil, coconut oil, natural & artificial flavors
Tryptophan Plus Gel (Horses Prefer, Menomonie, WI)	feed 1.5 to 2 hours prior to competition, strenuous exercise, racing, or shipping	not quantified	dextrose, cane molasses, propylene glycol, silicon dioxide, polysorbate 80, niacinamide, pork peptone, calcium chloride, magnesium oxide, potassium chloride, thiamine hydrochloride, pyridoxine hydrochloride, riboflavin, lactic acid, apple flavor, methylparaben, propylparaben, and ethoxyquin

*calculations based on 500 kg horse

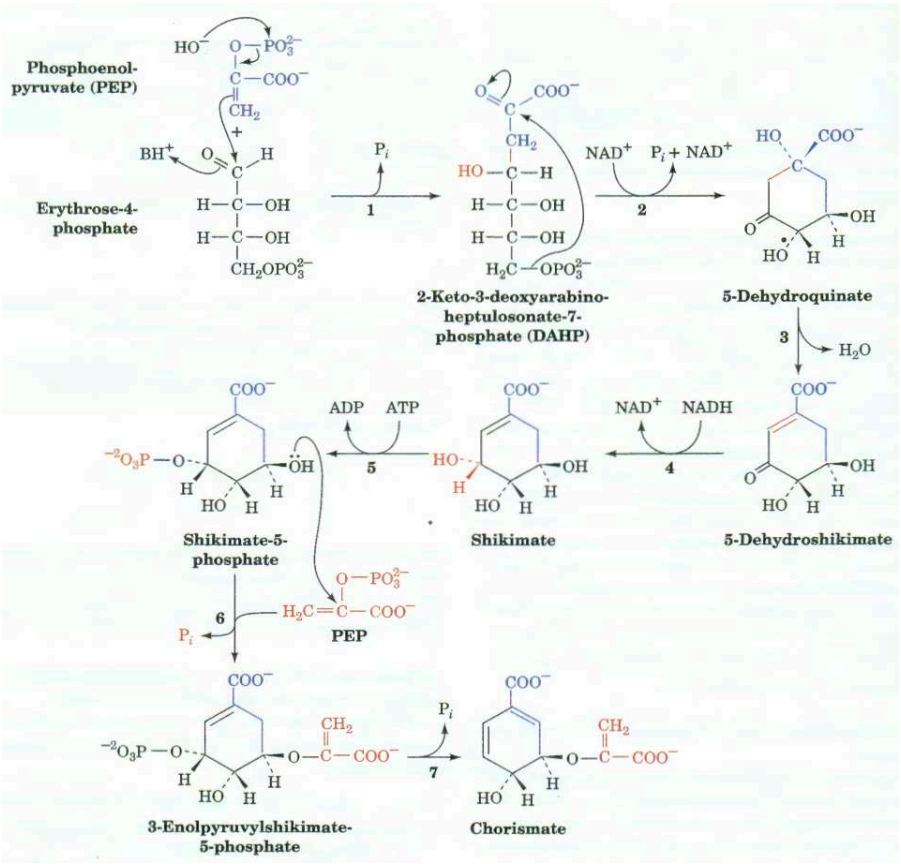

Fig. 1.1: The biosynthesis of chorismate. Pathway enzymes: (1) 2-keto-3-deoxy-D-arabinoheptulosonate-7phosphate synthase, (2) dehydroquinate synthase, (3) 5-dehydroquinate dehydratase, (4) shikimate dehydrogenase, (5) shikimate kinase, (6) 3-enoylpyruvylshikimate-5-phosphate synthase, (7) chorismate synthase. Reprinted from "Biochemistry" (p. 774), by D. Voet and J.G. Voet, 1995, New York: John Wiley & Sons, Inc. Copyright 1995 by John Wiley & Sons, Inc.. Reproduced with permission.

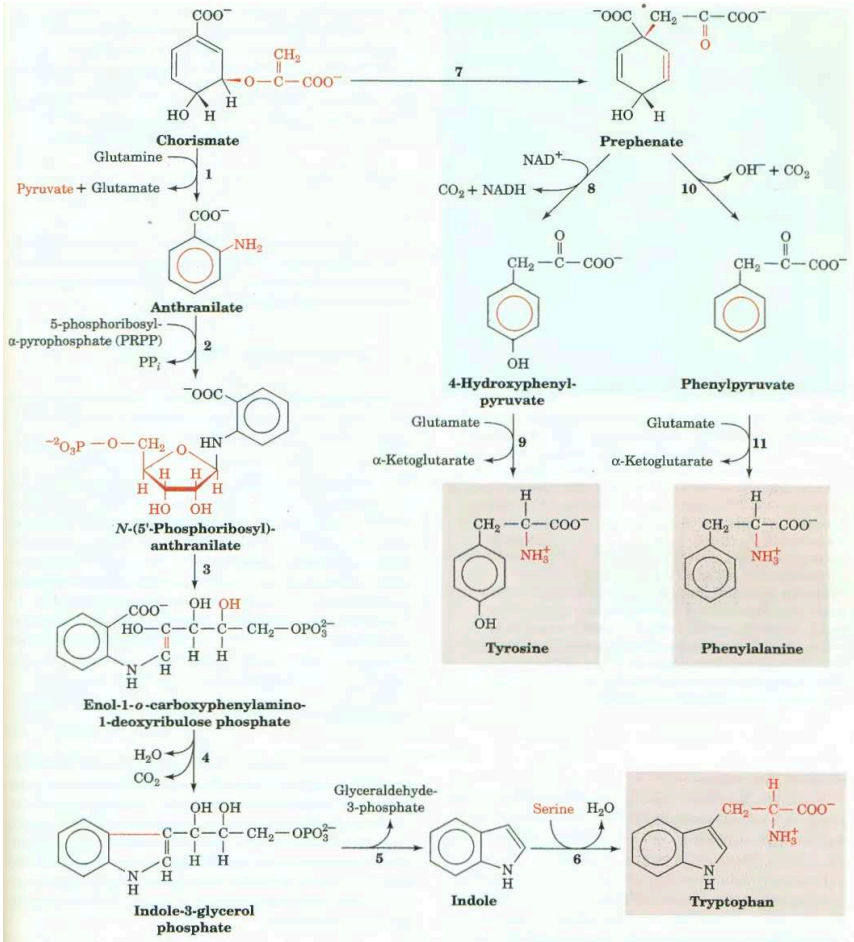

Fig. 1.2: Pathways for the synthesis of tryptophan. Pathway enzymes: (1) anthranilate synthase, (2) anthranilate-phosphoribosyl transferase, (3) N-(5'-phosphoribosyl)-antranilate isomerase, (4) indole-3-glycerol phosphate synthase, (5) tryptophan synthase, α subunit, (6) tryptophan synthase, β subunit. Reprinted from "Biochemistry" (p. 775), by D. Voet and J.G. Voet, 1995, New York: John Wiley & Sons, Inc. Copyright 1995 by John Wiley & Sons, Inc.. Reproduced with permission.

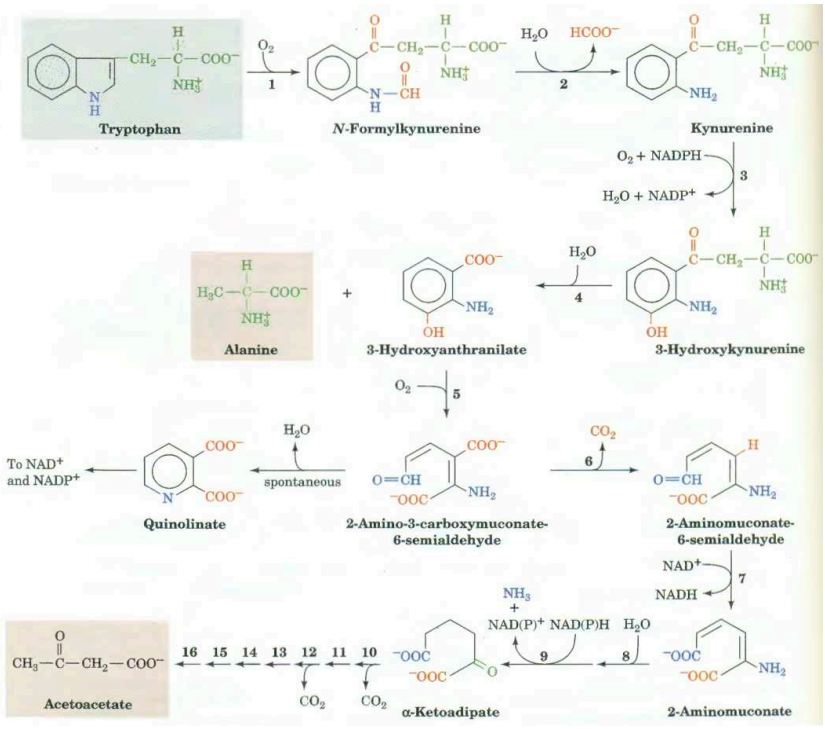

Fig. 1.3: Pathways of tryptophan degradation. Enzymes: (1) tryptophan-2,3-dioxygenase, (2) formamidase, (3) kynurenine-3-monooxygenase, (4) kynureninase, PLP dependent, (5) 3-hydroxyanthranilate-3,4,-dioxygenase, (6) amino caroxymuconate semialdehyde decarboxylase, (7) aminomuconate semialdehyde dehydrogenase, (8) hydratase, (9) dehydrogenase, (10) α-keto acid dehydrogenase, (11) glutaryl-CoA dehydrogenase, (12) decarboxylase, (13) enoyl-CoA hydratase, (14) β-hydroxyacyl-CoA dehydrogenase, (15) HMG-CoA synthase, (16) HMG-CoA lyase. Reprinted from "Biochemistry" (p. 744), by D. Voet and J.G. Voet, 1995, New York: John Wiley & Sons, Inc. Copyright 1995 by John Wiley & Sons, Inc.. Reproduced with permission.

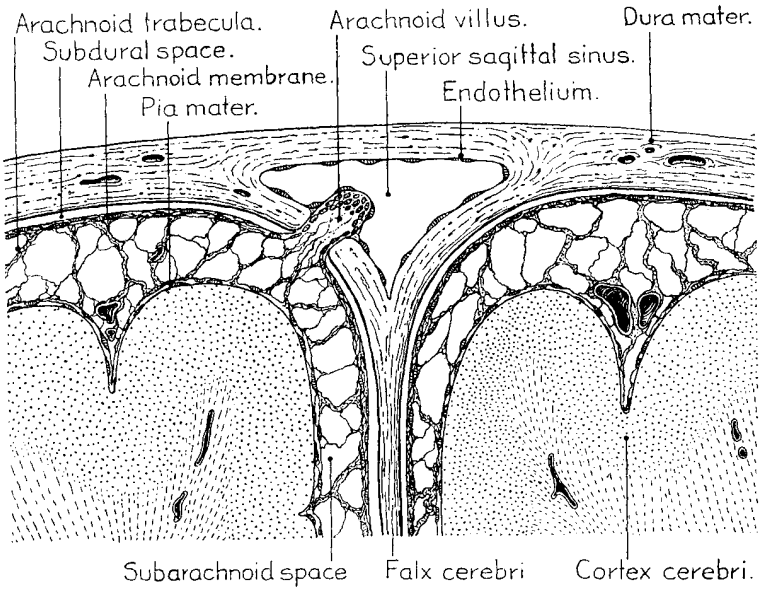

Fig. 1.4: The subarachnoid space. When there is no difference in pressure between the subarachnoid space and the venous blood, there are numerous microvilli made up of layers of overlapping endothelial cells on the arachnoid membrane. Reprinted from "The absorption of cerebrospinal fluid into the venous system," by L.W. Weed, 1923, American Journal of Anatomy, 31(3), 202. Copyright 1923 by Wiley-Liss. Reproduced with permission.

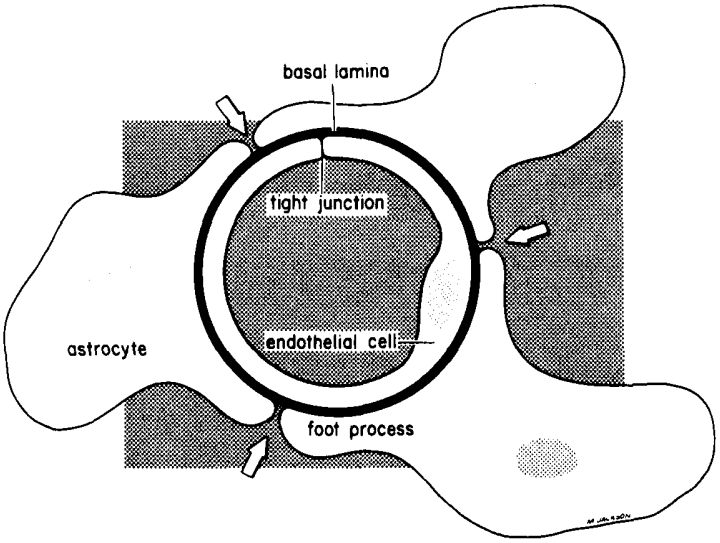

Fig. 1.5: Brain capillary-glial cell junction. The endothelial cells in the brain capillaries are closely associated with astrocytes. Interstitial fluid has access at the locations indicated by the arrows. Reprinted from "Endothelial cell-astrocyte interactions: a cellular model of the blood-brain barrier," by G.W. Goldstein, 1988, Annals of the New York Academy of Sciences, 529(1), 32. Copyright 2006 by John Wiley and Sons. Reproduced with permission.

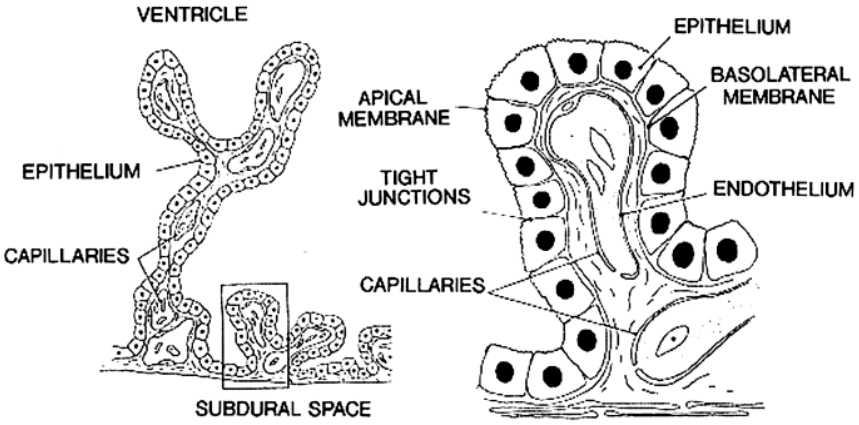

Fig. 1.6: The choroid plexus. The basal lamina of the choroid plexus faces highly vascularized connective tissue. There are microvilli on the apical surface, which face into the cerebrospinal fluid; tight junctions regulate what compounds diffuse from the capillaries into the cerebrospinal fluid. Reprinted from "Mechanisms of CSF secretion by the choroid plexus" by T. Speake, C. Whitwell, H. Kajita, A. Majid, and P.D. Brown, 2001, Microscopy Research and Technique, 52(1), 50. Copyright 2001 by Wiley-Liss. Reproduced with permission.

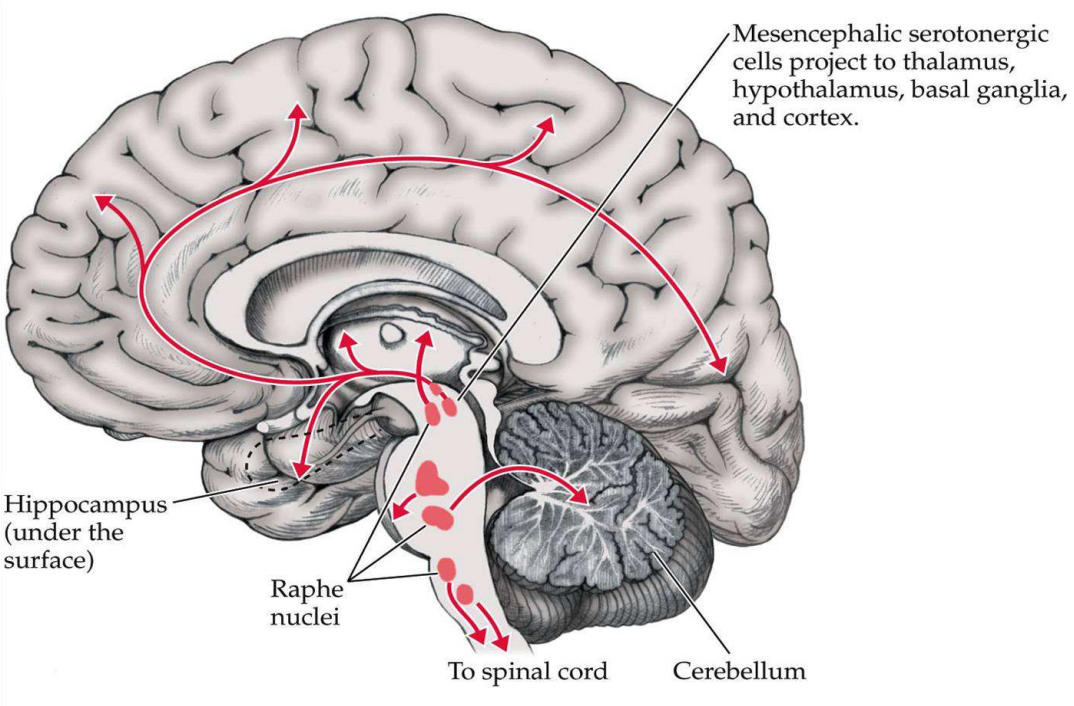

Fig. 1.7: Projections of serotonergic neurons. These neural pathways are involved in various aspects of cognition and behavior: prefrontal cortex (mood), basal ganglia (movement, potentially obsessions and compulsions), limbic area (anxiety and panic), and hypothalamus (appetite and eating behavior) (Corr, 2006). Reprinted from "Biological Psychology: An Introduction to Behavioral, Cognitive, and Clinical Neuroscience" (p. 93), by S.M. Breedlove, N.V. Watson, and M.R. Rosenzweig, 2010, Sunderland, MA: Sinauer Associates. Copyright 2010 by Sinauer Associates, Inc.. Reproduced with permission.

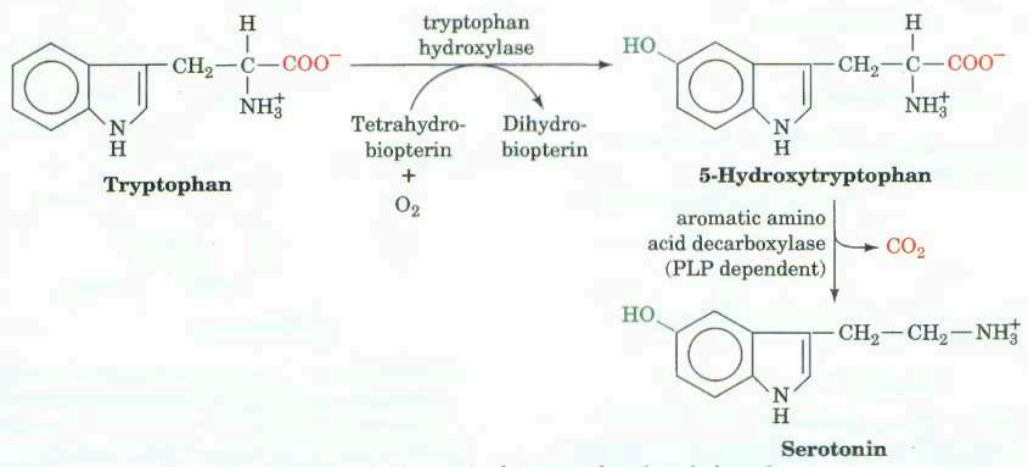

Fig. 1.8: Steps in serotonin biosynthesis. Reprinted from "Biochemistry" (p. 759), by D. Voet and J.G. Voet, 1995, New York: John Wiley & Sons, Inc. Copyright 1995 by John Wiley & Sons, Inc.. Reproduced with permission.

