Food supplementation affects gut microbiota and immunological resistance to parasites in a wild bird species

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Abstract

1. Supplemental feeding can increase the overall health of animals but also can have varying consequences for animals dealing with parasites. Furthermore, the mechanism mediating the effect of food supplementation on host-parasite interactions remains poorly understood.

2. The goal of the study was to determine the effect of food supplementation on host defenses against parasitic nest flies and whether host gut microbiota, which can affect immunity, potentially mediates these relationships. In a fully crossed design, I experimentally manipulated the abundance of parasitic nest flies (*Protocalliphora sialia*) and food availability then characterized the gut microbiota, immune responses, and nest parasite abundance of nestling eastern bluebirds (*Sialia sialis*).

3. Food supplemented birds had 75% fewer parasites than unsupplemented birds. Parasite abundance decreased throughout the breeding season for unsupplemented birds, but abundance did not change throughout the season for supplemented birds. Food supplementation increased overall fledging success. Parasitism had a sublethal effects on blood loss, but food supplementation mitigated these effects by increasing parasite resistance via the nestling IgY antibody response.

4. Food supplementation increased the gut bacterial diversity in nestlings, which was negatively related to parasite abundance. Food supplementation also increased the relative abundance of *Clostridium* spp. in nestlings, which was positively related to their antibody response and negatively related to parasite abundance.

5. *Synthesis and applications*. Overall, these results suggest that food supplementation, especially early in the breeding season, increases resistance to parasitism during the early life stage of the host, which could be mediated by the effect of supplementation on the gut

microbiota. Wildlife food supplementation is a common pastime for humans worldwide and therefore it is important to determine the consequences of this activity on animal health. Furthermore, supplemental feeding could induce resistance to detrimental parasites (e.g. invasive parasites) in hosts when management of the parasite is not immediately possible.

Keywords: bird feeding, blowfly, diet, food supplementation, host defenses, microbiome, resistance, tolerance

43 **1 | INTRODUCTION**

44 Environmental factors, such a food availability, can influence host-parasite interactions
45 (Becker, Streicker & Altizer 2015; Becker *et al.* 2018; Sánchez *et al.* 2018). Host defense
46 strategies against parasites, such as tolerance and resistance, are often condition-dependent and
47 regulated by resource availability (Lee *et al.* 2006; Cotter *et al.* 2011; Sternberg *et al.* 2012;
48 Howick & Lazzaro 2014; Knutie *et al.* 2017c). Tolerance mechanisms, such as tissue repair or
49 compensation for energy loss, reduce the damage that parasites cause without reducing parasite
50 fitness (Miller, White & Boots 2006; Råberg, Sim & Read 2007; Read, Graham & Råberg 2008;
51 Medzhitov, Schneider & Soares 2012). For example, avian parents from parasite-infested nests
52 reduce the cost of parasitism by feeding their offspring more than parents from non-parasitized
53 nests (Christe et al. 1996; Tripet and Richner 1997; Knutie et al. 2016). Consequently, despite
54 increasing parasite loads, offspring do not suffer a high cost of parasitism because they are able
55 to compensate for resources lost to the parasites.

56 Another condition-dependent defense mechanism is resistance, such as the immune
57 response, which reduces the damage that parasites cause by reducing parasite fitness (Read *et al.*
58 2008). Resistance can be condition-dependent because mounting immune responses can be
59 energetically costly and therefore only hosts with enough food resources may be able to invest in
60 immunity (Sheldon & Verhulst 1996; Svensson *et al.* 1998; Lochmiller & Deerenberg 2000;
61 Demas 2004; Sternberg *et al.* 2012; Howick & Lazzaro 2014; Cornet *et al.* 2014). One
62 explanation for the positive relationship between food availability and immunity is that the extra
63 nutrients directly increase the production of immune cells (Strandin, Babayan & Forbes 2018).
64 For example, supplemented protein can increase the concentration of cellular immune cells (e.g.

65 eosinophils, globule leukocytes and mast cells) (reviewed in Coop and Kyriazakis 2001) and
66 humoral immune cells (e.g. Ig antibodies) (Datta *et al.* 1998).

67 Food availability might also indirectly influence resistance by affecting the gut
68 microbiota of the host. Host diet composition can directly alter the gut microbiota of the host
69 (David *et al.* 2014; Carmody *et al.* 2015). For example, hosts that consume a plant-based diet
70 have significantly different bacterial communities than hosts that consume an animal-based diet
71 (David *et al.* 2014; Knutie *et al.* 2017a). Since the gut microbiota can affect the development and
72 maintenance of the immune system of the host (reviewed in Round and Mazmanian 2009;
73 Hooper et al. 2012), diet-based shifts in the host gut microbiota might also affect parasite
74 resistance. However, studies are still needed to determine whether the host microbiota plays a
75 role in mediating the effect of food availability on parasite resistance.

76 Humans can change resource availability for animals by increasing the abundance of
77 unnatural food using wild bird feeders and the disposal of human trash (Murray *et al.* 2016;
78 Bosse *et al.* 2017; Start *et al.* 2018). In fact, humans provide many wild bird species with a large
79 proportion of their food (Jones & James Reynolds 2008; Jones 2011; Cox & Gaston 2018),
80 which can play a key role in maximizing the birds' fitness (Tollington *et al.* 2019). In the United
81 States alone, approximately 50 million households provide over a half a million tons of
82 supplemental food to attract wild birds to their property (Robb *et al.* 2008; Cox & Gaston 2018).
83 Supplemental feeding of birds can have several benefits to birds and humans. Feeding wild birds
84 can improve the mental health of humans along with their connection with nature (Jones 2011;
85 Cox & Gaston 2016, 2018; Cox *et al.* 2016; Shaw, Miller & Wescott 2017). Birds that are
86 supplemented with food are often in better condition, which in turn can increase their
87 reproductive success (Tollington *et al.* 2019) and enhance some measures of immunity

88 (Lochmiller, Vestey & Boren 1993; Wilcoxen *et al.* 2015; Ruhs, Vézina & Karasov 2018;
89 Sánchez *et al.* 2018; Strandin *et al.* 2018). Because supplemental feeding can increase bird
90 abundance (Fuller *et al.* 2008), it can also increase pathogen transmission by increasing host
91 aggregation and contact at the feeders (Wilcoxen *et al.* 2015; Becker *et al.* 2015). Although
92 correlational studies have variably linked food availability and host-parasite outcomes, causal
93 tests are still needed to determine the effect of food supplementation on host-parasite interactions
94 and how the host microbiota could be affecting parasite resistance (Becker *et al.* 2015; Altizer *et*
95 *al.* 2018).

