THESIS

INJECTABLE ANESTHESIA AND EFFECTS OF ANALGESICS ON THE IMMUNE RESPONSE IN JAMAICAN FRUIT BATS

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Marissa Pollak

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Master's Committee:

Advisor: Lon Kendall

Tony Schountz
Pedro Boscan

ABSTRACT

INJECTABLE ANESTHESIA AND EFFECTS OF ANALGESICS ON THE IMMUNE RESPONSE IN JAMAICAN FRUIT BATS

Injectable anesthesia and immunomodulatory effects of buprenorphine and meloxicam in Jamaican fruit bats (*Artibeus jamaicensis*) (JFBs) have not been studied to date. Testing injectable anesthetic protocols in JFBs would allow for potential alternatives to isoflurane anesthesia which is relied upon in the field and in laboratory settings, but requires special equipment and can be difficult to manage in the field. Injectable anesthetic protocols in bats have been studied in limited capacity yet are valuable due to minimal equipment needs and the ability to induce multiple animals in succession. Four injectable anesthetic protocols (n=6) were compared with isoflurane inhalant anesthesia (control, n=5) in JFBs. Ketamine (K), alfaxalone (A), butorphanol (B), and nalbuphine (N), were each combined with dexmedetomidine (DX) and delivered intraperitoneally, followed by subsequent reversal with atipamezole. KDX and ADX induced anesthesia in 5 out of 6 bats, while NDX and BDX only induced mild to moderate sedation in all bats. All groups except for KDX resulted in return to flight within 60 minutes of injection. In summary, KDX and ADX induced anesthesia in the majority of bats tested and are viable alternatives compared to isoflurane alone. As KDX resulted in a prolonged return to flight time, it is not ideal for use in the field where timely recovery is needed to ensure the safety of bats.

Bats are frequently used as a model to study emerging infectious diseases and bat immune responses. These studies may result in clinical signs, such as flu-like symptoms, in the

bats. Minimizing pain and distress is a priority in laboratory animal medicine and is necessary to remain in compliance with regulations including the Guide for Care and Use of Laboratory Animals and the Animal Welfare Act. Opioids and non-steroidal anti-inflammatory drugs (NSAIDs) can be used to mitigate the potential pain and distress associated with infectious agents. Here we aim to understand how buprenorphine HCl (BUP), an opioid and meloxicam (MEL), an NSAID, affect the immune response in JFBs.

Three male and three female bats in each group were immunized subcutaneously with keyhole limpet hemocyanin (KLH) with complete Freund's adjuvant on day 1. Immediately prior to immunization, bats were bled for baseline IgG determination. Subcutaneous doses of meloxicam (MEL) at 5 mg/kg, buprenorphine (BUP) at 1 mg/kg, or 0.15 mL of saline (SAL) were given daily for 21 days. On day 21, bats were given a subsequent immunization with KLH and incomplete Freund's adjuvant. On day 28 bats were euthanized and blood was collected to assess IgG responses in the serum and spleens were isolated to assess gene expression responses in KLH stimulated splenocytes. Antibody response to KLH was measured by ELISA and gene expression profiling was evaluated by RT-qPCR. There were no antibodies detected prior to immunization in any group. After immunization and treatments, all groups had elevated antibody responses.

There were no statistical differences in the antibody responses of bats treated with either BUP or MEL compared to SAL treated bats. The only statistically significant difference in gene expression was decreased IL-13 expression in BUP treated bats compared to SAL treated bats. Overall, these results suggest that the use of BUP or MEL will have minimal impact on the bat immune response.

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CHAPTER 1: BACKGROUND ON INJECTABLE ANESTHESIA IN JAMAICAN FRUIT BATS

The first goal of this research is to test injectable drug combinations to determine whether they are suitable for anesthesia or immobilization in JFBs. Injectable combinations of anesthetic and sedative drugs have not been studied in this species. These protocols can provide alternatives to inhalant anesthesia and may provide analgesia, reduced equipment requirements, ability to anesthetize multiple bats simultaneously or in quick succession and elimination of personnel exposure to volatile compounds.

There are currently no publications outlining injectable immobilization or anesthetic protocols in JFBs, although there is published data on other bat species. Anesthesia in small laboratory animal species like JFBs can be achieved using volatile anesthetic agents, such as isoflurane, through injectable anesthetic protocols, or through a combination of both (Fish, 2008). Isoflurane allows for fast induction and recovery time in anesthesia and is a reliable method for inducing and maintaining anesthesia (Fish, 2008). Animals also do not need to be weighed prior to administration. However, their use does not provide an analgesic effect after recovery of the animal (Oh, 2024). In addition, specialized equipment is needed for their use and there can be risks associated with personnel exposure (Oh, 2024).

Injectable anesthetic protocols can provide flexibility to researchers because there are minimal equipment needs, making them advantageous for field applications. Other advantages include the ability to anesthetize animals in rapid succession and the potential to provide analgesia upon anesthetic recovery, depending on the drugs used. Some disadvantages include slower induction and recovery time compared to inhalant anesthetics and the need to weigh each

animal for accurate dose calculations (Misal, 2026). In addition, injectable options do not always reliably induce anesthesia (Machin, 1998).

Anesthesia is useful because it allows veterinarians and researchers to perform procedures on animals that could cause pain and distress in a conscious state. Anesthesia induces a state of unconsciousness, amnesia, immobilization, and analgesia (Dodds, 1999). An unconscious animal is not aware or able to perceive its surroundings. Methods of confirming unconsciousness in animals of similar size to JFBs include immobilization and loss of the righting reflex (Antognini, 2005). While it is difficult to assess loss of consciousness, there are correlations between concentrations needed to induce loss of consciousness in humans (no longer responsive to verbal cues) and loss of righting reflex in mice and rats (Franks, 2008). Therefore, loss of righting reflex is used in these animals to indicate loss of consciousness. Amnesia refers to the inability to recall a particular experience. While amnesia is also difficult to assess in animals, there is evidence that mice were unable to recall training events up to two hours after recovery from halothane anesthesia (Rosman,1992). Immobilization refers to the inability to move. Immobilization is simpler to assess for anesthetists because it can be tested directly during anesthesia by delivering a noxious stimulus (Rehberg, 2004). Analgesia refers to diminished or absence of pain.

In a conscious animal, the neural pathway involves nociceptors in the periphery that transmit a signal to the spinal cord through the dorsal horn, which then relays a signal both to the efferent peripheral neuron and to the brain that can be subsequently perceived as pain (D'Mello, 2008). The efferent peripheral neuron pathway transmits a signal back to the peripheral motor unit resulting in movement. Anesthetic agents depress or block the transmission of neural pathways, including those involved in nociception (Kendig, 1998). Spinal reflexes may be intact

depending on anesthetic depth and stimuli can affect physiologic parameters including blood pressure, heart rate and respiratory rate (Tranquilli, 2015). During anesthesia, noxious stimuli are frequently used to test anesthetic depth and analgesia in small laboratory animal species and are often tested via a toe pinch. However, other methods of mechanical stimulation include needle sticks and surgical stimuli.

There are four stages of anesthesia that have been described by Guedal that help characterize anesthetic depth. Stage 1 is characterized by a state of disorientation that can include the onset of analgesia. This stage occurs before loss of consciousness (Keys, 1975). Stage 2 is an excitatory phase where delirium and uncontrolled movements may occur. The reflexes of the airway remain intact and irregular breathing patterns are common in this stage, making intubation difficult (Karunarathna, 2024). Stage 3 marks the onset of surgical anesthesia and can be further divided into four planes. Through the progression of the surgical planes, additional reflexes are lost (Laycock, 1953). Palpebral reflex is lost in the first plane and there is slight muscle relaxation. In the second plane, the corneal reflex is lost, the pupillary light reflex is diminished and there is moderate muscle relaxation. Plane 3 is characterized by the absence of responses to surgical stimuli. In this plane the pupillary light reflex is lost and there is deep muscle relaxation (Guedal, 1937). In plane 4, apnea occurs. Stage 4 is a state of anesthetic overdose in which there is profound central nervous system depression. Hypotension is marked and both respiratory and cardiovascular support are necessary.

The goals of anesthesia are to maintain a plane at an adequate depth for a given procedure, stage 3 plane 3, where responses to stimuli are absent, and to ensure that cardiovascular and respiratory function are maintained and supported. As such, monitoring of vital parameters is crucial throughout the anesthetic period. Ideally, blood pressure, respiration

rate and character, heart rate, electrocardiogram, oxygen saturation (SpO2), body temperature, and end tidal carbon dioxide should be monitored throughout anesthesia. The ability to measure these depends on equipment availability and can also depend on the size of the animal. In small species, such as JFBs, blood pressure monitoring is difficult. JFBs do not have tails and their flight membranes prevent the use of indirect blood pressure monitors on their limbs. Small species also have faster respiratory rates and heart rates, making manual counts for these parameters more challenging compared to larger species. However, electrocardiograph monitoring is available for tracking heart rate and rhythm under anesthesia. Pulse oximetry is valuable in these animals because it can provide readings of oxygen saturation and heart rate. Temperature readings are also important due to increased risk of hypothermia in these animals from high surface area to volume ratios and high metabolic rates. End tidal carbon dioxide may be measured indirectly or directly (if intubated), but this equipment is not always available and likely will depict inaccurate numbers due to the small size of JFBs. Although methods and equipment exist to monitor vital parameters in JFBs, the anatomy and size of JFBs result in monitoring limitations.

Anesthetic drugs have variable mechanisms that ultimately cause inhibition of signaling within the nervous system. Glutamate is a neurotransmitter that activates the NMDA (N-methyl D-aspartate) receptor, a major excitatory receptor in the CNS. Whereas glycine and gamma-aminobutyric acid (GABA) are both major inhibitory neurotransmitters. It is of note that there are many other neurotransmitters involved as well that can modulate transmission of glutamate, GABA and glycine (Clarke, 2014). Cardiovascular and respiratory depression may occur as a result of neuronal inhibition, but anesthetics may also directly affect these systems.

Isoflurane affects multiple receptors within the CNS. It allosterically agonizes the $GABA_A$ receptor (Topf, 2003), potentiates the effects of glycine receptor activity (Grasshoff, 2006), and antagonizes NMDA receptors (Brosnan, 2011). Due to this multimodal action, isoflurane is an effective general anesthetic. Isoflurane induces a dose-dependent respiratory depression, leading to decreased tidal volume without an appropriate increase in respiratory rate (Doi, 1987). Isoflurane also causes a dose dependent decrease in mean arterial pressure; however, this is often partially offset by an increase in heart rate particularly in healthy individuals (Torri, 2010).

