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Research article

Dietary putrescine supplementation reduces faecal abundance of Clostridium perfringens and markers of inflammation in captive azure-winged magpies

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Abstract

Dietary supplementation allows aviculturists to correct for nutritional imbalances helping maintain peak health and welfare of captive populations. Supplementation with the polyamine putrescine (PUT) has been used widely in poultry production and is shown to promote and regulate multiple biological processes essential for growth, digestion and immunity. This novel investigation quantified the effect of PUT supplementation on gut health and behaviour in a new model passerine: the azure-winged magpie (*Cyanopica cyanus*). Faecal samples from 15 birds were processed over 10 weeks prior to, during and following a four week PUT supplementation period. Markers of relative gastrointestinal health were quantified using intestinal microbiota abundance from cultures, immunoassay of pro-inflammatory cytokine interleukin-6, and excreted polyamine concentrations from samples. Instantaneous focal sampling was used to highlight changes to group behavioural frequencies. PUT significantly reduced faecal interleukin-6 concentration ($P<0.0001$) and *Clostridium perfringens* abundance ($P<0.0001$). Lactic acid bacteria ($P=0.0011$) and enterobacteria ($P=0.0017$) abundance increased with PUT, with a subsequent decrease in colony count after supplementation ceased. Flying ($P=0.0006$) and perching ($P=0.0041$) frequencies significantly increased and decreased respectively with supplementation but could be attributed to chicks hatching. Frequencies of both self-grooming ($P=0.03$) and drinking ($P=0.0013$) increased. PUT supplementation here is shown to be beneficial to *C. cyanus* by reducing gut inflammation and colonisation of *C. perfringens*, subsequently allowing the proliferation of commensal bacterial populations into available niches and improved assimilation of nutrients across the epithelium, reducing nutritional stress. Such reductions in *C. perfringens* abundance may also indicate the relevance of using polyamines in combinatory treatments for chronic clostridiosis.

Introduction

Captive animal nutrition within ex-situ collections is a complex and dynamic process among all animal groups. Various dietary conditions can arise from providing a diet with a nutritional imbalance or allowing animals choice in selecting items leading to an imbalance in feed intake over time (Rees 2011; Hosey et al. 2013). Malnutrition often results in morbidity or obesity, with disadvantageous effects to fitness, behaviour and life expectancy if unresolved (Dierenfeld 1989; Rees 2011; Hosey et al. 2013). It is, therefore, crucial for zoological collections to provide and maintain adequate nutrition in naturalistic modes to promote peak health and welfare in often-threatened species.

Dietary supplementation, to correct for nutritional imbalances, is a valued tool to aviculturists and used widely within zoological collections. This, combined with the use of commercially available species-specific complete foods, minimises the prevalence of dietary conditions (Nijboer 2016). However, even with carefully managed diet plans, some bird species still show evidence of nutritional stress with noted effects to an animal's breeding success, cognitive development and immune system function, among others (Ullrey 1993; Allen and Ullrey 2004; Crissey 2005; Klasing 2007; Fidgett and Gardner 2014).

Nutritional stress effects have been studied widely in poultry, due to its relevance to improving yields in commercial food production (Girdhar et al. 2006; Powers and Angel 2008). Methods to increase yields by improving body condition and

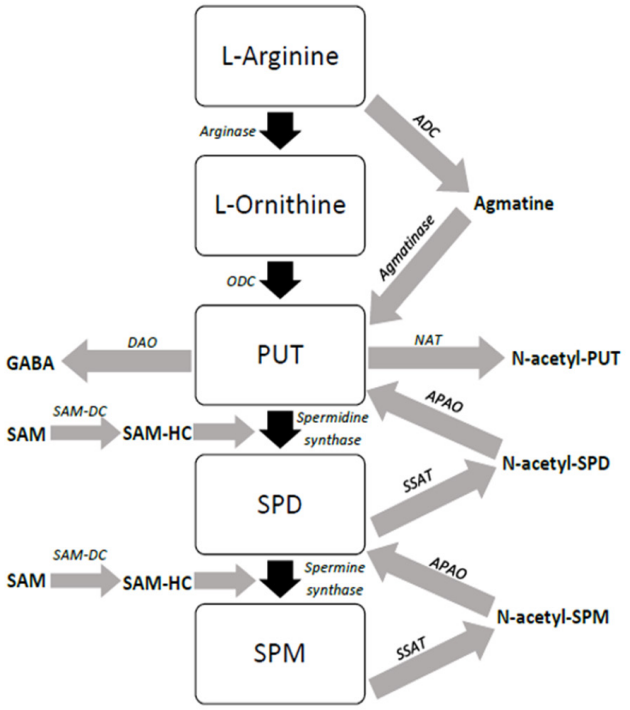

Figure 1. Higher polyamine biosynthetic pathway. Simplified schematic representing the higher polyamine biosynthesis pathway from L-arginine and L-ornithine (adapted from, Kalač and Krausová 2005; Larqué et al. 2007; Di Martino et al. 2013). ADC, arginine decarboxylase; APAO, acetylpolyamine oxidase; DAO, diamine oxidase; GABA, gamma-aminobutyric acid; NAT, N-acetyl transferase; ODC, ornithine decarboxylase; PUT, putrescine; SAM, S-adenosylmethionine; SAM-DC, S-adenosylmethionine decarboxylase; SAM-HC, S-adenosylmethionine homocysteamine; SPD, spermidine; SPM, spermine; SSAT, spermidine/spermine acetyltransferase.

reducing mortality are at the forefront for investigation (Powers and Angel 2008; Pan and Yu 2013). Developing our understanding of key biochemical pathways that underpin growth and immune function in birds, will identify target areas where supplementation could have a beneficial effect (Grimble and Grimble 1998; Awad et al. 2009; Pan and Yu 2013). Targeting supplements to directly influence key organic reactants within these processes, or providing more of the metabolites themselves, is the main method to be impactful.