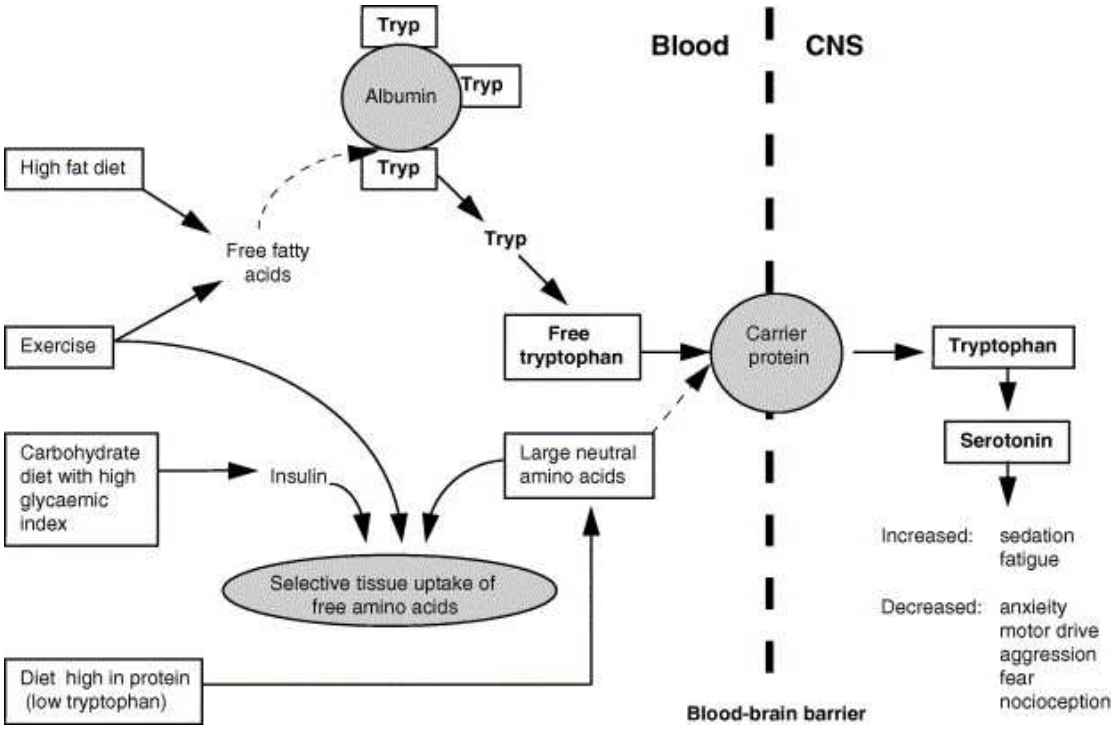

Fig. 1.9: Exercise and diet composition impact the rate of serotonin biosynthesis. Reprinted from "Calmatives for the excitable horse: A review of L-tryptophan," by A. Grimmett and M.N. Sillence, 2005, The Veterinary Journal, 170(1), 26. Copyright 2004 Elsevier Ltd.. Reproduced with permission.

CHAPTER II: EVALUATING THE EFFECTIVENESS OF VARYING DOSES OF SUPPLEMENTAL TRYPTOPHAN AS A CALMATIVE IN HORSES

INTRODUCTION

Tryptophan is often marketed in the horse industry as a calmative supplement, claiming to encourage focus and relaxation while reducing tense, nervous, and spooky behavior. Horse owners frequently utilize calming products in situations including transportation, competition, and other novel training events. Tryptophan is the amino acid precursor to serotonin, a neurotransmitter involved in mood, appetite, sleep, memory, and learning in many species, including humans. Psychiatrists utilize serotonin-enhancing drugs in people to treat conditions such as depression, anxiety, and obsessive-compulsive disorder. Tryptophan supplementation has been shown to increase serotonin production (Fernstrom, 2013). However, while supplementation has been linked to reduced aggression and fearfulness in species other than the horse, there is no current research within the scientific community to support any behavioral effects of tryptophan supplementation in horses (Grimmett and Sillence, 2005). The use of this supplement for calming purposes in horses remains unproven and somewhat controversial among horse owners and professionals. The objective of the following study is to evaluate behavioral and physiological responses resulting from differing dosages of L-tryptophan supplementation in the horse. Identification of a safe and effective dose of tryptophan in horses could give owners a non-prescriptive, non-invasive tool to manage potentially dangerous situations. In addition, feed and supplement companies may benefit from the results of this study, recognizing new dosages or potential additives in products marketed to horse owners.

Because tryptophan competes with other amino acids to bind to transport proteins and cross the blood-brain barrier, scientists are now using the ratio of tryptophan to other large neutral amino

acids (LNAA) to estimate serotonin production in the central nervous system (Wilson et al., 2007). Research has shown that serum tryptophan levels increase when horses are supplemented at a dose comparable to those used in commercial supplements but that there are no significant behavioral effects (Malmkvist and Christensen, 2007; Noble et al., 2008). Other studies have noticed a potential effect of diet and time of sampling on serum tryptophan levels in horses (Alberghina et al., 2010b; Wilson et al., 2007). While it is possible that these studies may not have seen behavioral effects when supplementing tryptophan because researchers were administering doses too low, there are also health concerns when supplementing tryptophan at doses too high. High doses of tryptophan may cause hemolytic anemia and symptoms of respiratory distress in horses. Researchers predict that these symptoms are the effect of the toxic indole metabolite produced in the hindgut of horses when large doses of tryptophan are administered orally (Paradis et al., 1991). There is a large range, from approximately 12.9 mg/kg bodyweight (Malmkvist and Christensen, 2007) to 350 mg/kg bodyweight (Paradis et al., 1991), of oral tryptophan supplementation that has not been scientifically tested for behavioral or physiological effects in horses. Nevertheless, horse owners and trainers frequently utilize supplements containing tryptophan and/or ingredients like magnesium, vitamin B_6, and a variety of herbs to promote a calming effect. One benefit to supplementing natural ingredients versus using prescription drugs is that many common dietary components are legal to use in show circuits whereas sedatives are banned substances (USEF, 2015). We hypothesized that, within the untested range of tryptophan supplemented to horses, there is a safe and behaviorally effective dose.

MATERIALS AND METHODS

Horses and Care

The study took place December 2014-January 2015 at Colorado State University's Equine Teaching and Research Center. Ten geldings and two mares were included in the experiment. Three of the horses were Quarter Horse type and the other nine were draft horse crosses. Horses ranged in age from 2.5-16 years (average 9.1 years), height from 150-169 cm (average 158.7 cm), weight from 462-626 kg (average 541.4 kg), and body condition score from 3-8 (average 5.2) (Henneke et al., 1983). Horses were divided into three groups based on bodyweight. One horse from Group 2 was removed from the study during the acclimation period due to handling difficulties. A description of the remaining horses is provided in Table 2.1. Variance between subjects is displayed in Table 2.2. All horses were used for trail riding and leased from a private owner. Informed client consent was obtained prior to the beginning of the experiment. In addition, all animal care and experimental procedures were approved by the Colorado State University Animal Care and Use Committee under protocol #14-5191A.

All horses were dewormed with 1.87% ivermectin (MWI, Boise, ID) and evaluated by a veterinarian prior to being accepted into the study. Throughout the study, horses were allowed ad libitum access to water and salt. Expected body weight at a body condition score of 5 was calculated for each horse (Henneke et al., 1983). NRC (2007) requirements were calculated based on expected body weight. Horses were fed 0.5% of their expected body weight in concentrate, on a dry matter basis, per day. The concentrate provided was a commercially-available senior feed (Purina Animal Nutrition LLC, Shoreview, MN). Each horse's remaining energy requirements were met with grass hay. Horses were housed in stalls and their daily ration divided into two

feedings at approximately 07:00 and 19:00 hours. Grain and hay nutrient profiles are reported in Table 2.2.

In order to monitor horse health status, resting respiratory rates were monitored at feeding and blood collection times (three times daily) to safeguard against any signs of respiratory distress going unnoticed. Resting heart rate and rectal temperature were recorded during the effective dose time for horses receiving treatment.

Study Design

Horses were given a 7-day acclimation period before treatment began. There were four treatment levels: a negative control (CON), in which horses received no supplemental tryptophan; LOW, in which horses were given 20 mg Trp/kg BW; MED, in which horses were given 40 mg Trp/kg BW; and HIGH, in which horses were given 60 mg Trp/kg BW. Pharmaceutical grade L-tryptophan (Ajinomoto North America, Raleigh, NC) was mixed with approximately 50 cc applesauce and water to create a paste-like consistency and administered orally before morning feeding. CON horses received an oral dose of only applesauce and water. Each treatment period lasted three days and there was a four-day washout period between treatments. Each horse received all treatments. Horses were assigned treatments in a random order so that all horses in one group were receiving different treatments. To ensure accurate dosing, each horse was weighed on a scale the morning prior to beginning a new treatment. In order to maintain consistent dose, sample collection, and behavior test timing, groups began the experiment on staggered start dates, spaced one to three days apart. Daily procedures, broken down by group, are described in Table 2.3.

Behavior Testing

Horses underwent a reactivity test similar to one described by Noble et al. (2013). The setup for the behavior test is shown in Figure 2.1. Footing within the chute was raked between

startle tests in order to try to maintain consistency despite weather conditions. Temperature, wind speed, and precipitation were recorded at the time of each test so that the effect of environmental conditions could be quantified. Each horse was individually led into the chute and held at a standstill for 10 seconds at a location 1 meter into the chute. Upon release, a startling sound and movement were made from a constant location behind the horse. A blind was set up to ensure that horses did not see the startling visual cue prior to stimulus presentation. The rate (m/s) at which the horse exited the chute (ExitSpeed) was measured using electronic timers (FarmTek, Wylie, TX) placed 6 meters apart on the sides of the chute. Sensors were placed 1 meter off the ground so that the horses' legs or chest may break the light beam. In order to reduce habituation, the startling stimuli rotated between a whoosh paired with a waving flag, an alarm sound paired with an opening umbrella, and machine gun fire paired with a flapping plastic bag. The auditory stimuli were pre-recorded and consistent in volume and intensity. To minimize habituation to the startle test, null tests, in which there are no startling stimuli, were performed during the acclimation period, on day two of each treatment, and on washout days. Startle tests performed 2-3 hours after morning administration of tryptophan supplement on days one and three of each treatment. Each horse experienced either a startle or null test each day. All tests on treatment days were video recorded.

Heart rate data was collected during the behavior tests using a Polar RS800CX training computer, which received heart rate data from a Polar WearLink W.I.N.D. transmitter attached to a chest strap that fitted around the horses' heart girth (Polar Electro OY, Kempele, Finland). Hair around the location of the electrodes was shaved and water and/or electrode gel were used to improve contact with the horses' skin. Horses remained in the stall until a consistent baseline heart rate was found by the heart rate monitor. After the behavior test, each horse was brought back to

their stall. Handlers waited for heart rate to return to baseline before removing the heart rate monitor.

Heart rate data was uploaded daily via infrared communication to Polar WebLink and transferred to polarpersonaltrainer.com for storage. In addition to the baseline heart rate information, the following measurements were taken: (1) HR30: heart rate (bpm) 30 seconds post-startle stimuli, (3) HR_Diff: HR30 minus baseline heart rate, and (4) TimetoBL: time (sec) from presentation of startle stimuli until baseline heart rate resumed.

Blood Sample Collection and Analysis

Blood samples were collected into evacuated blood collection tubes containing either a clot activator or sodium fluoride (BD Vacutainer evacuated blood collection tube, Fisher Health Care, Chicago, IL). Blood samples were taken before morning feeding or supplementation (BC1), at approximately 0600, and again before horses preformed the behavior test (BC2). This was done on the last day of the acclimation period as well as throughout the treatment periods. Blood obtained from BC1 was analyzed for packed cell volume in order to monitor the health of each individual horse, ensuring that no horses became anemic during the study. On days one and three of each treatment, additional blood samples were taken both before the startle test was performed (BC2) and again approximately 15 minutes after the test (BC3). These samples were analyzed to compare glucose, lactate, and cortisol levels before and after the startle test. Blood samples were allowed to clot at room temperature for 45-60 minutes before being centrifuged at 2,500 rpm for 15 minutes. Serum was then removed and stored in 0.5 mL aliquots in microcentrifuge tubes at -20°C until further analysis.

BC2 and BC3 samples were analyzed for serum glucose and lactate using a YSI Model 2700 SELECT Biochemistry Analyzer (YSI Life Sciences, Yellow Springs, Ohio). Serum glucose

(mg/dL) was analyzed from sodium fluoride vacutainers. Each sample was run 2-4 times, until glucose concentrations read within 1% of each other. The closest two readings were then averaged together to obtain pre- and post-startle test serum glucose values (from BC2 and BC3, respectively). Post-startle serum glucose (GluPostAvg) was considered as a response variable. Additionally, the difference in glucose values (GluDiff) was calculated by subtracting pre-startle (BC2) values from post-startle (BC3) values. Serum lactate (mmol/L) was analyzed from vacutainers containing only a clot activator. Samples were run 2-4 times, until values within 2% of each other were obtained. In the same way glucose response variables were obtained, post-startle serum lactate (LactPostAvg) and the difference between lactate pre- and post-startle values (LactDiff) were calculated response variables. The YSI auto-calibrated every five samples. Standards were analyzed before any samples, after every fifty samples, and once all samples had been analyzed. The date each sample was analyzed was recorded and considered as a covariate in the appropriate model (i.e. glucose sample run date (GluRunDate) was considered as a covariate for GluPostAvg and GluDiff models while lactate sample run date (LactRunDate) was considered as a covariate for LactPostAvg and LactDiff models).

Serum cortisol (mg/dL) was analyzed from BC2 and BC3 red top vacutainers using ELISA kits (Rocky Mountain Diagnostics, Colorado Springs, CO). Samples were analyzed in duplicates. Average post-startle serum cortisol values were calculated from BC3 samples (CortPostAvg). In addition, differences in serum cortisol (CortDiff) were calculated by subtracting BC2 values from those found in BC3 on the startle test days of each treatment. Intra-assay precision averaged an 11.36% coefficient of variation. The ELISA plate that each sample was run on (CortPretestPlate or CortPosttestPlate) were considered as potential significant predictor variables for CortPostAvg and CortDiff models.

A subset of samples (from horses in Group 1) were analyzed for serum amino acid content via gas chromatography using the procedures described by Zhang et al. (2005). This was done in order to verify treatment effect and to compare values with those found in previous studies. Concentrations (mmol/L) of serum free tryptophan (Trp), isoleucine, leucine, tyrosine, phenylalanine, and valine were obtained from pre- and post-supplementation blood draws (BC1 and BC2, respectively) on Day 1 and Day 3 of treatment. Difference in serum free tryptophan (TrpDiff) was calculated by subtracting values in BC1 from those found in BC2. Additionally, the ratio of tryptophan to other large neutral amino acids (Trp:LNAA) was calculated for BC1 and BC2 samples. The difference between the ratio seen in BC1 and BC2 (TrpDiff) was considered as another response variable.

Statistical Analysis

All analyses were carried out using SAS (version 9.4, SAS Inst. Inc., Cary, NC). Significance was set at $p \leq 0.05$ while trends were identified at $p \leq 0.10$.

Day 1 and Day 3 data were analyzed separately. Correlations were run to identify potential covariates for each response variable (ExitSpeed, HR30, HR_Diff, TimetoBL, GluDiff, GluPostAvg, LactDiff, LactPostAvg, CortDiff, and CortPostAvg) using PROC CORR. Potential covariates included the following: Group (1-3); TimeLag, which was the time (in minutes) from tryptophan supplementation until the behavior test was performed; TrtNum, which was the sequence (1-4) of treatments; as well as Temperature (°C) and WindSpeed (m/s) outside when each behavior test was performed. Correlations between these variables and the response variables were examined; a covariate was included in the models for both Day 1 and Day 3 if a significant correlation to the response variable was found on either day, as long as significance was maintained once added to the model. Additional covariates (GluRunDate, LactRunDate, CortPretestPlate, and

CortPosttestPlate) were considered for response variables requiring lab analysis. ANOVA and ANCOVA models were constructed for each response variable using PROC MIXED. In all models, Horse and Treatment were identified as class variables. If Group, TrtNum, GluRunDate, LactRunDate, CortPretestPlate, or CortPosttestPlate were included in any model, they were also categorized as class variables. Additionally, Horse was treated as random effect. Treatment means, standard errors, and differences between treatments were determined using the PDIFF option in LSMEANS.

The following models were used to evaluate treatment effect on Day 1 and Day 3:

ExitSpeed = Group + Temperature + Treatment
HR30 = TrtNum + Treatment
HR_Diff = Group + TrtNum + Treatment
TimetoBL = Temperature + Treatment
GluDiff = GluRunDate + TimeLag + Treatment
GluPostAvg = TimeLag + Treatment
LactDiff = Treatment
LactPostAvg = Treatment
CortDiff = Treatment
CortPostAvg = CortPosttestPlate + Treatment

The subset of the blood samples ($n \leq 4$ samples per treatment, day, and blood collection) analyzed for amino acid content was also examined using PROC MIXED. Data points were excluded from analysis if they were both (1) outside of the range of serum amino acids found in horses based on previous literature and (2) more than three standard deviations away from the sample mean. Time between supplementation and BC2 was considered as a covariate but was not significant and, therefore, not included in the models. Horse, Treatment, and Day were recognized as class variables while Horse and Horse*Treatment were random effects. Day 1 and Day 3 data were considered separately using the SLICEBY=DAY statement. Treatment means were evaluated using the PDIFF option. Effect of treatment number (to evaluate the effectiveness of washout periods) and comparisons between blood collections and days were analyzed in PROC

MIXED using the PDIFF option in LSMEANS. In these models, Horse, Treatment, TrtNum, Day, and blood collection were class variables while Horse and Horse*Treatment remained random effects.

The following models were used to evaluate serum amino acid content in Group 1:

TrpDiff = Treatment | Day
RatioDiff = Treatment | Day

RESULTS

Horses remained healthy throughout the study; all resting heart rate, respiratory rate, body temperature, and packed cell volume measurements remained within normal limits. Individual horse body weight varied somewhat throughout the study and feed/treatment doses were adjusted accordingly. Serum glucose, lactate, cortisol, and amino acid levels are compared to expected ranges based on previous literature in Table 2.4.

Correlations between model covariates and response variables, along with covariate effects in the models, are presented in Table 2.5. Means and standard deviations for each response variable by treatment and day are displayed in Table 2.6.

LOW

While heart rate was elevated above baseline at 30 seconds post-startle for both the low dose and control on Day 1, heart rate difference was less on Day 1 of the low treatment compared to Day 1 of control ($P = 0.05$). Day 1 lactate difference was also significantly less on the low dose than on control ($P = 0.03$). On the low dose Day 1, average serum lactate was less post startle test compared to values before the startle test. On control Day 1, however, lactate increased after the startle test. These results indicate that the low treatment had a sedative effect on Day 1 in terms of changes in heart rate and serum lactate levels.

No significant effects or trends were seen at the low treatment on Day 3.

MED

Day 1 cortisol difference on the medium treatment was less compared to control ($P = 0.01$). Serum cortisol decreased post-startle test on the medium dose while it increased post-startle on control. This seems to indicate that the medium treatment had a sedative effect on cortisol levels on Day 1.

Time for heart rate to return to baseline post-startle was greater on Day 3 of medium dose than control ($P = 0.03$). These results show that the medium dose had an excitatory effect on heart rate on Day 3.

HIGH

Cortisol difference before and after the startle test tended to be different on Day 1 of the high dose compared to control ($P = 0.10$). Numerically, serum cortisol levels were lower post-startle than pre-startle on high treatment Day 1 where levels actually increased after the startle test on control Day 1. This trend implies that the high dose may have had a sedative effect on cortisol levels on Day 1.

Post-startle serum lactate on Day 3 of the high dose showed a trend of being higher than on Day 3 of control ($P = 0.10$), suggesting somewhat of an excitatory effect on this response variable.

Amino Acids

Mean differences in serum free tryptophan and Trp:LNAA by treatment and day are presented in Figures 2.2 and 2.3, respectively. Significance for pairwise comparisons of means between treatments are shown in Tables 2.7 and 2.8.

On Day 1, serum Trp and Trp:LNAA in BC1 did differ significantly from levels in BC2 ($P < 0.0001$ and $P < 0.0001$, respectively). However, on Day 3, neither Trp nor Trp:LNAA differed

significantly from BC1 to BC2 (P = 0.98 and P = 0.99, respectively). In addition, Trp and Trp:LNAA on Day 3 BC1 were not significantly different from those seen on Day 1 BC2 (P = 0.93 and P = 0.87, respectively).

The efficacy of the washout periods was analyzed by comparing Trp and TRP:LNAA in BC1 on Day 1 of each treatment. Treatment number was not significant for serum Trp levels (P = 0.54) or Trp:LNAA (P = 0.37).

DISCUSSION

We did see serum cortisol levels higher than those previously reported in horses. Serum free tryptophan was also seen at higher concentrations than those previously reported but we provided oral tryptophan supplement to horses at doses that have not been previously evaluated.

It is interesting to note that on Day 1, all treatments had some sort of sedative effect on at least one of the response variables whereas on Day 3, either no effect was seen (LOW) or some sort of excitatory effect was seen (MED, HIGH) on one of the response variables. The reason for this cannot be determined based on this experiment, but one possibility is that horses started to experience symptoms of serotonin syndrome and became more excitable on Day 3 at the MED and HIGH doses. However, we did not see any of the symptoms of the syndrome as they present in other species (Boyer and Shannon, 2005; Gillman, 1999).

Another potential explanation for the results on Day 3 conflicting with those seen on Day 1 could be that it is the actual change in the Trp:LNAA ratio that produces the desired sedative behavioral effects noted in other species. Some evidence for this may lie in the serum amino acid analysis of Group 1 horses, as treatment on Day 3 had little effect on serum free tryptophan concentration or Trp:LNAA. The lack of significant changes in amino acid profiles on Day 3 and from Day 1 to Day 3 may provide reasoning as to why no significant calming effects were seen in

any of the response variables on Day 3. It is possible that serum amino acids would have dropped between the time of BC1 and BC2 on Day 3, had the horses not received another treatment during that time. Again, we are unsure as to why this is; without further amino acid analysis, we do not know how long free tryptophan or Trp:LNAA remain elevated in the serum. We can see that after the four-day washout period, levels returned to baseline and treatment number had no significant effect.