Ketamine (KET) is a non-competitive inhibitor of the NMDA receptor. Ketamine binds to the calcium ion channel pores of the NMDA receptor blocking the influx of calcium, preventing the normal action of glutamate binding and resulting in loss of consciousness (Smith, 2002). Ketamine also antagonizes muscarinic receptors, leading to sympathomimetic effects (Hirota, 1996). Thus, ketamine results in increased heart rate and blood pressure, bronchodilation, and preservation of the respiratory drive (Kurdi, 2014). An advantage of using ketamine in injectable protocols is its wide therapeutic margin (Fish, 2008); however, ketamine use can result in prolonged recovery time and muscle rigidity (Bhatia, 2022; Vien, 2017).

Alfaxalone (3-alpha-hydroxy-5- alpha-pregnane-11,20-dione, ALX) is a synthetic neuroactive steroid that binds to the $GABA_A$ receptor in the central nervous system causing a decrease in neuronal excitability that results in loss of consciousness and muscle relaxation (Pérez, 2023). Although alfaxalone leads to muscle relaxation, muscle rigidity is a reported side effect during induction and recovery, particularly when used as a sole agent (White, 2019; Maney; 2013; Lau, 2019). Alfaxalone is a positive allosteric modulator of the receptor (Lambert,1996). Specifically, binding of the receptor leads to influx of chloride into the neuron,

hyperpolarizing the membrane, and ultimately making it less likely to fire a neuronal response, potentiating the effects of GABA (Lambert, 2003). Alfaxalone causes dose dependent respiratory depression through the $GABA_A$ receptor (Yamada, 1982; Maddern, 2010). Alfaxalone also has dose dependent cardiovascular depressive effects resulting in decreases in mean arterial pressure (Muir, 2008).

Dexmedetomidine (DEX) is an α2 adrenergic receptor agonist that acts in the central nervous system and inhibits the release of norepinephrine through binding the α2 receptor (Lu, 2021). It is distinct from other drugs used in anesthesia due to its mechanism of action. It has been shown to decrease glutamate release in the brain (Lu, 2021). Dexmedetomidine has sedative, analgesic, anesthetic, and muscle relaxant effects. Its use is advantageous because it is reversible with atipamezole (Flecknell, 2009). In addition, it has biphasic effects on cardiovascular parameters leading to increased peripheral vascular resistance and a reflex bradycardia (Weerink, 2017). This is followed by a decrease in peripheral vascular resistance. Dexmedetomidine has variable effects on respiration, which is species dependent. In rabbits, dexmedetomidine causes dose dependent respiratory depression, with lower doses inducing more profound effects, but does not cause marked hypercapnia or hypoxemia (Nishida, 2002). In dogs, studies have shown that dexmedetomidine decreases respiratory rate but maintains minute volume, suggesting that respiratory depressive effects are absent in this species (Pleyers, 2020; Dugdale, 2020). Although there are reports of successful sole use of α2 adrenergic agonists for anesthesia, these require continuous rate infusions and can have deleterious effects on the cardiovascular system due to high dosages used (Adami, 2023). When used with ketamine, dexmedetomidine counters the sympathomimetic effects of ketamine and provides muscle relaxation (Magalhães, 2016). In combination with alfaxalone, dexmedetomidine reduces muscle

rigidity associated with the use of alfaxalone while providing analgesia (Ferrini, 2020). Furthermore, dexmedetomidine reduces the dose requirements of other injectable drugs, leading to faster recovery (Magalhães, 2016).

Butorphanol (BUT) and nalbuphine (NAL) are agonist-antagonist opioids that partially agonizes the μ receptor and are full agonists at the κ receptor; these opioids are also considered μ receptor antagonists because they displace full μ receptor opioids from the μ receptors (Flecknell, 2009). Their action at the κ and μ receptors result in analgesia as well as sedation and muscle relaxation. The analgesic effects of the opioid receptors occur through hyperpolarization of pain modulating neurons in the central nervous system and periphery, leading to decreased likelihood of generating action potentials (Al-Hasani, 2011). The partial agonist effects of butorphanol and nalbuphine at the μ receptor prevent dose-dependent respiratory depression, which is profound in full μ agonist opioids (Gress, 2020; Pachter, 1985). Butorphanol can also result in increased blood pressure, but this is not the case with nalbuphine (O'Hair, 1988).

Multiple injectable protocols using ketamine and an α2 adrenergic agonist have been tested in bats and are outlined in Table 1. These protocols were tested in *Pteropus hypomelanus*, *Carollia perspicillata*, and *Rousettus aegyptiacus*, using variable routes of administration. The mean induction time was under five minutes in five protocols (KET + xylazine (X) (11/2); KET + medetomidine (MED) (6/0.06); KET + MED (5/0.05); KET + MED (18/0.18); KET + DEX (7/0.04). However, in the study using KET + MED (18/0.18) where mean induction time was not defined, 323 of 328 bats were induced in roughly ten minutes after injection (Reitl, 2024). Mean recovery times with KET + DEX (7/0.04) and KET + MED (5/0.05) were 5.8 minutes and 12.7 minutes, respectively, with recovery defined as the ability to fly and return of reflexes (pedal withdrawal, palpebral and bite reflexes) (Amari, 2022; Epstein, 2011). In the KET +DEX

(7/0.04) protocol where the mean recovery time was the shortest, isoflurane was supplemented for gonadectomy procedures that lasted over 45 minutes (Amari, 2022). The time needed for the procedures likely provided additional time for metabolic processing of the KET and DEX, leading to a faster recovery time. Mean heart rates recorded in bats injected with KET + DEX (7/0.04) (Amari, 2022) were lower compared to baseline measurements in the same species (Noll, 1979). Respiratory drive was maintained for all bats in these studies throughout anesthesia and recovery, except for one bat that died under anesthesia from presumed septicemia (Reitl, 2024).

Alfaxalone has been used in combination with α2 adrenergic receptor agonists intraperitoneally to successfully induce surgical anesthesia in mice in a dose dependent manner (Erickson, 2019; Siriarchavatana, 2016). As shown in Table 1, alfaxalone has been evaluated in *R. aegyptiacus* and was used in combination with midazolam, which is a benzodiazepine (Tuval, 2021). The drug combination was delivered subcutaneously (s.c.) and the mean induction time was 4.2 minutes. Flumazenil was administered for reversal in half of the bats. Mean recovery time was faster in bats that were given flumazenil (10 minutes) compared to saline controls (45 minutes). Recovery was defined as an attempt to fly. There was variation in anesthetic depth and duration among individual bats. Bats also exhibited twitching and generalized tremors during induction and similarly exhibited excitatory behavior upon recovery. Mean heart rates recorded under anesthesia were increased compared to mean resting heart rates recorded in this species (Noll, 1979) and the respiratory drive was maintained in all bats throughout anesthesia and recovery.

Butorphanol (along with other opioids) is often combined with α2 adrenergic agonists because they enhance the analgesic and sedative effects of opioids (Cardoso, 2014). Butorphanol

has been documented in anesthetic protocols in bats but was not paired with α2 adrenergic agonists alone. These protocols used butorphanol, an α2 adrenergic agonist, and midazolam and were documented in *R. aegyptiacus* using intramuscular (i.m.) and s.c. routes of administration, as shown in Table 1. Mean induction times were under ten minutes for both protocols tested

Table 1. Characteristics of injectable immobilization or anesthetic protocols documented in bats are described. In Tuval's 2018 study, ability to fly was not assessed in 50% of bats due to prolonged recovery. Abbreviations: i.m., intramuscular; i.v., intravenous; s.c., subcutaneous.

	Dose/Route	Species	Induction Time (mean)	Induction Definition	Recovery Time	Recovery definition	Study
Ketamine + xylazine	11 mg/kg + 2 mg/kg i.m.	*Pteropus hypomelanus*	1.3 min	Loss of withdrawal reflex	3-4 hr	Able to climb tree	Sohayati, 2008
Ketamine + medetomidine	6 mg/kg + 0.06 mg/kg i.m.	*P. hypomelanus*	1.3 min	Loss of righting reflex	3 hr	Not defined	Heard, 2006
Ketamine + medetomidine	5 mg/kg + 0.05 mg/kg i.v.	*P. hypomelanus*	2.6 min	Absence of reflexes (not righting)	12.7 min (mean)	Return of reflexes	Epstein, 2011
Ketamine + medetomidine	18 mg/kg + 0.18 mg/kg s.c.	*Carollia perspicillata*	Not provided	Absent or reduced palpebral + no movement	Not provided	Ability to climb or fly	Reitl, 2024
Ketamine + dexmedetomidine	7 mg/kg + 0.04 mg/kg i.m.	*Rousettus aegyptiacus*	2.5 min	No movement induced by foot palpation	5.8 min (mean)	Able to fly	Amari, 2022
Alfaxalone + midazolam	15 mg/kg + 2 mg/kg s.c.	*R. aegyptiacus*	4.2 min	Recumbency without movement	10 min + 45 min (mean)	Flying attempt	Tuval, 2021
Butorphanol + dexmedetomidine + midazolam	0.3 mg/kg + 0.04 mg/kg + 0.3 mg/kg i.m.	*R. aegyptiacus*	2.8 min	No movement induced by foot palpation	7 min (mean)	Able to fly	Amari, 2022
Butorphanol + medetomidine + midazolam	1 mg/kg + 0.15 mg/kg + 1.5 mg/kg s.c.	*R. aegyptiacus*	7 min	No movement and no response to face mask (O2)	159 min (50%) (mean)	Ability to fly	Tuval, 2018

(BUT/DEX/MID (0.3/0.04/0.3) and BUT/MED/MID (1/0.15/1.5)). Mean recovery time in the BUT/DEX/MID (0.3/0.04/0.3) protocol was 7 minutes, in which reversal agents were

administered for both the α2 adrenergic agonist and midazolam (Amari, 2022). The bats in this protocol were supplemented with isoflurane to achieve surgical anesthesia for gonadectomy, as mentioned previously. In the protocol using BUT/MED/MID (1/0.15/1.5), half of the bats were not provided with reversal agents, and the mean recovery time was 159 minutes from the time of injection (Tuval, 2018). The other half of the bats in this protocol were given atipamezole at 180 minutes but were not monitored for full recovery. Recovery was defined in both protocols as the ability to fly. Mean heart rates measured in bats treated with BUT/DEX/MID (0.3/0.04/0.3) were lower compared to baseline heart rate measurements (Amari, 2022; Noll, 1979), and the respiratory drive was maintained in all bats throughout anesthesia and recovery.

