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The effect of ambient temperature on the gut microbiota diversity and composition of pied flycatcher (*Ficedula hypoleuca*) nestlings

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Vertebrate guts harbor a diverse community of microbes, which have significant effects on their host's body functions. Multiple external and internal factors influence the composition of gut microbiota, but the role of ambient temperature in shaping microbial assemblages is still largely unknown. To shed new light on this topic I explored whether temperature drop changes the diversity and composition of gut microbiota of pied flycatcher (*Ficedula hypoleuca)* nestlings. In addition to my main interest, temperature effects, I also investigated the role of age, the rearing environment, and genetic background in shaping gut microbiota. The role of environment and genetics was surveyed by conducting a partial cross-fostering experiment for 2 days old nestlings. To examine the temperature effects, the experimental cooling of nest boxes was initiated when nestlings were 7 days old and finished when nestlings were 13 days old. The cloacal swab samples were collected before and after the treatment, and the microbiota was surveyed using 16S rRNA sequencing. The cooling experiment did not influence the diversity or composition of nestlings' gut microbiota and neither did age, even though the relative abundance of Firmicutes increased with age. The rearing environment explained slightly more the variation in gut microbiota than the genetic background. The lack of effect of the cooling treatment on the gut microbiota diversity and composition might be explained by only a minor change in temperature or quite short cooling period. In addition, uncontrolled factors, such as diet, might override the effects of temperature drop.

Key words: Avian gut microbiota, *Ficedula hypoleuca*, temperature effects on microbiota, gut microbiota development, 16S rRNA sequencing

Contents

1 INTRODUCTION

1.1 Effects of gut microbiota on vertebrates

Vertebrates maintain both internal and external microbial communities that contain at least 10 times as much genetic information as the vertebrate host's genome (Colston & Jackson 2016). This entity that includes microorganisms, their genomes and habitat, is called microbiome. Microbiota, in turn, refers more concisely to microorganisms in a certain environment (Marchesi & Ravel 2015). Particularly the guts of vertebrates harbor a diverse community of microbes whose coevolution with vertebrate hosts has had fundamental impacts on the evolution of animal behavior and physiology (Evans et al. 2017). Of those gut microbes especially bacteria have profound effects on their host's body function. They can act as pathogens, but they also have several beneficial impacts on the health and physiological homeostasis of the host organism (Suzuki 2017).

Symbiotic gut microbes facilitate digestion processes, for example, by degrading cellulose and other fibers through fermentation. Especially ruminants have developed very complicated digestive system and their microbial communities involved in energy uptake have been studied extensively due to their importance in agriculture (Suzuki 2017; Youngblut et al. 2019). Microbes also play a key role in detoxification processes, where specialized gut bacteria detoxify secondary compounds or dietary toxins produced by plants. Therefore, toxin-degrading microbes are crucial for herbivorous hosts, which would otherwise absorb high levels of plant-based toxins (Kohl & Dearing 2016; Suzuki 2017).

There is lots of evidence that microbiota is an important part of the function of the immune system as well (Grond et al. 2018). For example, studies have revealed that germ-free mice are more vulnerable to different pathogenic infections than mice with normal microbiota (Kamada et al. 2013b; Uzbay 2019). Commensal gut microbes can directly affect pathogens via competition for nutrients and space or through production of specific toxins, which inhibit the growth of related bacterial species (Kamada et al. 2013a). In addition, host's microbiota can modify the environmental conditions, such as pH, and hence prevent pathogens from colonizing the gut (Shin et al. 2002). Microbes also have some indirect mechanisms for pathogen resistance: they can stimulate their host's immune responses by promoting mucosal barrier function and enhancing the production

of cytokines, which are essential in mediating the immune responses (Kamada et al. 2013a).

During the past decade scientists have become increasingly interested in the links between gut microbiome and behavior (Cryan & Dinan 2012). It has been shown that gut microbiota affects the brain development of mammals as well as the brain function through the gut-brain axis (Cryan & Dinan 2012; Sampson & Mazmanian 2015). The studies conducted on humans and mice have indicated that individuals treated with probiotics (putatively health benefits providing microbes), exhibited less anxiety and depression-like behavior (Bravo et al. 2011; Steenbergen et al. 2015). Moreover, it seems that intestinal microbes can change the odor of a host, which may have effects on animal's mate choice and kin recognition (Lize et al. 2013).

However, the studies on gut microbiota have mainly focused on humans and other mammals, while less attention has been paid to the intestinal microbial communities of non-mammalian species, such as birds (Colston & Jackson 2016). The research on avian gut microbiota has lagged behind that of other vertebrates despite the highly unique life history traits of birds, which may affect the microbial composition of avian gut (Kohl 2012; Waite & Taylor 2015). Although some studies have also been conducted on birds, most of them have been carried out with commercially important species, such as turkeys and broilers (Waite & Taylor 2015). In contrast to poultry and other captive-bred birds, wild birds inhabit a wide range of environments and vary substantially in their physiology and life-history traits (Kohl 2012; Grond et al. 2018), which means that results from poultry studies cannot be directly applied on wild birds. Thus, our knowledge on gut microbiota of non-captive birds in natural habitats remains remarkably poor (Grond et al. 2018).

