Increasing serotonin bioavailability in preweaned dairy calves impacts hematology, growth, and behavior
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Abstract
Peripheral serotonin has been shown to regulate important physiological functions such as energy homeostasis and immunity, particularly in rodent and humans, but its role is poorly understood in livestock species. Herein, we tested the safety and effectiveness of increasing serotonin bioavailability in preweaned dairy calves by oral supplementation of a serotonin precursor (5-hydroxytryptophan, 5-HTP) or a serotonin reuptake inhibitor (fluoxetine, FLX). Bull Holstein calves (21 ± 2 d old; N = 24) were fed milk replacer (8 L/d) supplemented with either saline as control (CON, 8 mL/d, n = 8), FLX (40 mg/d, approx. 0.8 mg/kg; n = 8), or 5-HTP (90 mg/d, approx. 1.8 mg/kg; n = 8) for 10 consecutive days in a complete randomized block design. Heart rate (HR), respiration rate, rectal temperature, and health scores were recorded daily. Hip height and body weight were measured at d 1, 5, and 10 relative to initiation of supplementation. Blood samples were collected once before the supplementation period (d 1), during the 10-d supplementation period (daily), and during a 14-d withdrawal period (d 2, 3, 4, 7, and 14 relative to initiation of withdrawal). Cerebrospinal fluid and muscle tissue were collected from a subset of calves (n = 12) that were euthanized after the 10-d supplementation or 14-d withdrawal period. Whole blood serotonin concentrations increased in 5-HTP calves and decreased in FLX calves compared with CON (P < 0.001), indicating that serotonin bioavailability was increased in both groups. Whole blood serotonin concentrations of 5-HTP and FLX calves returned to CON levels after 7 d of withdrawal. All calves grew and were considered healthy throughout the study. In fact, calves fed 5-HTP had higher average daily gain compared with CON (0.87 vs 0.66 ± 0.12 kg/d, P = 0.05). Calves fed FLX had lower HR (P = 0.02) and greater red blood cells and hemoglobin counts on d 10 of supplementation compared with CON (P < 0.01). After the 14-d withdrawal period, FLX was not detected in circulation of FLX calves, but was still present in the muscle tissue. Our results demonstrate that manipulation of the serotonin pathway by supplementing FLX or 5-HTP is a feasible and safe approach in preweaned dairy calves; however, it takes more than 14 d for FLX to be completely withdrawn from the body.

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1. Introduction
Serotonin is a monoamine synthesized in two steps: first, the rate-limiting enzyme tryptophan hydroxylase (TPH) catalyzes the conversion of the essential amino acid L-tryptophan to 5-hydroxytryptophan (5-HTP), and second, a ubiquitous enzyme, aromatic L-amino acid decarboxylase, converts 5-HTP to serotonin. Tryptophan hydroxylase is encoded by two different genes: TPH1 present in peripheral tissues and TPH2 present in the central nervous system (CNS), resulting in two independent serotonergic systems [1]. Less than 5% of the body’s total serotonin is found in the CNS, where it acts as a neurotransmitter regulating behaviors such as appetite, mood, cognition, and learning. Approximately 95% of serotonin is stored in platelets and regulates a variety of biological functions [2,3]. Peripheral serotonin derives
primarily from the enterochromaffin cells of the gastrointestinal tract but also from organs such as liver, bones, and mammary glands. Serotonin research has been primarily focused on its role as a neurotransmitter in the CNS; however, more recently, research has focused on its role in peripheral tissues of various species, including dairy cattle.

Peripheral serotonin is involved in bone metabolism by primarily stimulating bone resorption [4]; for example, mice lacking the rate-limiting enzyme, TPH1, experience impaired bone remodeling because of decreased osteoclastogenesis [5]. Several lines of evidence suggest a role for serotonin in the regulation of energy balance [6], particularly for mediating glucose and insulin metabolism in rodents [7,8]. Infusion of the serotonin precursor 5-HTP into late-lactation, nonpregnant dairy cows promoted glucose synthesis [9–11] and adipose tissue metabolism [11–13]. Serotonin has been implicated in the regulation of mammary gland homeostasis [14]. Elevated blood serotonin concentrations increase mammary gland tight junction permeability, potentially accelerating the involution process [15]. Indeed, intramammary infusions of fluoxetine (FLX) or 5-HTP disrupt mammary gland tight junctions and decrease milk yield [16]. More recently, serotonin has been proposed as an immunomodulatory molecule. In fact, multiple serotonin receptors have been identified in human and rodent immune cells including monocytes, lymphocytes, and dendritic cells [17]. Oral supplementation of 5-HTP to neonate dairy calves for the first 5 d of life upregulated the gene expression of haptoglobin, interleukin-1β, and nuclear factor kappa light chain in whole blood [18]. In addition, recruitment of neutrophils to sites of inflammation has been shown to be enhanced by serotonin [19].