One such group of compounds is the polyamines; a group of polycationic biogenic amines attributed with assisting in multiple intracellular roles linked with growth and metabolism (Bardócz et al. 1993; Kalač and Krausová 2005; Di Martino et al. 2013). These aliphatic compounds are fundamental in a variety of biological processes including: DNA stabilisation, gene transcription, mRNA translation, cellular structure, ion channel function, cell growth and proliferation, and apoptosis modulation (Kalač and Krausová 2005; Di Martino et al. 2013; Hashemi et al. 2014; Ramani et al. 2014). The importance of polyamines to maintain normal cellular function is evident as they are produced by a variety of organisms including plants, prokaryotes and eukaryotes (Kalač and Krausová 2005; Di Martino et al. 2013). Sources of polyamines in birds come from endogenous de novo synthesis, or from exogenous origin introduced via the diet or from intestinal microbiota excreta (Girdhar et al. 2006; Larqué et al. 2007). Dietary supply is the biggest contributor to the bodily polyamine pool (Larqué et al. 2007).

Putrescine (PUT), spermidine (SPD) and spermine (SPM) are the most abundant and biologically active polyamines in vertebrates; known as the higher polyamines (Larqué et al. 2007). Higher polyamines are derivatives of L-ornithine and L-arginine, formed in an enzyme-regulated biosynthetic pathway in somatic cells (Figure 1) (Bardócz et al. 1993; Ali et al. 2011; Ramani et al. 2014). This reversible process allows cells to regulate the concentrations of each compound according to demand in a growth-dependent process; individual polyamine functions are not well differentiated in birds (Löser et al. 1999; Kalač and Krausová 2005; Di Martino et al. 2013).

Polyamines are shown as significant compounds regulating metabolic activity, specifically in rapidly dividing cells such as the leukocytes and gastrointestinal (GI) epithelium in higher vertebrates (Bardócz et al. 1998; Kalač and Krausová 2005; Girdhar et al. 2006; Ramani et al. 2014). Enterocytes have one of the highest rates of cellular regeneration and differentiation, where an increase in the demand for polyamines and other metabolites has been shown experimentally (Girdhar et al. 2006). Higher polyamines are essential for the maintenance and development of the GI tract, as they are integral to mucosal renewal by epithelial proliferation and maturation (Bardócz et al. 1998; Larqué et al. 2007; Ramani et al. 2014). They maintain paracellular GI barrier function in poultry by increasing the expression of tight cell junction proteins, such as E-cadherin and occludin (Kalač and Krausová 2005; Camilleri et al. 2012; Ramani et al. 2014). In many bird species, polyamines act to increase villus length and crypt depth of the intestinal brush-border, expanding the surface area for peak nutrient absorption (Ali et al. 2011; Ramani et al. 2014). This additional space creates more ecological niches for commensal intestinal bacteria to colonise, further improving digestion efficiency (Rinttilä and Apajalahti 2013).

In the inflammatory response, polyamines support the accelerated metabolism of mature immune cells and upregulate lymphopoiesis (Larqué et al. 2007; Ramani et al. 2014). Similarly, many cytokines produced by leukocytes have key roles within the inflammation cascade (Martin et al. 2011). Like polyamines, cytokines such as transforming growth factor-beta (TGF-ß), interleukins one (IL-1) and six (IL-6), and tumour necrosis factor-alpha (TNF-α) are some of the biggest initiators of increased cellular metabolism during inflammation (Grimble and Grimble 1998; Leng et al. 2008). Interactions between polyamines and cytokines remain poorly understood (Grimble and Grimble 1998; Leng et al. 2008).

Azure-winged magpies (AWM; *Cyanopica cyanus*; Pallas 1776) are a small corvid species endemic to eastern Palearctic Asia, with a diverging population in the Iberian Peninsula (*C. cooki*) (Avilés 2004; Canário et al. 2004). These highly social passerines are most notably studied due to their unusual communal nesting strategy and cooperative breeding within Corvidae (Avilés 2004; Canário et al. 2004). As omnivores, these birds eat a variety of foodstuffs ranging from fruits and nuts, to insects and carrion (Canário et al. 2004). Citrus fruits, legumes and carrion are noted as foods with the highest PUT content and are staple components of wild AWM diets, with cultivated items likely not to include similar quantities (Canário et al. 2004; Kalač and Krausová 2005; Larqué et al. 2007; Ali et al. 2011). The use of passerine pellets within collections likely reduces the quantities of exogenous higher polyamines as PUT is not listed in the nutritional composition with testing not readily undertaken. This impacts the bodily polyamine pool and the biological processes regulated by them.

This within-subject longitudinal investigation aims to quantify the effect of exogenous dietary PUT supplementation on behaviour and markers of GI health in a novel exotic passerine. Relative GI health was assessed by monitoring changes to the GI microbiota and to pro-inflammatory cytokine concentrations within faecal

Table 1. Azure-winged magpie ethogram. Novel ethogram with comprehensive descriptions of behaviours exhibited by captive adult AWM at Bristol Zoological Gardens (BZG), England (adapted from, Kenward et al. 2006; Logan et al. 2012; Kozlowski et al. 2016; Smith and Wassmer 2016).

Category	Name	Behaviour description
Locomotion	Chasing	Bodily movement in direct pursuit of a moving conspecific.
	Flying	Bodily movement through the air with no contact with the enclosure furniture or substrate with wings fully extended.
	Hopping	Bodily movement through the air with no contact with the enclosure furniture or substrate with wings closed; appears to be 'jumping'.
	Perching	Body stationary with both feet in contact with enclosure furniture off the enclosure floor e.g. branch, cage exterior, etc.
	Sleeping	Body stationary and contracted in length with eyes closed; may be perched with head curled into body or within nest box.
	Standing	Body stationary with both feet in contact with the ground substrate with wings closed.
	Walking	Bodily movement with one or more feet in contact with enclosure substrate or furniture with wings closed.
Social	Allogrooming	Moving beak within the feathers of a conspecific that does not cause harm or the fellow bird to move away (non-aggressive).
	Nest tending	Bird alert with body not contracted in length and eyes open within or at a nest box entrance.
	Vocalisation	Production of sound.
Consumptive	Caching	Storing or hiding of food items within the substrate or enclosure.
	Drinking	Consumption of water.
	Eating	Consumption of food.
	Food washing	Submersion of food items within water prior or during eating, or prior to caching.
	Pecking	Manipulation of an object with the enclosure using beak e.g. removing bark from trees, removing twigs from branches, etc.
Self-maintenance	Beak rubbing	Brushing or scraping the beak against the enclosure furniture, typically a branch.
	Ruffling	Shaking of body and feathers when stationary while either perched or standing.
	Self-grooming	Moving beak within own feathers to clean and maintain plumage condition.
Miscellaneous	Grasping	Using feet to manipulate or hold onto a small item (usually food or a loose enclosure furniture item).
	Mouth holding	Using beak to hold an item off the substrate floor (usually food or loose enclosure furniture item) when stationary or in motion.
	Out of sight	Animal is not visible at point of sampling.