1.2 What shapes the avian gut microbiota?

The factors that affect the makeup of avian and generally animal gut microbiota can be divided into intrinsic and extrinsic factors (Fig 1.) (Grond et al. 2018; Fan et al. 2021). Those factors are tightly connected, and they have many interaction effects on gut microbial communities. Intrinsic factors are the innate features of a host, such as genetic background and age, whereas extrinsic factors include food resources provided by environment, breeding/nesting environment, and social interactions, for example (Grond et al. 2018).

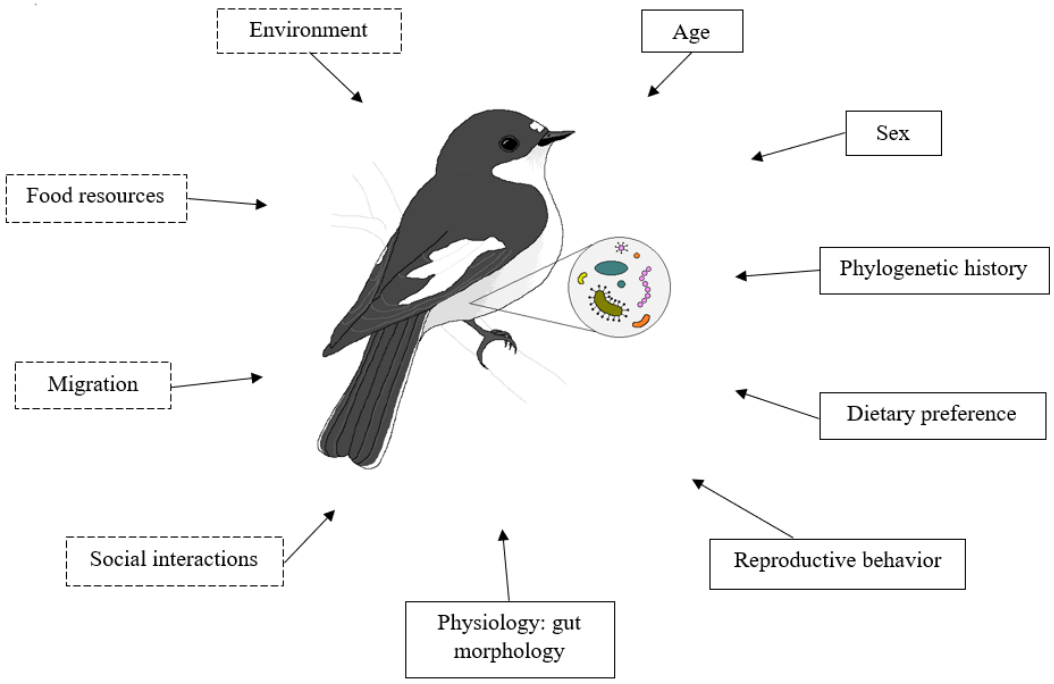

Figure 1. Different factors shaping the avian gut microbiota. Intrinsic factors are represented by solid-lined boxes and extrinsic factors by dash-lined boxes (pied flycatcher and gut microbes drawn by Noora Metsäranta).

However, such a division into intrinsic and extrinsic factors is somewhat artificial, given that some of them, such as diet, can be regarded both as an intrinsic and extrinsic factor depending on the context: an environmental point of view makes diet an extrinsic factor since environment affects the microbial content of food, but diet can be considered an intrinsic factor as well due to species-specific preferences for different food sources (Grond et al. 2018). However, it is crucial to know which and how different factors shape gut microbiota to gain a more comprehensive understanding of microbial ecology and evolution (Hird et al. 2015). In the following two sections I will focus on those intrinsic and extrinsic factors that are most relevant for my study.

1.2.1 Intrinsic factors

One of the intrinsic factors that shapes the animal gut microbiota is dietary preference. For example, the composition of gut microbiota differs between herbivorous and carnivorous birds: a high relative abundance of Bacteroidetes in the gut is typical for herbivores, while Proteobacteria and Firmicutes dominate the carnivore gut (Grond et al. 2018). Bacteroidetes are known for their role in polysaccharide, such as cellulose

degradation (Thomas et al. 2011), which explains their high abundance in herbivore gut (Grond et al. 2018). A quite recent study (Bodawatta et al. 2018) conducted on New Guinean passerine birds indicated, in turn, that the gut of insectivorous species was dominated by Firmicutes, such as *Lactobacillus* and *Enterococcus*. Those lactic acid bacteria are suggested to be an important part of energy uptake from protein-rich food due to their ability to hydrolyze amino acids and reduce harmful by-products resulted from protein hydrolysis (Bodawatta et al. 2018).

The phylogenetic history of host species is another intrinsic factor, which determines the composition of gut microbiota in animals. Thus, closely related species share more similar gut microbiota than distantly related species (Grond et al. 2018). Hird et al. (2015) indicated that factors associated with host taxonomy were the major determinant of gut microbiota of Neotropical birds. Waite and Taylor (2014) gained similar results as they found out that host bird species was the most significant factor shaping microbial communities, even though other determinants, such as diet and sampling site, contributed. The effects of genetic relatedness have also been studied within one species through partial cross-fostering experiments: in great tits (*Parus major*), however, foster siblings raised in a same nest shared more similar gut microbiota than separated biological siblings (Teyssier et al. 2018). The extent to which host phylogeny affects the diversity and composition of microbial communities is still uncertain, but it has been suggested that phylogenetic history shapes more the mammalian gut microbiota due to the vertical transmission of microbes during birth (Colston & Jackson 2016), whereas extrinsic factors have more profound impact on avian microbiota (Lucas & Heeb 2005; Hird et al. 2014; Grond et al. 2018).