96 The eastern bluebird (*Sialia sialis*) is a North American bird species that is unnaturally
97 fed by humans. In the 1970s, populations of eastern bluebirds declined, which was thought to be
98 linked to a loss of suitable foraging and nesting habitat (Gowaty & Plissner 2015). In response,
99 humans built and established artificial nest boxes and started supplementing the birds' natural
100 diet of insects, spiders, and small fruits (Pinkowski 1977a) with mealworms. Since the 1970s, the
101 eastern bluebird population size has rebounded (Sauer & Droege 1990) but humans continue to
102 maintain nest boxes and provide bluebirds with supplemental food, such as mealworms.
103 Bluebirds are also infested with parasitic nest flies (*Protocalliphora* sp.) throughout their range.
104 Past studies have found highly variable blowfly abundances along with both a negative and no
105 effect of blowflies on fledging success of bluebirds (Johnson 1929; Pinkowski 1977b; Roby,
106 Brink & Whitmann 1992; Wittmann & Beason 1992; Smar 1996; Hannam 2006). One
107 explanation for the variable parasite load and relationship between bluebirds and their parasites
108 is that the bluebird populations have access to different resources (natural and supplemental),
109 which in turn, could affect their ability to defend themselves.

110 The goal of this study was to determine the effect of food supplementation on host
111 defenses against parasites, whether these effects differ across the breeding season, and whether
112 the gut microbiota of the host could be mediating the effect of food on host defense
113 strategies. Across the breeding season, I experimentally manipulated parasite abundance and
114 food availability (by supplementing birds with mealworms (*Tenebrio molitor*) or not) then
115 quantified growth, hemoglobin levels (proxy of blood loss), glucose levels (proxy of energy),
116 and survival (fledging success) of nestling bluebirds. I also quantified the *Protocalliphora*-
117 binding antibody response, as a measure of resistance, and the gut microbiota of nestlings.
118 Bluebird nestlings are naturally tolerant of *Protocalliphora sialia* in relation to growth
119 and survival but suffered sublethal effects (blood loss) from the parasite (Grab *et al.* 2019). If
120 food supplementation supports host tolerance without affecting resistance, then parasitized birds
121 that are supplemented will maintain good health (i.e. growth, survival) without changes in their
122 parasite load. However, because food supplementation can enhance the immune response of
123 birds (Strandin *et al.* 2018), birds with supplemented food are predicted to shift their host
124 defense strategy from tolerance to resistance in order to reduce the sublethal effect of the parasite
125 on blood loss. Therefore, I predicted that food supplementation would reduce parasite load in the
126 naturally parasitized control nests. Because insect (food) availability can increase throughout
127 breeding season (Bowlin & Winkler 2004), I predicted that supplementation would be less
128 effective at reducing parasite abundance later in the breeding season.
129 Food supplementation could either directly or indirectly increase host resistance to
130 parasitism. More nutrients could provide additional resources to directly speed up immune
131 system development, such as the IgY antibody response or haptoglobin production, in nestlings.
132 When a host is bitten by an ectoparasite, a series of immune pathways is activated by the host to

133 induce an inflammatory response that acts to prevent or reduce feeding by the ectoparasite
134 (reviewed in Owen, Nelson & Clayton 2010). Briefly, tissue damage and the introduction of
135 antigens from the parasite stimulate the release of pro-inflammatory cytokines, which can
136 produce acute phase proteins (e.g. haptoglobins). These chemical substances signal cells of the
137 innate immune system, such as macrophages, to travel to the damaged tissue to degrade antigens
138 and cells of the adaptive immune response induce the production of antigen-specific antibodies
139 (e.g. IgY antibodies), which form lasting memory cells that can be activated quickly when the
140 host is re-exposed to the parasite (antigen). The immune cascade described above can negatively
141 affect ectoparasites by causing edema (tissue swelling), which prevents the parasites from
142 feeding from capillaries, and can damage the parasite's tissue with proteolytic molecules.
143 Consequently, these actions can reduce the fitness of the parasite by reducing blood meal size
144 (Owen *et al.* 2009). However, an immune response can be energetically costly to produce and
145 therefore only well-fed nestlings might be able to produce this response (Sheldon & Verhulst
146 1996; Lochmiller & Deerenberg 2000; Cornet *et al.* 2014).
147 Alternatively, diet can affect the gut microbiota (e.g. particular taxa) of the host (Knutie
148 *et al.* 2017a), which could impact parasite resistance. Therefore, I predicted that food
149 supplementation would increase the gut bacterial diversity of the nestlings, which would be
150 positively correlated with the immune responses and negatively correlated with parasite
151 abundance. Additionally, a particular species of symbiont (e.g. gut bacterial pathogens) could
152 affect another species due to its earlier arrival by modulating the immune response (Sousa 1992;
153 de Roode *et al.* 2005; Devevey *et al.* 2015). Therefore, I also explored the relationship between
154 ectoparasite abundance and the relative abundance of bacterial genera that included avian
155 pathogens, such as *Campylobacter spp., Clostridium spp., Enterococcus spp., Escherichia spp.,*

Lactobacillus spp., Listeria spp., Mycobacteria spp., Pasteurella spp., Salmonella spp., Staphylococcus spp., Streptococcus spp., and *Vibrios spp.* (reviewed in Benskin *et al.* 2009).

2 | MATERIAL AND METHODS

2.1 | Study system

From May to July 2017, approximately 150 nest boxes were monitored in Clearwater and Hubbard County in Northern Minnesota, USA, near the University of Minnesota Itasca Biological Station and Itasca State Park (47°13’33” N, - 95°11’42” W). Eastern bluebirds are abundant at the site and nest in artificial cavities. *P. sialia* is the only ectoparasite that infests the nests of bluebirds at this site.

2.2 | Experimental manipulation of parasites and food availability

Parasite abundance and food availability were experimentally manipulated for each nest using a 2x2 factorial design. Boxes were checked once a week for nesting activity. Once eggs appeared, nests were checked every other day until nestlings hatched. When eggs were incubated for 10 days, mealworm feeders were placed 10 m from the nest box (Fig. S1), with the intention that the feeder would only provide food to the birds from this focal nest. The feeders were constructed from pine wood (2.54 cm by 10.16 cm) and the basin for the mealworms was an empty, sterilized aluminum cat food can. The can had pin sized holes in the bottom of the can to allow for drainage from the rain and the can was attached to the wood platform with Velcro. The feeders were attached to a green garden fence post with 18-gauge aluminum wire, approximately 1.5m above the ground.

178 Nests were assigned to the mealworm-supplemented or non-supplemented treatment. For
179 the supplemented treatment, 15 mealworms (Rainbow Mealworms, Compton, CA) per nestling
180 per day were added to the feeders until the bird eggs hatched, which was based on the
181 recommended feeding regime by the North American Bluebird Society. For the non-
182 supplemented treatment, the feeders were visited daily but mealworms were not added to the
183 feeders to control for the effect of disturbance on the birds. Parents were supplied with
184 mealworms a few days before nestlings hatched to increase the chances that parents would find
185 the feeders before their nestlings hatched. Birds were supplemented or not until nestlings reached
186 10 days old.

187 Nests were also assigned to the parasitized or non-parasitized treatment when eggs
188 hatched. The nestlings and top liner of the nest were removed in order to treat the nest with either
189 water to allow for natural parasitism or a 1% permethrin solution (Permacap) to remove all
190 parasites (Knutie *et al.* 2016; DeSimone *et al.* 2018). Nestlings never contacted the parasite
191 treatment since the liquid was sprayed under the nest liner.