samples prior to, during, and following supplementation (A-B-A design). Additionally, excreted quantities of the higher polyamines were compared to show how additional PUT is processed in vivo. Lastly, the impact of PUT on behaviour was assessed evaluating changes to common behavioural frequencies among conspecifics.

Materials and methods

Sample collection

A healthy population of nine male and six female AWM were sampled in a single-species enclosure at Bristol Zoological Gardens (BZG), UK in 2017 (ethical approval UB/17/025). Ages ranged from 11 months to 8 years at the start of testing.

Fresh faecal samples were collected twice weekly from randomly selected quadrants of the enclosure, at the same time each day for 10 weeks. Thirty droppings were collected during each sample date in sterile containers, of which 15 were randomly selected for processing. Experimentation was split into a two-week pre-supplementation phase (days 1–14), a four-week supplementation phase (days 15–42), and a four-week post-supplementation phase (days 43–70). Six samples throughout experimentation were chosen to evaluate trends in cytokine and higher polyamine concentrations from faecal samples: Days 1, 16, 29, 43, 60 and 67.

Putrescine supplementation

Group weight was calculated as 1.319 kg, as sourced from the group records held on ZIMS (Zoological Information Management Systems, Species360®). A conservative PUT dosage of 0.03% of body weight was selected equating to a group dose of 0.3957 g of PUT per day (Hashemi et al. 2014). Pure PUT (Sigma-Aldrich, Dorset) was used as received and doses placed into day-labelled containers. During diet preparation, PUT was dissolved in 1 ml of water. The solution was added to the prepared diet, mixed until distributed evenly, and fed out according to normal husbandry routines. The captive diet contained a variety of produce (fruits and vegetables) in addition to an omnivorous bird crumb and mixed pulses. Additional feeding enrichment was provided routinely, comprising of mealworms and/or boiled egg.

Negligible quantities of feed items were left uneaten daily during sampling. Four chicks hatched during PUT supplementation and fledged during the post-supplementation phase. Due to the hatchlings, an increased-protein diet was provided from faecal sample 9 for the remainder of the investigation. This consisted of scatter feeding mealworms (30 g maximum) or eight pinkie mice per day.

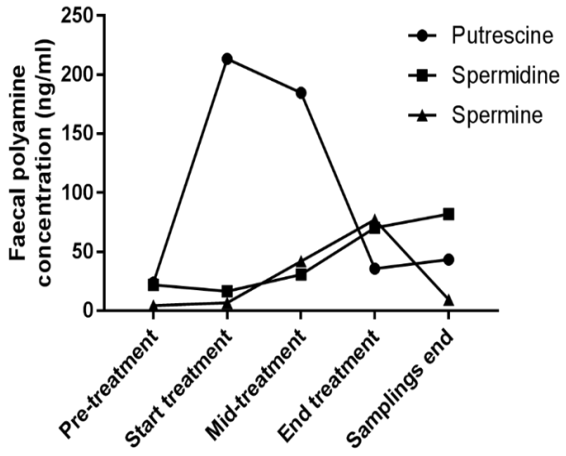

Figure 2. Change in concentration of excreted polyamines in faecal samples during sampling phases. Total detected concentration (ng/ml) of the higher polyamines expressed in collated group faecal samples of AWM population collected prior, during, and following PUT supplementation. PUT supplementation increased the concentration of PUT, SPD and SPM in faeces.

Quantification of bacteria in faeces

Faecal samples were dissolved in sterile phosphate-buffered saline (PBS) to a 1:10-dilution. Samples were repeatedly inverted until organic matter had fully separated. Resultant supernatant was decanted and diluted further with PBS to a 1:1000-dilution.

MacConkey (MAC), MRS and *perfringens* (PERF) agar plates were used to culture Gram-negative lactose-fermenting enteric bacilli (*Escherichia coli*-strains, *Enterobacter* and *Klebsiella*), Gram-positive lactic acid bacteria (LAB; Lactobacillales) and *Clostridium perfringens*, respectively (Hartemik and Roumbouts 1999). 10µL of the 1:1000-dilution was spread onto each labelled plate. Inoculated plates were incubated at 37±1°C for 24 hours. Microaerobic or anaerobic conditions were created for MRS and PERF plates using CampyGen and AnaeroGen sachets (ThermoFisher, Loughborough) respectively in sealed containers. After incubation, colony number was counted for each plate to a maximum value of 201.

Faecal collection dates were collated into five sampling phases each spanning two weeks (pre-treatment, start of treatment, end of treatment, after treatment, end of sampling). Mean colony count was calculated for each sampling day and phase. Mean values were log10 transformed, with 0.1 added to all raw data to include zero count samples in analyses.

Quantification of faecal interleukin-6

On select days (1, 16, 29, 43, 60 and 67), 1 ml of the supernatant from the 1:10-dilution from each sample was stored at −80°C until testing. An IL-6 (Abcam, Cambridge) ELISA kit was used per manufacturer's instructions. Absorbance values were read at 492 nm using a microplate reader and a standard concentration curve for comparison was fitted using a linear-regression line.