In addition to dietary preferences and host phylogeny, age affects the diversity and composition of vertebrate gut microbiota (Grond et al. 2018). Changes in microbiota diversity with age are suggested to result from the function of reproductive hormones (Leclaire et al. 2014) as well as changes in feeding strategies and diet (Godoy-Vitorino et al. 2010; Kohl et al. 2019). In most bird and mammalian species the diversity of gut microbiota is quite low at the early stages of life but increases during development, leading to more stable microbial communities at adulthood (Koenig et al. 2011; Kohl 2012; Kohl et al. 2019). Several studies conducted on wild birds (van Dongen et al. 2013; Kreisinger et al. 2017; Kohl et al. 2019) have indicated that adult birds harbor more diverse bacterial assemblages compared to nestlings. Studies have also provided evidence that microbial community structure changes with bird's age as certain bacterial groups

seem to increase in relative abundance, while others decrease (Teyssier et al. 2018; Kohl et al. 2019).

However, it is still unclear how early birds gain their first microbes and how the gut microbiota develops after first recruitment (Grond et al. 2017). The results on microbial colonization on birds have been contradictory: Some potentially pathogenic bacteria have been cultured from the gastrointestinal tract of domestic chicken (*Gallus gallus domesticus*) embryos (Kizerwetter-Swida & Binek 2008) supporting the hypothesis that the recruitment of gut microbes can initiate before hatching either via transovarian transmission or via penetration through eggshell after laying (Gantois et al. 2009; Martelli & Davis 2012). Conversely, a study performed on dunlins (*Calidris alpina*) and semipalmated sandpipers (*Calidris pusilla*) showed that gut microbial content was only minimal in the non-hatched embryos, and the microbiota became established only after hatching due to environmental exposure (Grond et al. 2017).

There is, even though scarce, some information about sex effects on avian gut microbiota. Lumpkins et al. (2008) noticed that gender affected the composition of gut bacteria in broilers, microbiota being less than 30 % similar between genders. Also some wild bird studies considering sex-driven differences in microbiota exist: northern bobwhite (*Colinus virginianus*) males and females exhibited differences in the abundances of three common bacterial genera, females having more *Enterococcus* in their gut while *Rothia* and *Streptococcus* were more common in males (Su et al. 2014). However, the cloacal microbiota did not differ between sexes in barn swallows (*Hirundo rustica*) (Kreisinger et al. 2015), neither did in dark-eyed junco (*Junco hyemalis*) (Whittaker et al. 2016).

1.2.2 Extrinsic factors

Microorganisms are present everywhere in nature (Gupta et al. 2017) and hence animals are constantly exposed to them. The microbes existing in soil, water, and animals' breeding environments have a potential to colonize animal gut. In addition, contacts with other animals within the same environment increase the chance of exchange of microbes (Grond et al. 2018). Therefore, environment is an extremely important modifier of the animal gut microbiota with its abiotic and biotic factors. It basically includes the other microbiota-altering extrinsic factors as well, such as food resources and social interactions (Grond et al. 2018). Due to the spatial and temporal heterogeneity of environments, their microbial communities are highly diverse. Therefore, animals

encounter a wide range of microbes depending on the location and time (Lewis et al. 2016; Grond et al. 2018).

The studies conducted on birds have shown that environment affects more the avian gut microbiota than intrinsic factors, such as host genetics (Lucas & Heeb 2005; Hird et al. 2014; Waite & Taylor 2015; Lewis et al. 2016; van Veelen et al. 2017). Nevertheless, as mentioned in the previous section, in some Neotropical birds the composition of gut microbiota is better explained by host taxonomy than by extrinsic factors (Hird et al. 2015). However, habitat quality might affect the relative contribution of genetic and environmental variation on microbiome composition: it has been suggested that variable environments can have significant impacts on the microbiota covering simultaneously the genetic influence, while in more homogenous habitats the host genetics may play more important role in shaping gut microbiota (Lewis et al. 2016).

Environment provides food that significantly affects the composition of intestinal microbiome as food contains a wide variety of microorganisms. Diet is strictly associated with location: different geographical areas provide different food resources and hence various microbial groups are ingested with food as well (Grond et al. 2018). There are, however, differences between vertebrate taxa in the extent to which diet determinates the gut microbiota. For instance, evidence suggests that in mammals gut microbiota might reflect more host phylogeny than diet and environment (Colston & Jackson 2016), whereas in birds available food resources seem to be more important modifiers of microbiome (Hird et al. 2014; Colston & Jackson 2016). The significant role of food in shaping avian gut microbiota was observed in three passerine species: the results indicated that the gut microbiota of these species became more similar when individuals spent time at the same stopover site and used similar resources (Lewis et al. 2017). However, researchers emphasized the possibility that similar diet does not exclusively explain the convergence of microbiota, due to the chance that birds' guts go through remodulation during migration as guts restructure to a more similar anatomical, chemical, and physiological environment. Most likely diet and gut remodulation together resulted in changes in avian microbiota during stopover (Lewis et al. 2017).

