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LETTER

Bacteria divert resources from growth for magellanic penguin chicks

Jaime Potti[1]*, Juan Moreno[2], Pablo Yorio[3], Víctor Briones[4], Pablo García-Borboroglu[3], Susana Villar[1] and Cristina Ballesteros[4]

[1]Departamento de Biología Animal, Universidad de Alcalá, E-28871 Alcalá de Henares, Madrid, Spain

[2]Museo Nacional de Ciencias Naturales-CSIC, Departamento de Ecología Evolutiva, J. Gutiérrez Abascal 2, E-28006 Madrid, Spain

[3]CENPAT-CONICET, Bv. Brown s/n, Puerto Madryn, Chubut, Argentina

[4]Departamento de Sanidad Animal, Facultad de Veterinaria, Universidad Complutense, E-28040 Madrid, Spain

**Correspondence: E-mail: jaime.potti@uah.es*

Abstract

The influence of bacteria on the growth of their wild avian hosts is unknown. We tested experimentally whether administration of a wide-spectrum antibiotic (cephalosporine) during early development of magellanic penguin (*Spheniscus magellanicus*) chicks had any effect on their growth rates in the wild. Chicks that were injected in two occasions with cephalosporine grew faster than control untreated chicks. The positive effect of medication on nestling growth disappeared after the treatment ceased, did not alter haematological indices indicative of health status, had no influence on chick survival until near independence and was related to a changed bacterial composition of the faecal microbiota of treated chicks when compared with that from control chicks. These results were similar to those obtained for poultry with antimicrobials promoting growth and chick nutrient assimilation rates. Gram-positive bacilli in the diphtheroid genus *Corynebacterium* are likely candidates to cause decreased growth rates in magellanic penguin chicks.

Keywords

Faecal flora, medication experiment, pathogens, host–symbiont interactions.

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INTRODUCTION

Except for disease outbreaks of bacterial origin, the influence of bacteria on many fitness components of their free-ranging avian hosts has been little explored, with a few recent exceptions (Lombardo *et al.* 1996; Burtt & Ichida 1999; Mills *et al.* 1999). Effects of bacteria on birds are thus mainly known for poultry, where continuous efforts are put in by the industry to minimize losses due to disease of bacterial origin (e.g. Boulianne 2000). Theory predicts that, similar to poultry, magellanic penguins (*Spheniscus magellanicus*) and other highly social avian species may also be good candidates for an important role of bacteria on the health status of individuals, due to the increased opportunities for horizontal transmission in the often highly crowded, fouled settings of their breeding colonies (Ewald 1994; Nuttall 1997). In addition, mode of chick feeding in penguins by regurgitation may also increase the likelihood of vertical transmission of bacterial flora from the parents' to the offspring's gut (Kyle & Kyle 1993) and adult penguins may acquire part of their bacterial communities from seawater that, unlike air, may function as a medium for both transport and growth of microorganisms (Hansen & Olafsen 1999). All these circumstances may provide a workable case for the detection of the roles of bacterial communities on the fitness of their hosts by enhancing bacterial diversity at the population and individual levels.

In wild populations the only studies to date of the effects of bacteria on avian growth have looked for correlations of nestling survival, growth rates and trait asymmetries with abundance scores of bacterial genera in cloacal samples of un-manipulated tree swallows (Lombardo *et al.* 1996; Mills *et al.* 1999). Here we test experimentally whether administration of a wide-spectrum antibiotic during early development to magellanic penguin chicks has any effect on their wild hosts' growth. The influence of some antibiotics as growth-promoters has been well established in the poultry industry (Lancini & Parenti 1982), although whether the conclusions reached in those highly unnatural settings can be applied to natural situations is unknown (Nuttall 1997). By analysing the composition of a part of the bacterial community in the chicks' faecal flora we also examine which

among the genera of bacteria that we detected may be responsible for putative effects on chick growth. Bacteria in the gut may entail positive (e.g. probiotics such as lactobacilli) but also detrimental (e.g. *Salmonella* infections) consequences, depending on equilibrium mediated by, among many other factors, competitive exclusion of intestinal pathogens (Batt *et al.* 1996; Caldwell *et al.* 2000). Life history and host–parasite theories posit that detrimental bacteria may sequester resources from hosts. This can be achieved directly by causing hosts to mount costly immune responses (e.g. Ilmonen *et al.* 2000), thereby slowing growth, or indirectly by competing with beneficial or mutualistic bacteria. Thus, we predicted that the administration of the antibiotic would enhance chick growth either by eliminating detrimental bacteria or by allowing beneficial non-pathogenic bacteria to outcompete pathogenic microbes, at least temporarily.