Quantification of faecal polyamines

Concentrations of PUT, SPD and SPM within faecal samples were evaluated using gas chromatography mass spectrometry (GC-MS) at the Biomolecular Sciences Research Centre, Sheffield Hallam University. On selected sampling days (1, 16, 29, 43, 60 and 67), 200 mg of each faecal sample was removed and collated, with samples stored at −80°C until testing. Prior to analysis, biogenic amines were converted to fluoroacetate derivatives to improve separation and detection (Chen et al. 2009). Corrected concentrations of polyamines were reported after applying an internal standard correction factor to account for recovery percentage.

Bacterial growth curves

Anaerobe basal, MRS and Lennox L growth media (ThermoFisher, Loughborough) were made according to manufacturer's instructions for *C. perfringens*, LAB and enteric bacilli respectively. A serial doubling-dilution of PUT-broth solution was created for each plate type (0.03–0.00047% PUT and negative control). 200 µL of each concentration was transferred into a sterile microtiter plate and inoculated with respective cultured bacterial colonies from 12 different samples. Plates were incubated for 24 hours at 37.5±1°C with optical density readings taken every 30 minutes. Microaerobic conditions (10% CO_2 and 2.5% O_2) were created for *C. perfringens*, with atmospheric air provided for enteric bacilli and LAB. Change in rate of growth was calculated for each plate type from absorbance values.

Behavioural sampling

A novel ethogram was adapted from the available literature and supplemented with pilot continuous behavioural sampling, as shown in Table 1. Instantaneous focal sampling was conducted during the same time of day on non-faecal collection days. Each individual was sampled every 30 seconds for 30 minutes twice-weekly for the duration of sampling. Behaviours conducted most frequently were selected for statistical analyses (nesting behaviours were excluded as individuals were not distinguishable within nest boxes). Fortnightly means were calculated for each behaviour into five sampling phases (as previously described for bacterial cultures).

Statistical analyses

Interactions between bacterial colony count and cytokine concentrations between sampling phases were evaluated using one-way ANOVA tests, with post-hoc Tukey tests to assess for multiple interactions. Behavioural frequencies were evaluated using Friedman ANOVA tests with Dunn's multiple correction test. Two-way repeated-measures ANOVA tests were conducted to analyse the effect of varying PUT concentrations on bacterial growth rates, with Tukey tests to measure for multiple interactions between factors. All results were analysed using GraphPad Prism software (version 7.03).

Results

Faecal polyamines were increased following putrescine supplementation

Prior to supplementation, all higher polyamines had similarly low basal concentration values ranging from 4.5–23.9 ng/ml. Figure 2 highlights the substantial increase in PUT to a peak value of 213.3 ng/ml at the start of supplementation and the decrease during supplementation which continued into the post-supplementation phases. At the start of supplementation, SPM concentration mildly decreased, where SPD concentration slightly increased. At the end treatment phase, SPD and SPM are still both continued to increase, with SPM reaching a peak concentration of 77.1 ng/ml. PUT concentration plateaued at the end of treatment phase to a similar value to the pre-treatment concentration. After sampling, SPD concentration reduced close to the starting value, where SPM continued to increase to a peak value of 82.0 ng/ml.

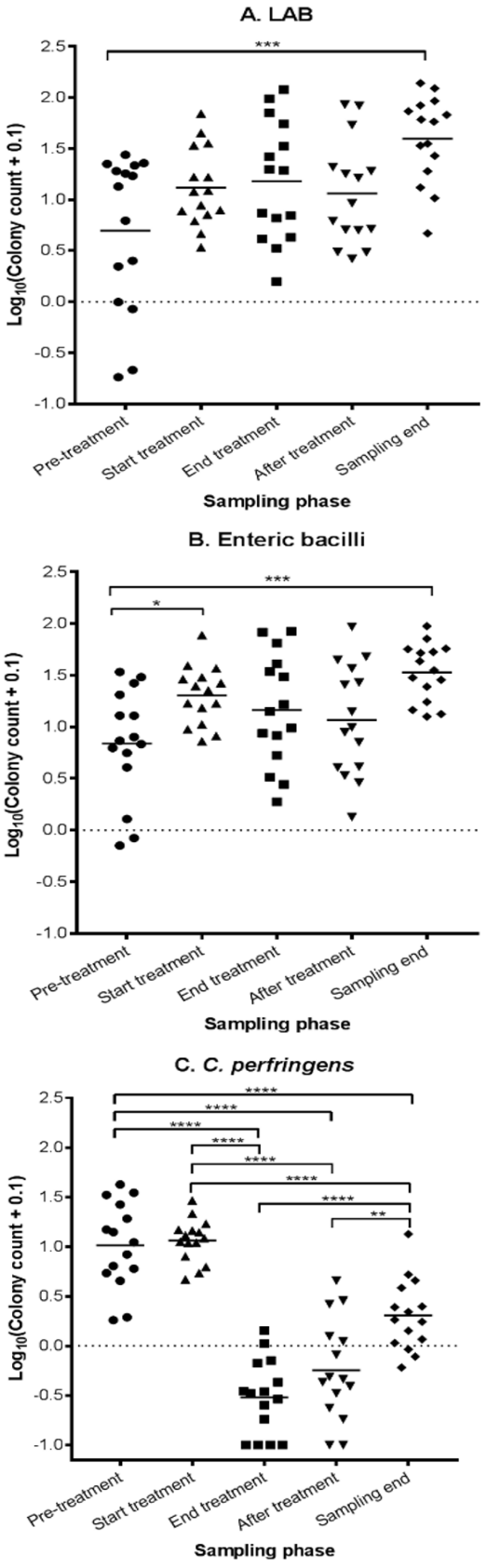

Figure 3. Change in mean bacterial colony count between sampling phases of different culture media. Effect of supplementation on microbial abundance between sampling phases; each point represents one sample and line represents mean. Lactic acid bacteria (LAB) abundance (A) and enteric bacilli abundance (B) significantly increased following PUT supplementation. *C. perfringens* abundance significantly decreased following PUT supplementation and started to increase following cessation of treatment (C). *=P<0.05; **=P<0.01; ***=P<0.001; ****=P<0.0001.