192

193 *2.3 | Nestling growth and survival*

194 Within two days of hatching, nestlings (g) were weighed using a portable digital scale
195 balance and tarsus length (mm), bill length (mm), and first primary feather length (mm) were
196 measured using dial calipers. When nestlings were ten days old, they were measured again and
197 banded with a numbered metal band. When nestlings were approximately 13 days old, the boxes
198 were checked every other day from a distance (to avoid pre-mature fledging) to determine the
199 fledging success and age at which the nestlings fledged or died (>10-day old nestlings are not
200 typically removed from the nest by the parents after they die, S.A.K. personal obs.).

201 *2.4 | Sample collection*

202 Feces and a small blood sample (<30 μL) were collected from nestlings when they were
203 10 days old. Hemoglobin was measured from whole blood using a HemoCue® HB +201
204 portable analyzer and glucose was measured using a HemoCue® Glucose 201 portable analyzer.
205 Blood samples were placed on ice until they were centrifuged for 3 minutes at 10000 rpm to
206 separate the plasma from the red blood cells. Plasma and red blood cells were then stored
207 separately in a -80°C freezer. Fecal samples were placed on ice until stored in a -80°C freezer.
208 Samples were then transported on dry ice to the University of Connecticut. Enzyme-linked
209 immunosorbent assays (ELISA) and Tridelta PHASE haptoglobin assay (TP-801) were then
210 performed to quantify *P. sialia*-binding antibody (IgY) and haptoglobin (acute phase protein)
211 levels, respectively, in the plasma of parasitized nestling. IgY antibody levels from
212 unsupplemented nestlings were first published in (Grab *et al.* 2019); see this paper for the
213 detailed protocol for the IgY ELISA.

214

215 *2.5 | Quantifying parasites*

216 Once nestlings died or fledged, nests were collected and stored in plastic bags. Within
217 eight hours of collection, nests were dissected and all larvae, pupae, and pupal cases were
218 counted to determine total parasite abundance for each nest. Eclosed flies were collected and
219 identified as *Protocalliphora sialia.*

220

221 *2.6 | Bacterial DNA extraction and sequencing*

222 Total DNA was extracted from nestling bluebird feces from parasitized nests and three
223 mealworms using a MoBio PowerFecal DNA Isolation Kit. DNA extractions were then sent to

224 the University of Connecticut Microbial Analysis, Resources and Services for sequencing with
225 an Illumina MiSeq platform and v2 2x250 base pair kit (Illumina, Inc). A laboratory blank was
226 also sequenced to control for kit contamination and found no detectable sequences. Bacterial
227 inventories were conducted by amplifying the V4 region of the 16S rRNA gene using primers
228 515F and 806R and with Illumina adapters and dual indices (Kozich *et al.* 2013). Raw sequences
229 were demultiplexed with onboard bcl2fastq and then processed in Mothur v1.39.5 (Schloss *et al.*
230 2009) according to the standard MiSeq protocol (Kozich *et al.* 2013). Briefly, forward and
231 reverse sequences were merged. All sequences with any ambiguities, that did not align to the
232 correct region, or that did not meet length expectations, were removed. Sequences were aligned
233 to the Silva nr_v119 alignment (Quast *et al.* 2013). Chimeric reads were also removed using
234 UCHIME (Edgar *et al.* 2011). Non-bacterial sequences that classified as chloroplasts,
235 mitochondria, or unknown (i.e. did not classify to the level of kingdom) were removed.
236 Sequences were grouped into operational taxonomic units (OTUs) based on a 97% similarity
237 level and identification of the OTUs was done using the Ribosomal Database Project Bayesian
238 classifier (Wang *et al.* 2007) against the Silva nr_v119 taxonomy database. Alpha (sobs,
239 Shannon index, Simpson index) and beta diversity statistics were calculated by averaging 1,000
240 random subsampling of 10,000 reads per sample. The resulting data sets included a total of
241 702,648 sequences and an average of 41,332 reads per sample (min: 17,582, max: 57,283).
242

243 *2.7 | Statistical analyses*

244 A generalized linear model (GLM) with zero-inflated Poisson errors (to control for the
245 number of zeros in the fumigated treatment) was used to determine whether parasite treatment,
246 food treatment, and their interaction affected parasite abundance. Within the sham-fumigated

247 treatment, a negative binomial GLM was used to determine whether food supplementation,
248 timing of breeding (Julian date), and their interaction affected parasite abundance.
249 A GLM with binomial errors for proportional data (i.e. logistic regression) was used to
250 determine the effect of the food treatment, parasite treatment, and their interaction on fledging
251 success. A GLM was also used to determine the effect of the interaction between the food
252 treatment and parasite abundance on fledging success to test whether food treatment affected
253 host tolerance of the parasite (Simms 2000); i.e. if the effect of the interaction on fledging
254 success is significant then nestlings from each food treatment differ in parasite tolerance. Julian
255 date was originally included as a covariate for the growth and fledging success models but it was
256 excluded from all models because it did not account for a significant amount of variation.
257 Nestling growth metrics (bill length, tarsus length, first primary feather length, body
258 mass) were not highly correlated (pairwise correlation coefficients ranged from 0.37 to 0.66;
259 Table S1); therefore, individual generalized linear mixed models (GLMM) with nest as a random
260 effect were used to determine the effect of parasite treatment, food treatment, and their
261 interaction on each $\log_{10}$ transformed nestling growth metrics. GLMMs with nest as a random
262 effect were also used to determine the effect of parasite treatment, food treatment, and their
263 interaction on $\log_{10}$ transformed hemoglobin levels (proxy of blood loss) and glucose levels
264 (proxy of energy levels).
265 Within the sham-fumigated treatment, GLMMs with nest as a random effect were used to
266 determine the effect of food treatment on immune responses (antibody levels) and alpha bacterial
267 diversity metrics (Shannon index and the $\log_{10}$ of sobs and Simpson index). A GLM was used to
268 analyze the effect of food treatment on haptoglobin levels because haptoglobin was measured in
269 only one nestling per nest. Analyses were conducted using the glm (GLM) and glmer (GLMM)

270 functions with the lme4 package or glmmTMB (zero-inflated Poisson GLM) function with the
271 glmmTMB package. Probability values were calculated using log-likelihood ratio tests using the
272 Anova function in the car package (Fox & Weisberg 2002).

