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Avian gut microbiota and behavioral studies

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As many wild birds move long distance with the seasons and show very distinctive behaviors during the mating and molting seasons, birds are one of the ideal taxa to study the relationship between microorganisms and host animals. Recently, inferring from the various molecular tools, it has been suggested that microbes play an important role in avian host behaviors. Many studies focus on the avian microbiota and its effects on animals in both genetic and environmental aspects. In this paper, research areas related to the gut microbiota and behavioral studies in wild birds are introduced. Here, I focus on ‘mating’, ‘recognition’ and ‘molt-fast’ behaviors and discuss possible mechanisms on how such behaviors are affected by the gut microbes. In addition, I report my observations on different colors and structures of Antarctic penguins (Gentoo penguin *Pygoscelis papua* and Chinstrap penguin *Pygoscelis antarctica*) feces between the periods (green and white during at molting and light red with undigested krill at the feeding period), which implies the possible changes of fecal microbiota by molt-fast.

key words: Avian gut microbiota, Behavior, Fasting, Recognition

Introduction

Although microorganisms are organisms too small to be seen, except with the aid of a microscope, they have built a relationship with host animals and adapted to the various environments in distinctive forms (McFall-Ngai *et al.* 2013). They live on most of the surface of animal’s body, including the skin, respiratory organs, digestive and secretory organs and influence animals, their hosts (Gilbert *et al.* 2012). While many biological studies have been mostly conducted on pathogenic bacteria related to diseases, the studies on how microorganisms build a relationship with animals and how they influence each other is recently on the rise from the viewpoint of ethology where animals are hosts of microorganisms (Archie and Theis 2011; Lewis and Lizé 2015; Yuan *et al.* 2015). For example, microbes play an important role when animals recognize one another by odor (Theis *et al.* 2013) or when they look for mates (Sharon *et al.* 2010). The change in microbial community composition on the surface of an egg also plays a role behind incubation behavior when birds incubate their eggs (Cook *et al.* 2005; Shawkey *et al.* 2009; Lee *et al.* 2014). Many studies had used conventional culture methods where microorganisms are inoculated into a medium. However, with these methods, less than one percent of microbial species

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can be identified (Amann *et al.* 1995). Indeed, when identifying coliform bacteria of penguin stomach the cultured species were very few and limited (Thouzeau *et al.* 2003a). As genetic analyses, such as the Next Generation Sequencing (NGS), have become popular these days, even the uncultured microorganisms have become identifiable at once (Mardis 2008).

As many wild birds move long distance with the seasons and show very distinctive behaviors during the mating and molting seasons, they are one of the most appropriate taxa to understand the correlation between microorganisms and their host and the adaptation of the host according to the environmental changes. Most studies on the behavior of microorganisms and their host have been conducted with poultry and mammals in laboratory conditions. However, studies on the function of the avian gut microbiota have started to emerge these days both in domestic and wild birds with various populations from vultures in mid-latitudes to penguins in Antarctica (see Table 1). The number of the studies not only on the interspecies differences but also on the diversity and genetic effects of microorganisms is now on the rise. It is known that various microorganisms colonize animal guts and play an important role in the immune function (Round and Mazmanian 2009) and fat metabolism (Bäckhed *et al.* 2004) of their host. Therefore, an understanding of how microorganisms and their host influence each other in terms of the environmental changes is important to confirm the evolution of behaviors and

Table 1. Previous studies on the avian gut microbiota and its effects on the host animals.