Nalbuphine has not been documented in injectable anesthetic or sedation protocols in bats but is valuable in field research because it is not a controlled substance.

There are many variations in the literature regarding injectable protocols for immobilization and anesthesia in bats and not many species have been used. The literature described varied widely in the methods for monitoring anesthetic depth. In the study described in this manuscript, righting reflex, pedal withdrawal reflex and movement response to intradermal and intramuscular pin pricks were chosen. These collectively assess the loss of consciousness (righting reflex) and analgesia in response to needle sticks. Negative responses would indicate that JFBs would likely tolerate suture placement, which may be needed for tagging, venipuncture, and other procedures such as rectal or oropharyngeal swabbing. Many of the studies reviewed did not consistently result in anesthesia (Epstein, 2011; Heard 2006; Tuval 2021; Amari, 2022; Reitl, 2024). The ketamine and medetomidine protocol in *C. perspicillata* was the most successful for anesthesia, with a vast majority of bats achieving surgical levels of anesthesia without isoflurane supplementation (Reitl, 2024). There was variability in recovery

assessment in the reviewed studies; return to flight was chosen for this study as a monitoring parameter because flight allows bats to resume foraging behavior and escape predation in field settings.

Despite the increasing use of JFBs in research, there is no published data on immobilization or anesthetic protocols in this species. The aim of this portion of the project is to test injectable protocols and determine how they compare to isoflurane anesthesia. Based on the literature review, the hypothesis is that fixed doses of ketamine combined with dexmedetomidine, and alfaxalone combined with dexmedetomidine will induce anesthesia in some but not all JFBs. It is hypothesized that bats in both groups will return to flight relatively quickly after initial injection. It is also hypothesized that protocols using fixed doses of butorphanol with dexmedetomidine, and nalbuphine with dexmedetomidine will result in sedation but not anesthesia in JFBs and result in a relatively quick return to flight time.

CHAPTER 2: COMPARISON OF FOUR INJECTABLE ANESTHETIC PROTOCOLS TO INHALANT ISOFLURANE IN JAMAICAN FRUIT BATS

The goal of this chapter is to determine the effects of four intraperitoneal (i.p.) injectable protocols in JFBs in comparison to isoflurane anesthesia. The hypothesis is that ketamine and dexmedetomidine (KDX) and alfaxalone and dexmedetomidine (ADX) injected i.p. will result in anesthesia in some but not all bats; butorphanol and dexmedetomidine (BDX) and nalbuphine and dexmedetomidine (NDX) will not induce anesthesia but will have sedative effects. It is hypothesized that bats in all injectable groups will return to flight in under 60 minutes. Isoflurane has been documented for use in JFBs and is considered the standard of care in small animals such as mice and rats (Strumpf, 2020; Oh, 2024); it is expected that isoflurane anesthesia will successfully induce anesthesia in all bats and result in return to flight time in under 60 minutes.

Methods.

JFBs were acquired from the Colorado State University on-site colony. All animal work was performed at an AAALAC International accredited program and approved by the Institutional Animal Care and Use Committee. Bats were housed in bird-type cages (0.76 m × 0.45 m × 0.45 m) for the duration of the study. Landscape fabric was secured to the top and sides of the cage in strips to allow for roosting. Food and water were provided ad libitum daily. Food included watermelon, cantaloupe, banana, blueberries, and honeydew. Protein supplementation (Mazuri Softbill Diet) was provided at approximately 20 grams per pound of fruit.

Male JFBs were manually caught at random from the colony. Dose ranges were determined through allometric scaling (Sedgewick, 1994) using established doses in sheep and cats (Nagore, 2013; Kastner, 2007; Posner, 2009; Flecknell, 2009; Berry, 2015; Abrahamsen,

2008; Kim, 2016; Kahn, 2010). The dose ranges were determined to be 10-15 mg/kg for ketamine (KET), 10-40 mg/kg for alfaxalone (ALX), 0.2-1.6 mg/kg for butorphanol (BUT), 25-38 mg/kg for nalbuphine (NAL) and 0.05-0.075 mg/kg for dexmedetomidine (DEX).

A pilot study was performed to determine the efficacy of s.c. and i.p. injectable protocols using between 1 and 5 bats. The pilot protocols used were ketamine at 10 mg/kg with dexmedetomidine at 0.05 mg/kg s.c. (n=3), KET at 13.5 mg/kg with DEX at 0.075 mg/kg i.p. (n=5), ALX at 12 mg/kg with DEX at 0.075 mg/kg s.c. (n=5), ALX at 15 mg/kg with DEX at 0.05 mg/kg i.p. (n=2), ALX at 25 mg/kg with DEX at 0.05 mg/kg i.p. (n=2), and BUT at 1.2 mg/kg with DEX at 0.075 mg/kg i.p. (n=1). For s.c. injections, drugs were combined and delivered between the shoulder blades while being manually restrained. After bats received injections, they were individually placed in a small container with a metal grating set on top. Each bat was observed for loss of ear movement, hind feet gripping, and wing flapping. When loss of this voluntary movement was noted, each bat was manually placed into dorsal recumbency to assess for loss of righting reflex. Once loss of the righting reflex was observed, bats were held in dorsal recumbency, and the pedal withdrawal reflex on the right hind foot was tested, followed by an intradermal (i.d.) pin prick and intramuscular (i.m.) pin prick, both over the left pectoral musculature using a 25-gauge needle. If no reaction was noted on these three tests, that was considered the start of anesthesia and time was recorded. If a response was noted, these three tests were repeated every 30 seconds until no response was noted. The three reflex tests were repeated at five minutes (T5) and ten minutes into anesthesia (T10). Ten minutes after the start of anesthesia, each bat was reversed with atipamezole i.m. in the right or left pectoral muscle and transferred to a clear mouse cage for observation. If an anesthetic plane was not reached, atipamezole was not administered. As soon as voluntary movement was noted, the bats

were considered recovered from anesthesia, time was recorded, and they were placed back in the roosting cage for monitoring. Bats were individually removed from their roosting cages to test for return to flight in an open flight area by releasing manual restraint and allowing bats to voluntarily fly away. Once the bats were able to maintain flight for at least ten seconds, return to flight time was recorded, and bats were recaptured and placed in roosting cage.

For the main study, six male bats were used for each experimental group and five male bats were used for the isoflurane (ISO) control group. In each experimental group, the two drugs were combined and administered i.p.. The drugs were diluted using sterile normal saline in sterile vials on the same day of use. Bats were individually restrained using leather gloves in a dorsal position with the head angled down approximately 45 degrees. The i.p. drug combination was administered in the lower left abdominal quadrant. Group 1 (KDX), received KET diluted to 7 mg/mL at a dose of 15 mg/kg (MWI, Boise, ID) in combination with DEX diluted to 0.04 mg/mL (all protocols) at 0.05 mg/kg (Dechra, Overland Park, KS). Group 2 (ADX), received undiluted ALX at a dose of 25 mg/kg (Jurox, North Kansas City, MO) in combination with DEX at 0.075 mg/kg. Group 3 (BDX), received BUT diluted to 0.8 mg/mL at a dose of 1.6 mg/kg (Dechra, Overland Park KS) in combination with DEX at 0.075 mg/kg. Group 4 (NDX), received undiluted NAL at a dose of 38 mg/kg (Hospira Inc, Lake Forest, IL) in combination with DEX at 0.075 mg/kg. The bats given KDX were reversed with atipamezole diluted to 0.08 mg/mL at a dose of 0.08 mg/kg, and the bats given ADX and BDX were reversed with atipamezole diluted to 0.16 mg/mL at a dose of 0.12 mg/kg (Modern Veterinary Therapeutics, LLC, Sunrise, FL). Atipamezole was given i.m.in the left or right pectoral muscle. Isoflurane was delivered to the control group (ISO) with a mask through a non-rebreathing system at a

range of 2-5% in 100% oxygen at 1L per minute (MWI, Boise, ID). Isoflurane was delivered continuously for 10 minutes after induction was confirmed.

After bats in the KDX, ADX, BDX and NDX groups received injections, they were assessed for anesthetic depth as described for the pilot study. In addition, at the start of anesthesia, a pulse oximeter was applied to the left wing and a temperature was obtained rectally. Heart rate, temperature and SpO2 were recorded, when possible, at the onset of anesthesia (T0), five minutes into anesthesia (T5), and ten minutes into anesthesia (T10). Bats that were not successfully anesthetized were reversed with atipamezole 15 minutes after i.p. injection. Isoflurane treated bats were assessed when there was a loss of movement observed, the righting reflex was tested and bats were placed in dorsal recumbency for anesthetic assessment. At ten minutes post- induction of anesthesia, isoflurane was discontinued, allowed to dissipate, and oxygen was delivered until voluntary movement was noted. Recovery time and return to flight was recorded. All bats were kept housed in the roosting cage overnight and tested again at 24 hours for flight ability.

For the pilot study, up to 5 bats were used to account for variability. If an injectable protocol was not successful at inducing anesthesia, additional bats were not used. GraphPad Prism was used for statistical analysis. To determine group sizes for the main study, Lenth's online power calculator was used corresponding to a two-sample t-test with an α of 0.05. Based on conjectured difference of 30 min comparing two protocol with a standard deviation of 15 min with 5 ISO control bats and 6 bats per treatment groups achieved a power of 0.84. For anesthesia success rate, Fisher's exact tests were run to compare proportions. For time to return to flight, a single log rank test was performed and a Kaplan-Meier curve was generated. Based on observed

data, bats that took longer than 60 minutes to return to fight are considered “censored” observations. Hence, the log rank test is preferred over the two-sample t-test.

Results.

Pilot data is summarized in Table 2. Bats that were successfully induced had negative pedal withdrawal reflexes and negative i.d. and i.m. pin pricks at T0, T5, and T10. The KDX and ADX injectable protocols were inconsistent in their ability to induce anesthesia in JFBs. Both

Table 2. Pilot study data in Jamaican fruit bats. Abbreviations: s.c., subcutaneous; i.p., intraperitoneal. Return to flight time was documented in all anesthetized bats, but not in all bats used.