Faecal abundance of* C. perfringens *was reduced following putrescine supplementation

In both LAB and enteric bacilli cultures, there was a significant overall increase in mean colony count from pre-treatment into the supplementation phase, which decreased mildly post-treatment (LAB, P=0.0011; enteric bacilli, P=0.0017) (Figure 3A, B). The greatest mean colony count for LAB and enteric bacilli was noted as the end of sampling. *C. perfringens* abundance remained unchanged during the start of treatment compared to the pre-treatment phase; however, abundance dropped significantly during the end of treatment phase (P<0.0001) (Figure 3C). Following cessation of putrescine supplementation, *C. perfringens* mean colony count increased.

Varying PUT concentrations in growth media in vitro had no significant effect on *C. perfringens* (P=0.2405), enteric bacilli (P=0.2214) or LAB (P=0.7171) growth rates over time (Figure 4).

Faecal IL-6 was reduced following putrescine supplementation

There was a significant reduction in faecal IL-6 concentration over time (P<0.0001). Mean IL-6 concentration remained stable prior to and at the start of treatment, with a significant reduction over the remaining treatment weeks. IL-6 concentration remained low throughout the post-treatment phase, with a significant increase noted at the end of sampling (Figure 5).

Effect of putrescine supplementation on behaviour

No caching or food washing was seen throughout sampling, therefore not reducing the group dosage of PUT. Vocalisations were omitted as individual calls could not be differentiated. Eating was excluded as could not be clearly seen within the indoor enclosure. Allogrooming, grasping and sleeping behaviours were not witnessed throughout sampling.

There was a significant increase in flying (P=0.0006) and decrease in perching (P=0.0041) behaviours during PUT supplementation. An increase in the mean frequency of flying in the end of treatment phase was mirrored by a reduction in perching behaviour at the same time, with both behaviours shown to correlate over time. Mean pre- and post-treatment locomotion frequencies were very similar, as shown in Figure 6A, B.

Self-grooming behaviours remained similar during the pre-treatment and treatment phases, which then significantly increased during the post-treatment phases (P=0.0301) (Figure 6C). There was a significant increase in the observed frequency of drinking during treatment from pre-treatment values (P=0.0013) (Figure 6F). Drinking was most frequent at the start of treatment, which decreased linearly until the post-treatment phase, where a slight increase was noted after sampling. Beak rubbing and ruffling were not significantly affected by PUT supplementation.

Discussion

The PUT supplement was clearly ingested by the birds from the large increase and subsequent decrease in faecal polyamines during and following supplementation (Figure 2). This assured that all birds were receiving exogenous PUT and subsequent effects described above therefore could be attributed to its action in vivo. SPD and SPM, after a lag time, were more readily found in the faeces during supplementation. This not only suggests PUT is quickly metabolised within the intestinal lumen, but that polyamines are either kept within the GI tract or are assimilated into the body from the gradual increase in SPM and SPD concentrations in the post-supplementation phase.

In this investigation, significant changes in the microbiota were observed following PUT supplementation. The reduction in *C. perfringens* abundance during the end of treatment phase was unlike that seen in enteric bacilli or LAB (Figure 3C). As PUT and

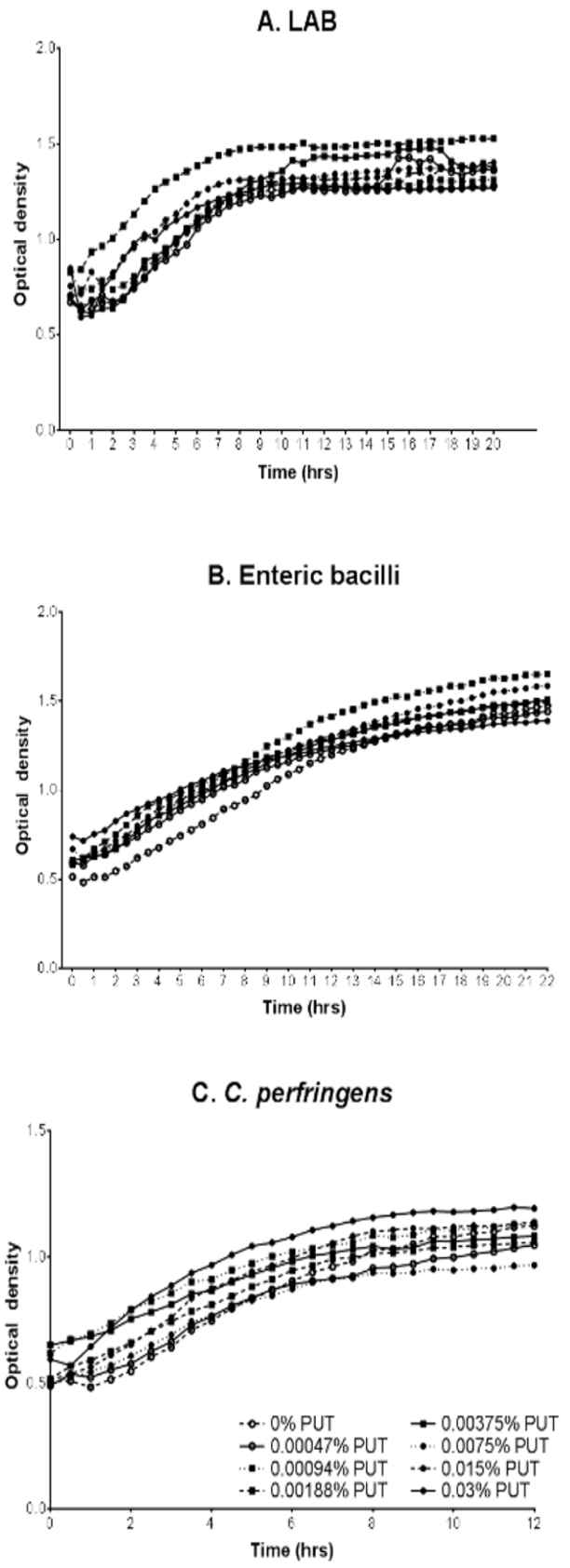

Figure 4. Bacterial growth rates over time under different concentrations of PUT. Bacterial growth rates of LAB (A), enteric bacilli (B), and *C. perfringens* (C) over time within media under different concentrations of PUT until bacteria reached stationary phase at 20, 22 and 12 hours respectively. Provision of exogenous PUT had no effect on the growth kinetics of LAB, enteric bacilli or *C. perfringens.*