Reference	Study species	Effects of avian gut microbiota
Vela <i>et al.</i> (2015)	Eurasian griffon vulture	Geographical and anatomical differences of cultivable bacterial diversity
Dewar <i>et al.</i> (2014a)	Little penguin, King penguin	Molt-induced bacterial changes
Dewar <i>et al.</i> (2014b)	Procellariiform species (short-tailed shearwater, common diving petrel and fairy prion)	Differences between oil-producing and non-oil-producing species
Dewar <i>et al.</i> (2013)	King, Gentoo, Macaroni, Little penguin	Interspecific differences
Waite and Taylor (2014)	Literature review	Species-specific similarity
Hird <i>et al.</i> (2014)	Brown-headed cowbird	Diversity and environmental influences
van Dongen <i>et al.</i> (2013)	Black-legged kittiwake	More diverse microbiota in chicks compared to adults
Kohl (2012)	Literature review	Discussions of the diversity and functions of the avian gut microbiota
Lize <i>et al.</i> (2012)	Literature review	Discussions of the possible roles of the gut microbiota in kin recognition
Archie and Theis (2011)	Literature review	Discussions of microbial studies with animal behavior
Banks <i>et al.</i> (2009)	Adelie penguin	Genetic inheritance
Ley <i>et al.</i> (2008)	Literature review	Discussions about evolution of the vertebrate gut microbiota
Thouzeau <i>et al.</i> (2003a)	King penguin	Molt-induced stomach bacterial changes
Muramatsu <i>et al.</i> (1993)	Domestic chicken	Energy metabolic changes with the gut microbiota

the adaptation of animals with regard to microorganisms (Carey and Duddleston 2014). Here, I aim to discuss the research fields related to behaviors of the avian gut microbiota and their host, wild birds (i.e. mating behavior, recognition, and fasting during the molting period). Furthermore, I report the possibility of changes in the avian gut microbiota during the molting and chick-feeding periods by observing the guano of penguins that breed in the Antarctic.

Mating and recognition

The microbial transmission can easily occur through a sexual contact in the process of mating behaviors of animals (Lockhart *et al.* 1996). As the genitals of birds, in particular, do not protrude, they come in contact with the cloaca in the process of mating. Thus, bacterial infection may occur during copulation and pathogenic bacteria can spread across the individuals (Sheldon 1993). Sexual and social behaviors have been hypothesized that these behaviors would increase the sexual transmission of bacteria between mates (Kulkarni and Heeb, 2007). As expected, bacterial transmission appeared to occur during copulation in the wild black-legged kittiwake (*Rissa tridactyla*) (White *et al.* 2010) and the bacterial diversity and community variability were higher in polyandrous females than that in monandrous females (White *et al.* 2011) in common lizards (*Zootoca vivipara*). Especially, recent findings suggest that the gut microbiota may play a role in mate preferences (Sharon *et al.* 2010) and this implies that microorganisms may influence even the speciation as a result. As the bacterial transmission caused by mating behaviors can cause disease infections and relate to the health condition of a host, this transmission may influence the mating behavior and sexual selection.

Recent studies reported that the gut microbiota may play a role in the recognition of animals (Lizé *et al.* 2013, 2014). This means that gut microbiota may be an important clue to which signs animals use to recognize each other, in connection with the foregoing discussion that it may influence animal mating behavior and mate choice. Especially, olfactory signals made by microorganisms (i.e. a transmission by an odor of symbiotic bacteria in glands; Thesis *et al.* 2013; Leclaire *et al.* 2014) can be significantly used for the communication among mammals, as well as insects having a keen sense of smell (Lizé *et al.* 2013). Although there have been several studies on the bio-chemical signaling, called ‘Gut-Brain axis’, which investigated how gut microbiota is transmitted to the brain and influences the behaviors by performing an experimental approach with mammals (Cryan and O’Mahony, 2011; Foster and Neufeld 2013), how the gut microbiota can influence the recognition behaviors of birds has yet to be determined. However, considering the research findings that birds also recognize one another through their olfactory signals (Bonadonna *et al.* 2007), it is still worth investigating the function of the gut microbiota.

Fasting

1. Fasting and energy metabolism

Mammals hibernating in winter are in the condition of no external nutrition supply for a long time. During hibernation, certain bacterial taxa decrease and the community composition changes (Dill-McFarland

et al. 2014). According to the result of a fasting experiment using experimental rats, the gut microbiota began to dramatically change after 24 hours since an interruption of food supply (Crawford *et al.* 2009). Compared to gnotobiotic mice, mice with the gut microbiota had a high expression of Peroxisome proliferator-activated receptor alpha (Ppara), a nuclear receptor transcription factor required for fatty acid metabolism after fasting and, consequently, they acquired energy through an increase in ketone body metabolism using internal body fat (Crawford *et al.* 2009). Through these results, it was revealed that the gut microbiota can contribute to the energy metabolism of its host, the mammals, during an interruption of nutrition supply.