Drugs	Dose/Route	n	Successful Induction	Time to Anesthesia (min)	Time to Return to Flight (min)
Ketamine + Dexmedetomidine	10 mg/kg + 0.05 mg/kg s.c.	3	2	3.3 - 12.4	49 - >60
Ketamine + Dexmedetomidine	13.5 mg/kg + 0.075 mg/kg i.p.	5	3	5.4 - 6	42 - >60
Alfaxalone + Dexmedetomidine	12 mg/kg + 0.075 mg/kg s.c.	5	4	2.5 - 20.8	20.8 - >60
Alfaxalone + Dexmedetomidine	15 mg/kg + 0.05 mg/kg i.p.	2	0	-	Not evaluated
Alfaxalone + Dexmedetomidine	25 mg/kg + 0.05 mg/kg i.p.	2	1	5.3	37.3
Butorphanol + Dexmedetomidine	1.2 mg/kg + 0.075 mg/kg i.p.	1	0	-	>60

KDX protocols tested resulted in prolonged return to flight in at least one bat and there was more variability in induction time in the s.c. protocol compared to the i.p. protocol. The s.c. ADX protocol required lower doses of ALX to induce anesthesia compared to i.p. ADX protocols but resulted in more variability with induction time and return to flight time. The BDX protocol did not result in anesthesia and resulted in a prolonged return to flight time, although atipamezole

was not administered. Based on the pilot data, i.p. protocols were used exclusively due to lower variability compared to s.c. protocols. The upper limits based on allometric scaling calculations were chosen for KET, BUT and NAL in order to maximize anesthetic depth or sedative effects. The ALX dose was not increased to minimize return to flight time. The 0.075 mg/kg dose of DEX was selected for combination with ALX because 0.05 mg/kg of DEX combined with 25 mg/kg ALX did not consistently result in anesthesia. The 0.05 mg/kg dose of DEX was selected for combination with KET because 15 mg/kg of KET was not tested in the pilot study and because the highest KET dose was selected based on allometric scaling calculations; it was suspected that a higher dose of DEX would not be needed to induce anesthesia. The 0.075 mg/kg dexmedetomidine doses were chosen for BDX and NDX groups to maximize sedative effects. This resulted in the following drug combinations to be tested: KDX (15 mg/kg of KET combined with 0.05 mg/kg DEX; ADX (25 mg/kg ALX combined with 0.075 mg/kg DEX), BDX (1.6 mg/kg BUT combined with 0.075 mg/kg DEX), and NDX (38 mg/kg NAL combined with 0.075 mg/kg DEX).

Table 3 shows the range, mean and standard deviations for bats in the main study for each time point of the anesthetic assessment. Anesthetic induction rates were variable across groups, with ISO resulting in the shortest induction time followed by KDX and ADX resulting in the longest induction time. Although there were variations in induction times between groups, these were not significant. Loss of righting reflex was not observed in any bats following the BDX or NDX treatment suggesting they did not reach an anesthetic plane.

Anesthetic depth was assessed using pedal withdrawal and response to i.d. and i.m. stimulus. Two bats in the ISO group had no responses at any of the time points tested. One bat had no response to the stimulus at T0; however, began to move prior to T5 and the isoflurane

was increased, resulting in no responses at T5 and T10. One ISO bat at 5% isoflurane had a positive pedal withdrawal reflex at T5 and subsequent positive responses to pedal withdrawal and i.d. and i.m. stimulus at T10. The final ISO bat at 5% had positive responses to pedal withdrawal and i.d. and i.m. stimulus at T10. Bats that were successfully induced in the KDX and ADX groups had negative pedal withdrawal reflexes and negative i.d. and i.m. pin pricks at

Table 3. The range (R), mean (M), and standard deviation (SD) of time to anesthesia, recovery and return to flight in minutes for all groups are shown. Only 5 of 6 bats were anesthetized in the KDX and ADX groups. Only anesthetized bats were included in this table for those groups. Mean and standard deviation of return to flight data were not calculated due to four bats exceeding 60 minute return to flight time in the KDX group. No bats were anesthetized in BDX and NDX groups, thus no data was collected for time to anesthesia and time to recovery. Injectable groups were compared to isoflurane for time to anesthesia and time to recovery (**, $p<0.01$).

Anesthetic	Time to Anesthesia (min)			Time to Recovery (min)			Time to Return to Flight (min)		
	R	M	SD	R	M	SD	R	M	SD
ISO	1-6.7	2.4	2.4	12.7-20.8	7.1	1.4	18.5-35.4	24.4	7.2
KDX	2.0-6.0	3.8	1.9	21.7-40**	28.7**	3.3**	49.7->60	—	—
ADX	2.3-9.3	5.6	2.6	16.7-29.3	22.3	4.6	32.7-59	42.7	10.4
BDX	—	—	—	—	—	—	27.3-37.7	33.7	4.8
NDX	—	—	—	—	—	—	25.5-47.5	33.5	8.1

T0, T5 and T10. None of the BDX or NDX bats were tested for anesthetic responses since they did not reach an anesthetic plane.

Anesthetic recovery time occurred the fastest in the ISO group, followed by ADX and finally KDX. KDX recovery times were significantly different from recovery times in the ISO group ($p=0.009$), but there was no significant difference between ADX and ISO bats ($p=0.19$).

Of the bats that became anesthetized, bats in the ISO group returned to flight the soonest, followed by ADX bats, and finally the KDX bats. Four of the bats in the KDX group did not return to flight within 60 minutes, while one bat returned to flight in 49.7 minutes. The NDX and BDX groups returned to flight sooner than ADX and KDX, but they took longer to return to flight compared to ISO. BDX and NDX were evaluated for return to flight to provide recovery information for future studies that may incorporate these drugs. Log rank tests showed a significant difference in return to flight between all treatment groups compared to ISO except for NDX (Table 4). All groups except for the KDX group had a 100% chance of return to flight within 60 minutes. The KDX group had a 20% chance of return to flight within 60 minutes (Figure 1).

Table 4. Log rank tests comparing return to flight times of treatment groups compared to isoflurane control. All injectable anesthetic groups had a statistically significant difference ($p < 0.05$) in return to flight time except for NDX.

	Log Rank Test p value Compared to Isoflurane Control
KDX	0.0007
ADX	0.006
BDX	0.02
NDX	0.12

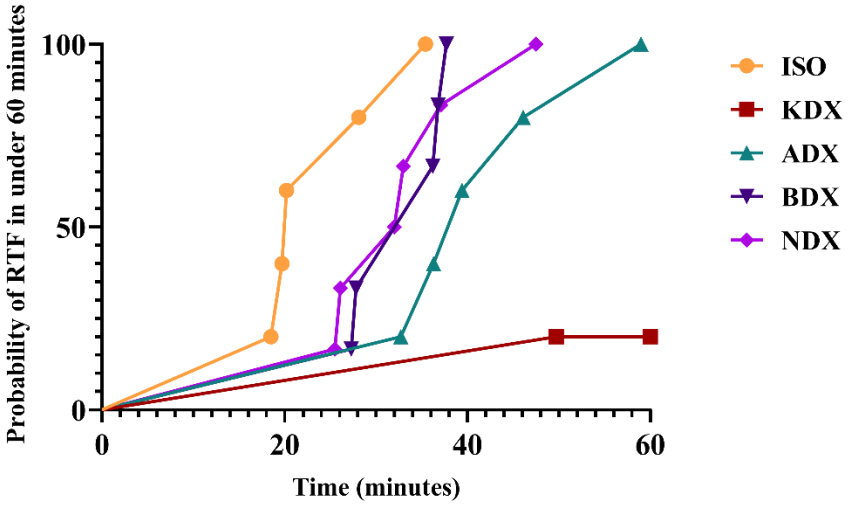

Figure 1. Kaplan-Meier curve depicting return to flight times in anesthetized bats in KDX, ADX and ISO groups and return to flight time in all bats in BDX and NDX groups. The data points for four bats in the KDX group are censored because there was no additional monitoring for return to flight after 60 minutes.

While all bats in the ISO control group were anesthetized in 1-6 minutes, they did not remain consistently anesthetized throughout the ten-minute period. There were periods of occasional movement or responses to pin prick stimuli. This occurred even when the maximum rate of delivery was used at 5%. As shown in Table 5, Fisher's exact tests indicated that there was not a statistical difference between the proportion of bats anesthetized with KDX or ADX in comparison to ISO (p value >0.999), indicating that KDX and ADX do not differ significantly in their ability to induce anesthesia compared to ISO. They also showed that there was a statistically significant difference between the BDX and NDX groups compared to ISO (p value=0.002), indicating they are not suitable for inducing anesthesia compared to ISO. Due to the small sample sizes used, these statistical analyses should be interpreted with caution.

Table 5. KDX and ADX had an equivalent proportion of anesthetized JFBs and were not significantly different compared to the ISO control, whereas the proportions of anesthetized JFBs in the BDX and NDX groups were significantly different compared to ISO.

	Proportion of JFBs Anesthetized Compared to Isoflurane	p value
ISO	5/5	—
KDX	5/6	>0.999
ADX	5/6	>0.999
BDX	0/6	0.002
NDX	0/6	0.002

There was an overall downward trend in heart rate for the ADX group and the KDX group throughout anesthesia. The number of data points acquired for heart rate in the ISO group was insufficient to determine a pattern over time. The majority of recorded heart rates across all groups ranged between 150 and 250 beats per minute. Due to overlapping time points between individual bats, vital parameter data were not consistently acquired for all time points in all bats.

There was a general downward trend in rectal temperature as anesthesia progressed, independent of the anesthetic protocol (Figure 2). The KDX and ADX groups were similar in temperature trends, whereas temperatures were lower across all time points in the ISO group.

Figure 3 shows recorded SpO2 under anesthesia. Values for these parameters were only consistently measured for the ADX group because bats in this group were anesthetized individually without time point overlaps. SpO2 was highly variable in KDX (range 78-100) and ADX (range 80-100) groups. The two readings acquired in the ISO group were 86 and 99.