polyamine-analogues are crucial metabolites for the development and maintenance of the GI epithelium, more bioavailable polyamines result in improved barrier function (Ramani et al. 2014). Additionally, PUT is shown to be a key driver for leukocyte synthesis and maturation, particularly within gut-associated lymphoid tissues (GALT) (Revolledo et al. 2006; Larqué et al. 2007). By PUT up-regulating the readiness of GALT, along with improving barrier function, some microbes are gradually displaced by more favoured taxa (Ramani et al. 2014). As *C. perfringens* secretes biotoxins, which result in substantial damage to enterocytes, these bacteria are readily expelled from the intestinal lumen (Berkes et al. 2003). Once removed, microbes then compete with better-adapted organisms who prevent re-colonisation by competitive exclusion (CE) and quorum sensing strategies (Hofacre et al. 2003; Revolledo et al. 2006).

Physiological stress causes the release of hormones, such as catecholamines, which work to up-regulate the sympathetic nervous system in response to an internal stressor (Boyanova 2017). These hormones have been shown to simultaneously accelerate the virulence of *C. perfringens* by up-regulating biotoxin production and increasing growth rates (Boyanova 2017). As PUT reduces nutritional stress by improving digestion across the better-functioning GI barrier, the secretion of these hormones could be reduced, possibly limiting the propagation of *C. perfringens*.

Conversely, there is an association between a high-protein diet and *C. perfringens* abundance. High-protein diets increase the production rate of pancreatic enzymes, which have roles in neutralising biotoxins released by *C. perfringens* (Rood 1998; Drew et al. 2004). Secreted toxins result in significant damage to the brush-border through necrosis (Drew et al. 2004). Therefore, increased dietary protein could increase the production of pancreatic enzymes and so the amount of biotoxins neutralised. This reduces the damage to the epithelium, maintaining GI barrier function. This subsequently allows better-adapted taxa to expel *C. perfringens* from crypts under CE and quorum sensing in the large intestine.

A combination of the above mechanisms likely explains the differences in *C. perfringens* abundance seen. However, as PUT is removed with the diet remaining unchanged, counts begin to increase (Figure 3C). This indicates that PUT is likely the largest contributor influencing changes in the microbiota compared to increased dietary protein, by encouraging the expulsion of pathogenic agents through evolved biological mechanisms (Larqué et al. 2007; Kamada et al. 2013).

LAB and enteric bacilli counts were shown to rise, plateau, drop and subsequently rise during sampling (Figure 3A, B). As most of the interactions between the phases were non-significant, this is attributed to sampling variation. However, there was a significant difference between pre-treatment and start of treatment phases for enteric bacilli (Figure 3A). Chattopadhyay et al. (2003) suggest that PUT has a role in protecting E. coli from hyperoxic conditions, where increased PUT could initially promote replication in vivo. This change, however, is not sustained as polyamines are metabolised and transported throughout the body via the systemic circulation; hence the drop in abundance at the end of supplementation (as shown in Figure 2) (Bardócz et al. 1998). Alternatively, additional polyamine-analogues within the intestinal lumen at the start of treatment could provide additional metabolites to accelerate E. coli metabolism and energy provision, increasing abundance (Yoshida et al. 2016).

The significant increase in LAB abundance at the end of sampling could be attributed to the reduction in *C. perfringens* within the large intestine. Expulsion of *C. perfringens* and other species from the crypts leave vacant spatial niches. These spaces are quickly filled to restore symbiosis between the host and metacommunity (Hofacre et al. 2003; Rinttilä and Apajalahti 2013). Similarly, as

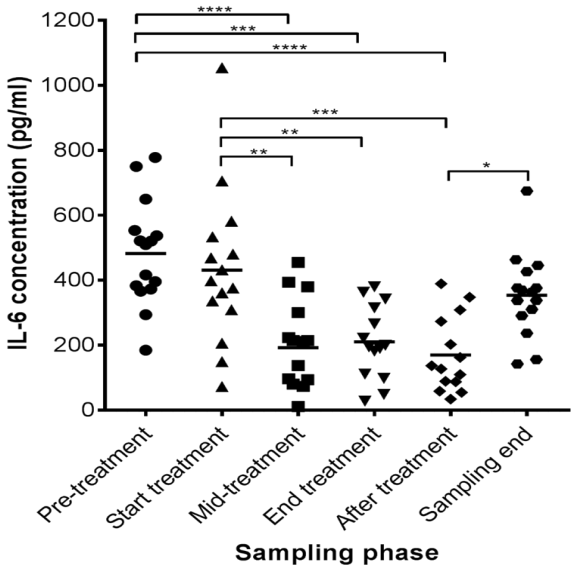

Figure 5. Change in IL-6 concentration between sampling phases. Effect of PUT supplementation on IL-6 concentration within faecal samples between sampling phases; each point represents one sample and bars represent mean. Faecal IL-6 was significantly reduced during and shortly after the supplementation phase. *=P<0.05; **=P<0.01; ***=P<0.001; ****=P<0.0001.

Lactobacillus accounts for 80–90% of the small intestinal flora of chickens, they readily out-compete enteric bacilli and other taxa under peak conditions (Rinttilä and Apajalahti 2013). By PUT providing optimal environmental conditions for commensal microbial growth, LAB numbers should increase to fill all available niches (La Ragione et al. 2004; Koutsos and Arias 2006).