Circulating levels of serotonin in neonatal calves range from 2,500 to 4,000 ng/mL [18] and are 1.5- to 2.3-fold higher than in lactating cows [20]. However, serotonin research is limited in livestock species and the physiological role of serotonin in dairy calves is still unknown. Hence, as a first step to understand the role of peripheral serotonin in dairy calves, we sought to determine the safety and feasibility of improving serotonin bioavailability by supplementing milk replacer with FLX, a selective serotonin reuptake inhibitor (SSRI), or 5-HTP, the serotonin precursor. Fluoxetine increases serotonin bioavailability by blocking the serotonin transporter (SERT, SLC6A4) in cell membranes and, thus, inhibits serotonin reuptake and degradation. Alternatively, 5-HTP bypasses the rate-limiting step (TPH) of serotonin synthesis, thus directly increasing its biosynthesis by cells. To our knowledge, this is the first study to manipulate the serotonin pathway through oral administration of FLX or 5-HTP, exploring their pharmacokinetics and impacts on physiological parameters, growth, health, and behavior of preweaned dairy calves.

2. Materials and methods

2.1. Animals and experimental design

All procedures performed in this study were approved by the Institutional Animal Care and Use Committee at the University of Florida (protocol # 201709851). Twenty-four Holstein bull calves (n = 24, 21 ± 2 d, 47 ± 3.2 kg) were transported to experimental pens (n = 8, 2.3 × 2.5 m; width × length; 3 calves/pen) at the University of Florida and randomly assigned to treatments in a complete randomized block design. All calves received 4 L of milk replacer (Southeast Milk Inc, Okeechobee, FL) twice a day (07:00 h and 17:00 h) for 5 d (acclimation period). Afterward, treatments were administered by supplementing milk replacer with 5-HTP (90 mg/d, approx. 1.8 mg/kg; n = 8, Sigma, St. Louis, MO; #H9772), FLX (40 mg/d, approx. 0.8 mg/kg; n = 8, Spectrum Chemical, Gardena, CA; #F1200) or saline (CON, 8 mL/d, n = 8) for 10 consecutive days (supplementation period). Herein abbreviations will be used when referring to treatments and spelled out when referring to physical substances. Fluoxetine and 5-hydroxytryptophan treatment doses were prepared the night before. Briefly, both powders were weighed and solubilized in sterile saline by stirring for approx. 30 min at 37°C aliquoted and stored at 4°C overnight. Milk replacer was prepared fresh in the morning and afternoon (4 L per calf per meal) and the corresponding volume of either FLX, 5-HTP, or saline was added to the replacer during the morning feeding only. The morning after the 10th day of supplementation, 4 calves per treatment (n = 4 randomly selected pens) were euthanized at the University of Florida abattoir for sample collection. Calves in the remaining 4 pens were kept for a 14-d withdrawal period, where milk replacer was no longer supplemented with FLX, 5-HTP, or saline, and were subsequently euthanized on d 14.

Calves were housed in a barn with shade curtains and straw bedding. The average temperature humidity index during the experimental period was 71.1 ± 9.9. Water and grain (Purina Animal Nutrition LLC, Shoreview, MN) were offered ad-libitum throughout the experiment and intakes were not recorded. Calf milk replacer and grain starter were sampled weekly for chemical composition analyses (Table 1).