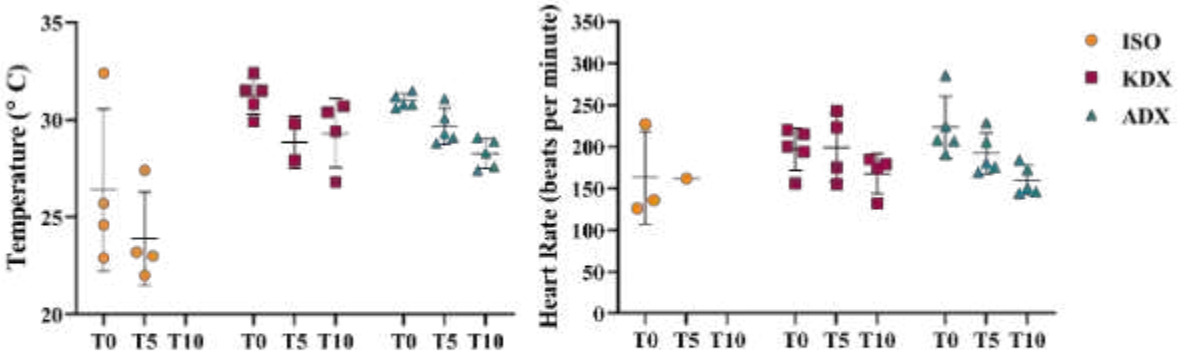

Figure 2. Heart rates and temperatures are shown for JFBs under anesthesia from the start of anesthesia (T0), 5 minutes (T5) and 10 minutes (T10) for the ISO, KDX and ADX bats. Individual data points, mean and SD are shown for each time point.

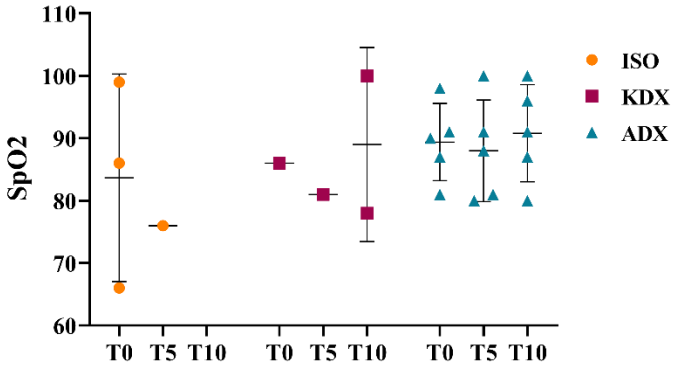

Figure 3. SpO2 measurements are shown for JFBs under anesthesia from the start of anesthesia (T0), 5 minutes (T5) and 10 minutes (T10) for the ISO, KDX and ADX bats. Individual data points, mean and SD are shown for each time point.

Discussion.

This study showed that ISO, KDX and ADX are variably efficacious in inducing anesthesia in JFBs. ISO did not result in consistent anesthesia over a ten-minute period when

using a vaporizer, even at the maximum rate of delivery of 5%. ISO anesthesia resulted in the fastest induction time and return to flight time. KDX and ADX resulted in anesthesia in 5 of 6 bats in each group. Neither BDX nor NDX were shown to be suitable for anesthesia, but both had sedative effects. KDX resulted in prolonged return to flight time, while ADX, BDX, NDX, and ISO resulted in a return to flight time under 60 minutes.

The use of ISO in bats has mixed results. It has been reported that big brown bats (*Eptesicus fuscus*) can be induced at 5% isoflurane and maintained at 2.5% (Heard, 2014). Others have reported that isoflurane alone does not induce surgical levels of anesthesia in *C. perspicillata* (Reitl, 2024). However, injectable protocols in combination with isoflurane have resulted in surgical levels of anesthesia in *R. aegyptiacus* (Amari, 2022). The ISO group in this study did not maintain anesthesia supporting that it may not induce a surgical level of anesthesia when delivered by a nose cone. Thus, a combination of injectable drugs with isoflurane supplementation may be needed to prolong anesthesia and achieve an appropriate anesthetic plane in JFBs.

All bats in the BDX and NDX groups displayed subjectively reduced and slower movements compared to normal bats and returned to flight within 60 minutes; there was also less resistance to manual restraint compared to normal bats, which was noted when administering atipamezole. These sedative effects may be suitable for procedures that do not require surgical anesthesia or complete immobilization, while still allowing for relatively fast recovery.

Challenges were experienced with monitoring vital parameters of the bats during anesthesia. Bats from the KDX and ISO groups were anesthetized in succession, making it difficult to acquire accurate readings for each time point while also ensuring correct timing for reversal agent administration. This was adjusted for the ADX group, where only one bat was

anesthetized at a time and it was feasible to accurately record vital parameters. Statistical analyses on vital parameters could not be performed due to missing data, thus significance between groups was not determined. Thus, conclusions drawn from these data should be interpreted with caution. The temperatures in the ISO group were lower compared to the injectable groups likely because of the cooling effect of air flow from the delivery of the anesthetic. Despite these low temperatures, the bats in this group recovered and returned to flight faster than the bats in the injectable groups. The heterothermic capabilities of bats (Hock,1951; Buckles, 2015) may influence their ability to tolerate low body temperatures during anesthesia. JFB body temperatures have been reported between 8 and 33.2 °C, depending on ambient temperature (Ortega, 2001). KDX and ADX both result in decreased heart rate over time during anesthesia, and this is expected due to the cardiovascular effects of DEX that result in peripheral vasoconstriction and bradycardia. SpO2 readings varied widely and may have been inaccurate at times due to difficulties in achieving proper placement of the probe on the bats, which was attributed to the wing anatomy of JFBs. Based on this study SpO2 probe placement on the wing is not recommended. There are no other ideal anatomical locations for SpO2 probe placement in JFBs. Although respiratory rates were not consistently recorded, all bats maintained respiratory function throughout anesthesia.

Dexmedetomidine was used at two different dosages across groups in this study, which coincided with the variable atipamezole dosages used. Atipamezole was delivered i.m. based on manufacturer recommendations in other species to avoid the potential for cardiac arrest (Zoetis Inc., n.d.). Small volumes were used for injections and no impairments in flight ability were noted immediately after anesthesia or 24 hours post anesthesia.

There were several limitations of this study. Only male JFBs were used in this study due to difficulty in identifying pregnancy status in females during early gestation. The estrous cycle and pregnancy status of females may affect the efficacy of these anesthetic protocols which is an important consideration. Female C57BL/6J mice have been shown to be more resistant to isoflurane anesthesia compared to males and recover faster than males (Wasilczuk, 2024). Intraperitoneal anesthetic protocols must be given with caution in pregnant female JFBs as they may result in fetal death. Another limitation was that there was no blinding for the treatment groups and blinding would not have been possible for the ISO control group due to the route of administration. Although efforts were made to standardize procedures and data collection, there is potential that the results are influenced by observer biases.

There are several factors that should be considered regarding recovery time and return to flight time in this study. Heat support was not provided but is valuable during recovery due to improved metabolic breakdown of drugs (Heldmaier, 1992). Bats have short gastrointestinal transit times and tend to have blood glucose levels that are at the low end of normal ranges prior to feeding (Widmaier, 1993). The JFBs were anesthetized just prior to their once daily feeding.

Providing injectable or oral dextrose during or after anesthesia in future studies may assist with recovery as normoglycemia improves cognitive function and reflex responses (Maran, 1995).

The variability noted in efficacy of anesthetic protocols may be explained in several ways. Individual stress levels likely play a role, as it has been shown that wild animals require higher doses of injectable anesthetic drugs compared to domesticated animals (Woolnough, 2012; Portas, 2003). Another factor includes potential age variation in the bats. Only adult JFBs

were used, but the age of the bats was not determined. There are also genetic variations that influence drug metabolism (Johnson, 2023).

It is unlikely that there are injectable immobilization or anesthetic protocols that would work across a wide range of bat species. This is due to interspecies variability in thermoregulatory and energy balance strategies, body mass, and ecology (Lyman, 1970; Stawski, 2011; Heard, 2014). However, captive breeding colonies are maintained within a specific temperature range and provide bats with consistent forage availability, which may make injectable protocols simpler to translate across species of similar sizes in laboratory settings.

Other combinations of injectable drugs or alternative doses of KET, ALX, and DEX should be investigated to determine more reliable injectable protocols in JFBs. Ketamine doses can likely be increased due to its wide safety margin (Fish, 2008), but this will likely prolong recovery time to a greater degree. Dexmedetomidine doses can potentially be increased in JFBs, as reported doses of DEX in mice range between 0.5 and 1 mg/kg (Oh, 2024). These doses should be increased with caution, as DEX has profound cardiovascular effects (Weerink, 2017) and its effects on these parameters in JFBs are not known. Injectable protocols using BUT, α2 adrenergic agonists (xylazine and DEX), and midazolam have been reported in bats (Tuval, 2018; Amari, 2022) and should be considered for use in JFBs. All of these agents can be reversed, which may improve recovery time (Fish, 2008). Future studies using combinations of injectable and inhalant isoflurane should be considered to produce a more reliable anesthesia. Similarly, BUT and NAL combinations with DEX and/or midazolam should also be tested alone and with isoflurane, due to efficacy in inducing anesthesia in other species using similar combinations.

While the i.p. route of administration was used in this study, other routes could be considered. The s.c. route of administration can also be used, but the pilot study data demonstrated high variability in induction time with s.c. administration. However, in *C. perspicillata*, a s.c. protocol resulted in surgical anesthesia in 323 out of 328 bats (Reitl, 2024). Intramuscular administration could be given in the large pectoral muscles of the JFB; however, that may result in some adverse effects as those are principal muscles used for flight, and injections volumes need to be considered. Intravenous anesthesia is not practical in JFBs due to their size. Additionally, dose response determination for each drug would be valuable to determine the highest effective doses that would not result in side effects in JFBs.

In conclusion, KDX and ADX protocols are suitable alternatives to isoflurane anesthesia in JFBs, whereas BDX and NDX protocols are not suitable alternatives. Induction times in the KDX and ADX groups were comparable to the ISO group with more consistent anesthesia compared to ISO. Recovery times and return to flight times were comparable between the ADX and the ISO group, whereas recovery and return to flight were prolonged in the KDX group. While BDX and NDX did not induce anesthesia in JFBS, both protocols resulted in consistent sedative effects and may be valuable for procedures that do not require complete immobilization or anesthesia. Although ISO resulted in anesthesia and quick recovery in all bats, the duration and depth of anesthesia were variable. Thus, it should be used with caution for surgical anesthesia in JFBs when used alone.

CHAPTER 3: BACKGROUND ON THE EFFECTS OF BUPRENORPHINE AND MELOXICAM ON IMMUNE RESPONSE IN JAMAICAN FRUIT BATS

The second goal of this research is to determine the effects of buprenorphine and meloxicam on the immune response in JFBs. It is advantageous to understand these effects as JFBs are valuable immunological research models and analgesia may be clinically indicated throughout the course of infectious disease studies.