Similarly to LAB, the terminal increase in enteric bacilli is likely attributed to the filling of empty ecological niches within the upper GI tract. Alternatively, Bäumler and Sperandio (2016) showed in mice that manipulation to reduce the abundance of Clostridia resulted in the depletion of the short-chain fatty acid (SCFA) butyrate. This reduction in bacteriostatic SCFAs drives the expansion of enterobacteria through mechanisms that are not yet fully understood (Bäumler and Sperandio 2016). This down-regulates the expression of key chemical messengers to prevent pathogen invasion and colonisation, such as in quorum sensing, allowing enteric bacilli numbers to rise (Bäumler and Sperandio 2016).

As PUT had no significant impact on growth rates for all bacterial types (Figure 4), this suggests that the changes seen in the microbiota were a result of adaptations to the intestinal environment, not a direct effect on microbial metabolism. This indirect effect is likely due to the reduced inflammation (Figure 5) and improved GI mucosa functioning from the increased bodily polyamine pool.

PUT supplementation significantly reduced the concentration of IL-6, indicating a decrease in relative gut inflammation. The intestinal mucosa is vital to preventing bacterial invasion and maintaining naivety of the immune system to intestinal symbionts (Duerkop et al. 2009). By reducing inflammation, enterocyte damage is limited, lowering the risk of dysbiosis and improving nutrient acquisition across the epithelium (Berkes et al. 2003; Holmes et al. 2011). Additionally, reduced inflammation lowers the activity of patrolling intraepithelial lymphocytes, limiting the displacement of commensal microbial populations colonising the crypts (Revolledo et al. 2006). This is then further enforced through a positive feedback system, where healthy commensals release byruvate, which inhibits pro-inflammatory cytokine action (Koutsos and Arias 2006).

The increased flying and decreased perching frequencies at the end of treatment phase, is accredited to the hatching of chicks during week four. As colonial nesters, rearing and guarding young is the sole priority of non-nesting individuals (Valencia et al. 2003; Canário et al. 2004). Conspecific duties entail; providing food for the laying adult and nestlings, supplying materials to maintain the nest, and patrolling the nest to identify and mob potential predators (Valencia et al. 2003). This would increase the activity of all individuals within the population, giving rise to the result in Figure 6A, B.

Conversely, the significant changes in locomotory behaviours could be attributed to an increase in energy acquisition from improved nutrient absorption (Koutsos and Arias 2006). As PUT is shown to improve GI functioning, improved digestion increases nutrient acquisition with more metabolites for energy production (Bäumler and Sperandio 2016). This too would explain the subsequent reduction in activity following cessation of supplementation (Figure 6A, B) but does not show a direct causal link. Although the former explanation is far more likely, the latter requires further investigation.

The linear increase in self-grooming frequencies over time could be attributed to increased growth following supplementation. As a promotor of growth, PUT may have increased the activity of feather follicles during the treatment phases (Prum 1999; Romero et al. 2005). Increased growth requires more amino acids to form proteins, which was provided from the high-protein diet change (Prum 1999). By the post-supplementation phase, these primary feathers would be emerging from the epidermis with birds preening the plumulaceous feathers to maintain condition (Prum 1999; Romero et al. 2005). This could give rise to the increased frequency of self-grooming noted. However, as behavioural frequencies were sampled rather than time budgets, the potential increase in time spent preening cannot be evaluated.

Drinking was shown to significantly increase during and following supplementation. Firstly, this could be attributed to PUT increasing the demand for water from the upregulated metabolism to remove of waste products. Secondly, as sampling was conducted during summer months, increased ambient temperature may result in increased thirst to regulate homeostasis or to compensate for water lost from the increased locomotory activity (O'Connor et al. 2017). Lastly, the increased efficiency of nutrient absorption from the action of PUT and the increased protein diet may have resulted in increased absorbance of dietary nitrogen compounds (Camilleri et al. 2012). More bioavailable nitrogen requires additional water to dilute and flush out from the body in the form of urea or uric acid (Koutsos et al. 2001).

Captive stress effects are historically quantified through monitoring behaviour changes, focusing on stereotypy frequencies or activity budgets, or using biochemical quantification of glucocorticoids in response to changing environmental conditions (Owen et al. 2004; Hill and Broom 2009). Physiological measures of welfare are often favoured as they are more precise and robust modes of quantifying stress (Hill and Broom 2009). However, physiological analysis often entails inappropriate invasive methods of sample collection (i.e. blood or urine sampling) which itself induces stress, directly skewing the obtained results (Owen et al. 2004; Hill and Broom 2009). Non-invasive methods such as