Social environment has effects on microbial assemblages as well (Grond et al. 2018). Birds exhibit a diversity of social groups as they form groups for breeding, roosting, foraging, migration, and wintering (Bodawatta et al. 2022). These groups expose birds to close physical contact and thus exchanges of gut microbes are possible among

conspecifics (Grond et al. 2018). Social environment, which consists of parents and their offspring, is even more important modifier of the avian gut microbiota. Especially altricial nestlings which stay in the nest for weeks and are strongly dependent on parental care, have higher potential for the parental effect on gut microbiota. Parents expose altricial nestlings to new microbes through their saliva and prey caught for young, for example (Grond et al. 2017, 2018). Furthermore, siblings affect each other's gut microbiota as they are in tight contact via their feathers and feces due to the limited space available during the nestling stage (Whittaker et al. 2016).

Although many of these extrinsic factors that shape animal gut microbiota are quite well-known, the knowledge on the effects of environmental temperature on the microbial communities is relatively poor. This is somewhat surprising given that temperature is known to be one of the most important abiotic factors that affect animal physiology and behavior (Sepulveda & Moeller 2020). Changes in temperature especially affects the physiology of ectotherms as well as the structure and function of their gut microbiota (Fontaine & Kohl 2020). However, evidence on temperature effects on microbiota of vertebrates is generally scarce, and therefore I will elucidate in this study how temperature can affect the gut microbiota of avian nestlings, whose thermoregulation capacity is very limited after hatching (Dawson & Evans 1960; Rodriguez & Barba 2016a). Before focusing on the temperature impacts on gut microbial communities it is reasonable to consider first how temperature affects the physiology and development of birds overall.

1.3 Temperature and avian postembryonic development

Temperature has an important role in the postembryonic development and survival (Dawson et al. 2005; Andreasson et al. 2018). The physiological effects vary with age, depending on the homeothermic abilities of the chick (McCarty & Winkler 1999). Newly hatched altricial chicks have poor thermoregulation capacity (Dawson & Evans 1960; Howell 1964), and therefore they are very dependent on their parents for warmth at the beginning of life (Lynn & Kern 2014). Rise and drop in temperature affect the metabolism in a different way, but both can have positive or negative effects on the nestling growth and survival depending on the temperature change, duration of exposure as well as other environmental factors, for example. Moreover, temperature can either directly impact the nestlings' physiology, or it can have indirect effects on their development via food availability (McCarty & Winkler 1999).

A non-manipulative study conducted by McCarty & Winkler (1999) indicated that ambient temperature affects the body mass of tree swallow (*Tachycineta bicolor*) nestlings, especially the mass of younger chicks: maximum daily temperature correlated positively with the change in nestling body mass, having positive effects on nestling growth. In the older chicks temperature was observed to affect the nestling growth indirectly via insect abundance as well since maximum temperature had a positive impact on insect abundance, which in turn affected positively on the body mass of nestlings (McCarty & Winkler 1999). Dawson et al. (2005), in turn, conducted a heating experiment with tree swallows, where heated nests had 5 ºC higher temperature than control nests. Nestlings in heated nest boxes were heavier at 16 days and they also had longer ninth primary feathers. Moreover, their survival was higher during the nestling phase compared to control chicks.

Due to the undeveloped thermoregulation capacity of altricial nestlings (Dawson & Evans 1960; Howell 1964), unfavorable temperatures expose nestlings to impaired growth and reduced survival (Greno et al. 2008; Rodriguez & Barba 2016a). Ambient temperature that is above optimal thermal range of the species, increases risk of hyperthermia, which is a serious threat for nestling's development and survival (Rodriguez & Barba 2016a). The negative effect of temperature increase was detected in experimental studies in blue tits (*Cyanistes caeruleus*) (Andreasson et al. 2018) and great tits (*Parus major*) (Rodriguez et al. 2016), as their body mass gain was reduced during the heat stress.

The endocrine system has a crucial part in avian thermoregulation as it helps individuals to cope in changing environmental conditions. The most important regulators of thermogenesis are thyroid hormones, which have a key role in physiological cold response in birds (Zheng et al. 2014; Shang et al. 2021). Even though thyroid function is not fully developed in newly hatched altricial nestlings, Shang et al. (2021) showed that only 3-day-old Asian short-toed lark (*Alaudala cheleensis*) chicks were able to increase thyroid hormone secretion to cope with cold conditions. Corticosterone has also been indicated to participate in thermoregulation: in Asian short-toed lark nestlings corticosterone levels correlated negatively with nest temperature, addressing that corticosterone concentrations increased in response to cold conditions (Shang et al. 2021). In birds, corticosterone secretion is known to be a response to a stressful situation, which helps them to cope with challenging conditions. However, increased corticosterone levels usually impair the function of immune system, which can have deleterious effects on survival (Stier et al. 2009).

The effects of cold exposure on nestling growth were investigated in a great tit study (Rodriguez & Barba 2016b), where nest temperatures were experimentally lowered by almost 5 ºC. Rodriguez & Barba (2016b) showed that nestlings in cooled nests had smaller tarsi at the age of 15 days. Since thermoregulation under cold conditions is energetically costly, nestlings were suggested to allocate more resources to body temperature maintenance instead of skeletal growth, which resulted in smaller tarsus length (Rodriguez & Barba 2016b). However, experimental studies trying to find out the effects of cool temperatures on altricial nestling development are scarce (Rodriguez & Barba 2016b), as most of them have been performed during the laying or incubation stage (Nager & Van Noordwijk 1992; Nilsson et al. 2008). Therefore, only little is known so far how cold exposure affects the postembryonic development, not forgetting that consequences of temperature treatments on post-fledging survival are poorly known and yet to be studied as well (Rodriguez & Barba 2016b).