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<th>Item</th>
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b Prepared by Purina Animal Nutrition, LLC (Shoreview, MN). Contained plant protein products, processed grain by-products, grain products, roughage products, molasses products, forage products, animal and vegetable fat, vitamins, amino acids, and minerals.
2.2. Sample and data collection

2.2.1. Vital signs, health, and growth parameters

Calves’ vital signs and health scores were evaluated during treatment administration as a measurement of health status. Heart rate (HR), respiration rate (RR), and rectal temperature (RT) were recorded once daily throughout the 10-d supplementation period 3 h after the morning feeding. On d 1 and 2, HR, RR, and RT were recorded at 1, 4, 7, and 10 h after feeding to monitor calves’ initial reaction to the administered doses. Hip height and body weight (BW) were recorded on d 1, 5, and 10 of the supplementation period and on d 7 and 14 of the withdrawal period. Average daily gain (ADG) during supplementation and withdrawal periods was calculated. Health scores were determined using the University of Wisconsin-Madison’s Calf Health Scoring Sheet [21] daily during the adaptation period, supplementation period and on d 1, 2, 3, and 14 of the withdrawal period. Calves’ lying activity was monitored throughout the 10-d supplementation period using electronic data loggers (Hobo Pendant G Data Logger: Onset Computer Corp., Pocasset, MA) placed on the inner left rear leg.

2.2.2. Blood sampling

Blood samples were collected from the jugular vein before (d 1, baseline) and daily 4 h after the morning feeding during the 10-d supplementation period. To monitor initial fluctuations of circulating serotonin in response to 5-HTP and FLX supplementation, serial blood samples were collected on d 1 and 2 at 3, 6, and 9 h after the morning feeding. During the withdrawal period, blood samples were collected on d 2, 3, 4, and 14 to monitor the timing of serotonin clearance from circulation. Blood was collected using K2 EDTA, Vacutainer Serum Plus, and Sodium Heparin blood collection tubes (BD, Franklin Lakes, NJ, #368047, 366430, 366480, respectively). Sodium Heparin tubes were immediately placed on ice and Serum Plus tubes were allowed to clot for 20 min at room temperature. Tubes were centrifuged at 3,000 × g for 20 min at 4°C, for collection of serum and plasma fractions. Whole blood from K2 EDTA tubes was transferred to disposable culture tubes (Fisher Scientific; #14–961-26) with 10 mg/mL of ascorbic acid and mixed gently to stabilize and protect serotonin against oxidative loss. Whole blood, serum, and plasma aliquots were stored at −20°C until analysis.

2.2.3. Cerebrospinal fluid, fecal and muscle tissue collection

During both euthanasia days, after 10 d of treatment supplementation or 14 d of withdrawal, calves were sedated by intravenous administration of 0.2 mg/kg of xylazine. Approximately, 1.5 mL of cerebrospinal fluid (CSF) was collected using a 2-inch 18-gauge spinal needle, placed on ice and stored at −80°C until analysis. Fecal samples were collected using cotton swabs and stored at −20°C until analysis. Calves were humanely euthanized using a captive bolt pistol followed by jugular exsanguination. Afterward, a sample (~1 cm³) of biceps femoris muscle was collected, rinsed in sterile PBS, and snap-frozen in liquid nitrogen. Muscle tissue was stored at −80°C until protein extraction.

2.3. Laboratory analysis

2.3.1. Serotonin and fluoxetine analyses

Whole blood, CSF, and muscle serotonin concentrations were measured using a serotonin enzyme immunoassay kit (IM1749; Immunotech, Beckman Coulter, Marseille Cedex 9, France) according to manufacturers instructions. Whole blood samples were diluted 1:100 in kit dilution buffer. Protein was isolated from 80 mg of muscle tissue collected at euthanasia using radioimmunoprecipitation assay buffer with 10 µL/mL of Halt Protease and Phosphatase Inhibitors Cocktail (Thermo Scientific, Grand Island, NY; #788441). Protein concentrations were determined by bicinchoninic acid assay (Pierce Chemicals, Grand Island, NY; #23227). Fifty milligrams of protein per sample was used to measure muscle serotonin concentration. All assays were performed in duplicate. Concentrations were determined using a semilogarithmic curve fit. The intra- and inter-assay CV for all serotonin assays averaged 6.1% and 11.0%, respectively.