We were surprised at how little significance treatment had on most of the response variables and how trends did not seem to be reliable between treatments or days. One exception can be seen in the decrease in serum cortisol post-startle on Day 1 of the MED and HIGH treatments. These results agree with the response Koopmans et al. (2005) saw in swine supplemented with tryptophan; although no behavioral effects were seen in that study, tryptophan supplementation reduced plasma cortisol and noradrenaline in pigs exposed to social stress.

There was a lot of variability among individual horses used in this study. Perhaps testing a more homogenous group of horses (in which individuals were of the same breed, sex, and similar age) would generate more clear results. Previous research provides evidence that there are differences in male and female serotonergic systems and responses to changes in dietary Trp:LNAA (Albonetti et al., 1994; Dickinson and Curzon, 1986; Henry et al., 1992; Henry et al., 1996; Kennett et al., 1986; Kim and Camilleri, 2000; Rouvinen et al., 1999). Alberghina et al. (2014) as well as Ferlazzo et al. (2012) saw differences in serum tryptophan in horses of different ages. Other studies have observed breed differences (Alberghina et al., 2014; Bagshaw et al., 1994; Grimmett and Sillence, 2005). While all of these factors may play a role in the permeability of the blood-brain barrier the effectiveness of supplemental tryptophan on serotonin biosynthesis, it is also worth considering that these types of treatments may be most effective in horses with

dysfunctioning serotonergic systems. This would be in line with research in other species, where tryptophan supplements and proserotonergic drugs were most effective in individuals that exhibited symptoms of disorder within the serotonin system (Argyropoulos et al., 2004; Bell et al., 2001; Weld et al., 1998).

The environment was another source of variability. Although researchers aimed to keep the timing of the tests and the footing within the test chute as consistent as possible, variations in temperature, wind, precipitation, and on-farm activities provided uncontrolled stimuli that may have impacted the results of the behavior tests. Performing these tests in a more controlled environment would be desirable and may provide more reliable results.

Including a positive control, with a sedative known to be effective in horses (such as acepromazine), may have helped elucidate treatment effects. It's possible that the startling stimuli were too effective at spooking the horses in this experiment and that any treatment effects were nullified as a result. Comparing treatment effects to that of a positive control could provide support or evidence against this hypothesis.

Supplementing tryptophan to horses at higher doses (40 mg/kg BW and 60 mg/kg BW) may be an effective way to reduce cortisol levels in stressful situations. We also saw some evidence of calming effects of tryptophan at a lower dose (20 mg/kg BW). However, these findings were seen in only a few of the results variables measured in this experiment. The horses we used responded to tryptophan supplementation more favorably on the first day they received the supplement. This finding does not support the directions found on some commercially available products that recommend providing the supplement to the horse 24 hours in advance and again several hours before a stressful event takes place. The subset of blood samples we analyzed for amino acid content provides evidence to refute this recommendation as well. We saw no additional

beneficial effects in terms of increasing serum free tryptophan levels or the ratio of tryptophan to other large neutral amino acids on Day 3 of supplementation versus Day 1. This experiment confirms that supplementing horses with tryptophan is effective at increasing serum free tryptophan and the ratio of tryptophan to certain amino acids. We found little evidence for the behavioral or physiological calmative effects of supplemental tryptophan in horses at the doses tested. However, from the results presented and discussed in this paper, it appears that supplementing horses with tryptophan may produce desired results only a few hours after administration and that longer term use may provide no additional benefit or may even have unwanted effects.

When evaluating the use of calming supplements or drugs, it's important to consider the welfare of the horse. While these compounds may be beneficial in alleviating short-term stress and anxiety (for example, when veterinary care needs to be provided) the cause of such emotions should be evaluated. Horses kept in unnatural environments, managed poorly, or asked to perform beyond their level of training may show signs of stress and anxiety. Chronic health issues such as ulcers or lameness may also be the culprit. Oftentimes, sedative drugs and supplements are utilized to limit unwanted behaviors such as spooking, bolting, rearing, or bucking. Looking into the potential causes of unwanted behaviors should be the first step before owners turn to calming drugs or supplements. Providing more training, turnout time, or treatment for an underlying disease or condition could result in a more sustainable way to reduce a horse's unwanted behaviors and could improve welfare for the animal.

TABLE 2.1: Horse profile by group

	n	Mean age (years)	Mean body weight (kg)	Mean BCS (1-9)	Mean height (cm)	Number of mares: Number of geldings	Number of Quarter Horse type: Number of draft horse type
Group 1	4	6.5	463.0	4.4	152.8	0:4	3:1
Group 2	3	8.6	493.9	5.7	160.3	0:3	0:3
Group 3	4	11.0	618	5.8	162.8	2:4	0:4

TABLE 2.2: Variance between horses

	Day 1			Day 3		
Response Variable	Estimate	Standard Error	Ratio (horse : residual)	Estimate	Standard Error	Ratio (horse : residual)
ExitSpeed (m/s)	0.953	0.5521	1.773	0.04921	0.2089	0.03719
HR30 (bpm)	215.49	131.02	0.7418	208.61	144.05	0.5164
HR_Diff (bpm)	190.12	131.37	0.7159	34.1998	81.1269	0.07998
TimetoBL (sec)	732.31	1142.78	0.1449	7143.96	4253.96	0.8022
GluDiff (mg/dL)	8.6686	7.4146	0.4094	0	.	0
GluPostAvg (mg/dL)	0	.	0	2.0242	9.4039	0.03188
LactDiff (mmol/L)	0.0113	0.01392	0.1805	0.01986	0.01978	0.2628
LactPostAvg (mmol/L)	0.02363	0.01927	0.4152	0.02802	0.02414	0.3843
CortDiff (μg/dL)	0	.	0	1.046	3.3003	0.06633
CortPostAvg (μg/dL)	87.7944	41.2961	5.5207	48.3134	24.9931	2.2064

TABLE 2.3: Guaranteed analysis of feedstuffs on as-fed basis

	Hay: Timothy Brome Mix	Concentrate: Purina Equine Senior
Digestible Energy (Mcal/kg)	2.07	2.70
Dry Matter (%)	92.3	90.0
Crude Protein (%)	6.70	min 14
Estimated Lysine (%)	0.23	min 0.70
Acid Detergent Fiber (%)	33.5	*
Neutral Detergent Fiber (%)	55.5	*
Starch (%)	0.2	max 12
Crude Fat (%)	2.2	min 5.5
Ash (%)	4.9	*
Calcium (%)	0.41	0.5-1.00
Phosphorus (%)	0.17	min 0.4
Magnesium (%)	0.17	0.33
Potassium (%)	1.12	min 1.60
Sodium (%)	0.03	min 0.24
Iron (ppm)	64	min 220
Zinc (ppm)	14	min 220
Copper (ppm)	4	min 55
Manganese (ppm)	44	min 220

*Proprietary blend: information unavailable.

TABLE 2.4: Daily procedures

Day	Group A	Group B	Group C
1	Heart rate, temperature, weigh, null reactivity test		
2	Heart rate, temperature, null reactivity test	Heart rate, temperature, weigh, null reactivity test	
3	Heart rate, temperature, weigh, null reactivity test	Heart rate, temperature, null reactivity test	
4	Heart rate, temperature, null reactivity test	Heart rate, temperature, weigh, null reactivity test	
5	Heart rate, temperature, weigh, null reactivity test	Heart rate, temperature, null reactivity test	Heart rate, temperature, weigh, null reactivity test
6	Heart rate, temperature, null reactivity test	Heart rate, temperature, weigh, null reactivity test	Heart rate, temperature, null reactivity test
7	Heart rate, temperature, BC1 (PCV, TRP:LNAA), weigh, BC2 (AA, glucose, lactate, cortisol), null reactivity test, BC3 (glucose, lactate, cortisol)	Heart rate, temperature, null reactivity test	Heart rate, temperature, weigh, null reactivity test
8	BC1 (PCV, AA), weigh, begin first* treatment, heart rate, temperature, BC2 (AA, glucose, lactate, cortisol), startle reactivity test, BC3 (glucose, lactate, cortisol)	Heart rate, temperature, BC1 (PCV, TRP:LNAA), weigh, BC2 (AA, glucose, lactate, cortisol), null reactivity test, BC3 (glucose, lactate, cortisol)	Heart rate, temperature, null reactivity test
9	BC1 (PCV, AA), treatment, heart rate, temperature, BC2 (AA), null reactivity test	BC1 (PCV, AA), weigh, begin first* treatment, heart rate, temperature, BC2 (AA, glucose, lactate, cortisol), startle reactivity test, BC3 (glucose, lactate, cortisol)	Heart rate, temperature, weigh, null reactivity test

10	BC1 (PCV, AA), treatment, heart rate, temperature, BC2 (AA, glucose, lactate, cortisol), startle reactivity test, BC3 (glucose, lactate, cortisol)	BC1 (PCV, AA), treatment, heart rate, temperature, BC2 (AA), null reactivity test	Heart rate, temperature, null reactivity test
11	Washout day: heart rate, temperature, BC1 (PCV), null reactivity test	BC1 (PCV, AA), treatment, heart rate, temperature, BC2 (AA, glucose, lactate, cortisol), startle reactivity test, BC3 (glucose, lactate, cortisol)	Heart rate, temperature, BC1 (PCV, TRP:LNAA), weigh, BC2 (AA, glucose, lactate, cortisol), null reactivity test, BC3 (glucose, lactate, cortisol)
12	Washout day: heart rate, temperature, BC1 (PCV), null reactivity test	Washout day: heart rate, temperature, BC1 (PCV), null reactivity test	BC1 (PCV, AA), weigh, begin first* treatment, heart rate, temperature, BC2 (AA, glucose, lactate, cortisol), startle reactivity test, BC3 (glucose, lactate, cortisol)
13	Washout day: heart rate, temperature, BC1 (PCV), null reactivity test	Washout day: heart rate, temperature, BC1 (PCV), null reactivity test	BC1 (PCV, AA), treatment, heart rate, temperature, BC2 (AA), null reactivity test
14	Washout day: heart rate, temperature, BC1 (PCV), null reactivity test	Washout day: heart rate, temperature, BC1 (PCV), null reactivity test	BC1 (PCV, AA), treatment, heart rate, temperature, BC2 (AA, glucose, lactate, cortisol), startle reactivity test, BC3 (glucose, lactate, cortisol)
15	BC1 (PCV, AA), weigh, begin second* treatment, heart rate, temperature, BC2 (AA, glucose, lactate, cortisol), startle reactivity test, BC3 (glucose, lactate, cortisol)	Washout day: heart rate, temperature, BC1 (PCV), null reactivity test	Washout day: heart rate, temperature, BC1 (PCV), null reactivity test
16	BC1 (PCV, AA), treatment, heart rate, temperature, BC2 (AA), null reactivity test	BC1 (PCV, AA), weigh, begin second* treatment, heart rate, temperature, BC2 (AA, glucose, lactate, cortisol), startle reactivity test, BC3 (glucose, lactate, cortisol)	Washout day: heart rate, temperature, BC1 (PCV), null reactivity test

17	BC1 (PCV, AA), treatment, heart rate, temperature, BC2 (AA, glucose, lactate, cortisol), startle reactivity test, BC3 (glucose, lactate, cortisol)	BC1 (PCV, AA), treatment, heart rate, temperature, BC2 (AA), null reactivity test	Washout day: heart rate, temperature, BC1 (PCV), null reactivity test
18	Washout day: heart rate, temperature, BC1 (PCV), null reactivity test	BC1 (PCV, AA), treatment, heart rate, temperature, BC2 (AA, glucose, lactate, cortisol), startle reactivity test, BC3 (glucose, lactate, cortisol)	Washout day: heart rate, temperature, BC1 (PCV), null reactivity test
19	Washout day: heart rate, temperature, BC1 (PCV), null reactivity test	Washout day: heart rate, temperature, BC1 (PCV), null reactivity test	BC1 (PCV, AA), weigh, begin second* treatment, heart rate, temperature, BC2 (AA, glucose, lactate, cortisol), startle reactivity test, BC3 (glucose, lactate, cortisol)
20	Washout day: heart rate, temperature, BC1 (PCV), null reactivity test	Washout day: heart rate, temperature, BC1 (PCV), null reactivity test	BC1 (PCV, AA), treatment, heart rate, temperature, BC2 (AA), null reactivity test
21	Washout day: heart rate, temperature, BC1 (PCV), null reactivity test	Washout day: heart rate, temperature, BC1 (PCV), null reactivity test	BC1 (PCV, AA), treatment, heart rate, temperature, BC2 (AA, glucose, lactate, cortisol), startle reactivity test, BC3 (glucose, lactate, cortisol)
22	BC1 (PCV, AA), weigh, begin third* treatment, heart rate, temperature, BC2 (AA, glucose, lactate, cortisol), startle reactivity test, BC3 (glucose, lactate, cortisol)	Washout day: heart rate, temperature, BC1 (PCV), null reactivity test	Washout day: heart rate, temperature, BC1 (PCV), null reactivity test

23	BC1 (PCV, AA), treatment, heart rate, temperature, BC2 (AA), null reactivity test	BC1 (PCV, AA), weigh, begin third* treatment, heart rate, temperature, BC2 (AA, glucose, lactate, cortisol), startle reactivity test, BC3 (glucose, lactate, cortisol)	Washout day: heart rate, temperature, BC1 (PCV), null reactivity test
24	BC1 (PCV, AA), treatment, heart rate, temperature, BC2 (AA, glucose, lactate, cortisol), startle reactivity test, BC3 (glucose, lactate, cortisol)	BC1 (PCV, AA), treatment, heart rate, temperature, BC2 (AA), null reactivity test	Washout day: heart rate, temperature, BC1 (PCV), null reactivity test
25	Washout day: heart rate, temperature, BC1 (PCV), null reactivity test	BC1 (PCV, AA), treatment, heart rate, temperature, BC2 (AA, glucose, lactate, cortisol), startle reactivity test, BC3 (glucose, lactate, cortisol)	Washout day: heart rate, temperature, BC1 (PCV), null reactivity test
26	Washout day: heart rate, temperature, BC1 (PCV), null reactivity test	Washout day: heart rate, temperature, BC1 (PCV), null reactivity test	BC1 (PCV, AA), weigh, begin third* treatment, heart rate, temperature, BC2 (AA, glucose, lactate, cortisol), startle reactivity test, BC3 (glucose, lactate, cortisol)

27	Washout day: heart rate, temperature, BC1 (PCV), null reactivity test	Washout day: heart rate, temperature, BC1 (PCV), null reactivity test	BC1 (PCV, AA), treatment, heart rate, temperature, BC2 (AA), null reactivity test
28	Washout day: heart rate, temperature, baseline blood draw (PCV), weigh, null reactivity test	Washout day: heart rate, temperature, BC1 (PCV), null reactivity test	BC1 (PCV, AA), treatment, heart rate, temperature, BC2 (AA, glucose, lactate, cortisol), startle reactivity test, BC3 (glucose, lactate, cortisol)
29	BC1 (PCV, AA), weigh, begin fourth* treatment, heart rate, temperature, BC2 (AA, glucose, lactate, cortisol), startle reactivity test, BC3 (glucose, lactate, cortisol)	Washout day: heart rate, temperature, BC1 (PCV), null reactivity test	Washout day: heart rate, temperature, BC1 (PCV), null reactivity test
30	BC1 (PCV, AA), treatment, heart rate, temperature, BC2 (AA), null reactivity test	BC1 (PCV, AA), weigh, begin fourth* treatment, heart rate, temperature, BC2 (AA, glucose, lactate, cortisol), startle reactivity test, BC3 (glucose, lactate, cortisol)	Washout day: heart rate, temperature, BC1 (PCV), null reactivity test

31	BC1 (PCV, AA), treatment, heart rate, temperature, BC2 (AA, glucose, lactate, cortisol), startle reactivity test, BC3 (glucose, lactate, cortisol)	BC1 (PCV, AA), treatment, heart rate, temperature, BC2 (AA), null reactivity test	Washout day: heart rate, temperature, BC1 (PCV), null reactivity test
32	Washout day: heart rate, temperature, baseline blood draw (PCV), null reactivity test	BC1 (PCV, AA), treatment, heart rate, temperature, BC2 (AA, glucose, lactate, cortisol), startle reactivity test, BC3 (glucose, lactate, cortisol)	Washout day: heart rate, temperature, BC1 (PCV), null reactivity test
33	Washout day: heart rate, temperature, baseline blood draw (PCV), null reactivity test	Washout day: heart rate, temperature, BC1 (PCV), null reactivity test	BC1 (PCV, AA), weigh, begin fourth* treatment, heart rate, temperature, BC2 (AA, glucose, lactate, cortisol), startle reactivity test, BC3 (glucose, lactate, cortisol)
34	Washout day: heart rate, temperature, baseline blood draw (PCV), null reactivity test	Washout day: heart rate, temperature, BC1 (PCV), null reactivity test	BC1 (PCV, AA), treatment, heart rate, temperature, BC2 (AA), null reactivity test

35	Washout day: heart rate, temperature, baseline blood draw (PCV), weigh, null reactivity test	Washout day: heart rate, temperature, BC1 (PCV), null reactivity test	BC1 (PCV, AA), treatment, heart rate, temperature, BC2 (AA, glucose, lactate, cortisol), startle reactivity test, BC3 (glucose, lactate, cortisol)
36		Washout day: heart rate, temperature, BC1 (PCV), null reactivity test	Washout day: heart rate, temperature, BC1 (PCV), null reactivity test
37			Washout day: heart rate, temperature, BC1 (PCV), null reactivity test
38			Washout day: heart rate, temperature, BC1 (PCV), null reactivity test
39			Washout day: heart rate, temperature, BC1 (PCV), null reactivity test

*Fixed treatments (CON, LOW, MED, HIGH) were assigned in a random order so that no horses within a group were receiving the same treatment at the same time

TABLE 2.5: Serum values in horses

Compound	Ranges found in previous literature	Ranges found in current study
Glucose (mg/dL)	62[1] - 134[1]	67.5 - 112.5
Lactate (mmol/L)	0.56[2] - 2.32[3]	0.47 - 2.78
Cortisol (μg/dL)	0.08[4] - 25.5[4]	0.02 - 56.81
Free tryptophan (μmol/L)	8.0[5] - 118.1[6]	19.3 - 142.3
Isoleucine (μmol/L)	36.1[5] - 171.8[5]	72.3 - 120.1
Leucine (μmol/L)	60.1[6] - 371.3[5]	122.4 - 148.2
Valine (μmol/L)	115.0[5] - 311.8[5]	164.1 - 201.3
Penylalanine (μmol/L)	14.7[6] - 103.4[6]	31.1 - 57.3
Tyrosine (μmol/L)	22.1[6] - 125.4[6]	68.6 - 88.2
Trp:LNAA	0.09[7] - 0.13[7]	0.04 - 0.28

[1]Kahn and Line, 2010
[2]Stull and Rodiek, 2000
[3]Nogueira et al., 2002
[4]Alenka et al., 2008
[5]Assenza et al., 2004
[6]Bergero et al., 2005
[7]Wilson, 2007

TABLE 2.6: Model covariates—correlation to response variables and influence in models

Response Variable	Covariate in Model	DAY 1			DAY 3		
		r*	*P***	estimate ± SE***	r*	*P***	estimate ± SE***
ExitSpeed (m/s)							
	Group	-0.42097	0.0068		-0.44902	0.0022	
	Temperature (°C)	0.54964	0.0002	0.080 ± 0.017	0.11043	0.4755	0.002 ± 0.029
HR30 (bpm)							
	TrtNum	0.03286	0.8363		0.31953	0.0391	
HR_Diff (bpm)							
	Group	-0.31893	0.0395		-0.42359	0.0052	
	TrtNum	0.12101	0.4452		0.42941	0.0045	
TimetoBL (sec)							
	Temperature (°C)	0.39175	0.0136	4.114 ± 1.586	0.46337	0.0023	10.950 ± 2.523
GluDiff (mg/dL)							
	GluRunDate	-0.06339	0.7014		-0.38298	0.0161	
	TimeLag (sec)	-0.43873	0.0052	-0.048 ± 0.032	-0.42515	0.0070	-0.105 ± 0.032
GluPostAvg (mg/dL)							
	TimeLag (sec)	-0.38014	0.0142	-0.074 ± 0.029	-0.39329	0.0110	-0.106 ± 0.044
CortPostAvg (μg/dL)							
	CortPosttestPlate	0.13747	0.3914		0.31034	0.0483	

*PROC CORR: Pearson correlation coefficient
**PROC CORR: Prob > |r| under H0: Rho=0
***PROC MIXED: Solution for Fixed Effects

TABLE 2.7: Effect of tryptophan treatment on physiological and behavioral response variables: least squares means ± standard error

	CON		LOW		MED		HIGH	
Response Variable	Day 1	Day 3	Day 1	Day 3	Day 1	Day 3	Day 1	Day 3
ExitSpeed (m/s)	5.09 ± 0.38	5.19 ± 0.36	4.77 ± 0.38	5.54 ± 0.36	5.16 ± 0.37	5.12 ± 0.36	5.44 ± 0.39	5.59 ± 0.35
HR30 (bpm)	102.83 ± 6.79	108.36 ± 7.48	97.60 ± 7.05	103.66 ± 7.48	104.04 ± 7.06	99.91 ± 7.80	109.78 ± 6.80	99.02 ± 7.80
HR_Diff (bpm)	69.95 ± 6.48	71.87 ± 6.52	55.33 ± 6.72	68.56 ± 6.53	68.79 ± 6.77	68.19 ± 6.94	74.51 ± 6.48	65.29 ± 6.94
TimetoBL (sec)	224.00 ± 24.15	228.69 ± 38.21	213.53 ± 25.51	224.28 ± 39.69	249.62 ± 24.14	325.88 ± 39.58	209.59 ± 24.19	247.11 ± 39.67
GluDiff (mg/dL)	-4.34 ± 1.96	-1.78 ± 1.74	-8.27 ± 1.97	1.79 ± 1.69	-4.96 ± 2.07	1.98 ± 1.76	-0.64 ± 2.03	0.54 ± 1.90
GluPostAvg (mg/dL)	83.36 ± 2.33	85.17 ± 2.57	81.71 ± 2.20	85.41 ± 2.45	83.89 ± 2.25	86.90 ± 2.44	87.88 ± 2.43	87.00 ± 2.73
LactDiff (mmol/L)	0.17 ± 0.09	0.17 ± 0.10	-0.10 ± 0.09	0.30 ± 0.10	0.21 ± 0.08	0.24 ± 0.10	0.22 ± 0.09	0.19 ± 0.11
LactPostAvg (mmol/L)	1.14 ± 0.09	1.09 ± 0.11	0.98 ± 0.09	1.18 ± 0.10	1.25 ± 0.09	1.25 ± 0.11	1.11 ± 0.09	1.35 ± 0.11
CortDiff (μg/dL)	2.79 ± 1.27	-0.29 ± 1.30	1.09 ± 1.27	0.33 ± 1.24	-1.86 ± 1.22	-0.07 ± 1.30	-0.38 ± 1.34	-0.38 ± 1.38
CortPostAvg (μg/dL)	12.81 ± 3.11	12.65 ± 2.62	11.95 ± 3.08	10.55 ± 2.53	11.87 ± 3.08	13.50 ± 2.55	13.35 ± 3.16	10.73 ± 2.71

TABLE 2.8: Differences of least squares means for serum free tryptophan (μmol/L) Group 1 horses

DAY 1			DAY 3		
Treatment		*P**	Treatment		*P**
CON[a]	LOW[b]	0.0011	CON[a]	LOW[a]	0.7150
CON[a]	MED[a]	<0.0001	CON[a]	MED[a]	0.5878
CON[a]	HIGH[a]	<0.0001	CON[a]	HIGH[b]	0.6035
LOW[b]	MED[a]	0.0474	LOW[a]	MED[a]	0.3675
LOW[b]	HIGH[a]	<0.0001	LOW[a]	HIGH[b]	0.8554
MED[a]	HIGH[a]	0.0013	MED[a]	HIGH[b]	0.3114

[a] *n*=4. [b] *n*=3. *Difference of Least Squares Means.