MATERIALS AND METHODS

The study was carried out in the austral summer of 2000–01 in a rookery of magellanic penguins in Estancia San Lorenzo, Península Valdés, Argentina (42°05′S, 63°51′W). We randomly marked for the study 60 nests, with the restriction that the nest should contain two recently hatched chicks (estimated age 1–3 days; P. Yorio, unpublished data). This was intended to eliminate biases in chick growth rates due to variation among nests in sibling competition for food depending on number of young and brood asynchrony. Chicks were made identifiable for later visits by marking them with adjustable numbered plastic flipper bands. To study chick growth the same researcher measured both chicks at ages 1, 8, 15 and 22 days of nestling age. All analyses herein refer to body measurements and bacterial communities of the largest chick in each brood, which was the unit of comparison among treatments. This is justified on the grounds that the smaller, second-hatched chicks are more likely to die at early and late ages than first-hatched chicks. We measured flipper and foot length with callipers to the nearest 0.5 mm. Chick weight was recorded with a spring balance to the nearest 5 g. As chick growth rates are affected by within-nest competition for food, chick measurement and treatment (see below) were suspended when one chick had died or disappeared between dates of measurement. Until that eventuality, all available measurements for a given chick were used, which accounts for unequal sample sizes (Fig. 1). Although chicks were not measured beyond day 22 of age, we controlled for more long-term effects of our experiment by measuring nest success on days 57–60, which is very close to the age at which most chicks become independent (Williams 1995).

Experimental treatment

To our knowledge, no previous study of this kind has ever been conducted in the wild, hence we were conservative in drug administration and restricted the experiment to two dosages, with a weekly interval to control for the possibility of mortality induced by the experiment. We chose to inject the medicament, because although some cephalosporines

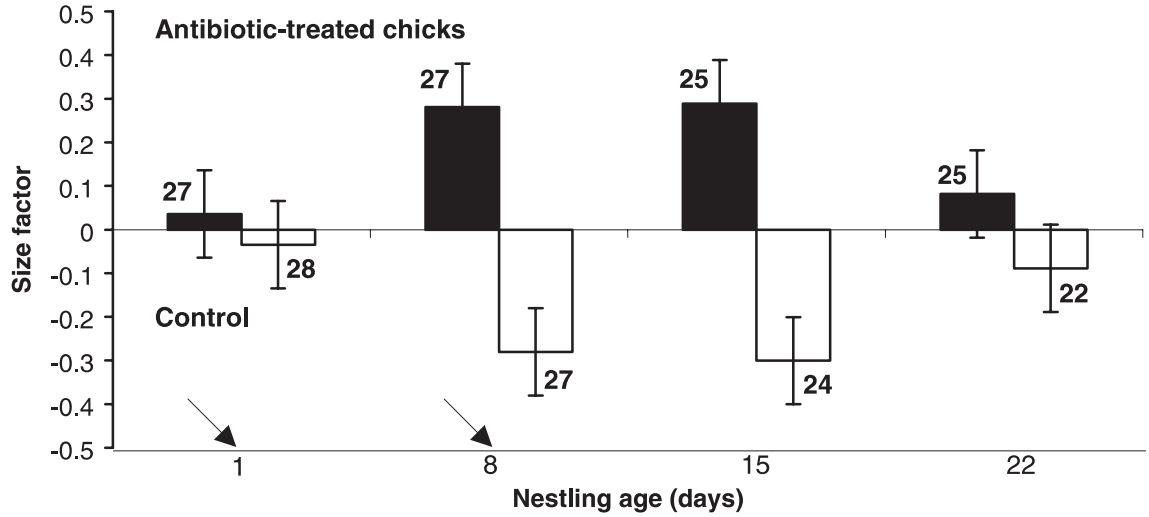

Figure 1 Mean (± 1 SE) body size factor of the largest magellanic penguin chick in two-chick broods at weekly intervals from first (day 1) to last (day 22) measurement in relation to treatment (dark bars: antibiotic-treated chicks; white bars: control chicks). Size factors were separately constructed for each sampling date, hence only between-group comparisons are meaningful. Figures besides bars are numbers of chicks. Arrows indicate dates of ceftiofur administration.