Fluoxetine concentrations in whole blood, CSF, and fecal samples were measured using a Fluoxetine ELISA kit (Fluoxetine ELISA, Neogen Corporation, Lexington, KY; #107619). To measure whole blood FLX concentrations, a standard curve was created using a Fluoxetine Standard (Sigma-Aldrich, St. Louis, MO; #F-918) and whole blood as the matrix, per manufacturer’s recommendations. Whole blood samples were diluted 1:5 in kit ELA buffer. For fecal FLX analysis, fecal swabs were diluted in 1 mL of buffer (1:5) and analyzed following the manufacturer’s meconium protocol. For CSF FLX analysis, CSF samples were tested and compared with the positive and negative controls provided in the kit. Fluoxetine presence in fecal and CSF samples were tested and analyzed as a binary response (presence or absence). All assays were performed in duplicate and mean optical density were log-log transformed to fall within the standard curve. The intra- and inter-assay CV for all FLX assays averaged 4.7%, and 12.0%.

2.3.2. Hematology analysis

Whole blood collected in tubes containing K2 EDTA anticoagulant were transported to the laboratory within 2 h of collection to analyze hematology parameters including platelets (PLT), hemoglobin (HGB), and red blood cell count (RBC) using the Idexx ProCyte Dx analyzer (Idexx Laboratories Inc, Westbrook, ME).

2.3.3. Total protein and hematocrit analyses

Total protein was quantified from plasma using a Digital Brix Refractometer (MA871; Rocky Mount, NC) and blood hematocrit (HCT) was analyzed from whole blood. For HCT, capillary tubes (Fisher Scientific, Pittsburgh, PA; #22–362–566) of whole blood were centrifuged at 10,000 RPM for 5 min in a microhematocrit centrifuge and read using a circular hematocrit reader.

2.4. Statistical analysis

Data were analyzed using repeated measures or one-way ANOVAs in R programming 3.5.1 (R Foundation for Statistical Computing; Vienna, Austria). Depending on the variable analyzed, statistical models included treatment, day (or
hour), and their interactions as fixed effects. Pen was included as a fixed effect and calf ID as a random effect in all models. Supplementation and withdrawal periods were analyzed separately. Normality of residuals was evaluated using Shapiro-Wilk tests, and outliers and influential points were evaluated using Cook’s distance test. Serotonin and FLX data were log-transformed and statistical results were back-transformed for data visualization. Presence of FLX in CSF or feces was tested using chi-square test. Statistical significance was declared at \( P \leq 0.05 \) and tendencies at 0.05 < \( P \leq 0.10 \). All values are reported as LS means ±SEM.

3. Results

3.1. Serotonin concentrations in whole blood, muscle, and cerebrospinal fluid

Whole blood serotonin concentrations were measured on d 1 and 2 at 3, 6 and 9 h to monitor initial serotonin fluctuations in response to 5-HTP and FLX supplementation. Serotonin concentrations were similar between 5-HTP and CON calves on d 1, but there was a treatment effect on d 2 (\( P < 0.001 \)), whereby 5-HTP calves had greater serotonin concentrations compared with CON calves at 3, 6, and 9 h (Fig. 1A). Whole blood serotonin concentrations were also measured daily during the 10-d supplementation period. There was a treatment by day interaction (\( P < 0.001 \)) whereby blood serotonin concentrations increased continuously across days in 5-HTP starting on d 2, but decreased in FLX starting on d 6, compared with CON calves (Fig. 1B).

To monitor circulating serotonin clearance after cessation of supplementation, whole blood samples were collected during the 14-d withdrawal period. There was a treatment-by-day interaction (\( P < 0.001 \)), whereby serotonin concentrations in 5-HTP-supplemented calves decreased, whereas serotonin concentrations of FLX-supplemented calves increased over time. Blood serotonin concentrations during a 14-d withdrawal period (n = 4 per group). Whole blood serotonin concentrations during a 14-d withdrawal period (n = 4 per group). Whole blood serotonin concentrations of FLX and CON calves during 10-d supplementation period at 3, 6, and 9 h on d 1 and 2 (D), d 1 to 10 (E) and d 2, 3, 4, 7, and 14 of withdrawal period (F). Data are presented as LS mean ± SEM. * = FLX versus CON; # = 5-HTP versus CON. \(* P < 0.05; ** P < 0.001; \# P < 0.05; \## P < 0.01; \### P < 0.001.\)