According to the previous studies using hen for livestock, it has been hypothesized that the gut microbiota causes a change of energy deposition and heat production of its host, hen, when the nutrition supply is interrupted; then, their energy metabolism is changed, consequently leading to the reduction in energy loss of the host during the period of no food supply (Muramatsu *et al.* 1988, 1994).

For wild birds, penguins from the order of Sphenisciformes are known to have fasting behavior. As many marine birds including penguins take their feed in the sea and breed on land, they perform a spontaneous fast with no food supply during the incubation period (breeding season) and the molting period (before their migration). Therefore, it is important for their survival to consume the accumulated body fat and protein during this spontaneous fasting period with no nutrition supply and to maintain their body condition until they re-start their feeding activity. For example, a research study that compared the retinol (vitamin A) levels of Rockhopper penguin (*Eudyptes crestatus*) and Magellanic penguin (*Spheniscus magellanicus*) before and after molting, observed the increase of vitamin A levels in the birds' liver tissues that are believed to be related to fatty acids caused by their fasting after molting (Ghebremeskel and Williams 1989). Thus, how and by what physiological reaction birds endure and adapt to the condition of having no nutrition supply during the molting period implies important ecological and physiological questions (see Groscolas and Robin 2001 for a review).

2. Changes in the gut microbiota during the fasting period and functional contribution of the gut microbiota to its host animal

As molting is required to consume much energy in the process of making new feathers, it belongs to an extremely stressful physiological stage (Murphy 1996). The findings of previous studies on hens for livestock suggest that artificially induced molt fasting causes the proliferation of their *Salmonella enteritidis* and further inflammation (Holt and Porter 1992). Therefore, during this period, animals are required to keep their bodies in a healthy condition until they begin feeding activity after the end of molting. These facts show that it would help animals later to keep their energy metabolism efficient during this period. Several studies put forward a hypothesis the host birds have symbiotic relationships with microorganisms in their digestive organs to perform an antimicrobial activity (Dearing *et al.* 2005; Kohl 2012). Hoatzins (*Opisthocomus hoazin*) use bacteria in their crop to reduce toxicity of saponin after they intake a plant containing a toxic material, saponin (Garcia-Amado *et al.* 2007). Similarly, male King penguins have a feature of preserving the stored food in their stomachs by secreting spheniscins, which performs antimicrobial activity during a three-week incubation fasting period

(Thouzeau *et al.* 2003ab). Thus, the avian gut microbiota may be helpful to resolve health problems that occurred during a fasting period, through the decomposition of harmful materials in the body. Meanwhile, the gut microbiota is closely related to the dietary nutrition and fat metabolism of its host (Cani *et al.* 2008; Murphy *et al.* 2010; Leser and Mølbak 2009). Therefore, it is expected that the gut microbiota may be also related to the energy optimization of its host in preparation for the interruption of nutrition supply during the molting period (Tremaroli *et al.* 2010).

When observing Gentoo penguin (*Pygoscelis papua*) and Chinstrap penguin (*Pygoscelis antarctica*) breeding in the same region of King George Island alongside the Antarctic Peninsula, some differences in the color and appearance of their feces were confirmed during the molting period (see Fig. 1). During the molting period of 2-3 weeks both species did not perform feeding activities and stayed on land excreting green feces (Fig. 1A and 2A). Meanwhile, during the period when they feed chicks, they produced light red feces in which some shells of Antarctic krill (*Euphausia superba*), their main food source, were observed (Fig. 1B and 2B). Bird dropping consists of white opaque liquid urates, clear urine, and feces where the remained food after digestion in the large intestine and microorganisms are lumped and its green color originates in the bile (Denbow 2015). Even though further DNA analysis of feces microorganisms is needed, it is expected, based on these observation results including the feces color change due to the bile, that the supply of krill, penguins' main food source, was interrupted, since they did not perform any feeding activity during the molting period and there was a change in the gut environment of the host animal (e.g. pH scale). As this gut environmental change provides the conditions under which microorganisms preferring the changed environment may increase, the gut microbiota composition can be changed (e.g. dominance and bile-tolerance of *Lactobacillus* at