TABLE 2.9: Differences of least squares means for Trp:LNAA Group 1 horses

DAY 1			DAY 3		
Treatment		*P**	Treatment		*P**
CON[b]	LOW[b]	0.0037	CON[b]	LOW[a]	0.5465
CON[b]	MED[a]	<0.0001	CON[b]	MED[a]	0.6022
CON[b]	HIGH[b]	<0.0001	CON[b]	HIGH[c]	0.9097
LOW[b]	MED[a]	0.0632	LOW[a]	MED[a]	0.2320
LOW[b]	HIGH[b]	0.0006	LOW[a]	HIGH[c]	0.6789
MED[a]	HIGH[b]	0.0252	MED[a]	HIGH[c]	0.5631

[a] *n*=4. [b] *n*=3. [c] *n*=2. *Difference of Least Squares Means.

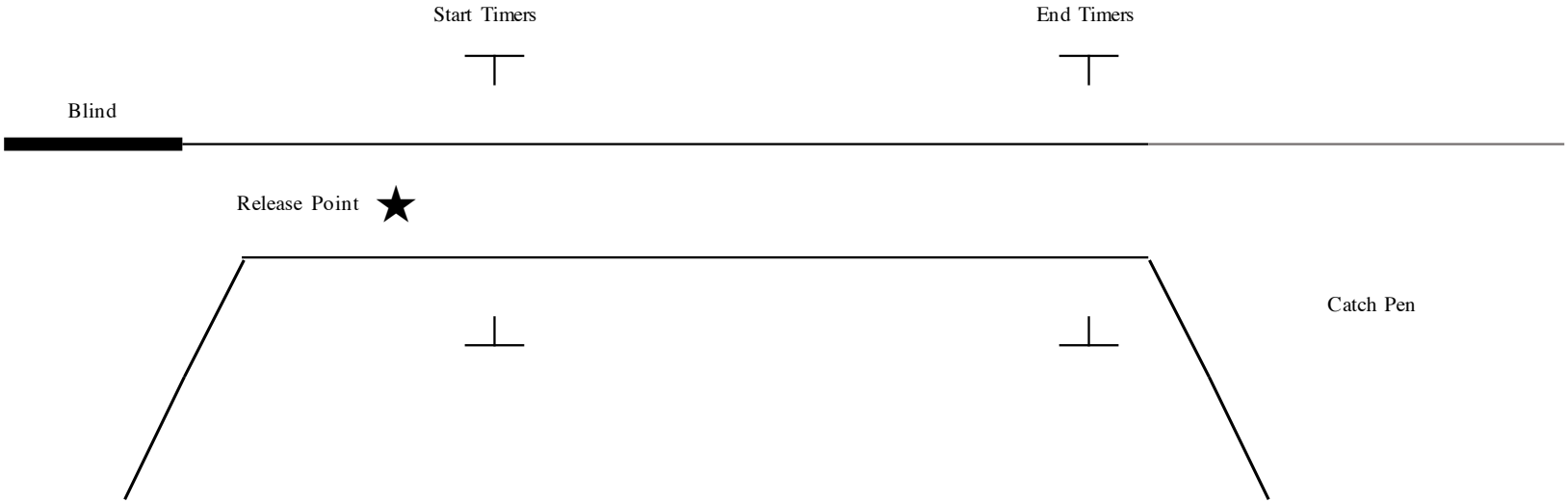

Fig. 2.1: Behavior test setup. The chute measured 1.5 meters wide and 7 meters long; not to scale.

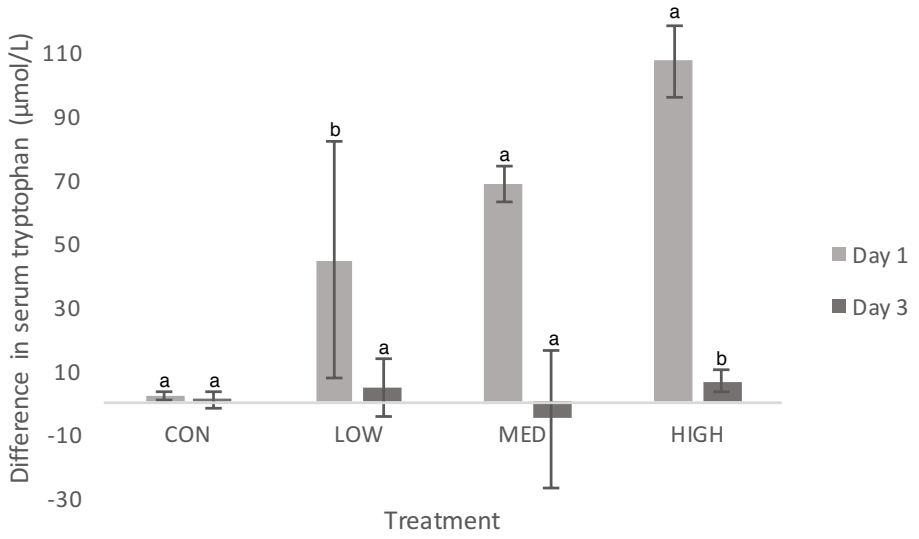

Fig. 2.2: Average (± s.d.) difference in serum free tryptophan (µmol/L) for Group 1 horses calculated by subtracting levels in BC1 sample from levels BC2 sample. [a]n=4. [b]n=3.

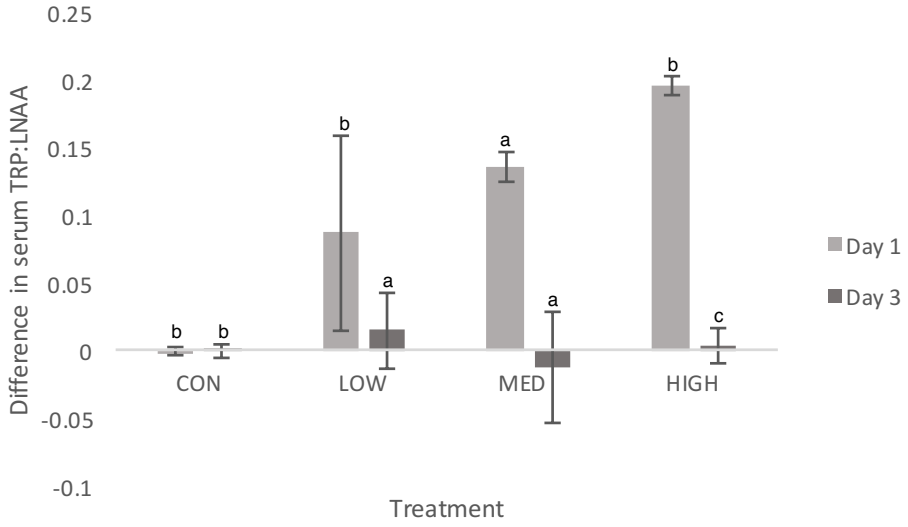

Fig. 2.3: Average (± s.d.) difference in serum Trp:LNAA for Group 1 horses calculated by subtracting ratio in BC1 samples from ratio in BC2 sample. [a]n=4. [b]n=3. [c]n=2.

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APPENDIX 1

SAS CODE

Analyze Treatment Effects on Behavioral and Physiological Response Variables on Day 1

```
PROC IMPORT OUT=WORK.AllDay1Data DATAFILE="/home/britdav/AllDay1Data.xlsx"
        DBMS=XLSX REPLACE;
        GETNAMES=YES;
run;

proc sort data=AllDay1Data;
        by Horse;
run;

*Summary Plots;

Proc Means data=AllDay1Data nway noprint;
        class Treatment Day;
        var ExitSpeed HR30 HR_Diff TimetoBL GluDiff GluPostAvg LactDiff LactPostAvg
                CortDiff CortPostAvg;
        output out=SumStats mean=;
run;

Proc Transpose data=SumStats out=SumStatsTr (rename=(Col1=Y));
        by Treatment Day _TYPE_ _FREQ_;
run;

Proc sort data=SumStatsTr;
        by _LABEL_;

*Simple interaction plot for each response variable;

Proc Sgplot ;
        by _LABEL_;
        series X=Treatment Y=Y;
run;

*proc corr data=AllDay1Data plots=scatter;
*       var treatment;
*       with exitspeed;
*run;

*proc corr data=AllDay1Data plots=scatter;
*       var treatment;
*       with HR30;
```

```
*run;

*proc corr data=AllDay1Data plots=scatter;
*	var treatment;
*	with HR_Diff;
*run;

*proc corr data=AllDay1Data plots=scatter;
*	var treatment;
*	with TimetoBL;
*run;

*proc corr data=AllDay1Data plots=scatter;
*	var treatment;
*	with GluDiff;
*run;

*proc corr data=AllDay1Data plots=scatter;
*	var treatment;
*	with GluPostAvg;
*run;

*proc corr data=AllDay1Data plots=scatter;
*	var treatment;
*	with LactDiff;
*run;

*proc corr data=AllDay1Data plots=scatter;
*	var treatment;
*	with LactPostAvg;
*run;

*proc corr data=AllDay1Data plots=scatter;
*	var treatment;
*	with CortDiff;
*run;

*proc corr data=AllDay1Data plots=scatter;
*	var treatment;
*	with CortPostAvg;
*run;

*Covariate tests;

proc corr data=AllDay1Data plots=scatter;
	var Age;
```

```
        with ExitSpeed HR30 HR_Diff TimetoBL GluDiff GluPostAvg LactDiff LactPostAvg
                CortDiff CortPostAvg;
run;

proc corr data=AllDay1Data plots=scatter;
        var BCS;
        with ExitSpeed HR30 HR_Diff TimetoBL GluDiff GluPostAvg LactDiff LactPostAvg
                CortDiff CortPostAvg;
run;

proc corr data=AllDay1Data plots=scatter;
        var Group;
        with ExitSpeed HR30 HR_Diff TimetoBL GluDiff GluPostAvg LactDiff LactPostAvg
                CortDiff CortPostAvg;
run;

proc corr data=AllDay1Data plots=scatter;
        var TimeLag;
        with ExitSpeed HR30 HR_Diff TimetoBL GluDiff GluPostAvg LactDiff LactPostAvg
                CortDiff CortPostAvg;
run;

proc corr data=AllDay1Data plots=scatter;
        var TrtNum;
        with ExitSpeed HR30 HR_Diff TimetoBL GluDiff GluPostAvg LactDiff LactPostAvg
                CortDiff CortPostAvg;
run;

proc corr data=AllDay1Data plots=scatter;
        var Temperature;
        with ExitSpeed HR30 HR_Diff TimetoBL GluDiff GluPostAvg LactDiff LactPostAvg
                CortDiff CortPostAvg;
run;

proc corr data=AllDay1Data plots=scatter;
        var WindSpeed;
        with ExitSpeed HR30 HR_Diff TimetoBL GluDiff GluPostAvg LactDiff LactPostAvg
                CortDiff CortPostAvg;
run;

proc corr data=AllDay1Data plots=scatter;
        var Breed;
        with ExitSpeed HR30 HR_Diff TimetoBL GluDiff GluPostAvg LactDiff LactPostAvg
                CortDiff CortPostAvg;
run;
```

```
proc corr data=AllDay1Data plots=scatter;
	var GluDiff GluPostAvg;
	with GluRunDate;
run;

proc corr data=AllDay1Data plots=scatter;
	var LactDiff LactPostAvg;
	with LactRunDate;
run;

proc corr data=AllDay1Data plots=scatter;
	var CortDiff CortPostAvg;
	with CortPretestPlate CortPosttestPlate;
run;

*Overview Stats;

proc means data=AllDay1Data mean std;
	class treatment;
	var exitspeed HR30 HR_Diff TimetoBL GluDiff GluPostAvg LactDiff LactPostAvg
		CortDiff CortPostAvg;
run;

proc univariate data=AllDay1Data plots;
	var ExitSpeed HR30 HR_Diff TimetoBL GluDiff GluPostAvg LactDiff LactPostAvg
		CortDiff CortPostAvg;
run;

title 'Day 1 Exit Speed';
Proc Mixed data=AllDay1Data covtest ratio;
	class Treatment Group Horse;
	model ExitSpeed=Group Temperature Treatment/ ddfm=kr solution residual;
	random Horse;
	lsmeans Treatment / pdiff;
	lsmestimate Treatment 3 -1 -1 -1/ divisor=3;
run;

title 'Day 1 HR 30';
Proc Mixed data=AllDay1Data covtest ratio;
	class Treatment TrtNum Horse;
	model HR30=TrtNum Treatment/ ddfm=kr solution residual;
	random Horse;
	lsmeans Treatment / pdiff;
	lsmestimate Treatment 3 -1 -1 -1/ divisor=3;
run;
```

```
title 'Day 1 HR Difference';
Proc Mixed data=AllDay1Data covtest ratio;
	class Treatment Group TrtNum Horse;
	model HR_Diff=Group TrtNum Treatment/ ddfm=kr solution residual;
	random Horse;
	lsmeans Treatment / pdiff;
	lsmestimate Treatment 3 -1 -1 -1/ divisor=3;
run;

title 'Day 1 Time to Return to Baseline';
Proc Mixed data=AllDay1Data covtest ratio;
	class Treatment Horse;
	model TimetoBL= Temperature Treatment/ ddfm=kr solution residual;
	random Horse;
	lsmeans Treatment / pdiff;
	lsmestimate Treatment 3 -1 -1 -1/ divisor=3;
run;

title 'Day 1 Glucose Difference';
Proc Mixed data=AllDay1Data covtest ratio;
	class Treatment GluRunDate Horse;
	model GluDiff=GluRunDate TimeLag Treatment/ ddfm=kr solution residual;
	random Horse;
	lsmeans Treatment / pdiff;
	lsmestimate Treatment 3 -1 -1 -1/ divisor=3;
run;

title 'Day 1 Glucose Post-Startle Average';
Proc Mixed data=AllDay1Data covtest ratio;
	class Treatment Horse;
	model GluPostAvg= TimeLag Treatment/ ddfm=kr solution residual;
	random Horse;
	lsmeans Treatment / pdiff;
	lsmestimate Treatment 3 -1 -1 -1/ divisor=3;
run;

title 'Day 1 Lactate Difference';
Proc Mixed data=AllDay1Data covtest ratio;
	class Treatment Horse;
	model LactDiff=Treatment/ ddfm=kr solution residual;
	random Horse;
	lsmeans Treatment / pdiff;
	lsmestimate Treatment 3 -1 -1 -1/ divisor=3;
run;

title 'Day 1 Lactate Post-Startle Average';
```

```
Proc Mixed data=AllDay1Data covtest ratio;
	class Treatment Horse;
	model LactPostAvg=Treatment/ ddfm=kr solution residual;
	random Horse;
	lsmeans Treatment / pdiff;
	lsmestimate Treatment 3 -1 -1 -1/ divisor=3;
run;

title 'Day 1 Cortisol Difference';
Proc Mixed data=AllDay1Data covtest ratio;
	class Treatment Horse;
	model CortDiff=Treatment/ ddfm=kr solution residual;
	random Horse;
	lsmeans Treatment / pdiff;
	lsmestimate Treatment 3 -1 -1 -1/ divisor=3;
run;

title 'Day 1 Cortisol Post-Startle Average';
Proc Mixed data=AllDay1Data covtest ratio;
	class Treatment Horse CortPostTestPlate;
	model CortPostAvg=CortPosttestPlate Treatment/ ddfm=kr solution residual;
	random Horse;
	lsmeans Treatment / pdiff;
	lsmestimate Treatment 3 -1 -1 -1/ divisor=3;
run;
```