1.4 Temperature and microbiota

There is lots of evidence that temperature changes affect a variety of physiological functions in animals, but only recently scientists have started to show more interest in the temperature effects on animal microbiota. Whether the effects of environmental temperature on microbiota are direct or indirect depends at least partly on the taxonomic group of the host species (Sepulveda & Moeller 2020). Endothermic animals, as mammals and birds, maintain a relatively constant body temperature (Crompton et al. 1978) that is not so strictly dependent on surrounding conditions. Hence, their gut microbiota is not as directly modified by environment as that of ectotherms (Liu et al. 2019). Unfavorable climatic conditions, however, cause many physiological and behavioral responses in endotherms that help them to cope with challenging circumstances. Under cold exposure, for example, non-hibernating endotherms try to minimize heat loss through narrowing of the blood vessels and huddling, while shivering and non-shivering thermogenesis are used to enhance heat production and thus to maintain thermal homeostasis (Wen et al. 2020). In addition, endothermic animals, particularly small mammals, increase their food intake and metabolic rate to maintain metabolic homeostasis as well (Bo et al. 2019).

In endothermic animals, hot and cold exposures cause alterations in gut microbiota via changes in metabolism. A recent study showed that cold exposure and diet-induced obesity together changed the composition of gut microbiota in mice (Wen et al. 2020).

The other study where Shaoxing ducks (*Anas platyrhynchos*) were exposed to high temperature (30–40 °C) for 15 days indicated that heat exposure altered many metabolic pathways in the three parts of gastrointestinal tract as well as taxonomic composition of gut microbiota (Tian et al. 2020). Similar results were obtained in the study conducted with laying hens (Zhu et al. 2019). Heat stress may also contribute to gut colonization by pathogenic bacteria, which can cause serious problems in poultry production (Zhu et al. 2019; Sepulveda & Moeller 2020).

In contrast to endotherms, ectothermic animals, such as amphibians and reptiles, adjust their body temperature to the current environment and therefore temperature changes affect more directly the environment of microbiota by changing conditions in the gut (Fontaine et al. 2018; Sepulveda & Moeller 2020). Fontaine et al. (2018) conducted a study on the red-backed salamanders (*Plethodon cinereus*) and showed that the efficiency of digestive performance and the structure of their gut microbiota were dependent on ambient temperature: when salamanders were exposed to higher temperature (20 ºC), the relative abundance of pathogenic bacteria increased while disease-resistant taxa reduced. Also the study conducted with tadpoles of the northern leopard frog (*Lithobates pipiens*) revealed that microbial community structure in the gut differed significantly between tadpoles exposed to cool and warm conditions (Kohl & Yahn 2016).

It is possible that temperature might have similar direct effects on young altricial birds as well. Although salamanders and frogs are ectothermic animals, and their physiology cannot be directly compared to that of birds, it is noteworthy that the thermoregulation of newly hatched nestlings is not fully developed (Dawson & Evans 1960; Howell 1964). Due to the high body surface-to-mass ratio of young birds and their undeveloped plumage (Dawson & Evans 1960; Jenni & Winkler 2020), nestlings' capability to conserve heat is very limited (Dawson & Evans 1960; Rodriguez & Barba 2016a), and thus their thermoregulation system resembles that of ectotherms (Price & Dzialowski 2018). Nevertheless, the impacts of temperature on the microbiota of young birds might also be more indirect through changes in physiology and behavior.

1.5 Study questions and hypotheses

The objective of this study is to shed new light on the significance of temperature on the early development of avian gut microbiota since studies investigating the effects of ambient temperature have remained overshadowed by the studies focusing on the roles

of other environmental factors. However, it is crucial to explore the effects of changing temperatures as well due to ongoing climate change, for example. Climate change is known to cause significant global climatic and environmental changes, of which temperature rises can already be detected. Knowledge on the impacts of temperature changes on animal microbiota will help us to understand and even predict how different organisms will respond to climate change (Sepulveda & Moeller 2020).

Since extreme weather conditions, such as sudden drops and rises in temperatures, will become more common due to climate change (Wuebbles et al. 2014; Turner et al. 2020), I will examine whether sudden temperature drop affects the diversity and composition of the gut microbiota of pied flycatcher (*Ficedula hypoleuca*) nestlings. In this study the temperature of the half of the inhabited nest boxes was experimentally lowered for 7 days (treatment group), while the temperature of the other half of the nest boxes was not manipulated (control group). To find out the effects of temperature drop on nestlings' gut microbiota, cloacal swabs were collected both before and after the cooling experiment. My assumption is that a decrease in temperature will change the microbial composition and diversity of nestlings' gut microbiota. The most dominant bacteria taxa in wild birds are Firmicutes, Proteobacteria, Bacteroidetes, and Actinobacteria (Waite & Taylor 2015; Grond et al. 2018), and I expect to see changes in the relative abundances of these phyla after the temperature treatment. Since the results of previous microbiota studies have been controversial, that is, the dominant bacteria taxa after temperature manipulation have varied significantly between studies, I do not make directional predictions of the dominant and minor bacterial groups. It is worth mentioning that even though microbes include many other taxa as well, I will only focus on the bacterial taxa.