Effective pain management in laboratory animals is a crucial component of ensuring animal welfare in accordance with The Guide for Care and Use of Laboratory Animals and The Animal Welfare Act (Committee for the Update of the Guide for the Care and Use of Laboratory Animals, 2011) (Animal Welfare Act, 1966). Opioids and nonsteroidal anti-inflammatory drugs (NSAIDs) are commonly used clinically to relieve pain in multiple species (Flecknell, 2009). However, both categories of drugs have been shown to have immunomodulatory effects which can impact research in immunology (Carrigan, 2004; Piersma, 1999; Sun, 2017; Mikawa, 2006; Banerjee, 1997; Ryan, 2005; Bancos, 2009; Yamaki, 2003; Chen, 2021).

Buprenorphine is a partial agonist at the μ opioid receptor, but it has been shown to antagonize the κ and δ opioid receptors, which is species dependent (Leander,1988; Negus, 2002). Buprenorphine is used to manage mild to moderate pain in many species (Fish, 2008). Dosing intervals are highly variable across species, ranging between 4 and 12 hours (Gades, 2000; Fish, 2008). The side effects are generally minimal and include hyporexia, sedation, mild respiratory depression, and decreased gastrointestinal motility (Jirkof, 2017; Dahan, 2006; Martin-Flores, 2017).

NSAIDs, such as meloxicam, block prostaglandin (PG) synthesis through inhibition of cyclooxygenase (COX) enzymes. PGs are short-lived compounds that cause erythema, edema, pyrexia, and increased pain sensation through stimulation of peripheral nociceptors (Khan, 2012) (Jang, 2020). Through PG inhibition, NSAIDs have anti-inflammatory, antipyretic, and analgesic effects (Khan, 2012). NSAIDs can be selective for the isoenzymes COX-1 or COX-2, or they can inhibit both non-selectively (Khan, 2012). COX-1 is produced by cells in the gastric mucosa, intestinal epithelium, and renal tubules (Khan 2012). Side effects are associated with COX-1 inhibition and include gastric or intestinal ulceration and azotemia (Khan, 2012; Fish, 2008). COX-2 is produced by inflammatory cells and is not associated with these side effects (Khan, 2012). Meloxicam is generally considered a selective COX-2 inhibitor, but it is nonselective in cats (Fish, 2008). Its elimination half-life of 12-24 hours makes it advantageous for clinical use (Fish, 2008).

Bats are of particular interest in viral immunology because they harbor viruses that are highly pathogenic in humans and other mammals, but they are immunotolerant to these viruses (Irving, 2021). The adaptive immune response to viruses in bats is not well understood and is an active area of investigation for immunologists. It is challenging to examine this due to a lack of developed bat-specific immunological tools (Burke, 2023).

The adaptive immune response is complex and involves the coordination of many immune cell types. When a T cell dependent antigen is used for immunization the first time, naïve CD4+ T cells interact with antigen presenting cells (APCs) (primarily dendritic cells) that present antigenic peptides on MHC II (Sallusto, 2002). The interaction occurs through the T cell receptor (TCR) on the CD4+ T cell and the MHC II complex on the APC. CD28, which is also expressed on the T cell surface interacts with B7-1 and B7-2 on APCs; this has a costimulatory

effect in T cell activation (Zhang, 1997). After these initial interactions, naïve CD4+ T cells begin a cascade of signaling pathways that lead to their differentiation into several different effector cell types. This is dependent on costimulatory effects from APCs and cytokine profiles within the immediate vicinity (Sallusto, 2002). CD4+ T cells can differentiate into several types of effector cells that include Th1, Th2, Th17, T follicular helper (Tfh), and T regulatory (Treg) cells.

Th1 cells combat intracellular pathogen including viruses and bacteria by releasing the cytokines IFN-γ and IL-2. IFN-γ activates macrophages and IL-2 promotes CD8+ T cell proliferation and formation of CD8+ memory cells (Williams, 2006). Th1 cell differentiation requires IL-12 and IFN-γ which are released by APCs and NK cells respectively (Luckheeram, 2012). The Th1 cell response also requires the transcription factor T-bet which controls the expression of genes needed for Th1 cell differentiation and function. T-bet also inhibits differentiation of CD4+ T cells to Th2 cells and other CD4+ cell subsets (Lazarevic, 2013).

Th2 cells mediate immunity against helminths and incite inflammatory responses to allergens. Th2 cell activation results in isotype class switching to IgE (via IL-4) and inhibition of macrophage functionality, while also promoting eosinophil, basophil, and mast cell functionality (Paul, 2010). The IgE complexes that are produced bind to basophils and mast cells resulting in their activation. The major cytokines released by Th2 cells are IL-4, IL-5, IL-9, IL-13, and IL-25 (Luckheeram, 2012). For Th2 cell differentiation, IL-2 and IL-4 are needed in addition to the transcription factor GATA3. Other cytokines promote Th2 cell differentiation including IL-6 and IL-21 (Luckheeram, 2012).

T follicular helper (Tfh) cells play an important role in the production of high-affinity antibodies and memory B cells (Crotty, 2014). They are also involved in germinal center

formation in lymphoid tissues (Crotty, 2014). IL-21 is the major effector cytokine of Tfh cells. It is involved in isotype class switching in B cells, especially to IgG (Ozaki, 2004). It also promotes B cell proliferation and B cell differentiation to plasmablasts (Ozaki, 2004). In addition, it plays an important role in affinity maturation (Zotos, 2010). Tfh cell differentiation depends on IL-6, IL-21 and the transcription factor Bcl6 (Luckheeram, 2012). The expression of the chemokine receptor CXCR5 also occurs during the early stages of the Tfh differentiation process (Crotty, 2014).

Th17 cells are important in immunity towards extracellular bacteria and fungi (Awasthi, 2009). Th17 effector cytokines include IL-17 and IL-22. The cytokines involved in Th17 differentiation are IL-6, IL-21, IL-23 and TGF-β (Luckheeram, 2012). IL-17 and IL-22 induce the production of antimicrobial peptides (AMPs) that are involved in fungal and bacterial immunity; these AMPs result in the destruction of fungal cell walls and suppress fungal and bacterial growth through ion sequestration (Vylkova, 2007; Valeri, 2016). IL-17 and IL-22 also recruit neutrophils to mucosal barriers by inducing chemokine production (Valeri, 2016).

Treg cells prevent autoimmunity and suppress the function of other CD4+ T effector cells to prevent excessive inflammatory responses. They release IL-10 and/or TGF-β (Luckheeram, 2012). IL-10 is an anti-inflammatory cytokine and has a role in suppressing the Th1 response (Tian, 2016). TGF-β and the transcription factor FoxP3 are needed for induced Treg differentiation (Yoshimura, 2011; Yagi, 2004).

T-dependent B cell interactions are important components of the adaptive immune response. Tfh cells interact with activated B cells in B cell follicles and germinal centers in secondary lymphoid organs (Smith, 2000). This incites B cells to undergo clonal expansion and differentiation and promotes B cell isotype switching and antibody production (Smith, 2000).

These interactions also drive B cell somatic hypermutation (SM) and affinity maturation which are needed to produce high affinity antibodies that principally account for high titer serum antibodies (Steiner, 1967). This process is the most effective during the secondary T-dependent immune response because memory B cells generated in the primary T-dependent response have already undergone SM (Steiner, 1967).

Full μ agonist opioids, such as morphine, have been shown to have immunosuppressive effects, affecting both innate and adaptive immune responses. Morphine has been shown to inhibit function of natural killer (NK) cells and macrophages (Sacerdote, 1997; Casellas, 1991; Roy, 2011; Szabo, 1993). It has also been shown to suppress the T helper 1 cell (Th1) response (Liang, 2016; Sacerdote, 1997), promote the T helper 2 cell (Th2) response (Liang, 2016), and decrease T cell proliferation (Sacerdote, 1997). Furthermore, morphine decreases B cell presentation of class II major histocompatibility complexes (MHC II), causing suppression of the T cell response (Beagles, 2004). Finally, morphine has been shown to suppress the IgG response in mice and rats (Lockwood, 1994; Eisenstein, 1990).

In contrast with full μ opioids, buprenorphine has been shown to have mixed immunomodulatory effects. Buprenorphine has been shown to suppress the activity of neutrophils, macrophages and NK cells (Mikawa, 2006; Sun, 2017; Piersma, 1999; Carrigan, 2004). In addition, buprenorphine was shown to decrease lymphocyte proliferation in rats (Carrigan, 2004). Lastly, chronic daily administration of buprenorphine in mice resulted in a progressive leukopenia that developed by day 30 (Banerjee, 1997). Conversely, studies have shown that buprenorphine has no effect on macrophage and NK cell activity (Allen, 2019; Franchi, 2007) and no effect on the IgG response (Allen, 2019; Kolstad, 2012).

NSAIDs have also been shown to have immunomodulatory effects. Meloxicam has been shown to suppress both the Th1 and Th2 responses (Yamaki, 2003) and multiple studies demonstrate that NSAIDs suppress the IgM and IgG responses (Chen, 2021; Ryan, 2005; Bancos, 2009; Yamaki, 2003).

The goals of this study were to determine whether buprenorphine and meloxicam affect the IgG response and whether they affect the balance of the CD4+ T helper cell responses. The ability to use analgesia in bats throughout studies can alleviate pain associated with disease progression, thus improving welfare for these animals. In bats immunized with keyhole limpet hemocyanin (KLH) followed by 21 days of treatment with buprenorphine or meloxicam, differences in IgG, Th1, Th2, Treg, and Tfh cell responses were evaluated. It is hypothesized that buprenorphine treatment will not significantly affect the immune response in JFBs and that meloxicam treatment will significantly affect the immune response in JFBs.

CHAPTER 4: EFFECTS OF BUPRENORPHINE AND MELOXICAM ON IMMUNE RESPONSE IN JAMAICAN FRUIT BATS

The goal of this study is to determine the effects of buprenorphine and meloxicam on the immune response in JFBs. The hypothesis is that buprenorphine treatment will not have a significant effect on the immune response in JFBs, whereas meloxicam treatment will have a significant effect on the immune response.

Methods.