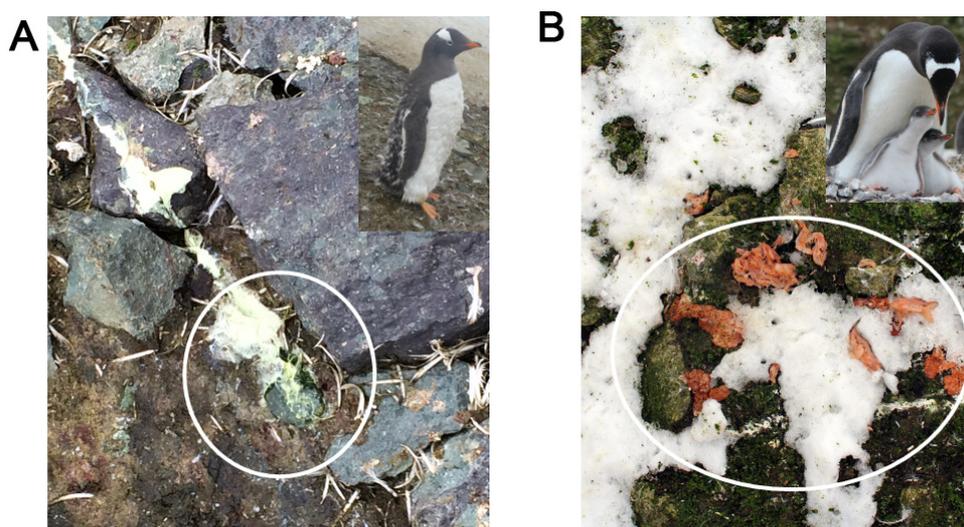


Fig. 1. Examples of Gentoo penguin phenotypes (upper right) and fresh feces (white circle) on the ground at molting stage (A) and at chick-feeding (B) on King George Island, Antarctica (photographs by Jin-Woo Jung, January and February 2014).

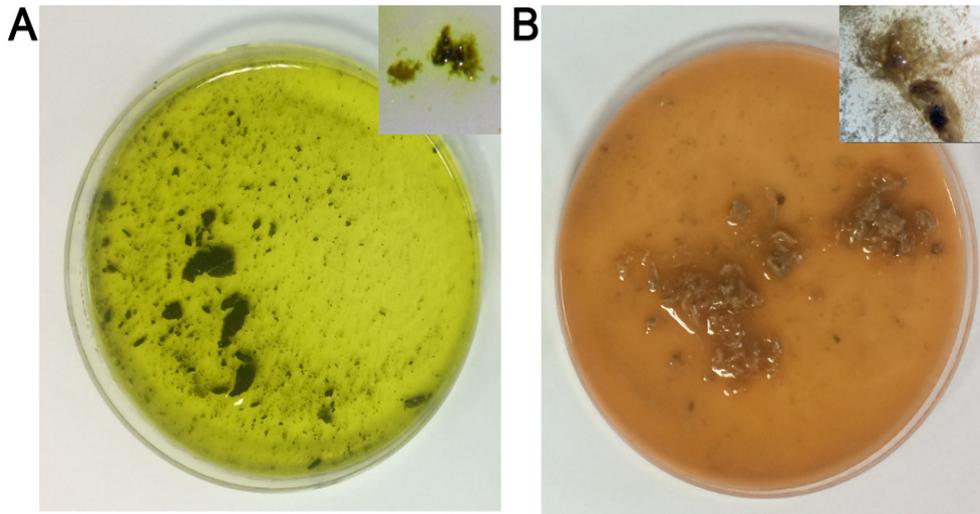


Fig. 2. Gentoo penguin feces and microscopic pictures (upper right; $\times 7.8$, Leica 205C) sampled from cloaca in 99% ethanol solution at molting stage (A) and at chick-feeding (B) on King George Island, Antarctica (photographs by Won Young Lee, February 2015). The feces showed different colors and compositions: green and white at molting (A) and light red containing undigested krill at feeding (B).