A further general question concerns the relative significance of environmental (in broad sense, beyond specific factors like temperature) and genetic factors in shaping the nestling's gut microbiota. Some previous studies have shed light on the effects of genetic factors and rearing environment on the development of gut microbiota of passerine nestlings. Teyssier et al. (2018) conducted a cross-fostering experiment with great tit nestlings and showed that foster siblings raised in the same nest shared more similar gut microbiota than biological siblings raised separately. Similar results were reported by Lucas & Heeb (2005) in great tit and blue tit broods. Thus, I predict, based on the studies of Lucas & Heeb (2005) and Teyssier et al. (2018), that the impacts of rearing environment will override the effects of genetic background on the composition of nestlings' gut microbiota. In other words, I predict that foster siblings of pied flycatcher

raised in the same nest will share more similar gut microbiota than biological siblings raised in separate nests.

In addition, I am interested in the age-associated impacts on the nestling gut microbiota: Does the composition of nestling's gut microbiota change with age? Since bacterial assemblages are highly dynamic and transient at the early stages of life, I predict that I will detect changes in relative abundances of different bacterial phyla during the nestling stage. Teyssier et al. (2018) and Kohl et al. (2019) showed in their wild bird studies that relative abundance of Firmicutes increased with age, whereas Proteobacteria decreased. Therefore, based on these quite recent studies, I assume that I will detect similar changes in these two bacterial phyla.

2 MATERIALS AND METHODS

2.1 Species and study area

The study species was pied flycatcher, a small secondary cavity nesting bird. It is a migratory species, which spends only its breeding time in the North. The first individuals arrive in Finland at the end of April, and the start of laying usually occurs by the turn of May/June (Lundberg & Alatalo 1992). This insectivorous bird was chosen for study species for many reasons: its ecology is well-known, it is a common species, and it accepts, even prefers, nest boxes as nesting site very easily. Additionally, they are quite tolerant of handling.

The study was conducted in the island of Ruissalo (60°26'N, 22°10'E), in the archipelago of southwestern Finland in Turku. Ruissalo is an environmentally unique and largely protected area with its rich deciduous and mixed forests that are inhabited by multiple species from different taxa. The island is known for its widest oak forests in Finland, due to which many threatened species dependent on oaks, such as beetles and fungi, are found in Ruissalo (Karhu et al. 1995).

The study site was divided into smaller plots that covered almost the whole Ruissalo island. The area contained approximately 300 nest boxes, hung at the height of 1,5 meters. The boxes are mainly inhabited by passerine species, such as pied flycatcher, great tit, and blue tit. Those nest boxes are maintained by researchers of Turku University and used in collecting data on bird populations. The experiments were conducted under license from the Animal Experiment Board of the Administrative Agency of South Finland (ESAVI/5718/2019) and South-Western Finland Centre for Economic Development, Transport and Environment (VARELY 924/2019).

2.2 Cross-fostering

All the experiments and data collection were conducted with the help of my supervisor Suvi Ruuskanen and her research group in summer 2019. Before initiating any experiments, we monitored the state of nest building, incubation, and hatching so that we could plan the timing of cross-fostering and cooling treatment. When the nestlings were 2 days old, we carried out a partial cross-fostering experiment between pied flycatcher nests by taking the nestlings to the "foster nests" to examine the effect of the rearing environment and genetic background (and prenatal maternal effects) on their gut

microbiota. Our aim was to exchange as same-aged nestlings as possible to minimize the potential competitive advantage of bigger nestlings. 54 nest boxes were involved in this cross-fostering experiment and each of them had 2–4 foster chicks in addition to their own chicks. In those nests that had four foster chicks the foster nestlings were from two different nests.

Since we could not ring 2-day-old nestlings due to their tiny size, but it was necessary to identify the nestlings, we had to create individual nail codes for the chicks. We created the nail codes by cutting the tip of the nail and, naturally, cut the different nail (or nails) from different chicks to enable later identification.

Before cross-fostering experiment, also hormone manipulations with thyroid hormones were performed on pied flycatcher eggs for the purpose of another study (not discussed here).

2.3 Temperature manipulation

My main interest in this study is to examine the effect of temperature on the diversity and composition of gut microbiota of pied flycatcher nestlings. To examine the impact of temperature, we performed a cooling treatment by using frozen freezer blocks of 500 grams. The number of successful pied flycatcher nests in the study area was 59, every nest including 2–8 fledglings. The cooling treatment was performed for 30 boxes, while the remaining 29 boxes served as control group. Nest boxes were assigned randomly to either treatment or control group, but in such a way that both treatment and control boxes were established during the same day to ensure their even distribution throughout the whole breeding season. We prepared fabric bags that we stapled to the outer walls of the nest boxes, after which we wrapped the freezer blocks with insulating cell foam and put them inside the fabric bags (Fig 2). The cell foam was used to slow down the melting of the freezer blocks. We set unfrozen blocks to control nest boxes to make sure that nestlings in the control group were exposed to the same amount of disturbance as the nestlings in the treatment group. For temperature monitoring we taped a temperature logging iButton to the inner side of the back wall above the nest rim. IButtons measured the inner temperature every third minute.

The cooling effect of the freezer blocks lasted until afternoon, and the blocks were replaced with colder ones every morning. However, we did not change the blocks for the control nests after the beginning of the experiment, but we visited control nest boxes daily

as well and shook the blocks to mimic the exchange situation. We initiated the cooling treatment on 7-day-old nestlings, and finished it after a week, when the nestlings were 13 days old.