![Fig. 1. Blood serotonin and fluoxetine concentrations in response to 5-hydroxytryptophan or fluoxetine 10-d dietary supplementation followed by a 14-d withdrawal period. Whole blood serotonin concentrations (A) at 3, 6, and 9 h on d 1 and 2, (B) daily (d 1 to 10 of supplementation) after feeding milk replacer supplemented with 5-hydroxytryptophan (5-HTP; ■), fluoxetine (FLX; △), or saline (CON; ○) to Holstein dairy calves (n = 8 per group). (C) Whole blood serotonin concentrations during a 14-d withdrawal period (n = 4 per group). Whole blood fluoxetine concentrations of FLX and CON calves during 10-d supplementation period at 3, 6, and 9 h on d 1 and 2 (D), d 1 to 10 (E) and d 2, 3, 4, 7, and 14 of withdrawal period (F). Data are presented as LS mean ± SEM. * = FLX versus CON; # = 5-HTP versus CON. \(* P < 0.05; ** P < 0.001; \# P < 0.05; \## P < 0.01; \### P < 0.001.\)
concentrations of 5-HTP and FLX calves were similar to those of CON calves after 7 and 14 d of withdrawal, respectively (Fig. 1C).

Serotonin concentrations in CSF did not differ among treatments after the 10-d supplementation or 14-d withdrawal period ($P > 0.18$; data not shown). Muscle serotonin concentrations were similar among treatments during the 10-d supplementation period ($P = 0.24$); however, FLX calves had higher serotonin concentrations in the muscle compared with CON (11.9 vs 6.3 ± 1.7 ng/mL, respectively; $P = 0.047$) after the 14-d withdrawal.

3.2. Fluoxetine concentrations in whole blood, muscle, cerebrospinal fluid, and feces

Whole blood FLX concentrations were measured on d 1 and 2 at 3, 6, and 9 h to quantify the initial fluctuations in blood after oral supplementation of FLX compared with CON. There was a treatment by hour interaction ($P < 0.03$) on both days. On d 1, FLX calves had greater FLX concentrations after 3 h of supplementation and concentrations increased further at 6 and 9 h compared with CON (Fig. 1D). On d 2, FLX calves had higher FLX concentrations when compared with d 1, and similarly to d 1, concentrations increased from 3 to 9 h (Fig. 1D). On d 2, FLX concentrations were higher compared with d 1, and the same pattern of increased whole blood FLX concentrations from 3 to 6 h was observed (Fig. 1D). Whole blood FLX concentrations were also measured daily during the 10-d supplementation period. There was a treatment by day interaction ($P < 0.0001$) whereby FLX calves had increased FLX concentrations from d 1 to 10 (65.7 to 972.43 ng/mL) compared with CON in which FLX was undetectable (Fig. 1E). There was also a treatment by day interaction during the 14-d withdrawal period ($P = 0.003$), whereby blood FLX concentrations decreased gradually in FLX calves at d 2, 3, and 4 ($P < 0.001$) until reaching CON levels at d 14 ($P = 0.81$, Fig. 1F).

A subset of FLX and CON calves (n = 4 per group) was euthanized after the 10-d supplementation or after the 14-d withdrawal period to determine FLX concentrations in muscle, CSF, and feces. Muscle FLX concentrations were higher in FLX compared with CON calves after 10-d supplementation and after 14-d withdrawal (4.28 vs 0.01 ± 0.5 ng/mL and 0.84 vs 0.00 ± 0.2 ng/mL, respectively; $P < 0.047$). After the 10-d supplementation period, an association was found between treatment and presence of FLX in feces and CSF ($x^2 = 8.0$, $P = 0.005$); however, after a 14-d withdrawal period, no association was found for either feces or CSF ($x^2 = 1.14$, $P = 0.29$).

3.3. Heath scores, vital signs, and blood hematology

Health scores and vital signs were recorded to monitor the safety of a 10-d oral supplementation of FLX or 5-HTP. No differences were observed in calves’ HR or RT among treatments on d 1 and 2 of treatment administration ($P > 0.33$). Animal vital signs were also recorded once per day through the 10-d supplementation period. Rectal temperature was similar among treatments ($P = 0.61$); however, there was a significant day effect ($P < 0.001$; Fig. 2C), reflecting temporal variation in temperature and humidity. Respiration rate was similar among treatments ($P = 0.71$; Fig. 2B), but FLX fed calves tended to have lower HR compared with CON (107 vs 114 ± 1.9 bpm, respectively, $P = 0.056$; Fig. 2A). After 10 d of supplementation, FLX calves had higher HGB compared with CON calves (7.5 vs 6.1 ± 0.2 M/L and 7.9 vs 7.14 ± 0.2 g/dL, respectively; $P < 0.005$; Fig. 3A), whereas 5-HTP calves had higher HCT compared with CON calves (24.76% vs 22.97%, respectively; $P = 0.006$). No significant differences in PLT count were observed among groups ($P = 0.62$, Fig. 3A). No significant differences were found for total protein among treatments ($P = 0.07$); however, there was a day effect for total protein, $P < 0.001$; Fig. 3B). Health scores were similar among treatments with a median score of 0, during the 10-
d supplementation and during the 14-d withdrawal period ($P > 0.46$), indicating that all calves were healthy throughout the study.