273 Microbiota data were also analyzed from mealworm samples and parasitized nestlings
274 from both food treatments. The effect of food treatment on bacterial community dynamics in
275 parasitized nestlings was analyzed with the Bray-Curtis Dissimilarity Matrices using
276 PERMANOVA+ (2008, version 1.0.1; with 999 permutations) in PRIMER (2008, version
277 6.1.11). Relative abundances (arcsine square root transformed; Shchipkova et al. 2010; Kumar et
278 al. 2012) of bacterial phyla and genera of birds and mealworms were analyzed using ANOVAs
279 with food treatment and mealworms as independent variables; false discovery rate (FDR) tests
280 were used to control for multiple analyses and Tukey post-hoc tests were used for pairwise
281 interactions. Relative abundances of six bacterial genera that include pathogenic species were
282 also compared between food treatments ANOVAs with FDR corrections. Analyses were
283 conducted in R (2017, version 3.4.3). All figures were created in Prism (2017, version 7).
284

285 **3 | RESULTS**

286 *3.1 | Effect of treatment and timing of breeding on parasite abundance*

287 Fumigation of nests with permethrin was effective at reducing parasite abundance to zero
288 (Fig. 1A) (Table S2) (GLM, $\chi^2 = 29.83$, $df = 1$, $P < 0.0001$). Food supplementation and the
289 interaction between food and parasite treatments also affected parasite abundance (Fig. 1A)
290 (GLM, food: $\chi^2 = 12.88$, $df = 1$, $P < 0.001$; interaction: $\chi^2 = 9.37$, $df = 1$, $P = 0.002$). Food
291 supplementation decreased parasite abundance within the sham-fumigated treatment: 100%
292 (11/11) of unsupplemented nests and 55.6% (5/9) of supplemented were infested with parasites

293 and unsupplemented nests had, on average, four times as many parasites as supplemented nests
294 (Fig. 1A). Overall, parasite abundance decreased with timing of breeding (Table S3) (Julian date:
295 $\chi^2 = 0.20$, $df = 1$, $P = 0.66$) but more specifically, there was an interaction between food
296 treatment and Julian date (interaction: $\chi^2 = 4.18$, $df = 1$, $P = 0.04$); parasite abundance decreased
297 throughout the season in unsupplemented nests, but not supplemented nests (Fig. 1B).
298
299 *3.2 | Fledging success and nestling growth*
300 Parasite and food treatment affected fledging success (GLM, parasite: $\chi^2 = 4.09$, $df =$
301 1, $P = 0.04$; food: $\chi^2 = 6.78$, $df = 1$, $P = 0.009$), with sham-fumigated nests having higher
302 fledging success than fumigated nests and supplemented nests having higher fledging success
303 than unsupplemented nests (Fig. 1C). However, the interaction between parasite and food
304 treatment did not affect fledging success ($\chi^2 = 2.35$, $df = 1$, $P = 0.13$). The relationship between
305 fledging success and parasite abundance did not differ across food treatments (Fig. 1D) (i.e.
306 tolerance to parasitism did not differ; $\chi^2 = 0.10$, $df = 1$, $P = 0.75$).
307 Parasite treatment, food treatment, and the interaction between the two factors did not
308 significantly affect bill length (Tables 1 and S4) (GLMM, parasite: $\chi^2 = 0.69$, $df = 1$, $P = 0.41$,
309 food: $\chi^2 = 0.99$, $df = 1$, $P = 0.32$, interaction: $\chi^2 = 0.23$, $df = 1$, $P = 0.63$). Parasite treatment had a
310 marginally non-significant effect on tarsus length (GLMM, parasite: $\chi^2 = 3.65$, $df = 1$, $P = 0.06$),
311 but food treatment and the interaction between the treatments did not affect tarsus length (food:
312 $\chi^2 = 0.99$, $df = 1$, $P = 0.32$, interaction: $\chi^2 = 0.72$, $df = 1$, $P = 0.40$). Food treatment affected
313 nestling body mass and first primary feather length (mass: $\chi^2 = 5.09$, $df = 1$, $P = 0.02$; feather: χ^2
314 $= 3.36$, $df = 1$, $P = 0.07$) with unsupplemented nestlings having lower body mass and slower
315 feather growth than supplemented nestlings (Table 1). Parasite treatment and the interaction

316 between parasite and food treatment did not affect body mass and first primary feather length
317 (Table 1) (mass: parasite, $\chi^2 = 0.04$, $df = 1$, $P = 0.83$; interaction, $\chi^2 = 0.01$, $df = 1$, $P = 0.92$;
318 feather: parasite, $\chi^2 = 1.13$, $df = 1$, $P = 0.29$; interaction, $\chi^2 = 0.38$, $df = 1$, $P = 0.54$).
319
320 *3.3 | Nestling hemoglobin and glucose levels*
321 Parasite treatment affected hemoglobin levels (Table S4) (GLMM, $\chi^2 = 5.55$, $df = 1$, $P =$
322 0.02) with parasitized nestlings having lower hemoglobin than non-parasitized nestlings (Table
323 1). Food treatment had a marginally non-significant effect on hemoglobin levels ($\chi^2 = 3.43$, $df =$
324 1, $P = 0.06$) with unsupplemented nestlings having lower hemoglobin levels than supplemented
325 nestlings (Table 1). These results were likely because hemoglobin levels were negatively related
326 to parasite abundance across parasite treatments ($\chi^2 = 18.60$, $df = 1$, $P < 0.0001$). The interaction
327 between parasite and food treatment did not affect hemoglobin levels ($\chi^2 = 1.29$, $df = 1$, $P =$
328 0.26). Parasite treatment, food treatment, and the interaction between the two factors did not
329 affect glucose levels (Tables 1 and S4) (parasite: $\chi^2 = 0.41$, $df = 1$, $P = 0.52$, food: $\chi^2 = 0.83$, $df =$
330 1, $P = 0.36$, interaction: $\chi^2 = 0.00$, $df = 1$, $P = 0.96$).
331
332 *3.4 | Immune responses of parasitized nestlings*
333 Food treatment affected nestling antibody levels (Table S5) (GLMM, $\chi^2 = 5.96$, $df =$
334 1, $P = 0.02$; supplemented, $n = 9$: 0.53 ± 0.15, unsupplemented, $n = 11$: 0.24 ± 0.07) with
335 supplemented nestlings having higher antibody levels than unsupplemented nestlings within the
336 sham-fumigated treatment (Fig. 2A). Mean nestling antibody levels within a nest were negatively
337 related to parasite abundance (Fig. 2B) (GLM, $\chi^2 = 5.86$, $df = 1$, $P = 0.02$). Food treatment did
338 not affect haptoglobin levels (Table S5) (GLM, $\chi^2 = 0.20$, $df = 1$, $P = 0.65$; supplemented: $0.36 \pm$