Before the start of the experiment, the effect of freezer blocks on nest box temperature was tested in a pilot test at University of Turku to make sure that they do cool down boxes, and thus they could be used in scientific research. Based on the results of the pilot test, the expected decrease in temperature was approximately 2–4 degrees Celsius, depending on the time of day and the temperature outside the nest boxes.

Figure 2. Nest box in cold treatment experiment in Ruissalo.

2.4 Data collection

To study the gut microbiota, we collected cloacal swab samples during June and July of 2019. The first cloacal samples were collected from 7-day-old nestlings before the initiation of temperature treatment, whereas the second sampling was performed after the cooling treatment, when the nestlings were 13 days old. The swab samples were taken from all those nestlings, which were big enough for sampling. The cloaca of a nestling

was sampled with a cotton swab, which was moistened with sterile phosphate-buffered saline (PBS) prior to sampling. The moistened cotton part of the swab was put into the nestling's cloaca and rotated for five seconds to get as comprehensive sample as possible. After this the swab was pulled out, cut short, and placed into a 1,5 ml Eppendorf tube. The tubes were stored at -20 °C until DNA extraction.

2.5 Laboratory procedures

The aim of my work in the laboratory was to prepare the samples for 16S ribosomal RNA sequencing, that is, metabarcoding of the most common bacterial gene for taxonomic identification. 16S rRNA sequencing is a widely used method for profiling prokaryotic, especially bacterial, communities in phylogenetic research due to the specific properties of the 16S rRNA genes: they are universal and known for their high conservation degree (Wang & Qian 2009). The 16S rRNA gene is composed of nine hypervariable subregions (V1–V9) that are separated by nine extremely conserved regions. Conserved regions enable the primer design, whereas hypervariable regions are used in species identification, and that is what makes the 16S rRNA gene the most popular marker gene in profiling of bacterial communities (Yang et al. 2016). The species identification is based on the specific DNA sequences found in hypervariable regions, and these sequences are highly unique to different species (Chakravorty et al. 2007).

2.5.1 Sample selection and protocol testing

Due to the huge number of collected samples, they all could not be sequenced. Therefore, I randomly chose samples of one biological and one foster chick from every nest. My aim was to utilize repeated measurements in this study, and therefore I picked both 7- and 13-day samples from the same individual if possible. In the end, I had 229 cloacal samples, of which 118 were from cool-treated nestlings (7d=58, 13d=60) and 111 from control nestlings (7d=56, 13d=55). Moreover, I had four negative controls: two from Ruissalo and two from extraction. The negative controls were needed for a control of possible contamination between samples and between samples and environment.

Before starting to operate on the samples, I tested the extraction protocol on test samples, which were the same cloacal swab samples from pied flycatchers. Based on the results of gel electrophoresis, it seemed that the protocol is suitable for my swab samples. However, there was contamination in the first test samples, and therefore I did the following extractions in the clean laboratory that is a more sterile environment. Moreover, I started

to use filter tips in pipettes to achieve as contamination-free result as possible. Despite the precautions, some contamination was still observed in test samples. The conclusion was that it might be impossible to get completely rid of the contamination, and the best option would be to try to diminish its effects later on when dealing with bioinformatics.

2.5.2 DNA extraction and amplification

The total DNA was extracted using the ZymoBIOMICS DNA Microprep Kit (Appendix 1) due to its suitability for small volume samples. I extracted the DNA in 14 batches, each of which included 10-24 samples. After the DNA extraction I ran two separate polymerase chain reactions, PCR1 and PCR2 (Appendix 2). For the PCRs I made two replicates for each sample to control the variation within the sample as well as pipetting errors. I used universal primers (Bakt_341F: CCTACGGGNGGCWGCAG, Bakt_805R: GACTACHVGGGTATCTAATCC) that were designed for V3–V4 regions of 16S rRNA gene (Herlemann et al. 2011). Shortly, in the PCR1 the V3–V4 regions of bacterial 16S rRNA gene were amplified, and the end product of this PCR1 (double-stranded DNA) was put into the next run (PCR2). Due to the huge amount of DNA copies after PCR1 and the effectiveness of PCR2, I diluted the PCR1-product before performing PCR2. In PCR2 DNA sequences from PCR1 were amplified, and each sample got a unique combination of index sequences (forward and reverse) so that they can be later identified.

After every PCR2 run I conducted agarose gel electrophoresis to ensure that extraction and PCR protocols had worked as planned. However, gel electrophoresis was not accurate enough to reliably indicate whether my samples were successfully extracted and amplified. Gel electrophoresis may erroneously show that samples have failed if they do not form bands in agarose gel, and this was the case with my samples, as they formed mainly weak bands or nothing in the gel. I assumed that most of my samples contained only small amounts of target DNA, and thus, the results of gel electrophoresis should be interpreted with caution.

2.5.3 Sample pooling, DNA purification and quantification

Due to the inaccuracy of gel electrophoresis, I used Qubit 2.0 Fluorometer for a more accurate quantitation of DNA concentrations. After measurements with Qubit I made two pools: one containing the replicate1-samples and one the replicate2-samples. Based on DNA concentrations I determined the pipetting volume for every sample to balance the content of the pool. If I had pipetted the samples of high DNA concentrations as much as

the samples with lower concentrations, the high-concentrated samples would have been overrepresented in sequencing while data from low-DNA samples would have remained poor.