APPENDIX 2

SAS CODE

Analyze Treatment Effects on Behavioral and Physiological Response Variables on Day 3

```
PROC IMPORT OUT=WORK.AllDay3Data DATAFILE="/home/britdav/AllDay3Data.xlsx"
DBMS=XLSX REPLACE;
	GETNAMES=YES;
run;

proc sort data=AllDay3Data;
	by Horse;
run;

*Summary Plots;

Proc Means data=AllDay3Data nway noprint;
	class Treatment Day;
	var ExitSpeed HR30 HR_Diff TimetoBL GluDiff GluPostAvg LactDiff LactPostAvg
		CortDiff CortPostAvg;
	output out=SumStats mean=;
run;

Proc Transpose data=SumStats out=SumStatsTr (rename=(Col1=Y));
	by Treatment Day _TYPE_ _FREQ_;
run;

Proc sort data=SumStatsTr;
	by _LABEL_;

*Simple interaction plot for each response variable;

Proc Sgplot ;
	by _LABEL_;
	series X=Treatment Y=Y;
run;

*proc corr data=AllDay3Data plots=scatter;
*	var treatment;
*	with exitspeed;
*run;

*proc corr data=AllDay3Data plots=scatter;
*	var treatment;
*	with HR30;
```

```
*run;

*proc corr data=AllDay3Data plots=scatter;
*	var treatment;
*	with HR_Diff;
*run;

*proc corr data=AllDay3Data plots=scatter;
*	var treatment;
*	with TimetoBL;
*run;

*proc corr data=AllDay3Data plots=scatter;
*	var treatment;
*	with GluDiff;
*run;

*proc corr data=AllDay3Data plots=scatter;
*	var treatment;
*	with GluPostAvg;
*run;

*proc corr data=AllDay3Data plots=scatter;
*	var treatment;
*	with LactDiff;
*run;

*proc corr data=AllDay3Data plots=scatter;
*	var treatment;
*	with LactPostAvg;
*run;

*proc corr data=AllDay3Data plots=scatter;
*	var treatment;
*	with CortDiff;
*run;

*proc corr data=AllDay3Data plots=scatter;
*	var treatment;
*	with CortPostAvg;
*run;

*Covariate tests;

proc corr data=AllDay3Data plots=scatter;
	var Age;
```

```
        with ExitSpeed HR30 HR_Diff TimetoBL GluDiff GluPostAvg LactDiff LactPostAvg
                CortDiff CortPostAvg;
run;

proc corr data=AllDay3Data plots=scatter;
        var BCS;
        with ExitSpeed HR30 HR_Diff TimetoBL GluDiff GluPostAvg LactDiff LactPostAvg
                CortDiff CortPostAvg;
run;

proc corr data=AllDay3Data plots=scatter;
        var Group;
        with ExitSpeed HR30 HR_Diff TimetoBL GluDiff GluPostAvg LactDiff LactPostAvg
                CortDiff CortPostAvg;
run;

proc corr data=AllDay3Data plots=scatter;
        var TimeLag;
        with ExitSpeed HR30 HR_Diff TimetoBL GluDiff GluPostAvg LactDiff LactPostAvg
                CortDiff CortPostAvg TrtNum;
run;

proc corr data=AllDay3Data plots=scatter;
        var TrtNum;
        with ExitSpeed HR30 HR_Diff TimetoBL GluDiff GluPostAvg LactDiff LactPostAvg
                CortDiff CortPostAvg TimeLag;
run;

proc corr data=AllDay3Data plots=scatter;
        var Temperature;
        with ExitSpeed HR30 HR_Diff TimetoBL GluDiff GluPostAvg LactDiff LactPostAvg
                CortDiff CortPostAvg;
run;

proc corr data=AllDay3Data plots=scatter;
        var WindSpeed;
        with ExitSpeed HR30 HR_Diff TimetoBL GluDiff GluPostAvg LactDiff LactPostAvg
                CortDiff CortPostAvg;
run;

proc corr data=AllDay3Data plots=scatter;
        var Breed;
        with ExitSpeed HR30 HR_Diff TimetoBL GluDiff GluPostAvg LactDiff LactPostAvg
                CortDiff CortPostAvg;
run;
```

```
proc corr data=AllDay3Data plots=scatter;
	var GluDiff GluPostAvg;
	with GluRunDate;
run;

proc corr data=AllDay3Data plots=scatter;
	var LactDiff LactPostAvg;
	with LactRunDate;
run;

proc corr data=AllDay3Data plots=scatter;
	var CortDiff CortPostAvg;
	with CortPretestPlate CortPosttestPlate;
run;

*Overview Stats;

proc means data=AllDay3Data mean std;
	class treatment;
	var exitspeed HR30 HR_Diff TimetoBL GluDiff GluPostAvg LactDiff LactPostAvg
		CortDiff CortPostAvg;
run;

proc univariate data=AllDay3Data plots;
	var ExitSpeed HR30 HR_Diff TimetoBL GluDiff GluPostAvg LactDiff LactPostAvg
		CortDiff CortPostAvg;
run;

title 'Day 3 Exit Speed';
Proc Mixed data=AllDay3Data covtest ratio;
	class Treatment Group Horse;
	model ExitSpeed=Group Temperature Treatment/ ddfm=kr solution residual;
	random Horse;
	lsmeans Treatment / pdiff;
	lsmestimate Treatment 3 -1 -1 -1/ divisor=3;
run;

title 'Day 3 HR 30';
Proc Mixed data=AllDay3Data covtest ratio;
	class Treatment TrtNum Horse;
	model HR30= TrtNum Treatment/ ddfm=kr solution residual;
	random Horse;
	lsmeans Treatment / pdiff;
	lsmestimate Treatment 3 -1 -1 -1/ divisor=3;
run;
```

```
title 'Day 3 HR Difference';
Proc Mixed data=AllDay3Data covtest ratio;
	class Treatment Group TrtNum Horse;
	model HR_Diff=Group TrtNum Treatment/ ddfm=kr solution residual;
	random Horse;
	lsmeans Treatment / pdiff;
	lsmestimate Treatment 3 -1 -1 -1/ divisor=3;
run;

title 'Day 3 Time to Return to Baseline';
Proc Mixed data=AllDay3Data covtest ratio;
	class Treatment Horse;
	model TimetoBL= Temperature Treatment/ ddfm=kr solution residual;
	random Horse;
	lsmeans Treatment / pdiff;
	lsmestimate Treatment 3 -1 -1 -1/ divisor=3;
run;

title 'Day 3 Glucose Difference';
Proc Mixed data=AllDay3Data covtest ratio;
	class Treatment GluRunDate Horse;
	model GluDiff=GluRunDate TimeLag Treatment/ ddfm=kr solution residual;
	random Horse;
	lsmeans Treatment / pdiff;
	lsmestimate Treatment 3 -1 -1 -1/ divisor=3;
run;

title 'Day 3 Glucose Post-Startle Average';
Proc Mixed data=AllDay3Data covtest ratio;
	class Treatment Horse;
	model GluPostAvg=TimeLag Treatment/ ddfm=kr solution residual;
	random Horse;
	lsmeans Treatment / pdiff;
	lsmestimate Treatment 3 -1 -1 -1/ divisor=3;
run;

title 'Day 3 Lactate Difference';
Proc Mixed data=AllDay3Data covtest ratio;
	class Treatment Horse;
	model LactDiff=Treatment/ ddfm=kr solution residual;
	random Horse;
	lsmeans Treatment / pdiff;
	lsmestimate Treatment 3 -1 -1 -1/ divisor=3;
run;

title 'Day 3 Lactate Post-Startle Average';
```

```
Proc Mixed data=AllDay3Data covtest ratio;
	class Treatment Horse;
	model LactPostAvg=Treatment/ ddfm=kr solution residual;
	random Horse;
	lsmeans Treatment / pdiff;
	lsmestimate Treatment 3 -1 -1 -1/ divisor=3;
run;

title 'Day 3 Cortisol Difference';
Proc Mixed data=AllDay3Data covtest ratio;
	class Treatment Horse;
	model CortDiff=Treatment/ ddfm=kr solution residual;
	random Horse;
	lsmeans Treatment / pdiff;
	lsmestimate Treatment 3 -1 -1 -1/ divisor=3;
run;

title 'Day 3 Cortisol Post-Startle Average';
Proc Mixed data=AllDay3Data covtest ratio;
	class Treatment Horse CortPosttestPlate;
	model CortPostAvg=CortPosttestPlate Treatment/ ddfm=kr solution residual;
	random Horse;
	lsmeans Treatment / pdiff;
	lsmestimate Treatment 3 -1 -1 -1/ divisor=3;
run;
```

APPENDIX 3

SAS CODE

Analyze Serum Amino Acids of Group 1 Horses

```
PROC IMPORT OUT=WORK.Group1 DATAFILE="/home/britdav/Group1.xlsx"
DBMS=XLSX REPLACE;
	GETNAMES=YES;
RUN;

*Summary Plots;
Proc Means data=Group1 nway;
	class Treatment Day;
	var TRP_Diff RatioDiff;
	output out=SumStatsGroup1 mean= ;
run;
Proc Transpose data=SumStatsGroup1 out=SumStatsTrGroup1 (rename=(Col1=Y));
	by Treatment Day _TYPE_ _FREQ_;
run;
Proc sort data=SumStatsTrGroup1;
	by _LABEL_;

*Simple interaction plot for each response variable;
Proc Sgplot;
	by _LABEL_;
	series X = Treatment Y = Y / group = Day;
run;

proc corr data=Group1 plots=scatter;
	var treatment;
	with TRP_Diff;
run;

proc corr data=Group1 plots=scatter;
	var treatment;
	with RatioDiff;
run;

title 'Difference in serum free Trp';
Proc Mixed data=Group1;
class Treatment Day Horse;
model TRP_Diff =  Treatment|Day / ddfm=kr solution residual ;
random Horse Horse*Treatment ;
slice Treatment*Day / sliceby = Day pdiff;
run;
```

```
title 'Difference in TRP:LNAA';
Proc Mixed data=Group1;
class Treatment Day Horse;
model RatioDiff =  Treatment|Day / ddfm=kr solution residual ;
random Horse Horse*Treatment ;
slice Treatment*Day / sliceby = Day pdiff;
run;

title 'Washout serum free Trp';
Proc Mixed data=Group1;
where Day=1;
class Treatment TrtNum Day Horse;
model PreTRP =  TrtNum / ddfm=kr solution residual ;
random Horse Horse*TrtNum ;
lsmeans TrtNum/pdiff;
run;

title 'Washout serum TRP:LNAA';
Proc Mixed data=Group1;
where Day=1;
class Treatment TrtNum Day Horse;
model PreRatio =  TrtNum/ ddfm=kr solution residual ;
random Horse Horse*TrtNum ;
lsmeans TrtNum/pdiff;
run;

PROC IMPORT OUT=WORK.AABCCompare
        DATAFILE="/home/britdav/AABCCompare.xlsx"
        DBMS=XLSX REPLACE;
        GETNAMES=YES;
RUN;

Proc Mixed data=AABCCompare;
class Treatment DBC Horse;
model TRP = DBC / ddfm=kr solution residual ;
random Horse Horse*Treatment ;
lsmeans DBC / pdiff;
run;

Proc Mixed data=AABCCompare;
class Treatment DBC Horse;
model Ratio = DBC / ddfm=kr solution residual ;
random Horse Horse*Treatment ;
lsmeans DBC / pdiff;
run;
```

APPENDIX 4

SAS SUMMARY OUTPUT

Day 1 and Day 3 Results

DAY 1

The MEANS Procedure

Treatment	N Obs	Variable	Label	Mean	Std Dev
0	11	ExitSpeed	ExitSpeed	5.1110000	1.1485880
		HR30	HR30	102.6363636	19.0329857
		HR_Diff	HR_Diff	69.5454545	20.2551901
		TimetoBL	TimetoBL	225.7000000	96.9227069
		GluDiff	GluDiff	-4.8388889	6.5327434
		GluPostAvg	GluPostAvg	84.2200000	8.7901207
		LactDiff	LactDiff	0.1703850	0.2849126
		LactPostAvg	LactPostAvg	1.1549550	0.3062569
		CortDiff	CortDiff	2.7914811	5.3027572
		CortPostAvg	CortPostAvg	13.8581452	10.9708711
20	11	ExitSpeed	ExitSpeed	4.6540000	1.7737982
		HR30	HR30	98.6000000	18.8455712
		HR_Diff	HR_Diff	56.4000000	12.2764454
		TimetoBL	TimetoBL	206.7777778	71.4086440
		GluDiff	GluDiff	-6.5900000	4.3247222
		GluPostAvg	GluPostAvg	82.0500000	7.6745684
		LactDiff	LactDiff	-0.1029833	0.2326554
		LactPostAvg	LactPostAvg	0.9701400	0.2181086
		CortDiff	CortDiff	1.0943183	3.3103723
		CortPostAvg	CortPostAvg	12.1232398	6.6245716
40	11	ExitSpeed	ExitSpeed	5.1036364	1.5033248
		HR30	HR30	103.4000000	26.1074702
		HR_Diff	HR_Diff	68.3000000	26.7168278
		TimetoBL	TimetoBL	246.5000000	89.5547629
		GluDiff	GluDiff	-4.4318182	7.1485059
		GluPostAvg	GluPostAvg	82.6000000	6.1487397
		LactDiff	LactDiff	0.2058545	0.3562690
		LactPostAvg	LactPostAvg	1.2467318	0.3483196
		CortDiff	CortDiff	-1.8589621	3.6007997
		CortPostAvg	CortPostAvg	11.9115737	10.5414673
60	11	ExitSpeed	ExitSpeed	5.6044444	1.0521420
		HR30	HR30	109.9090909	24.4763337
		HR_Diff	HR_Diff	74.3636364	23.6231781
		TimetoBL	TimetoBL	215.0000000	64.4773862
		GluDiff	GluDiff	-0.3777778	6.9744673
		GluPostAvg	GluPostAvg	88.0888889	8.5154557
		LactDiff	LactDiff	0.2294000	0.1388055
		LactPostAvg	LactPostAvg	1.1278833	0.2177054
		CortDiff	CortDiff	-0.3770851	3.6004427
		CortPostAvg	CortPostAvg	12.6833565	12.6058338

DAY 3

The MEANS Procedure

Treatment	N Obs	Variable	Label	Mean	Std Dev
0	11	ExitSpeed	ExitSpeed	5.2036364	1.3454090
		HR30	HR30	109.2727273	24.7390012
		HR_Diff	HR_Diff	72.8181818	29.1952674
		TimetoBL	TimetoBL	233.0000000	84.2531899
		GluDiff	GluDiff	-3.2750000	4.1620808
		GluPostAvg	GluPostAvg	84.7450000	9.6280392
		LactDiff	LactDiff	0.1782167	0.2547027
		LactPostAvg	LactPostAvg	1.1050667	0.3313360
		CortDiff	CortDiff	-0.3263981	6.3440143
		CortPostAvg	CortPostAvg	12.9971851	11.6014889
20	11	ExitSpeed	ExitSpeed	5.5545455	1.3703311
		HR30	HR30	103.8181818	19.4669884
		HR_Diff	HR_Diff	67.7272727	18.3580550
		TimetoBL	TimetoBL	230.1000000	135.8900291
		GluDiff	GluDiff	1.5050000	8.6916451
		GluPostAvg	GluPostAvg	85.0045455	3.9880104
		LactDiff	LactDiff	0.2972333	0.3745439
		LactPostAvg	LactPostAvg	1.1849250	0.3126254
		CortDiff	CortDiff	0.3276037	3.4494645
		CortPostAvg	CortPostAvg	10.7199882	5.7296725
40	11	ExitSpeed	ExitSpeed	5.1318182	1.2702033
		HR30	HR30	99.7000000	26.8661290
		HR_Diff	HR_Diff	65.6000000	26.8874196
		TimetoBL	TimetoBL	318.1000000	223.3380348
		GluDiff	GluDiff	-2.5700000	7.5269221
		GluPostAvg	GluPostAvg	87.1727273	11.7776985
		LactDiff	LactDiff	0.2281667	0.3879192
		LactPostAvg	LactPostAvg	1.2438167	0.3809181
		CortDiff	CortDiff	-0.1416802	3.1823009
		CortPostAvg	CortPostAvg	13.2783739	9.4159948
60	11	ExitSpeed	ExitSpeed	5.7054545	1.0771756
		HR30	HR30	97.9000000	30.2267358
		HR_Diff	HR_Diff	63.2000000	28.7162362
		TimetoBL	TimetoBL	251.5000000	111.6604476
		GluDiff	GluDiff	-0.9666667	5.3440387
		GluPostAvg	GluPostAvg	87.6055556	6.8875088
		LactDiff	LactDiff	0.1900875	0.1399264
		LactPostAvg	LactPostAvg	1.3715437	0.2088755
		CortDiff	CortDiff	-0.5091321	1.9982001
		CortPostAvg	CortPostAvg	9.7877718	5.1523127

Day 1 Exit Speed

The Mixed Procedure

Type 3 Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Group	2	8.15	0.59	0.5786
Temperature	1	25.2	21.47	<.0001
Treatment	3	25.3	1.25	0.3125

Least Squares Means Estimate						
Effect	Label	Estimate	Standard Error	DF	t Value	Pr > |t|
Treatment	Row 1	-0.03741	0.2726	25.3	-0.14	0.8919

Least Squares Means						
Effect	Treatment	Estimate	Standard Error	DF	t Value	Pr > |t|
Treatment	0	5.0861	0.3789	15	13.42	<.0001
Treatment	20	4.7708	0.3790	15	12.59	<.0001
Treatment	40	5.1639	0.3710	14	13.92	<.0001
Treatment	60	5.4357	0.3903	16.4	13.93	<.0001

Differences of Least Squares Means							
Effect	Treatment	Treatment	Estimate	Standard Error	DF	t Value	Pr > |t|
Treatment	0	20	0.3153	0.3332	25.3	0.95	0.3530
Treatment	0	40	-0.07785	0.3230	25.2	-0.24	0.8115
Treatment	0	60	-0.3496	0.3479	25.4	-1.01	0.3243
Treatment	20	40	-0.3931	0.3229	25.2	-1.22	0.2347
Treatment	20	60	-0.6649	0.3484	25.4	-1.91	0.0677
Treatment	40	60	-0.2718	0.3388	25.3	-0.80	0.4299

Day 3 Exit Speed

The Mixed Procedure

Type 3 Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Group	2	8.84	4.43	0.0465
Temperature	1	29.3	0.00	0.9452
Treatment	3	29	0.62	0.6048

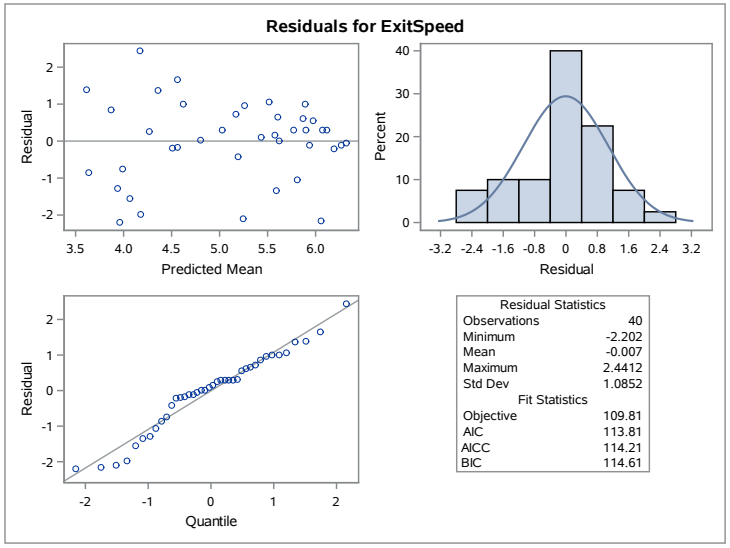
Least Squares Means Estimate						
Effect	Label	Estimate	Standard Error	DF	t Value	Pr > |t|
Treatment	Row 1	-0.2621	0.4013	29.01	-0.65	0.5189

Least Squares Means						
Effect	Treatment	Estimate	Standard Error	DF	t Value	Pr > |t|
Treatment	0	5.1867	0.3550	36.1	14.61	<.0001
Treatment	20	5.5371	0.3556	36.2	15.57	<.0001
Treatment	40	5.1191	0.3559	36.2	14.38	<.0001
Treatment	60	5.6902	0.3541	36.1	16.07	<.0001

Differences of Least Squares Means							
Effect	Treatment	Treatment	Estimate	Standard Error	DF	t Value	Pr > |t|
Treatment	0	20	-0.3503	0.4905	29	-0.71	0.4808
Treatment	0	40	0.06762	0.4942	29	0.14	0.8921
Treatment	0	60	-0.5034	0.4910	29	-1.03	0.3137
Treatment	20	40	0.4180	0.4953	29	0.84	0.4056
Treatment	20	60	-0.1531	0.4915	29	-0.31	0.7577
Treatment	40	60	-0.5711	0.4919	29	-1.16	0.2551

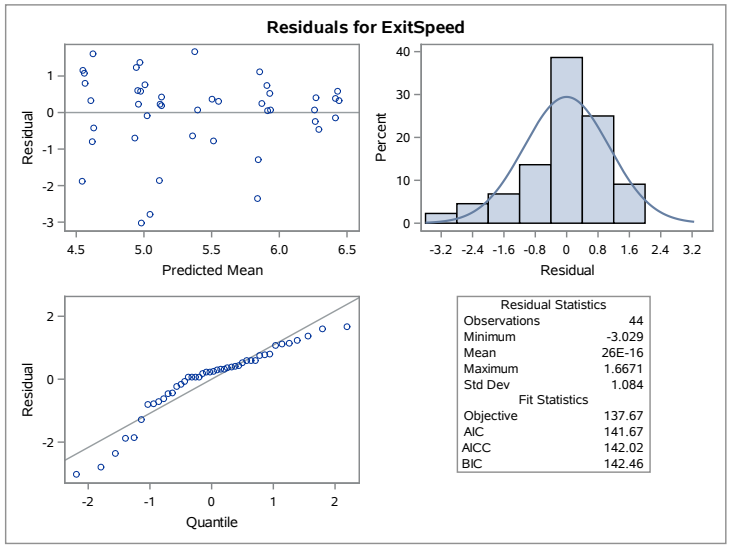
Day 1 Exit Speed

The Mixed Procedure



Day 3 Exit Speed

The Mixed Procedure



Day 1 HR 30

The Mixed Procedure

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
TrtNum	3	25.5	0.39	0.7613
Treatment	3	25.5	0.89	0.4586

Least Squares Means Estimate

Effect	Label	Estimate	Standard Error	DF	t Value	Pr > |t|
Treatment	Row 1	-0.9792	6.0371	25.33	-0.16	0.8724

Least Squares Means

Effect	Treatment	Estimate	Standard Error	DF	t Value	Pr > |t|
Treatment	0	102.83	6.7946	25	15.13	<.0001
Treatment	20	97.5991	7.0538	26.6	13.84	<.0001
Treatment	40	104.04	7.0556	26.6	14.75	<.0001
Treatment	60	109.78	6.7957	25	16.15	<.0001

Differences of Least Squares Means

Effect	Treatment	Treatment	Estimate	Standard Error	DF	t Value	Pr > |t|
Treatment	0	20	5.2280	7.5524	25.5	0.69	0.4950
Treatment	0	40	-1.2107	7.5530	25.5	-0.16	0.8739
Treatment	0	60	-6.9549	7.2998	25.2	-0.95	0.3497
Treatment	20	40	-6.4388	7.7839	25.8	-0.83	0.4157
Treatment	20	60	-12.1830	7.5089	25.5	-1.62	0.1170
Treatment	40	60	-5.7442	7.5578	25.5	-0.76	0.4542

Day 3 HR 30

The Mixed Procedure

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
TrtNum	3	25.4	2.08	0.1283
Treatment	3	25.4	0.46	0.7112