339 0.06, unsupplemented: 0.32 ± 0.04; n = 5 nestlings for both treatments). Haptoglobin levels were
340 not significantly correlated with antibody levels (GLM, $\chi^2 = 0.39$, $df = 1$, $P = 0.53$) or parasite
341 abundance (GLM, $\chi^2 = 0.08$, $df = 1$, $P = 0.78$).
342
343 *3.5 | Microbiota of parasitized nestlings*
344 Bacterial community structure and membership differed between the parasitized nestlings
345 and mealworms, but did not differ between food treatments for the nestlings (Fig. 3A and B)
346 (PERMANOVA, structure: $F_{2,16} = 2.15$, $P = 0.002$, membership: $F_{2,16} = 2.09$, $P = 0.001$).
347 Supplemented nestlings had higher bacterial diversity (sobs) compared to unsupplemented
348 nestlings, but this difference was not significant for sobs (Fig. 4A) (Tables S5 and S6) (GLMM,
349 $\chi^2 = 3.07$, $df = 1$, $P = 0.08$). Bacterial diversity (sobs) was not significantly related to antibody
350 levels (Fig. 4B) (GLM, $\chi^2 = 0.85$, $df = 1$, $P = 0.36$) but negatively related to parasite abundance
351 (Fig. 4C) (GLMM, $\chi^2 = 4.17$, $df = 1$, $P = 0.04$). Food treatment did not significantly affect the
352 Shannon (GLMM, $\chi^2 = 2.02$, $df = 1$, $P = 0.15$) and Simpson index (Tables S5 and S6) (GLMM,
353 $\chi^2 = 1.41$, $df = 1$, $P = 0.24$). Neither indices were significantly correlated with parasite abundance
354 (GLMM, Shannon: $\chi^2 = 0.29$, $df = 1$, $P = 0.59$, Simpson: $\chi^2 = 0.13$, $df = 1$, $P = 0.71$) or antibody
355 levels (GLM, Shannon: $\chi^2 = 0.43$, $df = 1$, $P = 0.51$, Simpson: $\chi^2 = 0.30$, $df = 1$, $P = 0.59$).
356 The relative abundance of several bacterial phyla differed between the birds and
357 mealworms. Birds in both treatments had higher relative abundances of phyla Planctomycetes
358 (ANOVA, $F = 8.61$, $P = 0.04$) compared to the mealworms, but mealworms had higher relative
359 abundances of phyla Cyanobacteria (ANOVA, $F = 20.96$, $P = 0.001$) and Tenericutes (ANOVA,
360 $F = 7.65$, $P = 0.04$) than the birds ($P < 0.05$ for each pairwise test). Mealworms were dominated
361 by phyla Firmicutes (36.03%), Proteobacteria (25.78%), Cyanobacteria (20.07%), Actinobacteria

362 (10.14%), Tenericutes (5.25%), and Bacteroidetes (2.44%). Nestling feces were dominated by
363 phyla Proteobacteria (28.24%), Actinobacteria (28.05%), Firmicutes (21.91%), Bacteroidetes
364 (8.07%), Cyanobacteria (6.14%), Planctomycetes (1.47%), and Saccharibacteria (1.04%).
365 Several bacterial genera differed between the nestlings and mealworms (Table S7).
366 Genera with known pathogenic bacterial species were then compared specifically between
367 supplemented and unsupplemented birds. *Campylobacter spp., Listeria spp., Mycobacteria spp.,*
368 *Pasteurella spp., Salmonella spp., and Vibrios spp.* were not found in the feces of nestlings.
369 Food treatment did not affect relative abundances of *Enterococcus spp., Escherichia spp.,*
370 *Lactobacillus spp., Staphylococcus spp., and Streptococcus spp*. Supplemented birds had higher
371 relative abundances of genus *Clostridium* (Fig. 4D) (ANOVA, $F = 7.92$, $P = 0.02$) than
372 unsupplemented birds. Relative abundance of *Clostridium spp.* correlated positively with
373 antibody levels (Fig. 4E) (GLM, $\chi^2 = 21.39$, $df = 1$, $P < 0.0001$) and negatively with parasite
374 abundance (Fig. 4F) (GLM, $\chi^2 = 3.59$, $df = 1$, $P = 0.058$), but did not correlate significantly with
375 haptoglobin levels (GLM, $\chi^2 = 0.11$, $df = 1$, $P = 0.74$).
376
377 **4 | DISCUSSION**
378 The study showed that parasitism did not significantly affect growth or fledging success
379 but negatively affected hemoglobin levels. Although nestlings maintained parasite tolerance
380 across food treatments, food supplementation combatted the sublethal effects of parasitism by
381 increasing nestling resistance to the parasites. However, food supplementation primarily
382 benefited the birds (i.e. reduced parasite abundance) early in the breeding season. The extra food
383 resources increased the nestling antibody response in parasitized nestlings, which decreased
384 parasite abundance. Interestingly, haptoglobin levels did not relate to parasite abundance, nor

385 were they affected by food supplementation, suggesting that these acute phase proteins are not
386 related to blowfly resistance. Supplementation also increased gut bacterial diversity in
387 parasitized nestlings, which was negatively related to parasite abundance, but not the antibody
388 response. However, specifically, supplementation increased the relative abundance of genus
389 *Clostridium* spp., which was positively related to the antibody response and negatively related to
390 parasite abundance. The mealworms had a distinct bacterial community from the birds, which
391 suggests that the nutritional composition of the mealworms influenced the gut microbiota of the
392 birds. Overall, these results suggest that food supplementation facilitates an increase in gut
393 *Clostridium* spp., which potentially primes the non-specific antibody response in nestlings to
394 resist parasitic nest flies.

395 Without the extra food, bluebird nestlings are tolerant to the parasitic nest flies, at least in
396 relation to survival (Grab *et al.* 2019). Therefore, why does food supplementation increase
397 resistance when the birds are already relatively well-defended against the parasite? Parasitism
398 negatively affected hemoglobin levels, which could have had lasting effects after the young left
399 the nest. For example, blowflies did not affect nestling mass or fledging success of ovenbirds
400 (*Seiurus aurocapilla*), however, fledgling survival and minimum distance traveled the first day
401 after fledging was significantly lower when the ovenbirds were parasitized (Streby, Peterson &
402 Kapfer 2009). Thus, when extra food is available resource-dependent resistance is likely
403 beneficial to ameliorate the long-term or subtle adverse effects of parasitism.

404 Traditionally, food supplementation is thought to have a direct effect on the antibody
405 response produced by the host. Immune function can be condition-dependent because the
406 immune response can be energetically costly to produce and therefore only hosts in good
407 condition may be physiologically able to invest in these responses (reviewed by Sheldon and

408 Verhulst 1996; Lochmiller and Deerenberg 2000; Svensson *et al.* 1998). Other studies have
409 found that supplemented nutrients can increase immunity (e.g. Ig antibodies) to parasites (Datta
410 *et al.* 1998). For example, hosts fed a high-protein diet produced more IgG antibodies to
411 parasitic worms compared to hosts fed a low-protein diet (Datta *et al.* 1998). Supplemented
412 mealworms could provide the necessary additional protein to induce or increase the development
413 of the IgY response in nestlings.

414 Food supplementation might have also or alternatively affected the nestling antibody
415 response through changes in the gut microbiota. Host diet can affect the gut microbiota (David *et*
416 *al.* 2014; Carmody *et al.* 2015; Knutie *et al.* 2017a). Specifically, my study found that food
417 supplementation increased the relative abundance of *Clostridium* spp. and other studies have also
418 found varying effects of host diet on *Clostridium* spp. abundance (e.g. in chickens) (Stutz &
419 Lawton 1984; Mitsch *et al.* 2004; Jia *et al.* 2009). For example, Drew *et al.* (2004) found that
420 protein supplementation increased *Clostridium perfringens* abundance. The high protein content
421 of mealworms (46.44%) (Ravzanaadii *et al.* 2012) could be responsible for the increase
422 *Clostridium spp.* abundance, but this idea requires further study.