are well assimilated by oral administration, most require intramuscular, subdermical or parenteral administration (García-San Miguel 1988). On day 1 we injected subdermically in the abdomen the heaviest chick in each brood of the group of antibiotic-treated nests (group A hereafter) with 12.5 mg of ceftiofur sodium cephalosporine (commercial name Excenel®; Pharmacia & Upjohn, Kalamazoo, MI, U.S.A.) as sterile powder dissolved in 0.25 mL of injectable apyrogenic water. A sample of other nests, where first-hatched chicks were injected with 0.25 mL of PBS buffer, served as a control group (C hereafter). No signs of increased mortality in A-nests in comparison with C-nests were observed, hence we proceeded with the treatment. For nests where both chicks survived, the same chick in the A- and C-groups that was previously injected was given a further injection of antibiotics or injectable water, respectively, in day 8 of its life. Ceftiofur sodium is a third generation, broad-spectrum semi-synthetic cephalosporine with an excellent bactericidal efficacy against a variety of gram positive, gram negative and anaerobic pathogens of veterinary importance (Brown *et al.* 1991; Salmon & Watts 2000). Its use with young poultry and domestic cattle (Salmon & Watts 2000) has been approved by the Federal Drug Administration of the U.S.A. for treatment of disease associated with *Escherichia coli* (Brown *et al.* 1991).

Chicks' health

We used haematological measurements to evaluate the health state of chicks included in the experiment. On day 22 of chick age, we took a sample of blood from the web patagium of focal chicks in both groups to obtain smears for haematological inspection. Blood was smeared on individually marked slides, air-dried and fixed in absolute ethanol for 5 min and stained with Giemsa pH 7.2 for 30 min. All smears were examined by S.V. at 1000× and the proportions of different types of leucocytes were obtained from examination of 50 fields (Fair *et al.* 1999). As measures of health state, two clinical screening variables, the total white blood cell count (WBC) and the heterophile/lymphocyte (H/L) ratio (Ots *et al.* 1998) were used. High values of H/L indicate poor health.

Bacterial sampling

On day 23 of chick growth we sampled the cloacae of all surviving chicks in both groups (47 chicks, 25 from the A-group and 22 from C-group) with sterile swabs with transport medium. After reception in the laboratory, samples were plated by duplication onto 5% Blood Sheep Agar (BioMérieux, Spain) and incubated aerobically and anaerobically at 37 °C for 48 h. They were also inoculated into peptone broth, incubated aerobically at 37 °C for 16 h (Difco) and then a small aliquot was transferred into selective enrichment broth and/or plates as appropriate for selected bacteria, enterococci, salmonella and *E. coli* as described elsewhere (Goyache *et al.* 2001; Téllez *et al.* 2002). Other bacteria were isolated from blood agar plates following standard bacteriological procedures. Identification was carried out following routine bacteriological tests, plus ad hoc multi-substrate identification systems (API System, BioMérieux, Spain): API 20E, API Coryne, API 50CH, API 20^{a} and API Rapid32Strep. Semi-quantitative estimates, as percentages of abundance for the different bacterial species in cloacae isolates, were used.

Statistical analyses

The effects of antibiotics on the prevalence of the three more common and abundant bacterial genera were tested with chi-squared tests of their frequencies in plates from swabs of first chicks in the C and A groups. Because of significant departures from normal distributions, scores of bacterial abundance and haematological counts were compared between groups with nonparametric statistical tests.

Our three measurements of chick development were highly inter-correlated, hence we used Principal Components Analyses (PCA) to construct a 'size' component at each sampling date (i.e. nestling ages 1, 8, 15 and 22 days) that summarized most of the variance in chick growth (variance explained = 74–80%). Factor scores of individual chicks on these principal components (i.e. the first component in four separate PCA analyses, one for each sampling date) were our point estimates of chick growth. These were subjected to repeated measures ANOVAs in relation to experimental treatment. Statistics are two-tailed. Means are given ± 1 SD.

RESULTS

Bacteria prevalence, diversity and abundance

Our tests were positive for seven genera of gram-positive bacteria, distributed in 15 species. *Staphylococcus, Enterococcus* and *Corynebacterium* were the most frequently found genera (Table 1). Among staphylococci, *Staphylococcus sciuri* was the most prevalent species, being detected in 11 C-chicks and 22 A-chicks; two C-chicks were positive for *Staphylococcus intermedius*, and two and one A-chick for *Staphylococcus lentus* and *Staphylococcus epidermidis* I, respectively. *Enterobacterium, Leuconostoc* and *Actynomices* were not detected in any chick of the A group, while chicks in both groups were positive for *Clostridium*, with a higher frequency in the C group. However, the differences in prevalence of bacterial genera in both groups were not statistically significant (Table 1).