### 3.4. Growth parameters

To evaluate the impact of oral supplementation of FLX and 5-HTP on growth parameters, BW was recorded and ADG was calculated. Calves fed 5-HTP had greater ADG during the 10-d supplementation period compared with CON calves (0.87 vs 0.66 ± 0.12 kg/d, respectively; $P = 0.05$). However, during the 14-d withdrawal period, ADG was similar among treatments (0.86, 0.72, and 0.77 ± 0.1 kg/d for 5-HTP, FLX and CON, respectively; $P = 0.26$). Hip height was similar among treatments during supplementation and withdrawal periods ($P > 0.84$).

### 3.5. Calf activity

Electronic data loggers were attached to the calf’s left rear leg to test whether activity patterns might be influenced by FLX or 5-HTP supplementation. There was a treatment by day interaction for lying duration ($P = 0.046$), whereby FLX calves were lying for longer periods of time per lying bout on d 2, 6, 7, 8, and 9 compared with CON calves (Fig. 4A). There was a treatment-by-day interaction for lying bout frequency, whereby 5-HTP and FLX calves tended to lay down fewer times on d 2 and 8, respectively, compared with CON calves ($P = 0.054$; Fig. 4B). Total lying activity throughout the day was calculated by averaging the minutes per day or per hour that calves spent lying down. Total lying time per day was similar among treatments ($P > 0.50$; Fig. 4C), however, there was a significant effect of time (h) driven by an increase in calf activity at feeding times ($P < 0.003$; data not shown).

### 4. Discussion

Serotonin produced by the enterochromaffin cells in the gut and other peripheral tissues of various species has been demonstrated to regulate physiological functions including, but not limited to, tissue energetics and immunity. However, the role of peripheral serotonin has not been extensively studied and it is poorly understood in dairy calves. Herein, we sought to determine the feasibility of increasing serotonin bioavailability throughout oral administration of a serotonin precursor (5-HTP) or an SSRI (FLX), exploring their pharmacokinetics and safety of administration in dairy calves. Results demonstrate that our treatments are a feasible and safe approach to improve serotonin bioavailability in preweaned dairy calves.

Safety of administration was determined by monitoring health scores, vital signs, and physiological parameters of calves. We did not observe differences in health scores among treatments throughout the 10-d supplementation period, denoting that administration of 5-HTP or FLX did not impact the health status of calves. During the first two days of supplementation, RT, HR, and RR were similar among groups; however, FLX calves had lower HR compared with CON calves during the 10-d supplementation period. Lower HR is a common side effect observed in humans taking SSRIs. Indeed, FLX administration to human patients with cardiac disease was shown to be harmless despite a 6% reduction in patients’ HR [22]. It is important to note that regardless of the reduction in HR of FLX fed calves, the observed rates are within the physiological range of preweaned calves [23]. Oral supplementation of FLX increased hematology parameters such as RBC counts and HGB concentration. Amireault et al [24] reported that erythrocyte precursors in bone marrow express serotonin receptors and their activation leads to more efficient erythropoiesis. Thus, it is possible that increased serotonin bioavailability by FLX supplementation promoted RBC proliferation and consequently increased blood HGB concentrations. Hematocrit and total protein concentrations in 5-HTP or FLX fed calves were within established healthy physiological ranges [23]. Given that all calves were healthy, and serotonin did not impact vital signs and hematology parameters negatively, we propose that oral administration of FLX and 5-HTP to
preweaned dairy calves for 10 d is safe. However, further studies are needed to explore the long-term effects of supplementation.