423 A remaining question is whether the presence of *Clostridium spp.* in the gut is priming a
424 non-specific antibody response to ectoparasitic nest flies. Pathogenic *Clostridium spp.* can
425 activate the innate and adaptive immune system (including the IgY antibody response) in
426 chickens (Kulkarni *et al.* 2007), which can be influenced by food supplementation (Yitbarek *et*
427 *al.* 2012). Furthermore, priority effects, or the effect of one species on another species due to its
428 earlier arrival, can influence the fitness of the multiple symbionts (Sousa 1992; de Roode *et al.*
429 2005; Devevey *et al.* 2015). For example, initial infection by parasitic worms can negatively
430 affect the establishment of subsequent infections by other parasitic species in the host

431 (Hoverman, Hoye & Johnson 2013; Wuerthner, Hua & Hoverman 2017), which could either be
432 mediated by direct competition between the parasites or by the priming of the immune response
433 of the host. Although gut bacteria and ectoparasites occupy different spaces on and in their host,
434 the organisms are potentially exposed to the same circulating molecules related to the immune
435 system (e.g. plasma IgY antibodies). However, future experimental studies are needed to
436 determine whether increases in *Clostridium* spp. in hatchlings causally affects their IgY antibody
437 response and resistance to *P. sialia*.

438 Between 1920-1970, bluebird populations declined throughout North America due, in
439 part, to habitat destruction and the introduction of invasive species (Gowaty & Plissner 2015).
440 However, in the 1980s, bluebird populations started to rebound because the public established
441 nest boxes throughout the range of the bluebird. At study's field site, I established 70 nest boxes
442 in 2014-15, which increased to 150 boxes by 2017. Consequently, the number of nesting
443 bluebird pairs increased from six pairs in 2015 to 31 pairs in 2017, and this number continues to
444 increase. Across the past several decades, the public also became concerned about nest parasites
445 affecting the health of the birds and therefore, have implemented methods to eliminate or deter
446 the parasites (Zeleny 1976). Methods to remove parasites include removing old nests from the
447 box (Møller 1989) and placing deterrents in the box, such as vanilla extract and insecticides
448 (S.A.K. pers. comm.). My study suggests that supplying mealworms to bluebirds not only
449 increases the health of nestlings, but also reduces parasite loads in the nests. However, these
450 effects are most pronounced during the early part of the breeding season, likely because natural
451 insect (food) availability is lowest at this time (Bowlin & Winkler 2004).
452
453

454 **5 | CONCLUSION**

455 Determining the consequences of food supplementation is important because artificial
456 bird feeding is a common activity for humans throughout the world (reviewed in Cox & Gaston
457 2018). The results of this study show that increasing food availability for hosts can decrease
458 parasite pressure, especially during formative life stages. However, studies have shown that
459 having an epicenter of feeders where animals are directly or indirectly interacting can increase
460 transmission of more directly transmitted parasites (Becker *et al.* 2015). Therefore, the effect of
461 food supplementation on parasite resistance could vary when considering how feeders affect
462 parasite exposure. In this study, only the focal bluebirds were visiting the feeders (S.A.K. pers.
463 obs.), which reduced contact with other birds. Therefore, when feeding breeding birds, especially
464 box-nesting birds, placing individual feeders in the birds' territory could increase host defenses
465 without the risk of infection transmission.

466 Additionally, the results of the study might be particularly important for systems that are
467 dealing with detrimental parasites, such as Darwin's finches of the Galapagos Islands or
468 pardalotes of Australia and their parasitic nest flies (Fessl *et al.* 2010; Edworthy, Langmore &
469 Heinsohn 2019). When managing the parasites is not immediately possible, providing additional
470 food resources to the host could help increase their resistance (or even tolerance; (Knutie *et al.*
471 2016; McNew *et al.* 2019)) to parasites. However, assessing the potential risks of providing
472 additional resources to endangered or threatened species is required before attempting the
473 method.

474

475

476

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DATA ACCESSIBILITY

Sequences have been uploaded to GenBank (BioProject accession number: available upon acceptance) and data have been uploaded to FigShare (doi available upon acceptance).

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731

732 **Figure Legend**

733 Fig. 1. Effect of food and parasite treatment on parasite load and fledging success of bluebirds.
734 Birds that were supplemented with food were more resistant to parasites than birds that were not
735 supplemented (A). Parasite abundance decreased throughout the breeding season in the
736 unsupplemented treatment but not the supplemented treatment (B). Parasitism did not affect
737 fledging success, but supplemented birds had marginally higher fledging success than
738 unsupplemented bird (C). Within the parasitized treatment, birds from each treatment were
739 tolerant to their respective parasite abundances (D).

740

741 Fig. 2. Within the parasitized treatment, supplemented birds had higher antibody levels than
742 unsupplemented birds (A). Antibody levels were negatively related to parasite abundance (B).

743

744 Fig. 3. Within the parasitized treatment, bacterial community structure (A) and membership (B)
745 of nestlings did not differ between food treatments. The bacterial community of mealworms was
746 distinct from the bacterial community of the nestlings.

747

748 Fig. 4. Effect of food treatment on microbiota of nestlings and the relationships among
749 microbiota, immune response, and parasite abundance within the parasitized treatment.
750 Supplemented nestlings had marginally higher bacterial diversity than unsupplemented nestlings
751 (A). Bacterial diversity was not significantly related to antibody levels (B) but negatively related
752 to parasite abundance (C). Relative abundance of *Clostridium* spp. was higher in supplemented
753 nestlings than unsupplemented nestlings (D). *Clostridium* spp. abundance was positively related
754 to antibody levels (E) and negatively related to parasite abundance (F).