Least Squares Means Estimate

Effect	Label	Estimate	Standard Error	DF	t Value	Pr > |t|
Treatment	Row 1	7.4936	7.1308	25.22	1.05	0.3033

Least Squares Means

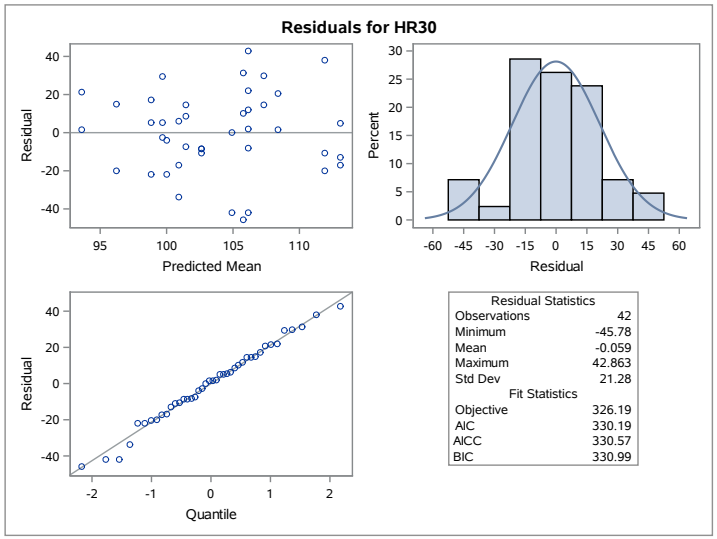
Effect	Treatment	Estimate	Standard Error	DF	t Value	Pr > |t|
Treatment	0	108.36	7.4783	27.9	14.49	<.0001
Treatment	20	103.66	7.4811	27.9	13.86	<.0001
Treatment	40	99.9135	7.8013	29.4	12.81	<.0001
Treatment	60	99.0201	7.8013	29.4	12.69	<.0001

Differences of Least Squares Means

Effect	Treatment	Treatment	Estimate	Standard Error	DF	t Value	Pr > |t|
Treatment	0	20	4.6956	8.6112	25.1	0.55	0.5904
Treatment	0	40	8.4459	8.9035	25.4	0.95	0.3518
Treatment	0	60	9.3393	8.9035	25.4	1.05	0.3041
Treatment	20	40	3.7502	8.8516	25.4	0.42	0.6754
Treatment	20	60	4.6437	8.8516	25.4	0.52	0.6044
Treatment	40	60	0.8935	9.1519	25.7	0.10	0.9230

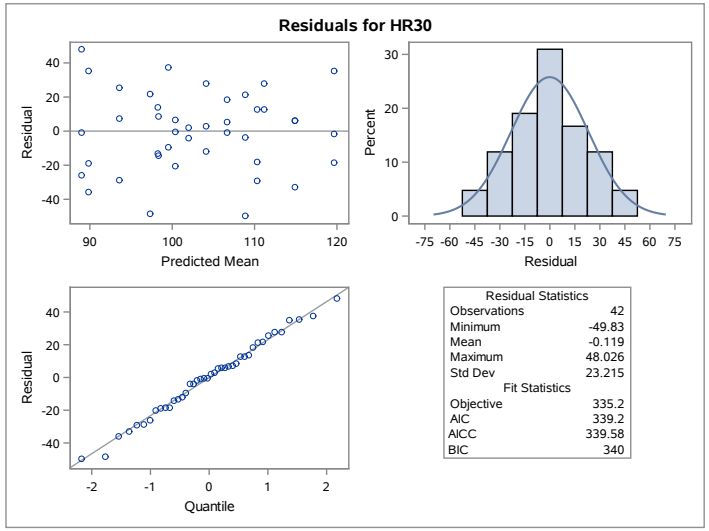
Day 1 HR 30

The Mixed Procedure



Day 3 HR 30

The Mixed Procedure



Day 1 HR Difference

The Mixed Procedure

Type 3 Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Group	2	8.09	0.89	0.4478
TrtNum	3	25.3	1.17	0.3393
Treatment	3	25.3	2.57	0.0762

Least Squares Means Estimate						
Effect	Label	Estimate	Standard Error	DF	t Value	Pr > |t|
Treatment	Row 1	3.7407	5.7735	25.15	0.65	0.5229

Least Squares Means						
Effect	Treatment	Estimate	Standard Error	DF	t Value	Pr > |t|
Treatment	0	69.9473	6.4825	20.7	10.79	<.0001
Treatment	20	55.3262	6.7246	22.3	8.23	<.0001
Treatment	40	68.7876	6.7672	22.6	10.16	<.0001
Treatment	60	74.5059	6.4824	20.7	11.49	<.0001

Differences of Least Squares Means							
Effect	Treatment	Treatment	Estimate	Standard Error	DF	t Value	Pr > |t|
Treatment	0	20	14.6211	7.2251	25.3	2.02	0.0537
Treatment	0	40	1.1597	7.2269	25.3	0.16	0.8738
Treatment	0	60	-4.5586	6.9800	25.1	-0.65	0.5196
Treatment	20	40	-13.4613	7.4537	25.5	-1.81	0.0827
Treatment	20	60	-19.1797	7.1829	25.3	-2.67	0.0131
Treatment	40	60	-5.7184	7.2319	25.3	-0.79	0.4365

Day 3 HR Difference

The Mixed Procedure

Type 3 Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Group	2	8.1	4.93	0.0397
TrtNum	3	25.1	4.03	0.0180
Treatment	3	25.3	0.17	0.9131

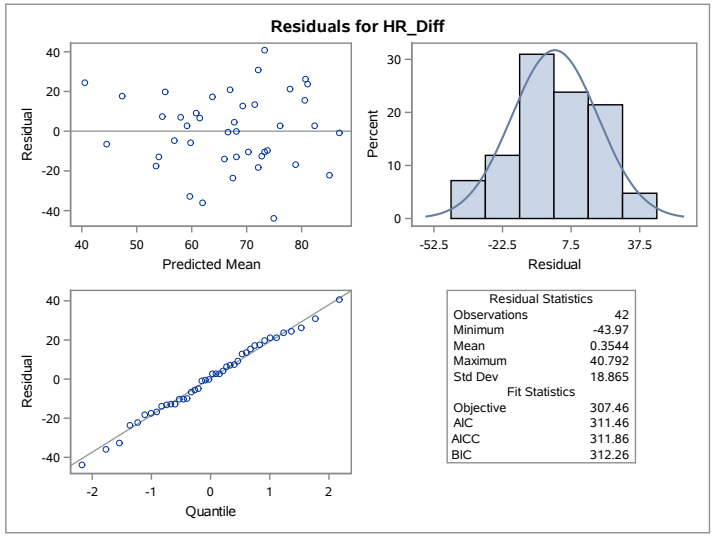
Least Squares Means Estimate						
Effect	Label	Estimate	Standard Error	DF	t Value	Pr > |t|
Treatment	Row 1	4.5217	7.3382	24.99	0.62	0.5433

Least Squares Means						
Effect	Treatment	Estimate	Standard Error	DF	t Value	Pr > |t|
Treatment	0	71.8709	6.5172	31.9	11.03	<.0001
Treatment	20	68.5627	6.5299	31.9	10.50	<.0001
Treatment	40	68.1947	6.9445	32.4	9.82	<.0001
Treatment	60	65.2902	6.9445	32.4	9.40	<.0001

Differences of Least Squares Means							
Effect	Treatment	Treatment	Estimate	Standard Error	DF	t Value	Pr > |t|
Treatment	0	20	3.3082	8.8597	24.9	0.37	0.7120
Treatment	0	40	3.6762	9.1533	25.3	0.40	0.6913
Treatment	0	60	6.5807	9.1533	25.3	0.72	0.4788
Treatment	20	40	0.3680	9.0990	25.3	0.04	0.9681
Treatment	20	60	3.2725	9.0990	25.3	0.36	0.7221
Treatment	40	60	2.9045	9.3783	26	0.31	0.7593

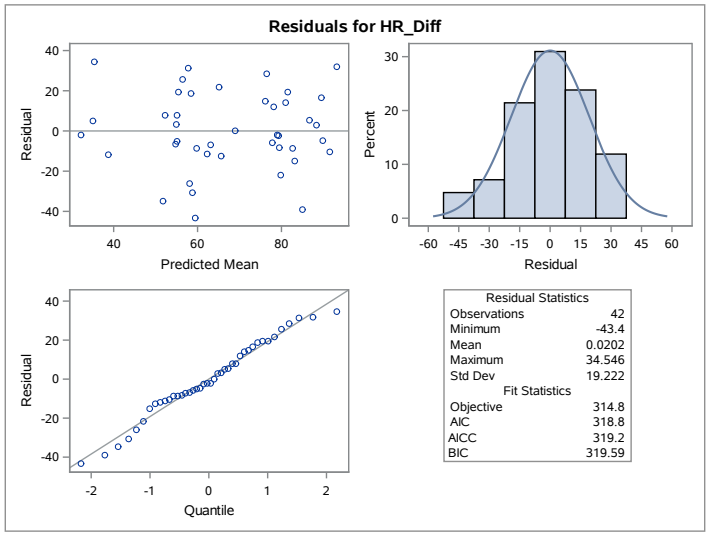
Day 1 HR Difference

The Mixed Procedure



Day 3 HR Difference

The Mixed Procedure



Day 1 Time to Return to Baseline

The Mixed Procedure

Type 3 Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Temperature	1	27.5	6.73	0.0150
Treatment	3	24.4	0.62	0.6060

Least Squares Means Estimate						
Effect	Label	Estimate	Standard Error	DF	t Value	Pr > |t|
Treatment	Row 1	-0.2434	26.4636	24.65	-0.01	0.9927

Least Squares Means						
Effect	Treatment	Estimate	Standard Error	DF	t Value	Pr > |t|
Treatment	0	224.00	24.1456	33	9.28	<.0001
Treatment	20	213.53	25.5115	33.4	8.37	<.0001
Treatment	40	249.62	24.1412	33	10.34	<.0001
Treatment	60	209.59	24.1871	33	8.67	<.0001

Differences of Least Squares Means							
Effect	Treatment	Treatment	Estimate	Standard Error	DF	t Value	Pr > |t|
Treatment	0	20	10.4741	33.2647	25	0.31	0.7555
Treatment	0	40	-25.6135	32.1909	24.4	-0.80	0.4339
Treatment	0	60	14.4093	32.1574	24.2	0.45	0.6581
Treatment	20	40	-36.0875	32.8550	23.7	-1.10	0.2831
Treatment	20	60	3.9353	33.3017	25	0.12	0.9069
Treatment	40	60	40.0228	32.2384	24.4	1.24	0.2262

Day 3 Time to Return to Baseline

The Mixed Procedure

Type 3 Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Temperature	1	29.7	18.83	0.0002
Treatment	3	26.9	2.44	0.0858

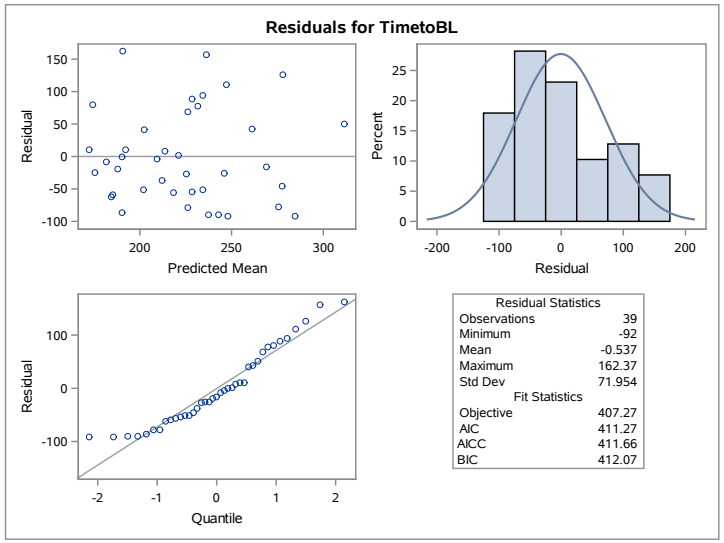
Least Squares Means Estimate						
Effect	Label	Estimate	Standard Error	DF	t Value	Pr > |t|
Treatment	Row 1	-37.0710	33.4777	26.77	-1.11	0.2780

Least Squares Means						
Effect	Treatment	Estimate	Standard Error	DF	t Value	Pr > |t|
Treatment	0	228.69	38.2087	24.2	5.99	<.0001
Treatment	20	224.28	39.6891	25.7	5.65	<.0001
Treatment	40	325.88	39.5782	25.9	8.23	<.0001
Treatment	60	247.11	39.6722	25.7	6.23	<.0001

Differences of Least Squares Means							
Effect	Treatment	Treatment	Estimate	Standard Error	DF	t Value	Pr > |t|
Treatment	0	20	4.4029	41.6165	26.9	0.11	0.9165
Treatment	0	40	-97.1953	41.6093	26.7	-2.34	0.0273
Treatment	0	60	-18.4206	41.6083	26.9	-0.44	0.6615
Treatment	20	40	-101.60	43.1177	27.3	-2.36	0.0259
Treatment	20	60	-22.8235	42.2024	26.5	-0.54	0.5932
Treatment	40	60	78.7747	43.0877	27.3	1.83	0.0785

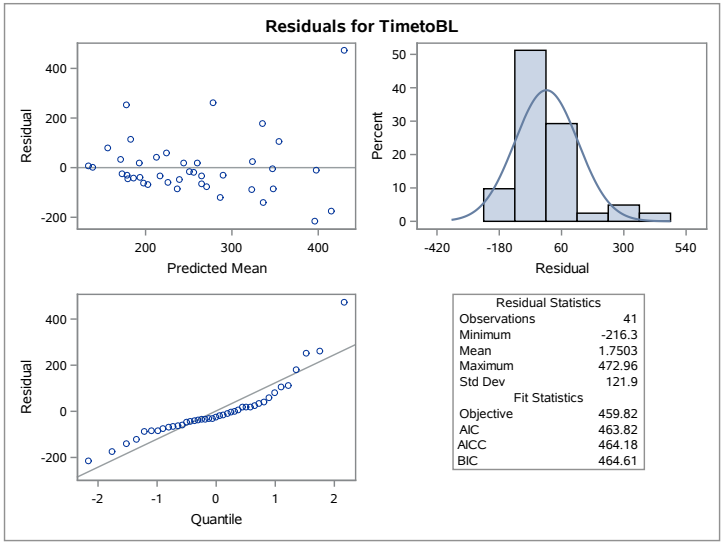
Day 1 Time to Return to Baseline

The Mixed Procedure



Day 3 Time to Return to Baseline

The Mixed Procedure



Day 1 Glucose Difference

The Mixed Procedure

Type 3 Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
GluRunDate	8	20.6	1.36	0.2695
TimeLag	1	24.9	2.30	0.1420
Treatment	3	19	3.81	0.0271

Least Squares Means Estimate						
Effect	Label	Estimate	Standard Error	DF	t Value	Pr > |t|
Treatment	Row 1	0.2812	2.1279	18.06	0.13	0.8963

Least Squares Means						
Effect	Treatment	Estimate	Standard Error	DF	t Value	Pr > |t|
Treatment	0	-4.3400	1.9570	25.6	-2.22	0.0357
Treatment	20	-8.2701	1.9689	25.2	-4.20	0.0003
Treatment	40	-4.9574	2.0685	25.7	-2.40	0.0241
Treatment	60	-0.6361	2.0332	25.4	-0.31	0.7569

Differences of Least Squares Means							
Effect	Treatment	Treatment	Estimate	Standard Error	DF	t Value	Pr > |t|
Treatment	0	20	3.9301	2.4298	18	1.62	0.1232
Treatment	0	40	0.6174	2.7307	18.8	0.23	0.8236
Treatment	0	60	-3.7039	2.3985	18.7	-1.54	0.1393
Treatment	20	40	-3.3127	2.2771	20	-1.45	0.1613
Treatment	20	60	-7.6340	2.2601	18.1	-3.38	0.0033
Treatment	40	60	-4.3213	2.5092	20.7	-1.72	0.0999

Day 3 Glucose Difference

The Mixed Procedure

Type 3 Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
GluRunDate	7	27	2.72	0.0284
TimeLag	1	27	10.59	0.0031
Treatment	3	27	1.17	0.3379

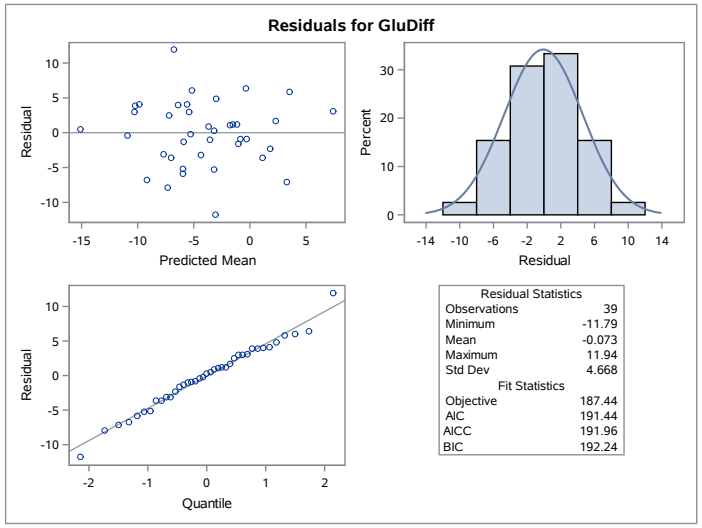
Least Squares Means Estimate						
Effect	Label	Estimate	Standard Error	DF	t Value	Pr > |t|
Treatment	Row 1	-1.9010	2.0013	27	-0.95	0.3506

Least Squares Means						
Effect	Treatment	Estimate	Standard Error	DF	t Value	Pr > |t|
Treatment	0	-1.7820	1.7395	27	-1.02	0.3147
Treatment	20	1.7944	1.6904	27	1.06	0.2979
Treatment	40	-1.9823	1.7606	27	-1.13	0.2701
Treatment	60	0.5449	1.9034	27	0.29	0.7769

Differences of Least Squares Means							
Effect	Treatment	Treatment	Estimate	Standard Error	DF	t Value	Pr > |t|
Treatment	0	20	-3.5764	2.4067	27	-1.49	0.1489
Treatment	0	40	0.2003	2.4302	27	0.08	0.9349
Treatment	0	60	-2.3269	2.5239	27	-0.92	0.3647
Treatment	20	40	3.7766	2.3988	27	1.57	0.1270
Treatment	20	60	1.2495	2.5131	27	0.50	0.6231
Treatment	40	60	-2.5272	2.4676	27	-1.02	0.3149

Day 1 Glucose Difference

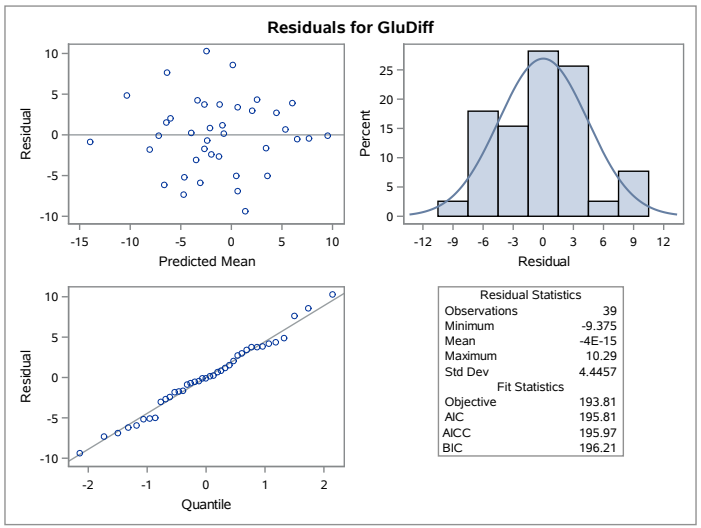
The Mixed Procedure



Residual Statistics	
Observations	39
Minimum	-11.79
Mean	-0.073
Maximum	11.94
Std Dev	4.668
Fit Statistics	
Objective	187.44
AIC	191.44
AICC	191.96
BIC	192.24

Day 3 Glucose Difference

The Mixed Procedure



Residual Statistics	
Observations	39
Minimum	-9.375
Mean	-4E-15
Maximum	10.29
Std Dev	4.4457
Fit Statistics	
Objective	193.81
AIC	195.81
AICC	195.97
BIC	196.21

Day 1 Glucose Post-Startle Average

The Mixed Procedure

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
TimeLag	1	36	6.39	0.0160
Treatment	3	36	1.24	0.3082

Least Squares Means Estimate

Effect	Label	Estimate	Standard Error	DF	t Value	Pr > |t|
Treatment	Row 1	-1.1391	2.6843	36	-0.42	0.6738

Least Squares Means

Effect	Treatment	Estimate	Standard Error	DF	t Value	Pr > |t|
Treatment	0	83.3564	2.3252	36	35.85	<.0001
Treatment	20	81.7106	2.1971	36	37.19	<.0001
Treatment	40	83.8935	2.2518	36	37.26	<.0001
Treatment	60	87.8824	2.4258	36	36.23	<.0001