Nine male and 9 female JFBs were randomly collected from the Colorado State University colony. Females suspected to be pregnant were not used (although on necropsy, 3 females in the saline group, 1 female in the buprenorphine group, and 2 females in the meloxicam group were found to be pregnant). The bats were kept on a 12/12 light dark cycle, at a minimum temperature of 21.1 °C and maximum temperature of 26.7 °C. Bats were fed a combination of fruit daily along with a protein supplement (Mazuri Softbill Diet). JFBs were housed in cages (0.51 m × 0.3 m × 0.46 m) with 6 bats per cage.

On day 1 each bat was anesthetized under isoflurane anesthesia. Isoflurane was delivered in oxygen at a flow rate of 2 L/min, ranging from 3-5% via mask induction. Once anesthetized, bats were weighed and their toenails were marked with nail polish for identification. Blood was collected from the wing vein by using a 22-gauge needle. Venipuncture sites were cleaned with 70% isopropyl alcohol saturated gauze prior to venipuncture. Blood droplets were collected in Eppendorf tubes up to a maximum of 200 μL per bat and dry gauze was used to achieve hemostasis. Three total groups were established with one group receiving buprenorphine (BUP) at 1 mg/kg subcutaneously (s.c.) once daily for 21 days, one group receiving meloxicam (MEL)

at 5 mg/kg s.c. once daily for 21 days and one group receiving 0.13 mL saline (SAL) s.c. once daily for 21 days. KLH (Sigma-Aldrich) was given to all bats s.c. on day 1 using 25 µL of a 5.5 mg/mL solution that had been filtered through a 0.22 um syringe filter and combined with 25 µL of complete Freund's adjuvant (Sigma-Aldrich). On day 21, KLH was given s.c. to previously immunized groups combined with 25 µL of incomplete Fruend's adjuvant (Sigma-Aldrich). Bats were weighed weekly for three weeks and doses of BUP and MEL were recalculated accordingly. On day 28, all JFBs were euthanized via isoflurane overdose using isoflurane saturated gauze in a conical tube. After cessation of breathing, blood was collected via cardiac puncture exsanguination. Spleens were collected in a sterile manner for splenocyte isolation and culture. All blood collected was permitted to clot and stored on ice until centrifugation. Blood was centrifuged at 3,200 x g for 10 minutes and serum was separated from clots and stored at -80°C until processing.

After terminal blood draw, bats were kept on ice. Sterile instruments and aseptic technique were used to isolate spleens. Spleens were removed and crushed with a sterile end of a syringe through a 100 µm filter that was primed with media (RPMI with 10% FBS) into a 50 mL conical tube, then flushed with 30 mL of media. The conical tubes were centrifuged for 10 min at 400 x g. The supernatant was decanted, keeping the cell pellet intact. The cell pellet was resuspended in the residual media by dragging the tube across a styrofoam tube rack. Two mL of AKC lysis buffer was added to the tube and incubated at room temperature for 10 min with gentle swirling every 3 min. Then 10 mL of media were added to the tube and the full volume was transferred to a 15 mL conical tube. The tubes were centrifuged for 10 min at 400 x g. The media was decanted leaving the pellet intact and the pellet was resuspended in the residual media as previously described. 10 mL of media were added and then the tubes were centrifuged at 400

x g for 10 min. The media was decanted off and the cell pellets were resuspended. The splenocytes were resuspended in 90% FBS and 10% DMSO, and frozen in cryovials in an insulated styrofoam box at -80 °C. The cryovials were subsequently transferred to liquid nitrogen for storage.

Enzyme-linked immunosorbent assays (ELISA) were used to determine IgG titers to KLH. A 96 well ELISA plate was coated with 100 µL of KLH at 1 µg/mL in each well and incubated overnight at 4 °C. The wells were emptied and washed three times with PBS. Two hundred µL of 0.25% gelatin blocking buffer was added to each well and incubated at room temperature for 1 hour. Serum was brought to room temperature and diluted 1:100 in antibody diluent (0.25% BSA-PBS/Pen-Strep, filter sterilized). The wells were emptied and washed three times with PBS-TWEEN, followed by 3 washings with PBS. One hundred µl of PBS were added to all wells except for the first row. Two hundred µL of the diluted sera were added to the first row. To make serial dilutions, 100 µL was transferred from the top row to the row below until the last row was reached. One hundred µL was discarded from the final row. The plates were incubated for 1 hour at room temperature, and the plates were washed three times with PBS-TWEEN followed by three times with PBS. 100 µL of protein A/G-Horse radish peroxidase at 1:5000 in antibody diluent was added to each well and incubated for an hour at room temperature. The wells were washed four times with PBS-TWEEN followed by four times with PBS. 100 µL of ABTS was added to each well and the plates were incubated at room temperature for 15 minutes. The plates were read at 405nm (Multiscan Spectrum, Thermofisher).

Splenocyte cultures were performed as described (adapted from Wang, 2001 and Allen, 2019). Cryovials were thawed quickly in a 37 °C water bath, until a sliver of ice remained.

Approximately 300 µL of prewarmed, medium (complete 5% FBS RPMI-1640) was added dropwise to the vial and then gently pipette mixed. The cells were then transferred dropwise to a 15 mL conical tube already containing 5 mL of prewarmed media. An additional 5 mL of media was added on top, and tubes were centrifuged at 350 x g for 10 min. The supernatant was decanted and the pellet was resuspended by dragging the conical tube containing the pellet across a plastic tube rack. An additional 10 mL of media was added to the conical tubes and the tubes were placed on ice.

In a 96 well plate, 1 µL of DAPI (5 mg/mL) and 5 µL of 7-AAD conjugated to Texas red (50 µg/mL) were combined with 20 µL of cells and pipette mixed. 20 µL of this suspension was loaded onto the appropriate slides, and cell count and viability were assessed using an automated cell counter (Countess 3 FI, Invitrogen). For samples exhibiting poor viability post-thaw, a density gradient was performed using Biowhittaker™ Lymphocyte Separation Medium.

Splenocytes were re-pelleted at 350 x g for 10 min. The supernatant was decanted, the pellet was resuspended 5 mL PBS. 5 mL lymphocyte separation media was added before adding the cell suspension dropwise on top with the conical tube at an angle to ensure the layers remained separated. The tubes were centrifuged for 15 minutes at 400 x g without brakes. The cell layer was then collected via pipette and filtered through a 70 µm filter into a 50 mL conical tube containing 5 mL of media. An additional 2 mL of media was added through the filter to wash any remaining cells. Cells from this step were then prepared for the automated cell counter and counted as previously described.

Based on cell count per mL and cell viability, 1 million splenocytes from each sample were placed into two Eppendorf tubes (1 million cells per tube) and centrifuged at 500 x g for 5 min. One million cells were plated in 1 mL of media with or without 20 µg of KLH. Cells were

cultured for 72 hours at 37 °C. For the samples from saline treated bats, RNA was extracted following the provided protocol from an RNeasy kit (Qiagen). This method was used for saline treated bats due to better saline viability compared to the buprenorphine and meloxicam groups.

For samples with poor cell viability, a Trizol RNA extraction method provided by Bradly Burke was adapted for use. After culture, cells were mixed in the wells and transferred to FACS tubes. They were centrifuged for 10 min at 340 x g. The supernatant was decanted, and 1 mL of Trizol (Invitrogen) was added. The tubes were vortexed, incubated at room temperature for 10 min and then transferred to 2 mL microfuge tubes. 200 µL chloroform (Sigma-Aldrich) was added, and the tubes were vortexed and incubated at room temperature for 5 min. The tubes were then centrifuged for 10 min at 12,000 x g. The aqueous phase was carefully removed and transferred to clean 2 mL microfuge tubes, with 1 µL glycogen added in each tube. 500 µL of Trizol and 100 µL of chloroform were added to the tubes containing Trizol, vortexed, incubated and centrifuged at 12,000 x g for 10 min. The aqueous phase was carefully removed again and transferred to the tubes containing aqueous phase and glycogen. Then 500 µL of cold isopropyl alcohol was added to the tubes containing the aqueous phase, vortexed and centrifuged for 20 min at 12,000 x g. The supernatant was removed with a pipettor, and then 1 mL of 70% ethanol was added to each tube and centrifuged for 10 min at 12,000 x g. The supernatant was pipetted off and the wash step was repeated again with 70% ethanol. After centrifuging, the supernatant was removed completely, leaving the pellet intact. A vacuum device was used to help dry the samples. Subsequently, 20 µL molecular grade water was added to each tube to proceed with RNA quantification.

NanoDrop was used to quantify RNA in each sample. Two µL of molecular grade water was used to blank the machine before reading samples. Two µL was used from each sample to

obtain a reading. After quantification 1 µL of Ribolock (Thermofisher) was added to each tube for storage at -80 °C.

Splenocyte cultures were evaluated for gene expression changes through a qPCR assay that has been previously described (Oko, 2006; Burke, 2024). cDNA was generated (QuantiTech RT, Qiagen) from isolated RNA using 100 ng per sample.

Next, qPCR was performed (QuantiTech SYBR Green, Qiagen) using previously published primers from Jamaican fruit bat genes (Burke, 2023). Gene expression within each sample was normalized to the Rps18 gene (ΔCq), and fold change was calculated by comparing gene expression in peptide-stimulated cells to unstimulated cells from the same bat (ΔΔCq).

Six bats per group were used based on a priori power analysis using one-way ANOVA, determining an α of 0.05, power of 0.80, and expected effect size of 0.9. GraphPad Prism was used to perform one-way ANOVA and Dunnett's multiple comparison test for optical density and titer values. The same tests were used to determine significance in gene expression for each individual gene.

Results.

Data from six bats in the BUP and MEL groups were collected, while only five data points were collected from the SAL group due to death of a bat during the study. No significant differences in anti-KLH IgG titers were noted in either BUP ($p=0.58$) or MEL groups ($p=0.70$) compared to the SAL control (Figure 4). The groups for titers were initially divided into male and female for each group. A one-way ANOVA showed no statistical differences in males versus females (data not shown), and the males and females were subsequently combined for each treatment group. Pre-immunization optical densities confirmed that IgG was absent in all bats on day 1. These findings imply that neither BUP nor MEL treated bats affected the immune

response in JFBs.