low pH; Jin *et al.* 1988). The hypothesis that the gut microbiota of birds and mammals in the Antarctic is related to the prey of its host has existed even in early microbial studies with culture (Soucek and Mushin 1970). Among recent studies, Dewar *et al.* (2014a) confirmed that the gut microbiota has changed in the process of molting of King penguin (*Aptenodytes patagonicus*) and Little penguin (*Eudyptula minor*), using qPCR (quantitative polymerase chain reaction) and pyrosequencing. They identified butyrate-producing microorganisms in both species (Fusobacteria and Clostridia in Little penguins; Psychrobacter in King penguins). Butyrate is known to enhance the body fat accumulation and the immune function of bird hosts (Panda *et al.* 2009). Butyrate-producing bacteria were observed to be dominant over the gut microbiota before the molting of King penguins and Little penguins. In this context, we can expect that the gut microbiota may be helpful to increase the survival rate of penguins by enhancing their fat accumulation before the beginning of the molting period. Similar to the example of King and Little penguins, the relationship between the gut microbiota and fasting of Gentoo and Chinstrap penguins during the molting period may be confirmed if the composition and functions of the gut microbiota are analyzed.

Future studies

Previous studies usually compared the similarity of the gut microbiota either on the species level (e.g. Dewar *et al.* 2013, 2014b; Waite and Talyor 2014) or based on the geographical distance on the population level (Banks *et al.* 2009, 2014). However, there is a limit to give out more ecological questions unless the sampling at the individual level is done. A recent study on Black-legged Kittiwake

(*Rissa tridactyla*) confirmed, through a comparison at the individual level, that there is a difference in the gut microbiota between adult and chick groups and especially that chicks show a higher diversity (van Dongen *et al.* 2013). This finding implies that various microorganisms temporarily appear in the guts of young animals and this condition gradually reaches a stable stage as they grow up to become adults.

Then, how can the gut microbiota appear in the clean gut of just hatched chicks? For mammals, the fetuses of mammals are influenced by their mothers both directly and indirectly. After birth, they absorb microorganisms from the surrounding environments or from breast-feeding, causing the formation of the gut microbiota (reviewed in Fuller 1989; Holzapfel *et al.* 1998). However, for birds, they are apart from their mothers before hatching. After hatching, chicks are fed with prey, not mother's milk, by receiving prey from their parents (in the case of altricial birds) or by searching it themselves (in the case of precocial birds). It has yet to be determined whether the avian gut microbiota is transmitted by parents or prey or whether microorganisms from both parents and prey simultaneously influence. To find an answer to this question with altricial bird species which receive prey from their parents, a further study is necessary to investigate the correlation between the gut microbiota and the crop and stomach of mother birds providing prey they once swallow to their chicks. In addition, a comparison of altricial bird species with precocial bird species which begin feeding activity themselves right after hatching may be a good research subject.

It is not yet clear whether the avian gut microbiota is influenced mainly by innate factors or by acquired environmental factors. A study on mammals like mice suggests that both innate factors of host animals and environmental factors influence the avian gut microbiota (Benson *et al.* 2010). On the other hand, a study on monovular and binovular human twins confirms that the similarity of the gut microbiota is not higher in monovular twins (Turnbaugh *et al.* 2009). A comparison of the formation of the avian gut microbiota with that of mammals' can be further investigated in future research. Further research using experimental approaches (e.g. methods to control the factors affecting a microorganisms' colonization and to inoculate/eliminate a specific colonization into/from animals) may be able to clearly identify the influence of a microbial colonization on its host.

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