Differences of Least Squares Means

Effect	Treatment	Treatment	Estimate	Standard Error	DF	t Value	Pr > |t|
Treatment	0	20	1.6458	3.1847	36	0.52	0.6085
Treatment	0	40	-0.5371	3.2904	36	-0.16	0.8712
Treatment	0	60	-4.5260	3.3519	36	-1.35	0.1854
Treatment	20	40	-2.1830	3.1678	36	-0.69	0.4952
Treatment	20	60	-6.1718	3.2695	36	-1.89	0.0671
Treatment	40	60	-3.9888	3.3225	36	-1.20	0.2378

Day 3 Glucose Post-Startle Average

The Mixed Procedure

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
TimeLag	1	34.8	5.86	0.0208
Treatment	3	27.8	0.15	0.9320

Least Squares Means Estimate

Effect	Label	Estimate	Standard Error	DF	t Value	Pr > |t|
Treatment	Row 1	-1.2672	2.9182	27.69	-0.43	0.6675

Least Squares Means

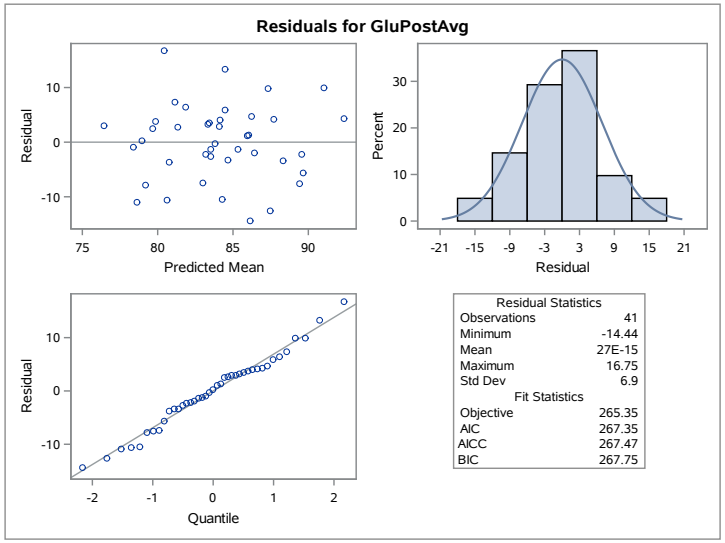
Effect	Treatment	Estimate	Standard Error	DF	t Value	Pr > |t|
Treatment	0	85.1687	2.5740	35.9	33.09	<.0001
Treatment	20	85.4068	2.4460	35.9	34.92	<.0001
Treatment	40	86.8982	2.4430	35.9	35.57	<.0001
Treatment	60	87.0029	2.7334	36	31.83	<.0001

Differences of Least Squares Means

Effect	Treatment	Treatment	Estimate	Standard Error	DF	t Value	Pr > |t|
Treatment	0	20	-0.2381	3.4900	27.5	-0.07	0.9461
Treatment	0	40	-1.7295	3.5024	27.7	-0.49	0.6253
Treatment	0	60	-1.8341	3.7018	28	-0.50	0.6241
Treatment	20	40	-1.4914	3.4089	27	-0.44	0.6652
Treatment	20	60	-1.5960	3.6304	28.5	-0.44	0.6635
Treatment	40	60	-0.1046	3.6066	28.3	-0.03	0.9771

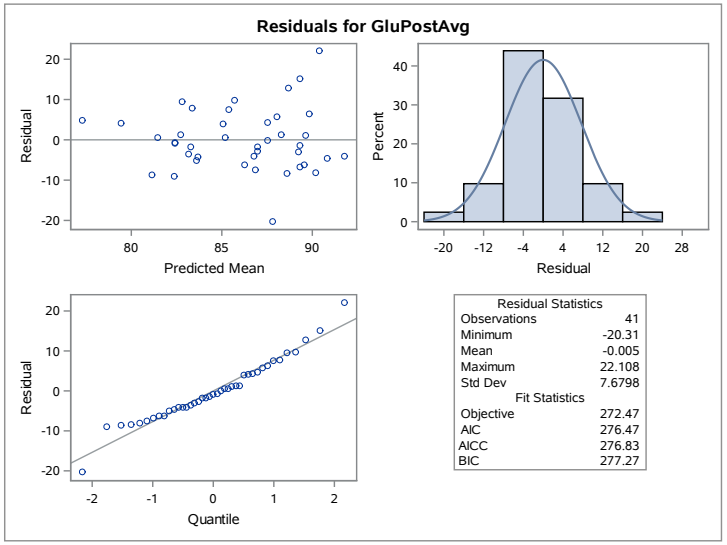
Day 1 Glucose Post-Startle Average

The Mixed Procedure



Day 3 Glucose Post-Startle Average

The Mixed Procedure



Day 1 Lactate Difference

The Mixed Procedure

Type 3 Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Treatment	3	26.3	3.25	0.0376

Least Squares Means Estimate						
Effect	Label	Estimate	Standard Error	DF	t Value	Pr > |t|
Treatment	Row 1	0.05637	0.09210	25.7	0.61	0.5459

Least Squares Means						
Effect	Treatment	Estimate	Standard Error	DF	t Value	Pr > |t|
Treatment	0	0.1661	0.08612	33.2	1.93	0.0623
Treatment	20	-0.09890	0.09090	33.8	-1.09	0.2843
Treatment	40	0.2059	0.08197	32.9	2.51	0.0171
Treatment	60	0.2224	0.09090	33.8	2.45	0.0198

Differences of Least Squares Means							
Effect	Treatment	Treatment	Estimate	Standard Error	DF	t Value	Pr > |t|
Treatment	0	20	0.2650	0.1156	25.9	2.29	0.0302
Treatment	0	40	-0.03972	0.1099	26.2	-0.36	0.7207
Treatment	0	60	-0.05622	0.1156	25.9	-0.49	0.6309
Treatment	20	40	-0.3048	0.1137	26.7	-2.68	0.0124
Treatment	20	60	-0.3213	0.1194	26.5	-2.69	0.0122
Treatment	40	60	-0.01650	0.1137	26.7	-0.15	0.8857

Day 3 Lactate Difference

The Mixed Procedure

Type 3 Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Treatment	3	23.8	0.39	0.7643

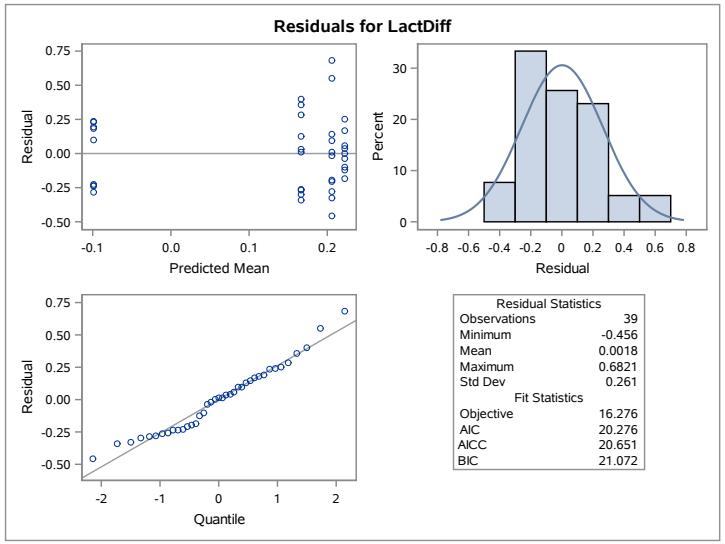
Least Squares Means Estimate						
Effect	Label	Estimate	Standard Error	DF	t Value	Pr > |t|
Treatment	Row 1	-0.07351	0.1079	23.65	-0.68	0.5024

Least Squares Means						
Effect	Treatment	Estimate	Standard Error	DF	t Value	Pr > |t|
Treatment	0	0.1691	0.1029	29.1	1.64	0.1111
Treatment	20	0.2994	0.1029	29.1	2.91	0.0069
Treatment	40	0.2391	0.1029	29.1	2.32	0.0273
Treatment	60	0.1892	0.1090	29.8	1.74	0.0931

Differences of Least Squares Means							
Effect	Treatment	Treatment	Estimate	Standard Error	DF	t Value	Pr > |t|
Treatment	0	20	-0.1304	0.1313	23.6	-0.99	0.3308
Treatment	0	40	-0.07003	0.1313	23.6	-0.53	0.5988
Treatment	0	60	-0.02012	0.1364	24.1	-0.15	0.8840
Treatment	20	40	0.06036	0.1313	23.6	0.46	0.6500
Treatment	20	60	0.1103	0.1364	24.1	0.81	0.4268
Treatment	40	60	0.04992	0.1364	24.1	0.37	0.7176

Day 1 Lactate Difference

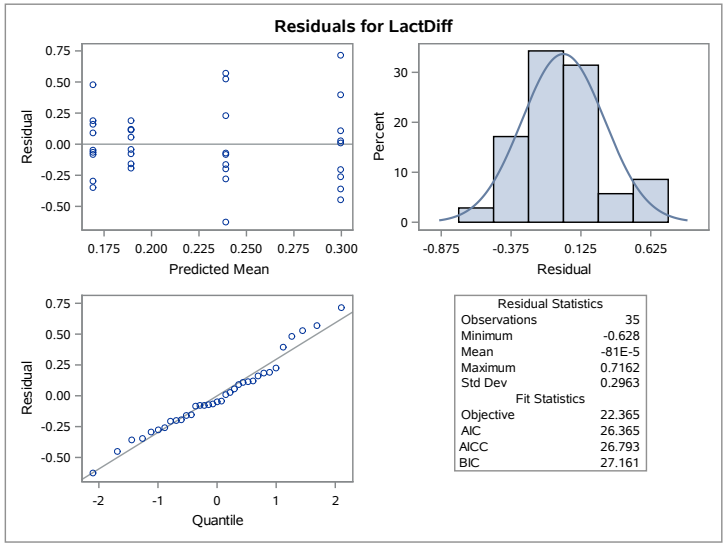
The Mixed Procedure



Residual Statistics	
Observations	39
Minimum	-0.456
Mean	0.0018
Maximum	0.6821
Std Dev	0.261
Fit Statistics	
Objective	16.276
AIC	20.276
AICC	20.651
BIC	21.072

Day 3 Lactate Difference

The Mixed Procedure



Residual Statistics	
Observations	35
Minimum	-0.628
Mean	-81E-5
Maximum	0.7162
Std Dev	0.2963
Fit Statistics	
Objective	22.365
AIC	26.365
AICC	26.793
BIC	27.161

Day 1 Lactate Post-Startle Average

The Mixed Procedure

Type 3 Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Treatment	3	26	2.16	0.1167

Least Squares Means Estimate						
Effect	Label	Estimate	Standard Error	DF	t Value	Pr > |t|
Treatment	Row 1	0.02538	0.08783	25.73	0.29	0.7749

Least Squares Means						
Effect	Treatment	Estimate	Standard Error	DF	t Value	Pr > |t|
Treatment	0	1.1373	0.08949	30	12.71	<.0001
Treatment	20	0.9811	0.08932	30.1	10.98	<.0001
Treatment	40	1.2467	0.08557	28.6	14.57	<.0001
Treatment	60	1.1079	0.09386	31.5	11.80	<.0001

Differences of Least Squares Means							
Effect	Treatment	Treatment	Estimate	Standard Error	DF	t Value	Pr > |t|
Treatment	0	20	0.1562	0.1083	26.2	1.44	0.1611
Treatment	0	40	-0.1095	0.1050	25.8	-1.04	0.3071
Treatment	0	60	0.02942	0.1104	25.6	0.27	0.7920
Treatment	20	40	-0.2656	0.1049	25.6	-2.53	0.0178
Treatment	20	60	-0.1268	0.1121	26.7	-1.13	0.2683
Treatment	40	60	0.1389	0.1088	26.2	1.28	0.2130

Day 3 Lactate Post-Startle Average

The Mixed Procedure

Type 3 Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Treatment	3	23.6	1.35	0.2821

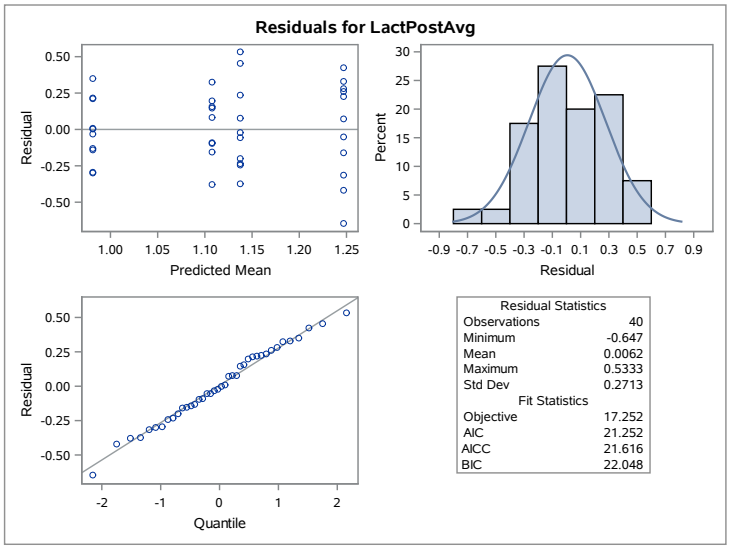
Least Squares Means Estimate						
Effect	Label	Estimate	Standard Error	DF	t Value	Pr > |t|
Treatment	Row 1	-0.1690	0.1059	23.16	-1.60	0.1241

Least Squares Means						
Effect	Treatment	Estimate	Standard Error	DF	t Value	Pr > |t|
Treatment	0	1.0895	0.1055	28.9	10.33	<.0001
Treatment	20	1.1771	0.1001	28.1	11.76	<.0001
Treatment	40	1.2497	0.1055	28.9	11.85	<.0001
Treatment	60	1.3487	0.1114	30	12.11	<.0001

Differences of Least Squares Means							
Effect	Treatment	Treatment	Estimate	Standard Error	DF	t Value	Pr > |t|
Treatment	0	20	-0.08764	0.1271	23.8	-0.69	0.4972
Treatment	0	40	-0.1602	0.1292	22.9	-1.24	0.2275
Treatment	0	60	-0.2592	0.1343	23.4	-1.93	0.0659
Treatment	20	40	-0.07256	0.1271	23.8	-0.57	0.5734
Treatment	20	60	-0.1716	0.1323	24.2	-1.30	0.2069
Treatment	40	60	-0.09902	0.1343	23.4	-0.74	0.4684

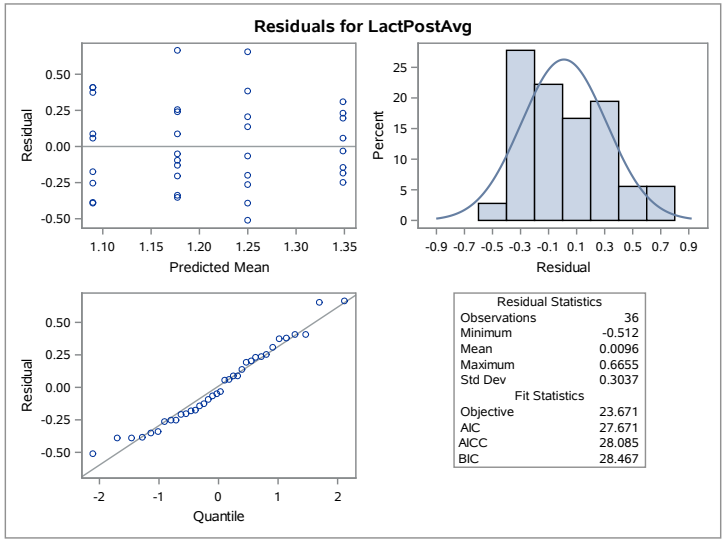
Day 1 Lactate Post-Startle Average

The Mixed Procedure



Day 3 Lactate Post-Startle Average

The Mixed Procedure



Day 1 Cortisol Difference

The Mixed Procedure

Type 3 Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Treatment	3	36	2.53	0.0722

Least Squares Means Estimate						
Effect	Label	Estimate	Standard Error	DF	t Value	Pr > |t|
Treatment	Row 1	3.1721	1.4733	36	2.15	0.0381

Least Squares Means						
Effect	Treatment	Estimate	Standard Error	DF	t Value	Pr > |t|
Treatment	0	2.7915	1.2748	36	2.19	0.0351
Treatment	20	1.0943	1.2748	36	0.86	0.3963
Treatment	40	-1.8590	1.2155	36	-1.53	0.1349
Treatment	60	-0.3771	1.3438	36	-0.28	0.7806

Differences of Least Squares Means							
Effect	Treatment	Treatment	Estimate	Standard Error	DF	t Value	Pr > |t|
Treatment	0	20	1.6972	1.8029	36	0.94	0.3528
Treatment	0	40	4.6504	1.7614	36	2.64	0.0122
Treatment	0	60	3.1686	1.8523	36	1.71	0.0958
Treatment	20	40	2.9533	1.7614	36	1.68	0.1023
Treatment	20	60	1.4714	1.8523	36	0.79	0.4322
Treatment	40	60	-1.4819	1.8120	36	-0.82	0.4188

Day 3 Cortisol Difference

The Mixed Procedure

Type 3 Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
Treatment	3	24.1	0.06	0.9783

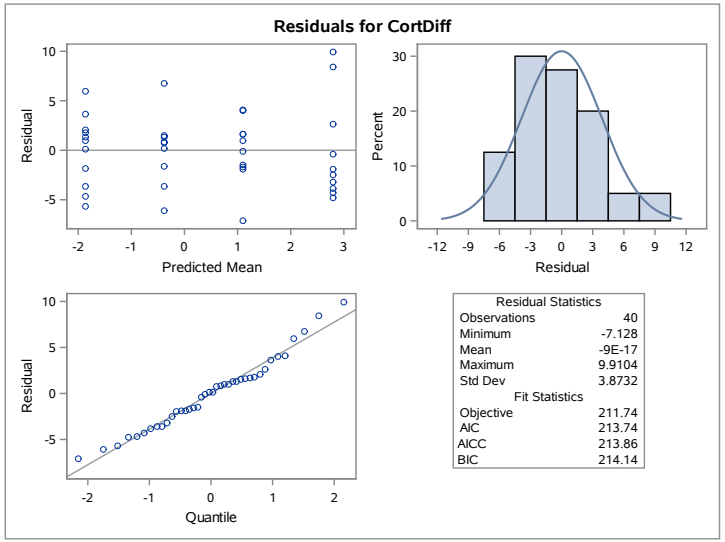
Least Squares Means Estimate						
Effect	Label	Estimate	Standard Error	DF	t Value	Pr > |t|
Treatment	Row 1	-0.2446	1.4604	24.17	-0.17	0.8684

Least Squares Means						
Effect	Treatment	Estimate	Standard Error	DF	t Value	Pr > |t|
Treatment	0	-0.2864	1.3037	35.6	-0.22	0.8274
Treatment	20	0.3276	1.2364	35.4	0.26	0.7926
Treatment	40	-0.06896	1.3037	35.6	-0.05	0.9581
Treatment	60	-0.3839	1.3820	35.7	-0.28	0.7828

Differences of Least Squares Means							
Effect	Treatment	Treatment	Estimate	Standard Error	DF	t Value	Pr > |t|
Treatment	0	20	-0.6140	1.7430	23.8	-0.35	0.7278
Treatment	0	40	-0.2174	1.7926	24.7	-0.12	0.9045
Treatment	0	60	0.09749	1.8339	23.9	0.05	0.9580
Treatment	20	40	0.3966	1.7430	23.8	0.23	0.8220
Treatment	20	60	0.7115	1.8023	24.7	0.39	0.6964
Treatment	40	60	0.3149	1.8339	23.9	0.17	0.8651

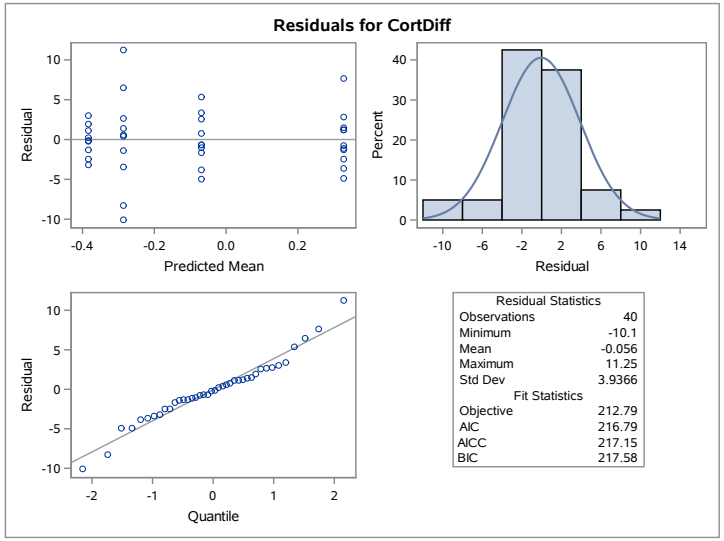
Day 1 Cortisol Difference

The Mixed Procedure



Day 3 Cortisol Difference

The Mixed Procedure



Day 1 Cortisol Post-Startle Average

The Mixed Procedure

Type 3 Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
CortPosttestPlate	4	23.5	2.85	0.0465
Treatment	3	23.2	0.28	0.8367