Table 1 Prevalences of seven genera of bacteria in cloacal isolates of magellanic penguin chicks, sorted by experimental treatment. Shown are the proportions of surveyed chicks in whose plates a given genus was scored; *n* is the number of chicks surveyed for bacteria in each group. The Yates-corrected Chi-squared statistic and associated probabilities are also given for genera with prevalence > 0.10.

Genus	Antibiotic-treated $n = 25$	Control $n = 22$	Chi-squared	*P*
Staphylococcus	0.88	0.68	1.69	0.19
Enterococcus	0.66	0.46	0.50	0.48
Corynebacterium	0.80	0.96	1.31	0.25
Clostridium	0.11	0.36	1.59	0.21
Leuconostoc	0.00	0.05		
Actynomices	0.00	0.06		
Enterobacterium	0.00	0.05		

Up to five and four bacterial species were obtained from individual birds in C- and A-chicks, respectively. The mean number of different species of bacteria within individual chicks was greater in the C (3.2 ± 0.9) than in the A group (2.8 ± 0.7), the difference lying just on the threshold of statistical significance ($F_{1,46} = 4.05$, $P = 0.05$). Though small, this difference indicates an effect of treatment on the diversity of cloacal bacteria in penguin chicks as sampled 15 days after last administration.

Table 2 summarizes the differences between scores of bacterial abundance among experimental groups. *Staphylococcus* spp. were more abundant in A-chicks, whereas C-chicks had significantly higher scores of *Corynebacterium*. There were no differences between groups in the abundance scores of either *Enterococcus* or *Clostridium*. Within chicks, there were significant negative correlations between the abundance scores of some genera of bacteria, suggesting that pre-emption by one genus, species or strain may be common. That was the case for the abundance scores of *Staphylococcus* and *Enterococcus* (Spearman rank correlation, $r_s = -0.62$, $n = 46$, $P < 0.0001$), *Staphylococcus* and *Corynebacterium* ($r_s = -0.77$, $n = 46$, $P < 0.0001$) and *Enterococcus* and *Clostridium* ($r_s = -0.37$, $n = 47$, $P = 0.01$). These correlations were of about the same sign and strength when computed separately within the A and C-groups, indicating that the negative relationships between abundance scores were not altered by the experimental treatment.

Table 2 Mean (SD) abundance scores of four different genera of cloacal bacteria in magellanic penguin chicks according to experimental treatment. The χ Mann–Whitney statistic testing for differences between groups and associated probabilities (*P*) are also given.

Genus	Antibiotic-treated	Control	χ	*P*
Staphylococcus	60.4 (32.8)	29.1 (29.8)	2.71	0.01
Enterococcus	19.2 (26.3)	20.7 (26.7)	−0.06	0.95
Corynebacterium	22.0 (17.2)	46.6 (25.9)	−3.33	0.001
Clostridium	0.8 (2.4)	1.6 (3.2)	−0.63	0.52

Chick growth and experimental treatment

As expected from random sampling, chicks in the A and C groups did not differ at the beginning of the experiment in either the size factor ($F_{1,53} = 0.07$, $P = 0.80$; Fig. 1) or any of the individual morphological measurements that were used to construct it (all $P > 0.70$). A global repeated measures ANOVA showed a strong effect of the experiment, as indicated by a significant treatment–age interaction ($F_{3,132} = 3.72$, $P = 0.01$; Fig. 1). The differences in size between groups had disappeared by day 22, i.e. 2 weeks after the last administration of ceftiofur to chicks in the A group (Fig. 1).

Although control nests contained more chicks (0.4 more chicks on average) that attained the age of 60 days than nests in the A group, the difference was not significant (*t*-test, $t = 1.63$, d.f. = 24, $P = 0.12$), indicating that the experiment did not affect chick viability until this age.

Chicks' health state

There were no differences between the A and C-chicks in any of the haematological variables we measured, and neither did the two types of chicks differ in the H/L ratio (Kruskal–Wallis ANOVAs, all $P > 0.10$). The variance in WBC within the A-group was significantly greater than in the C-group (*F* for variances = 3.66, $P = 0.003$), due to three chicks displaying much higher WBC counts than the rest of individuals in this group.