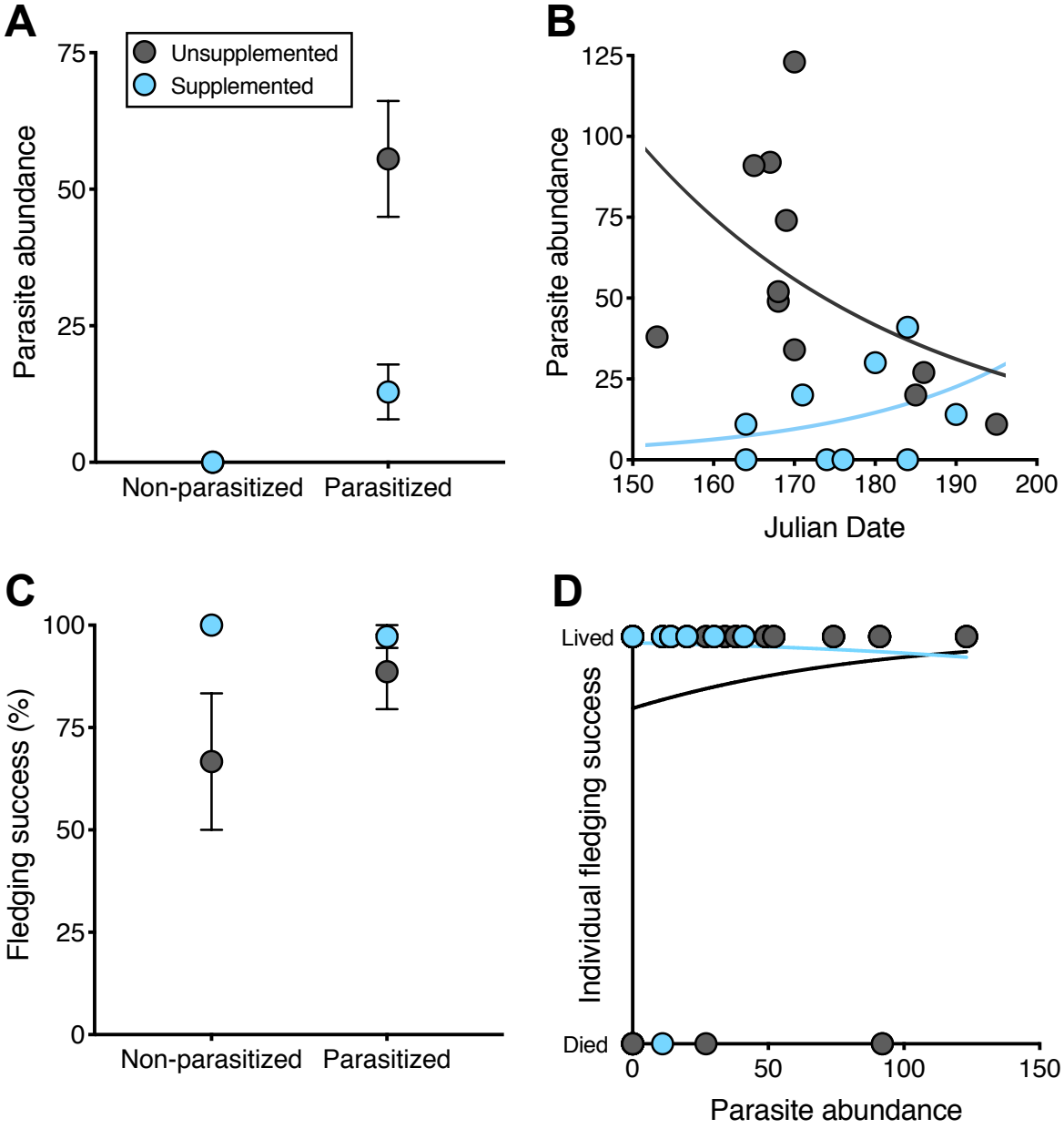
755 Table 1. Effect of parasite and food treatment on nestling growth, physiology, and fledging

756 success. Numbers are mean ± SE and numbers in parentheses are the number of nests.

	Non-supplemented		Supplemented	
Measurement	Non-parasitized	Parasitized	Non-parasitized	Parasitized
Parasite abundance	0.00 ± 0.00 (9)	55.55 ± 10.60 (11)	0.00 ± 0.00 (6)	12.89 ± 5.00 (9)
Bill length (mm)	5.00 ± 0.12 (6)	5.19 ± 0.14 (11)	5.26 ± 0.22 (6)	5.27 ± 0.10 (9)
Tarsus length (mm)	18.35 ± 0.19 (6)	18.60 ± 0.23 (11)	18.32 ± 0.25 (6)	18.93 ± 0.13 (9)
1st primary length (mm)	13.64 ± 1.06 (6)	16.41 ± 1.51 (11)	17.26 ± 1.43 (6)	17.81 ± 0.81 (9)
Mass (g)	25.71 ± 0.77 (6)	25.50 ± 0.77 (11)	27.21 ± 0.52 (6)	27.11 ± 0.56 (9)
Hemoglobin levels (g/dL)	11.21 ± 0.47 (5)	8.29 ± 0.83 (11)	11.55 ± 0.30 (6)	10.54 ± 0.88 (9)
Glucose levels (mg/dL)	282.50 ± 17.53 (5)	287.30 ± 32.50 (11)	321.30 ± 25.09 (6)	293.40 ± 18.80 (9)
Nestlings fledged	26/34 (77%)	48/51 (94%)	21/21 (100%)	38/39 (97%)

757

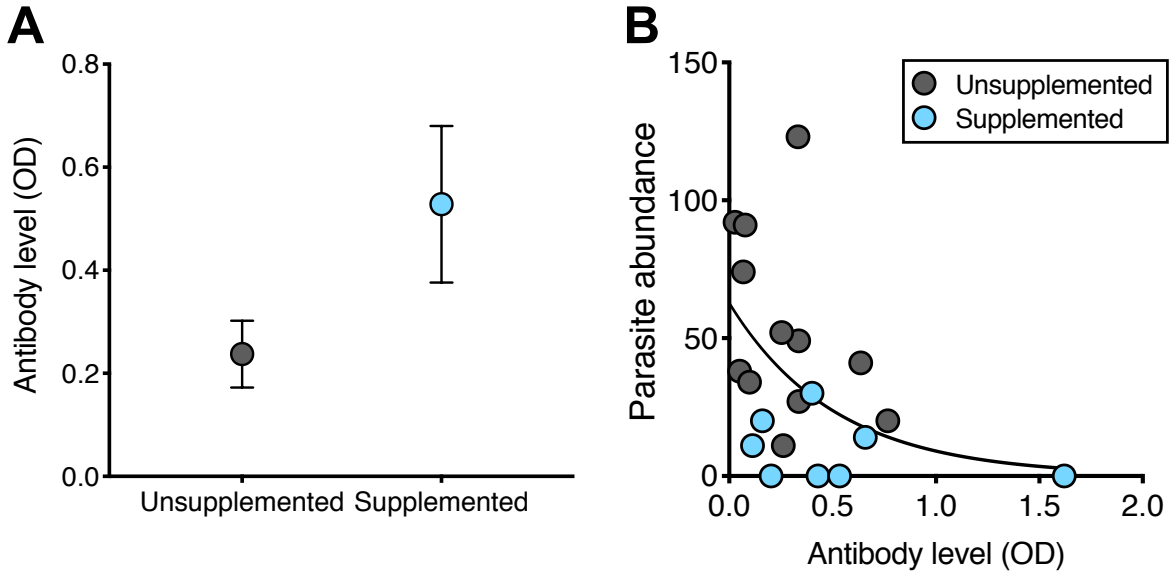
758 Fig. 1.