Least Squares Means Estimate						
Effect	Label	Estimate	Standard Error	DF	t Value	Pr > |t|
Treatment	Row 1	0.4193	1.5072	23.19	0.28	0.7833

Least Squares Means						
Effect	Treatment	Estimate	Standard Error	DF	t Value	Pr > |t|
Treatment	0	12.8127	3.1136	13.2	4.12	0.0012
Treatment	20	11.9544	3.0832	12.7	3.88	0.0020
Treatment	40	11.8746	3.0795	12.7	3.86	0.0021
Treatment	60	13.3511	3.1609	13.9	4.22	0.0009

Differences of Least Squares Means							
Effect	Treatment	Treatment	Estimate	Standard Error	DF	t Value	Pr > |t|
Treatment	0	20	0.8583	1.8025	23.1	0.48	0.6384
Treatment	0	40	0.9381	1.7996	23.1	0.52	0.6071
Treatment	0	60	-0.5384	1.9102	23.3	-0.28	0.7806
Treatment	20	40	0.07982	1.7226	23.1	0.05	0.9634
Treatment	20	60	-1.3967	1.8523	23.2	-0.75	0.4584
Treatment	40	60	-1.4765	1.8881	23.2	-0.78	0.4421

Day 3 Cortisol Post-Startle Average

The Mixed Procedure

Type 3 Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
CortPosttestPlate	4	23.4	1.70	0.1835
Treatment	3	22.9	0.96	0.4263

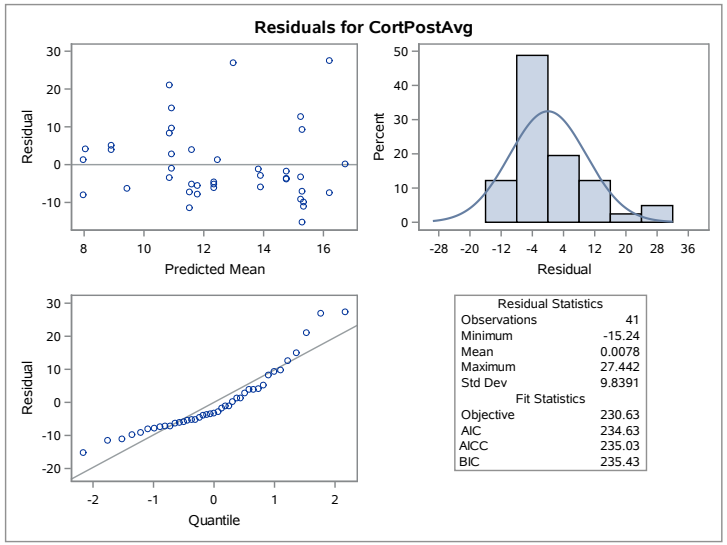
Least Squares Means Estimate						
Effect	Label	Estimate	Standard Error	DF	t Value	Pr > |t|
Treatment	Row 1	1.0556	1.7400	22.87	0.61	0.5500

Least Squares Means						
Effect	Treatment	Estimate	Standard Error	DF	t Value	Pr > |t|
Treatment	0	12.6517	2.6176	17.2	4.83	0.0001
Treatment	20	10.5505	2.5295	15.6	4.17	0.0008
Treatment	40	13.5042	2.5504	16	5.29	<.0001
Treatment	60	10.7338	2.7088	19.1	3.96	0.0008

Differences of Least Squares Means							
Effect	Treatment	Treatment	Estimate	Standard Error	DF	t Value	Pr > |t|
Treatment	0	20	2.1013	2.0974	23	1.00	0.3269
Treatment	0	40	-0.8525	2.0856	22.9	-0.41	0.6865
Treatment	0	60	1.9180	2.2207	22.8	0.86	0.3968
Treatment	20	40	-2.9537	2.0272	22.7	-1.46	0.1588
Treatment	20	60	-0.1833	2.2046	23.1	-0.08	0.9345
Treatment	40	60	2.7704	2.1962	23	1.26	0.2198

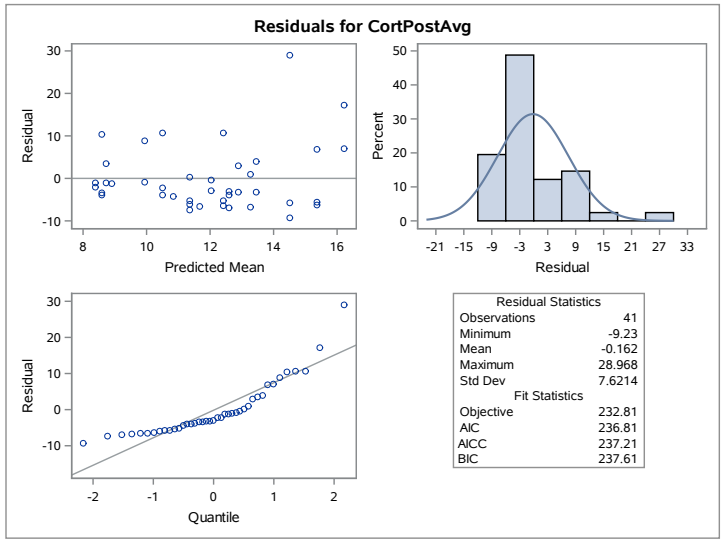
Day 1 Cortisol Post-Startle Average

The Mixed Procedure



Day 3 Cortisol Post-Startle Average

The Mixed Procedure



APPENDIX 5

SAS SUMMARY OUTPUT

Group 1 Amino Acid Data

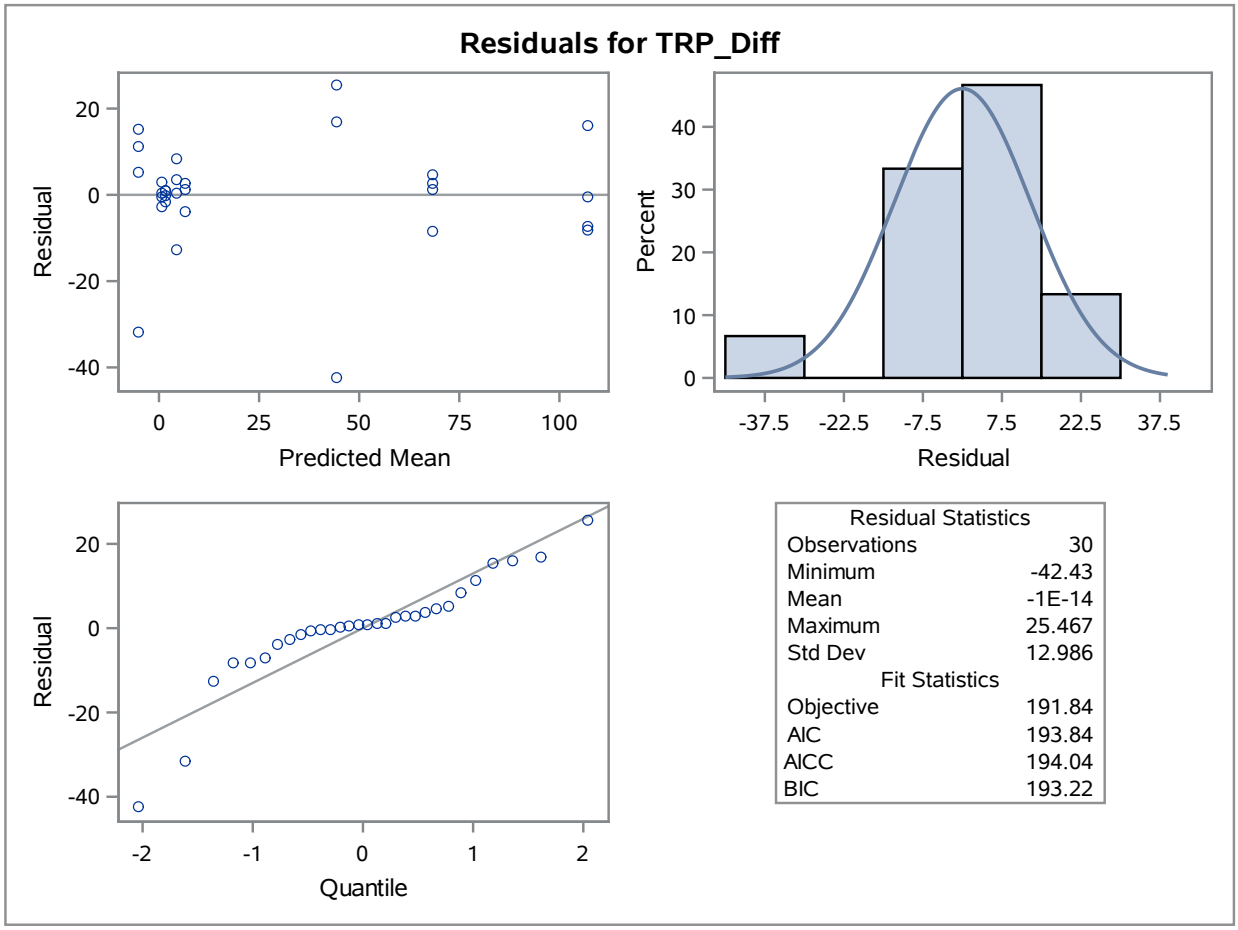
The MEANS Procedure

Treatment	Day	N Obs	Variable	Label	N	Mean	Std Dev	Minimum	Maximum
0	1	4	TRP_Diff RatioDiff	TRP_Diff RatioDiff	4 3	1.7750000 -0.000170638	1.1236103 0.0027832	0.3000000 -0.0030187	2.7000000 0.0025429
	3	4	TRP_Diff RatioDiff	TRP_Diff RatioDiff	4 3	0.6000000 0.000430384	2.3122860 0.0048699	-2.1000000 -0.0033927	3.5000000 0.0059132
20	1	4	TRP_Diff RatioDiff	TRP_Diff RatioDiff	3 3	44.4333333 0.0872213	36.9932877 0.0716457	2.0000000 0.0051378	69.9000000 0.1371981
	3	4	TRP_Diff RatioDiff	TRP_Diff RatioDiff	4 4	4.5000000 0.0154875	9.0225643 0.0279486	-8.1000000 -0.0243351	13.0000000 0.0394599
40	1	4	TRP_Diff RatioDiff	TRP_Diff RatioDiff	4 4	68.3500000 0.1357226	5.7494927 0.0112667	60.0000000 0.1193248	73.0000000 0.1448863
	3	4	TRP_Diff RatioDiff	TRP_Diff RatioDiff	4 4	-5.2000000 -0.0125671	21.5364807 0.0415631	-36.9000000 -0.0654264	10.1000000 0.0232422
60	1	4	TRP_Diff RatioDiff	TRP_Diff RatioDiff	4 3	107.0750000 0.1955426	11.1622504 0.0068534	98.8000000 0.1879360	123.0000000 0.2012364
	3	4	TRP_Diff RatioDiff	TRP_Diff RatioDiff	3 2	6.6000000 0.0037982	3.5552778 0.0137460	2.6000000 -0.0059217	9.4000000 0.0135181

Difference in serum free Trp

The Mixed Procedure

Type 3 Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
Treatment	3	22	17.45	<.0001
Day	1	22	96.09	<.0001
Treatment*Day	3	22	15.55	<.0001



DAY 1: TRP_DIFF

F Test for Treatment*Day Least Squares Means Slice				
Slice	Num DF	Den DF	F Value	Pr > F
Day 1	3	22	34.80	<.0001

Simple Differences of Treatment*Day Least Squares Means							
Slice	Treatment	Treatment	Estimate	Standard Error	DF	t Value	Pr > |t|
Day 1	0	20	-42.6583	11.3877	22	-3.75	0.0011
Day 1	0	40	-66.5750	10.5429	22	-6.31	<.0001
Day 1	0	60	-105.30	10.5429	22	-9.99	<.0001
Day 1	20	40	-23.9167	11.3877	22	-2.10	0.0474
Day 1	20	60	-62.6417	11.3877	22	-5.50	<.0001
Day 1	40	60	-38.7250	10.5429	22	-3.67	0.0013

DAY 3: TRP_DIFF

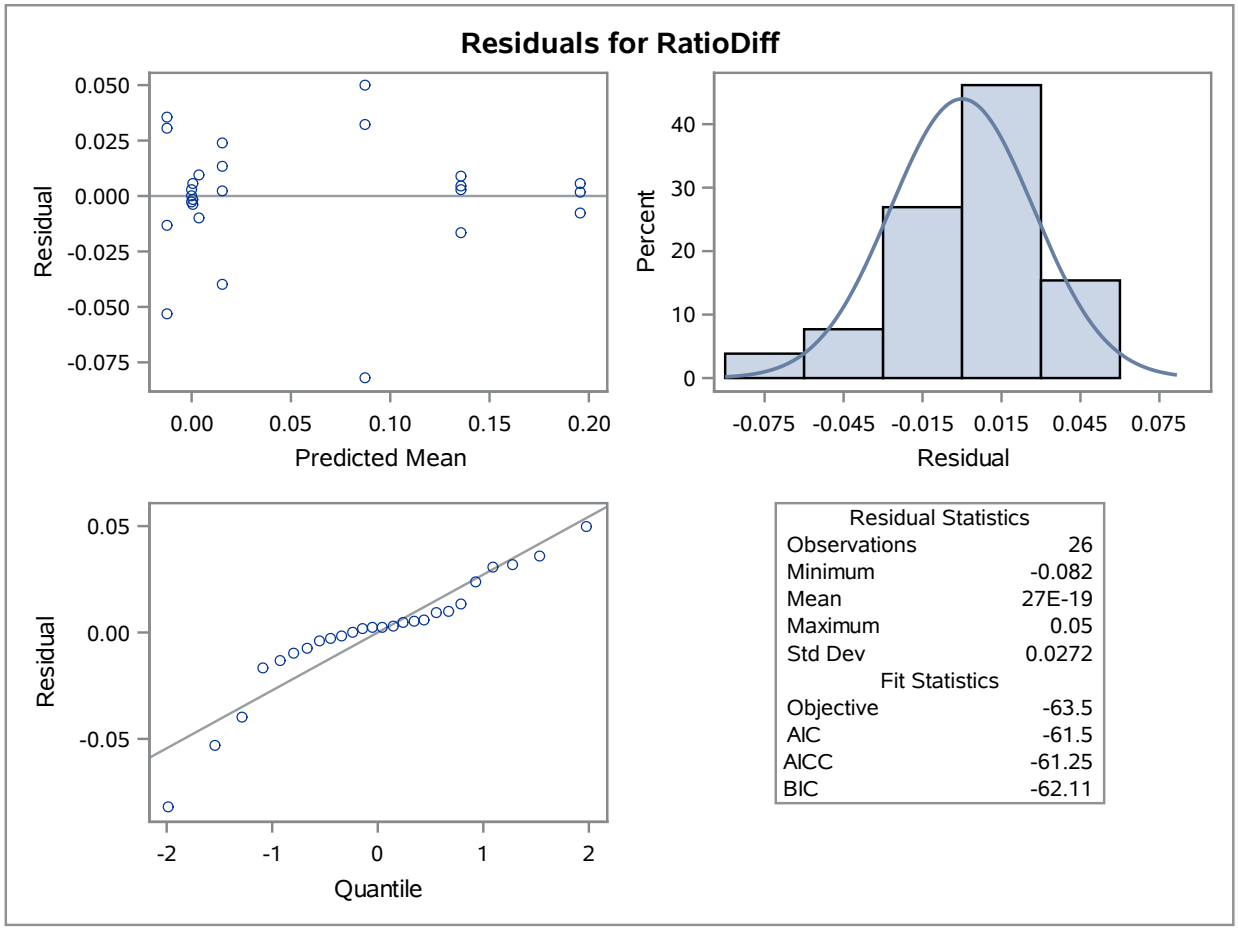
F Test for Treatment*Day Least Squares Means Slice				
Slice	Num DF	Den DF	F Value	Pr > F
Day 3	3	22	0.44	0.7239

Simple Differences of Treatment*Day Least Squares Means							
Slice	Treatment	Treatment	Estimate	Standard Error	DF	t Value	Pr > |t|
Day 3	0	20	-3.9000	10.5429	22	-0.37	0.7150
Day 3	0	40	5.8000	10.5429	22	0.55	0.5878
Day 3	0	60	-6.0000	11.3877	22	-0.53	0.6035
Day 3	20	40	9.7000	10.5429	22	0.92	0.3675
Day 3	20	60	-2.1000	11.3877	22	-0.18	0.8554
Day 3	40	60	-11.8000	11.3877	22	-1.04	0.3114

Difference in TRP:LNAA

The Mixed Procedure

Type 3 Tests of Fixed Effects				
Effect	**Num DF**	**Den DF**	**F Value**	**Pr > F**
Treatment	3	18	9.00	0.0007
Day	1	18	63.61	<.0001
Treatment*Day	3	18	10.14	0.0004



DAY 1: RATIO DIFF

F Test for Treatment*Day Least Squares Means Slice				
Slice	Num DF	Den DF	F Value	Pr > F
Day 1	3	18	20.23	<.0001

Simple Differences of Treatment*Day Least Squares Means							
Slice	Treatment	Treatment	Estimate	Standard Error	DF	t Value	Pr > |t|
Day 1	0	20	-0.08739	0.02619	18	-3.34	0.0037
Day 1	0	40	-0.1359	0.02450	18	-5.55	<.0001
Day 1	0	60	-0.1957	0.02619	18	-7.47	<.0001
Day 1	20	40	-0.04850	0.02450	18	-1.98	0.0632
Day 1	20	60	-0.1083	0.02619	18	-4.14	0.0006
Day 1	40	60	-0.05982	0.02450	18	-2.44	0.0252

DAY 3: RATIO DIFF

F Test for Treatment*Day Least Squares Means Slice				
Slice	Num DF	Den DF	F Value	Pr > F
Day 3	3	18	0.51	0.6775

Simple Differences of Treatment*Day Least Squares Means							
Slice	Treatment	Treatment	Estimate	Standard Error	DF	t Value	Pr > |t|
Day 3	0	20	-0.01506	0.02450	18	-0.61	0.5465
Day 3	0	40	0.01300	0.02450	18	0.53	0.6022
Day 3	0	60	-0.00337	0.02928	18	-0.12	0.9097
Day 3	20	40	0.02805	0.02268	18	1.24	0.2320
Day 3	20	60	0.01169	0.02778	18	0.42	0.6789
Day 3	40	60	-0.01637	0.02778	18	-0.59	0.5631

Comparing treatment numbers
Washout serum free Trp

The Mixed Procedure

Differences of Least Squares Means							
Effect	TrtNum	TrtNum	Estimate	Standard Error	DF	t Value	Pr > |t|
TrtNum	1	2	-0.4500	2.3466	12	-0.19	0.8511
TrtNum	1	3	0.8500	2.3466	12	0.36	0.7235
TrtNum	1	4	2.8000	2.3466	12	1.19	0.2558
TrtNum	2	3	1.3000	2.3466	12	0.55	0.5898
TrtNum	2	4	3.2500	2.3466	12	1.38	0.1913
TrtNum	3	4	1.9500	2.3466	12	0.83	0.4222

Comparing treatment numbers
Washout serum TRP:LNAA

The Mixed Procedure

Differences of Least Squares Means							
Effect	TrtNum	TrtNum	Estimate	Standard Error	DF	t Value	Pr > |t|
TrtNum	1	2	-0.00119	0.004447	12	-0.27	0.7938
TrtNum	1	3	0.002036	0.004447	12	0.46	0.6552
TrtNum	1	4	0.006502	0.004447	12	1.46	0.1694
TrtNum	2	3	0.003225	0.004447	12	0.73	0.4823
TrtNum	2	4	0.007690	0.004447	12	1.73	0.1094
TrtNum	3	4	0.004466	0.004447	12	1.00	0.3351

Comparisons between days and blood collections serum free Trp

The Mixed Procedure

Differences of Least Squares Means							
Effect	DBC	DBC	Estimate	Standard Error	DF	t Value	Pr > |t|
DBC	11	12	-56.2678	7.7451	43.2	-7.26	<.0001
DBC	11	31	-56.9563	7.5829	43.1	-7.51	<.0001
DBC	11	32	-57.1518	7.7451	43.2	-7.38	<.0001
DBC	12	31	-0.6884	7.7451	43.2	-0.09	0.9296
DBC	12	32	-0.8840	7.9101	43.4	-0.11	0.9115
DBC	31	32	-0.1956	7.7451	43.2	-0.03	0.9800

DBC = DayBC

Comparisons between days and blood collections serum TRP:LNAA

The Mixed Procedure

Differences of Least Squares Means							
Effect	DBC	DBC	Estimate	Standard Error	DF	t Value	Pr > |t|
DBC	11	12	-0.1069	0.01576	40	-6.78	<.0001
DBC	11	31	-0.1094	0.01465	39.2	-7.47	<.0001
DBC	11	32	-0.1091	0.01576	40	-6.93	<.0001
DBC	12	31	-0.00255	0.01576	40	-0.16	0.8720
DBC	12	32	-0.00227	0.01644	39.6	-0.14	0.8911
DBC	31	32	0.000289	0.01576	40	0.02	0.9855

DBC = DayBC