The effectiveness of 5-HTP in increasing serotonin bioavailability in preweaned dairy calves was determined herein. In our study, daily oral supplementation of 90 mg/d of 5-HTP increased whole blood serotonin concentrations starting on d 2 of administration, reaching a 5-fold increase on d 6 and remaining elevated thereafter compared with CON calves receiving saline. Using the same dose of 5-HTP in neonate dairy calves, Hernandez-Castellano et al [18] reported an increase in serum serotonin concentrations compared with nonsupplemented calves after 6 d of administration. Thus, dietary 5-HTP is absorbed in preruminant monogastric dairy calves and converted to serotonin, probably because of their undeveloped rumen at this age. In ruminants, daily intravenous administration of 1.5 mg/kg 5-HTP to late lactation, nonpregnant dairy cows increased serum serotonin concentrations by 2-fold after 2 d of administration [25]. Differences among these studies in the timing and the slope of the serotonin increase upon supplementation could be attributed to different routes of administration, age and/or physiological status of the animals, or the different blood fractions used to quantify serotonin (whole blood vs serum). Regardless of gender and physiological state, these studies showed a strong and relatively quick conversion of 5-HTP to peripheral serotonin in dairy cattle. In our study, whole blood serotonin concentrations returned to levels comparable with CON calves 7 d after cessation of 5-HTP administration.

Previously, FLX has been administered to livestock species via different routes. In sheep, administration of a single oral or subcutaneous injection of 40, 80, or 160 mg/d of FLX was reported to decrease milk yield in a dose-dependent manner [26]. Similarly, decreased milk yield was reported in dairy cows receiving 5 mg of FLX via intramammary injections for two days [16]. However, none of these studies addressed the pharmacokinetics of FLX postadministration. Herein, we collected serial blood samples to understand FLX dynamics after oral supplementation of 40 mg/d. Fluoxetine was detected in circulation 3 h after the first oral dose was administered, at concentrations greater than 80 ng/mL compared with undetectable circulating levels in CON calves. Whole blood FLX concentrations continued to increase gradually following daily oral FLX administration until d 6, when concentrations reached a plateau of approximately 900 ng/mL. In ewes, FLX absorption properties were tested by administering it orally or abomasally for 10 d. Higher absorption rates were reported for abomasally infused FLX, likely because of rumen bypassing [27]. In humans (ie, monogastrics), absorption of FLX is approximately 80%. Thus, our results show that preweaned dairy calves of 3–4 wk of age can efficiently absorb orally supplemented FLX, likely because of their undeveloped rumen. In our study, calves were subjected to a 14-d withdrawal period to monitor the clearance of FLX from circulation. In accordance with studies in humans, whole blood FLX decreased gradually reaching levels comparable with CON, approximately 0 ng/mL, after 7 d [28]. Fluoxetine clearance in ewes has been reported to be complete after 3 d of withdrawal [27]. Discrepancies observed might be attributed to dose, age, and/or physiological state of animals and species.

After confirming that oral FLX reaches the systemic circulation, we set out to determine its impact on blood serotonin concentrations. Oral supplementation of FLX for 6 consecutive days began to decrease whole blood serotonin concentrations, which remained 75% to 50% of values in CON calves until d 10. Fluoxetine binds to SERT, blocking serotonin reuptake, which precludes serotonin degradation and recycling, and subsequently increases its bioavailability.

![Fig. 4. Daily lying activity in response to 5-hydroxytryptophan (5-HTP) or fluoxetine dietary (FLX) supplementation for 10 d. (A) Lying duration (min/bout), (B) lying bout frequency (bouts/d), and (C) lying time per day (min/d) of Holstein bull calves supplemented with 5-hydroxytryptophan (5-HTP), fluoxetine (FLX), or saline (CON) for 10 d (n = 8 per group). Data are presented as mean ± SEM. * = FLX versus CON; # = 5-HTP versus CON. *P < 0.05; **P < 0.01; #P < 0.05; ##P < 0.01.](image-url)
In other words, it allows the available pool of serotonin to be used as a ligand molecule by serotonin receptors, and consequently, circulating serotonin concentrations will decrease [29]. Our results are in accordance with other studies supplementing FLX to various species. For instance, dogs supplemented with FLX for 12 wk have reduced whole blood serotonin concentrations in a dose-dependent manner [30]. Similarly, FLX fed rodents have lower brain serotonin concentrations and 5-hydroxyindoleacetic acid, the primary serotonin metabolite [31].