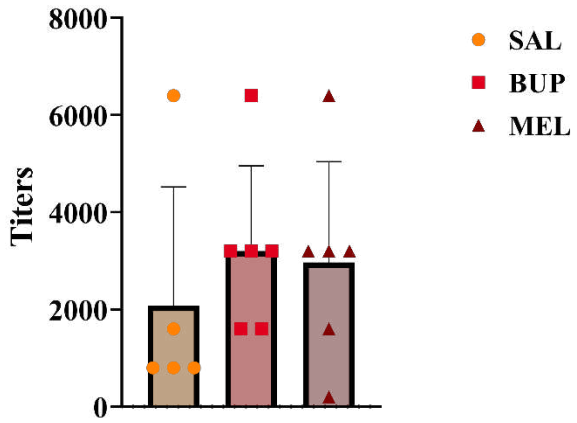

Figure 4. Anti-KLH IgG titers in SAL, BUP and MEL treated Jamaican fruit bats. Individual data points, mean and standard deviation are shown for each group.

There were no significant changes within the BUP group compared to SAL in the expression of IL-4, IL-2, IL-10, IL-21, IFN-γ, TGF-β, T-bet, FoxP3, or CXCR5 (Figure 5). There was a significantly lower level of IL-13 expression in the BUP treated bats compared to the SAL treated bats ($p=0.013$). No significant changes in gene expression were noted between MEL and SAL treated bats, but the sample sizes for both groups were low at 2 and 3 respectively. The sample size for the BUP group was 5. The p values for all other groups were as follows: IL-2 BUP ($p = 0.95$), IL-2 MEL ($p = 0.64$), IFN-γ BUP ($p = 0.41$), IFN-γ MEL ($p = 0.87$), T-bet BUP ($p = 0.71$), T-bet MEL ($p = 0.99$), IL-13 MEL ($p = 0.05$), IL-4 BUP ($p = 0.47$), IL-4 MEL ($p = 1.0$), IL-10 BUP ($p = 0.35$), IL-10 MEL ($p = 0.08$), TGF-β BUP ($p = 0.55$), TGF-β MEL ($p = 1.0$), FoxP3 BUP ($p = 0.95$), FoxP3 MEL ($p = 0.16$), CXCR5 BUP ($p = 0.49$), CXCR5 MEL ($p = 1.0$), IL-21 BUP ($p = 0.91$), and IL-21 MEL ($p = 0.99$).

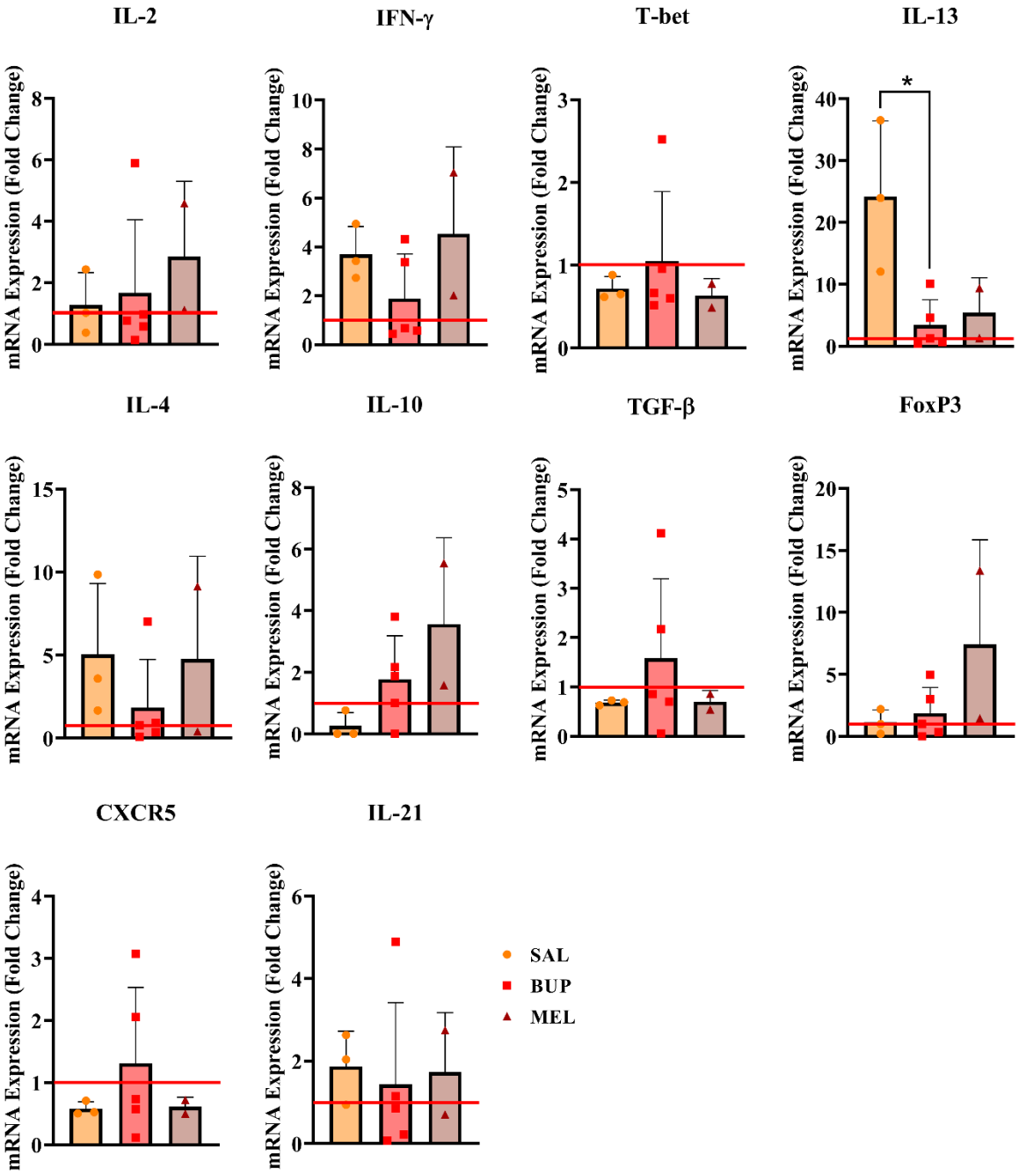

Figure 5. RT-qPCR quantification of gene expression in BUP and MEL treated JFBs compared to SAL control. A horizontal line at fold change = 1 indicates the baseline level, representing no change in gene expression. Individual data points, mean, and standard deviation are shown (*, $p < 0.05$).

Discussion.

Antibody responses are complex and involve the coordination of multiple cell types including APCs, T cells, and B cells. The primary immune response to an antigen results in the production of memory B cells, which then interact with T follicular helper cells to produce high affinity antibodies during the secondary immune response (Crotty, 2014). If there is impairment in the presentation of antigen from B cells or APCs, or dysfunction in cytokine responses of immune cells, the antibody response may be suppressed. The titers in the BUP and MEL groups were not significantly different from the control group, suggesting that long term treatment with either of these analgesics does not impact the adaptive immune response in JFBs. The high titer levels noted in the BUP and MEL groups indicate that high affinity antibodies were produced, which require a robust Tfh response in coordination with B cells and other CD4 + T cells (Steiner, 1967).

CD4+ T cells are necessary in orchestrating the adaptive immune response and drive humoral and cell mediated responses through various effector functions (Luckheeram, 2012). For example, Th1 cells release IL-2, which promotes the proliferation and function of CD8+ T cells, allowing for the destruction of cells infected with intracellular pathogens. Through assessment of immunogenic profiling, the effects of BUP and MEL on CD4+ effector function can be elucidated by looking at fold changes in expression of cytokines, transcription factors, and receptors. TGF-β and IL-10 are primarily secreted by Treg cells, which have an anti-inflammatory role, whereas FoxP3 is a transcription factor that is necessary for the differentiation and function of Tregs (Yoshimura, 2011; Yagi, 2004). IL-2 and IFN-γ are secreted by Th1 cells and T-bet is a transcription factor needed for Th1 differentiation and function (Williams, 2006; Luckheeram, 2012). IL-4 and IL-13 are effector cytokines of Th2

cells and CXCR5 is a chemokine receptor that is expressed on Tfh cells. IL-13 expression was significantly higher in the SAL control bats compared to the BUP treated bats, which suggests that BUP administration may dampen the Th2 response in the adaptive immune response. No other changes in gene expression were significant. Despite this finding, the effect of BUP on the immune response in JFBs is negligible based on the titer data.

The sample sizes for gene profiling were lower than anticipated due to low mRNA yields. It is also notable that mRNA was not isolated in the same manner for all bats. Trizol RNA extraction was performed on bats treated with BUP and MEL in attempt to isolate higher yields of mRNA. In addition, the splenocyte viability was lower for the BUP and MEL treated bats and these splenocytes were treated with lymphocyte separation media prior to culture. This is not expected to significantly affect gene expression because the CD4+ lymphocyte response to KLH drives changes in gene expression during splenocyte culture and the separation media selects for lymphocytes.

The responses in the BUP bats were more variable compared to the other groups. On average, TGF-β expression was greater in this group compared to the SAL control. One of the BUP treated bats had decreased expression of all genes compared to the unstimulated splenocytes from the same bat, with the highest expression of IL-10 and FoxP3. While these findings suggest increased Treg cell activity, these were not found to be significant.

Based on the data available from the gene profiling, only BUP shows statistically significant evidence of immunomodulation compared to the SAL controls. There is no statistically supported evidence of BUP affecting the Th1, Tfh or Treg responses. However, due to the small sample sizes used and the inherent genetic variability of individuals in the JFB

colony population, it is not feasible to draw conclusions regarding the CD4+ T cell response. There were not enough samples from the MEL treated bats to draw any meaningful conclusions.

Interestingly, TGF-β and CXCR5 were either upregulated or downregulated in the same direction when splenic lymphocytes were stimulated with KLH, which was apparent in all bats. This supports the finding that Tfh cell differentiation can be influenced by the presence of TGF-β, which is true in humans and mice (Schmitt, 2009; Chang, 2024).

The purpose of this study was to determine the effects of BUP and MEL administration on the immune response in JFBs. The 21 day treatment starting from the day of initial immunization was chosen for this study because this timeline is crucial for the primary adaptive immune response, setting the stage for the secondary adaptive immune response; in demonstrating that BUP and MEL did not have an impact on the immune response with this dosing regimen, shorter term treatment timelines which are typical in post operative treatment will have even less of an impact on the immune response. The results show that BUP and MEL have minimal effects on adaptive immunity in JFBs. However, additional gene expression data particularly in MEL and SAL treated bats would provide stronger evidence for this conclusion. Although there is evidence that BUP resulted in a suppressed Th2 response, the Th1, Treg, and Tfh responses were not significantly changed. In light of these findings, the use of BUP and MEL in JFBs should be strongly considered in infectious disease studies as clinically indicated.

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