759

760

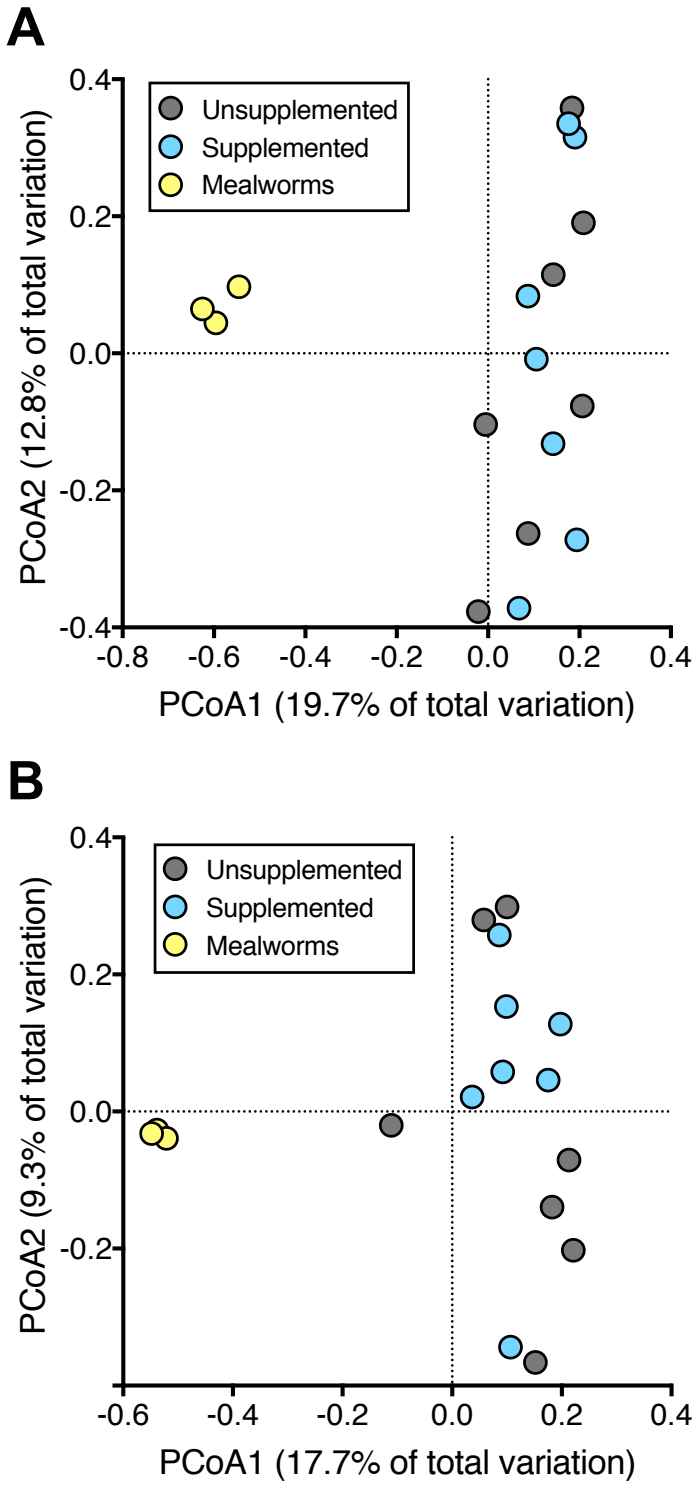
761 Fig. 2.



762

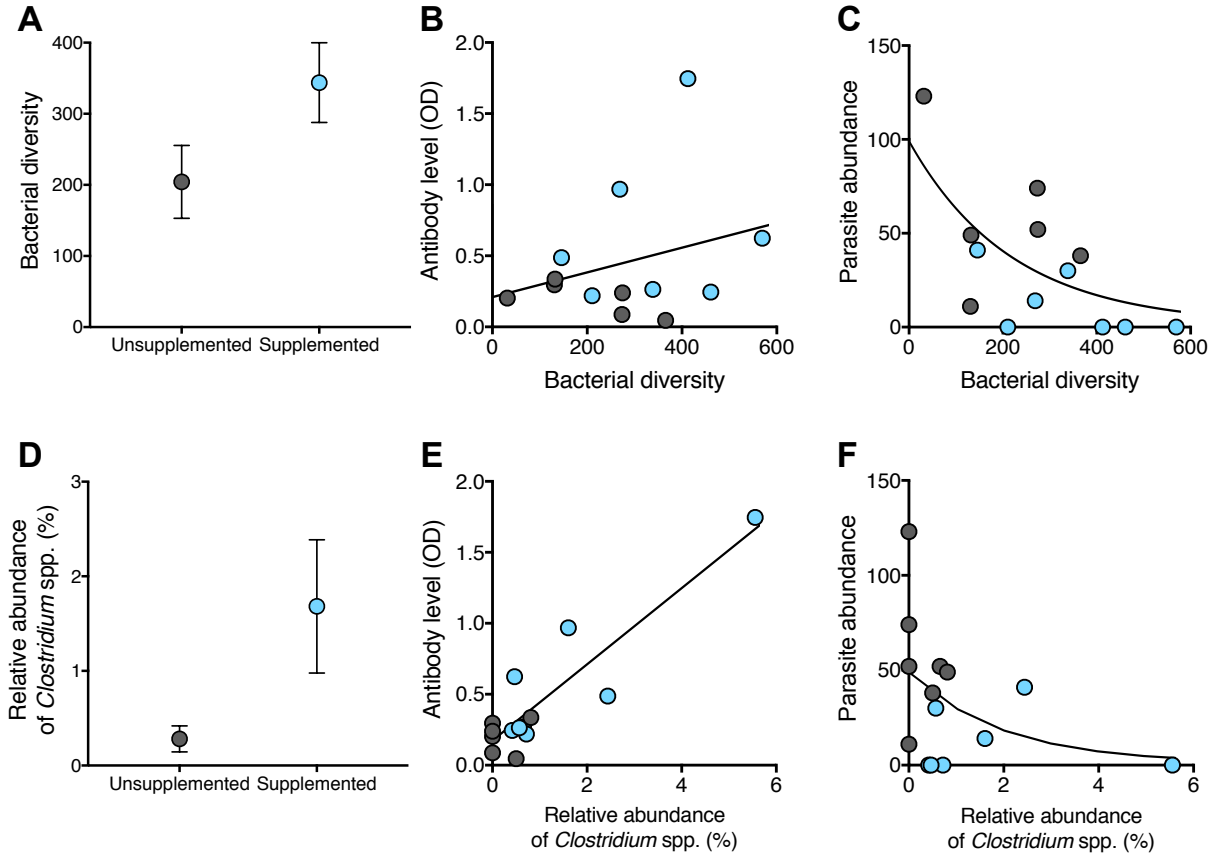
763

764 Fig. 3.


A
Unsupplemented
Supplemented
Mealworms
PCoA2 (12.8% of total variation)
PCoA1 (19.7% of total variation)
0.4
0.2
0.0
-0.2
-0.4
-0.8
-0.6
-0.4
-0.2
0.0
0.2
0.4
B
Unsupplemented
Supplemented
Mealworms
PCoA2 (9.3% of total variation)
PCoA1 (17.7% of total variation)
0.4
0.2
0.0
-0.2
-0.6
-0.4
-0.2
0.0
0.2
0.4

765

Fig. 4.


A
Bacterial diversity
400
300
200
100
0
Unsupplemented
Supplemented
B
Antibody level (OD)
2.0
1.5
1.0
0.5
0.0
0
200
400
600
Bacterial diversity
C
Parasite abundance
150
100
50
0
0
200
400
600
Bacterial diversity
D
Relative abundance of *Clostridium* spp. (%)
3
2
1
0
Unsupplemented
Supplemented
E
Antibody level (OD)
2.0
1.5
1.0
0.5
0.0
0
2
4
6
Relative abundance of *Clostridium* spp. (%)
F
Parasite abundance
150
100
50
0
0
2
4
6
Relative abundance of *Clostridium* spp. (%)