In this study, whole blood FLX concentrations decreased gradually in FLX fed calves during the 14-d withdrawal period. Consequently, a gradual increase in serotonin concentration was observed, reaching concentrations similar to those of CON calves at 14 d of withdrawal. Our results are comparable with human studies reporting a 7 to 15 d withdrawal period for FLX [28].

To understand the pharmacokinetics of FLX and 5-HTP, CSF, muscle and feces from all treatment groups were collected after the 10-d supplementation and 14-d withdrawal periods. Supplementation of FLX or 5-HTP did not impact CSF serotonin concentrations, as CSF serotonin levels from both groups were comparable with CON. Muscle serotonin concentrations were similar between 5-HTP and CON calves after the 10-d supplementation and 14-d withdrawal periods. However, FLX calves had higher muscle serotonin and FLX concentrations than CON calves after the 10-d supplementation period. Moreover, after the 14-d withdrawal period muscle, FLX concentration remained higher in the FLX calves compared with CON. To the extent of our knowledge, this is the first study to assess serotonin and FLX concentrations in muscle tissue after FLX supplementation or withdrawal. Fluoxetine was also present in feces of FLX calves after the 10-d supplementation period, but not after the 14-d withdrawal period. In humans, 15% of FLX is eliminated through feces [32] and the CSF of FLX-fed rats have detectable levels of FLX and its metabolite Norfluoxetine [33]. Our results indicate that FLX clearance from muscle takes longer than from blood. This information is particularly important in livestock species where meat is a product for human consumption, thus, determination of appropriate withdrawal periods should be established.

Even though peripheral serotonin does not cross the blood-brain barrier, peripheral FLX and 5-HTP do. Thus, increasing peripheral levels of FLX and 5-HTP by oral supplementation might consequently increase serotonin bioavailability at the level of the CNS. This, in turn, might impact endocrine pathways and possibly affect growth and behavior. Growth rates and activity patterns were measured and monitored during the 10-d supplementation period. Hip heights were similar among treatments, indicating that 5-HTP or FLX supplementation for 10 d is not detrimental to growth. During the 10-d supplementation period, 5-HTP calves had greater ADG compared with CON nonsupplemented calves. Increased serotonin bioavailability in humans and rodents has been associated with decreased feed intake and weight loss [34,35]. Furthermore, activation of hypothalamic serotonin 1A receptors has been shown to decrease food and water intake in rodents [36]. Hernandez-Castellano did not observe differences in BW or ADG in calves supplemented with 5-HTP during the first 3 wk of life [18]. Nevertheless, further investigation is needed to understand the mechanism through which peripheral 5-HTP supplementation might be enhancing calves’ growth. Fluoxetine administration had an effect on calf activity. Fluoxetine administration to mice and zebrafish resulted in reduced social stress and aggressive behavior, respectively [37,38]. In our study, FLX calves laid down for longer periods of time when compared with CON calves. Fluoxetine withdrawal in ewes has been shown to induce a depressed-like behavior such as decreased social behavior and attentiveness [27]; however, calves’ activity was not recorded during the 14-d withdrawal period in our study.

In summary, it is feasible and safe to increase serotonin bioavailability through oral supplementation of a serotonin precursor or an SSRI for 10 consecutive days in preweaned dairy calves. Even though these molecules have different mechanisms of actions, both resulted in increased serotonin bioavailability with no apparent adverse effects to dairy calf physiology. It is important to note that 5-HTP is a modified amino acid that is naturally produced by the body, whereas FLX is a prescription drug. Indeed, we showed that complete clearance of FLX in peripheral tissues is not fully achieved within 14 d of withdrawal. Hence, we propose the use of 5-HTP to increase serotonin bioavailability in dairy calves to further explore its biological function and potential downstream applications.

CRediT authorship contribution statement

M.G. Marrero: Project administration, Formal analysis, Writing - original draft, Writing - review & editing. B. Dado-Senn: Project administration, Writing - review & editing. S.L. Field: Project administration, Writing - review & editing. D.R. da Silva: Project administration, Writing - review & editing. A.L. Skibiel: Project administration, Writing - review & editing. J. Laporta: Conceptualization, Funding acquisition, Project administration, Formal analysis, Writing - review & editing.

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