DISCUSSION

Magellanic penguin chicks that were treated in two occasions with a wide-spectrum antibiotic grew faster than control untreated chicks. The effect of cephalosporine on nestling growth disappeared after the treatment ceased and was related to a changed bacterial composition of the faecal microbiota of treated chicks, when compared with control chicks, as sampled 15 days after last administration of the medicament. Control chicks presented more species of faecal bacteria than antibiotic-treated chicks and the abundance scores of several gram-positive genera also differed between groups. To our knowledge, this is the first experimental study showing that growth of an avian species in the wild may be related to the action of an

antimicrobial that did not seem to cause long-term secondary effects on chicks. Hence, although no previous information on a role of the cephalosporine as growth-promoter existed, and the mode of administration (parenteral vs. oral) differed from the standard used in animal husbandry, our results were similar to those obtained with poultry using antimicrobials such as avilamycine and avoparcine (Lancini & Parenti 1982; Greko 1999), substances that promote chick growth and chick nutrient assimilation rates by inhibiting intestinal bacteria that produce toxins, cause disease, even if asymptomatic, or destroy and sequester proteins and other essential nutrients in the diet. It is not known whether diminished body size may impinge on likelihood of survival and recruitment in magellanic penguin chicks. However, judging from what it is already known about the relationship between fledgling size and survival in penguin (Moreno *et al.* 1999) and other avian (Newton 1990) species, this may also be a plausible working hypothesis for magellanic penguin chicks. Were this to be true, in light of our results, bacterial loads might play a role as a selective factor in this species.

Differences in growth between the experimental and control groups were related to changes in the proportional abundances and, to a lower extent, the composition of the gram-positive flora in the normal chick's cloaca. For example, three genera with a low prevalence under normal conditions were unrecorded in treated chicks, *Clostridium* was (non-significantly) more frequent and abundant in the control group, and two genera, *Staphylococcus* and *Corynebacterium* reached significantly higher, differential abundance scores according to their hosts' inclusion in the treatment or control group. Overall, there was abundant evidence for pre-emption by just one bacterial genus in cloacal isolates of both treated and control subjects at our level of analysis.

What could be the proximate, physiological causes underlying the differences in growth among experimental chicks? Undoubtedly, the experimental treatment changed the relative proportions of several genera in cloacal isolates. The increased growth rate in treated chicks may have stemmed from elimination of detrimental bacterial strains, proliferation of mutualistic strains, or both. Regulation of the intestinal flora depends on complex interactions between many factors determined by the host environment, e.g. secretion of gastric acid, intestinal motility, biliary and pancreatic secretions, local immunity, structure of the inner gut and mucus layers, as well as diet. Important for hosts are interactions among bacteria, which can involve habitat alteration (e.g. role of pH), substrate depletion and production of bacteriocins inhibiting bacterial growth by some strains (Fons *et al.* 2000).

One problem of interpretation of differences among treated and control subjects is the time elapsed between treatment and data collection 2 weeks after the last drug administration. However, some differences in bacterial identity and abundance existed even after this time lag, in apparent contrast with the extended view among veterinarians (Dorrestein 1997) that avian bacterial composition of faecal microbiota reflects mainly that from current diet and environment. Magellanic penguins eat mainly fish and squid (Williams 1995), a diet for which no correlates as to bacterial composition have been described (Clark & Kerry 2000). The increased abundance of *Corynebacterium* spp. in C-chicks points to bacteria in this genus putatively having negative effects on the growth of magellanic penguin chicks. However, the literature refers to *Corynebacterium* spp. as to 'normal inhabitants' of the intestinal tract in several avian groups (Dorrestein 1997). It cannot be excluded that this diphtheroid genus, or some of its species/strains, such as the recently described from these samples *Corynebacterium spheniscorum* (Goyache *et al.* 2002), appear in normal proportions but conserve their well known potential for pathogenicity, even if asymptomatic. *Staphylococcus* spp. also are considered as normal inhabitants of the avian intestinal tract (Dorrestein 1997). The high prevalence of this genus in A-chicks may be most likely interpreted as due to a decrease in the abundance of other bacteria rather than a real increase of *Staphylococcus* spp.

Further research on the roles that intestinal *Corynebacterium* and *Staphylococcus* play on the growth of this and other avian species is required to confirm our findings. Alternative, related ways to test hypotheses on these effects may make use of inoculates (e.g. Kyle & Kyle 1993) or probiotics, microbial dietary supplements that beneficially affect the host through its effects in the intestinal tract (Guillot 1998). For example, bacteria with antagonistic effects against pathogens have been successfully administered to several fish species, resulting in decreased mortality or increased growth rate (Hansen & Olafsen 1999). Attention should also be paid to costs and benefits of particular interactions varying with a number of ecological and life-history factors and thus causing conditional outcomes (Bronstein 1994). One of the possible outcomes is adverse effects with local and systemic consequences, i.e. parasitism or disease. The present work has offered but a glimpse of the complexity of a part of the bacterial communities in the intestinal tract of their free-ranging avian hosts, and direct confirmation on what presumably are but a few of their costly effects in the wild.

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