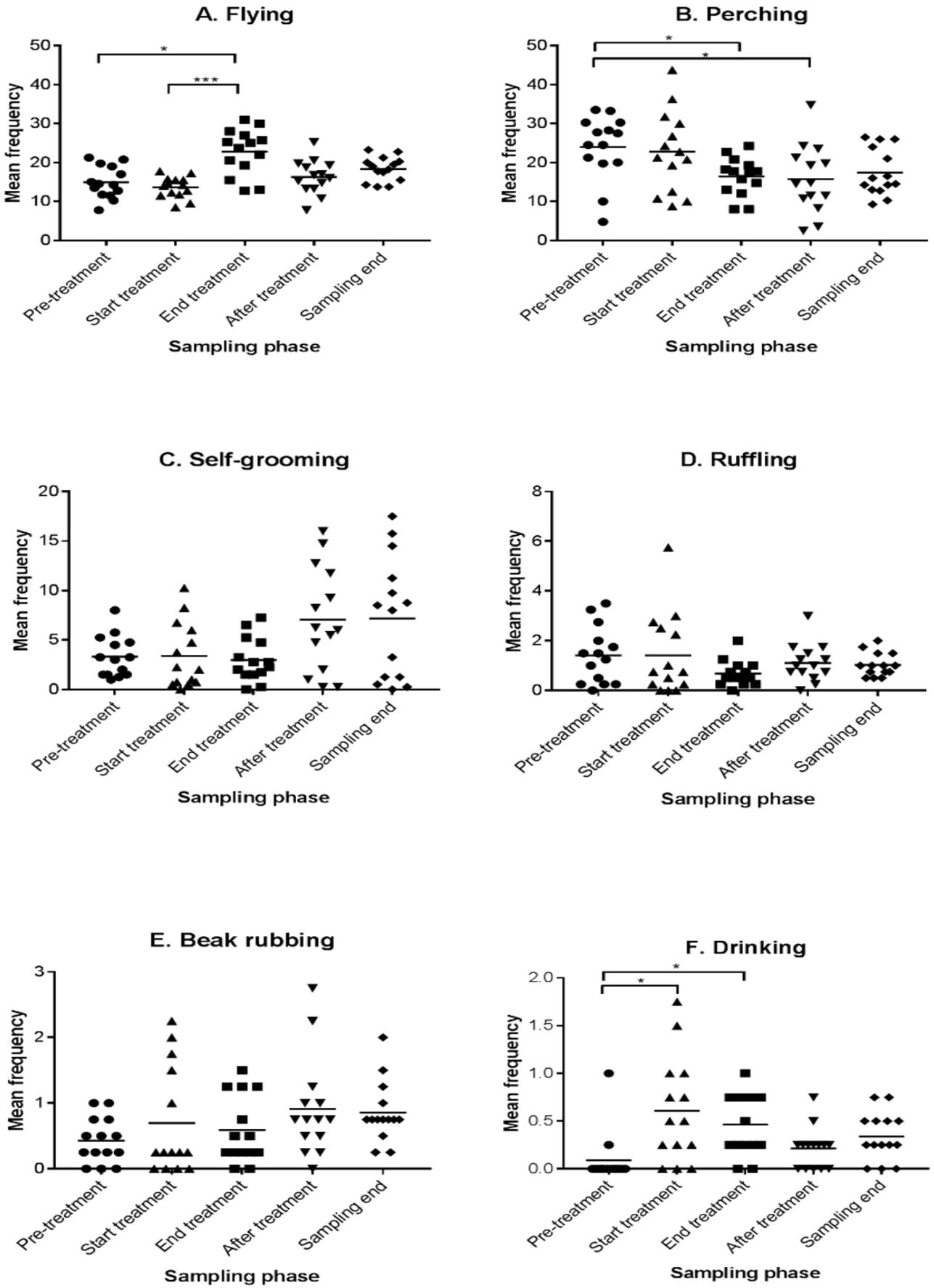

Figure 6. Change to mean behavioural frequencies between sampling phases of all adult AWM. Flying (A), perching (B), self-grooming (C), ruffling (D), beak rubbing (E), drinking (F) behavioural frequencies of captive adult AWM during different sampling phases; each point represents one bird and bars represent mean. *=P<0.05; ***=P<0.001.

analysis of shed feathers are difficult to determine with regard to the time of deposition of glucocorticoids. Therefore, finding non-invasive but accurate methods to quantify physiological effects from biochemical analyses in response to a changing stimulus is favoured (Whitham and Wielebnowski 2013).

Captive stress effects are a phenomenon in captive animals which contribute to suboptimal health and behaviour, thus impacting welfare (Crissey 2005). Quite how environmental stressors (e.g. limited spatial environment, nutritional stress, conspecific conflicts, etc.) impact on an individual's or population's ability to fight infections or reproduce is not well documented (Morgan and Tromborg 2007). Typically, environmental stress is quantified by looking into how visitor effects and the introduction of environmental enrichment reduces the frequencies of disadvantageous stereotypical behaviours (Carlstead and Shepherdson 2000). However, focusing on improving the psychological and physiological well-being of captive organisms is the best way to be truly impactful.

Methods to ameliorate nutritional and environmental stress effects in captive exotic birds are key in ex-situ conservation efforts. Martin et al. (2011) showed how captivity increases the amount of pro-inflammatory cytokines expressed in passerines compared to wild populations. Stress causes a heightened biological state through the release of glucocorticoids within the systemic circulation, which up-regulates the release of pro-inflammatory mediators (Sternberg 2007; Martin et al. 2011; Silverman and Sternberg 2012). Elevation of these and other hormones may be beneficial in the short-term to assist with an immune challenge, such as eliminating pathogens within the GI tract. However, during chronic stress, sustained inflammation distorts the epithelium resulting in GI barrier dysfunction (Sternberg 2007; Martin et al. 2011). As PUT is shown to reduce inflammation, supplementation may lessen the effect of glucocorticoids on the digestive system by inhibiting inflammatory damage under environmental stresses (Sears et al. 2011). This could improve captive health and welfare from an improved digestive system providing adequate nutrition for biological functions, such as growth and reproduction.

This investigation demonstrated significant benefits of PUT supplementation in captive AWM populations by improving relative GI health. By improving GI tract functionality by reducing GI inflammation and regulating the commensal intestinal metacommunity, this buffers the effect of environmental stresses on individuals. This will contribute to improving captive health, welfare and fecundity over time, key in ex-situ conservation efforts (Fidgett and Gardner 2014). Captive omnivorous birds like AWM, with citrus fruits, legumes and carrion as major constituents of wild diets, could truly benefit from PUT supplementation based on these findings from the proposed (Canário et al. 2004; Larqué et al. 2007).

Within exotic aviculture, published literature on captive nutrition is limited (Allen and Ullrey 2004). A comprehensive understanding and appreciation of the complexities of avian nutritional requirements remains in its infancy (Fidgett and Gardner 2014). Nutrition in zoological collections is becoming more crucial as an independent research field from the increased variety of species housed with varying dietary requirements, increased obesity prevalence, and the reliance of captive stock as reservoir populations for future reintroductions (Owen et al. 2004). As the results discussed cannot be compared to an experimental control group, the relevance of these conclusions to other captive taxa is limited but promising. The specificity and uniqueness of each captive group's dietary needs are wide-ranging and complex (Crissey 2005). Repeated and longitudinal investigations following a similar methodology to the above should be trialled in multiple avian species managed in captivity, to identify the true impact of PUT on bodily processes.

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