The last procedures in the laboratory were DNA purification and quantification. The objective of the purification was to remove all the extra DNA from my samples. This kind of "extra" DNA included primer dimers, which are by-products of PCR reaction. In favorable conditions primer dimers bind to each other due to complementary bases resulting in elongation (Garafutdinov et al. 2020). These primer dimers can be seen as blurry bands on the bottom edge of the gel after gel electrophoresis. The size of my target DNA was approximately 600 base pairs, and therefore I wanted to get rid of all the DNA fragments smaller than 600 bp. I purified the pools using Solid Phase Reversible Immobilization (SPRI) beads. The function of SPRI beads is based on their paramagnetic property: when the beads are mixed with samples and put on the magnetic field, they selectively bind DNA of desired size while other DNA-containing material remains in the solution (Beckman Coulter 2022).

After purification I performed DNA quantification with the Agilent 2100 Bioanalyzer, which is an analysis tool for the sample quality control of biomolecules. The purpose of DNA quantification was to ensure that purification was done successfully, that is, to ensure that there were only the DNA fragments of 600 bp left in my pools. DNA quantification also allowed me to assess if the DNA concentrations were sufficient for sequencing. The purified and quantified pools were delivered to Finnish Functional Genomics Centre in Turku for 16S rRNA sequencing. Sequencing was performed using the Illumina MiSeq Reagent Kit v3 (2x300 bp).

2.6 Statistics

2.6.1 Data modification

I received two separate sets of sequence data from Finnish Functional Genomics Centre, one for both pools. The data needed to be modified before analyses because they included primers, chimeric sequences, and negative control samples. In addition, taxonomic assignment and some filtering needed to be done prior to analyses. I started the data modification with supercomputer Puhti at CSC by removing primers and chimeric sequences. Chimeric sequences are usually PCR artifacts formed by at least two phylogenetically distinct sequences incorrectly joined together (Gonzales et al. 2005), so

they do not represent any real sequence existing in nature (National Center for Biotechnology Information 2018), and therefore they were removed from the data.

After primer and chimera removal I continued the data modification in R (version 4.0.2). I ran DADA2 workflow that produced an amplicon sequence variant (ASV) table, which shows the number of times each exact sequence variant is detected in each sample. Since I had two sequence datasets, I merged them into one large table, after which I filtered and combined the replicates, retaining an ASV if it was detected in both replicates. Replicate filtering was followed by taxonomic assignment, where ASVs were identified from phylum to genus level with certain probability. Also ASVs for which a high-rank taxonomy could not be assigned were filtered, followed by prevalence filtering, which removed those ASVs that were very low in frequency. The last filtering step was contamination identification. As mentioned earlier, contamination was observed both in test samples and in the actual samples in the final dataset. However, I was able to control the effect of contamination via negative control samples: a taxon was identified as contaminant if it was more prevalent in negative controls than in true biological samples. These taxa were then removed from the data.

This kind of 16S rRNA data poses special challenges to statistical analyses and interpretation because library sizes, i.e. sequence numbers, may vary substantially across samples (Weiss et al. 2017). The problem is that the different numbers of sequences tend to reflect more the efficiency of the sequencing process rather than real differences in taxonomic richness: more sequencing more observed species (Weiss et al. 2017). Due to the significant variability in the library sizes (sequence numbers) of samples, the data was normalized using rarefaction. Rarefaction is a commonly used normalization method that randomly selects sequences from the initial library to a certain library size (Cameron et al. 2021). In this case the data was rarefied to 1000 sequences per sample. Thus, the samples with less than 1000 sequences were filtered from the data, resulting in the loss of 39 samples. Consequently, the total number of samples after rarefaction was 190 (7 days old control=48, 7d cold treatment=51, 13d control=44, 13d cold treatment=47), including 110 different individuals.

2.6.2 Statistical analyses

The statistical analyses were conducted with R software (version 4.0.2). Temperature differences between cold treatment group and control group were analyzed using a linear

mixed model (function *lmer*, in package lme4), where group (treatment or control) acted as a fixed effect and nest box as a random effect. Nest box was included as a random effect because the data included seven temperature measurements per nest box (daily average temperature during 7-day experiment). Linear mixed model was also used to test whether the cooling experiment, age, the rearing environment, and genetic background impacted the abundances of the main bacterial phyla. Cold treatment, age, cross-fostering, a number of 2-day-old chicks in rearing nest, and cold*age interaction acted as fixed effects. As the same nestling was sampled at day 7 and 13, nestling identity (ring number) and nest box identity (nest of origin and nest of rearing) were modelled as random effects.

Microbiota α-diversity, which describes within-sample diversity, was characterized using Shannon index (H'), observed richness, and Pielou evenness index (J). Alpha diversity was calculated using the package microbiome and the function *alpha*. I conducted linear mixed models for these alpha diversity indices as well with the same fixed and random effects as in phylum-specific model. To study microbiota β-diversity, that is, whether the microbiota communities differ between two temperature groups, I conducted a non-metric distance scaling (NMDS) ordination based on Bray-Curtis dissimilarity indices. In addition, I used the permutational analysis of variance (PERMANOVA) to estimate the statistical significance of differences between the groups. The β-diversity calculations were performed with the vegan package and the function *adonis*.

3 RESULTS

3.1 Age and gut microbiota

Overall, the four most abundant bacterial phyla were Proteobacteria (65.6 ±SE=2.2%), Firmicutes (18.8 ±SE=1.9%), Actinobacteria (5.8 ±SE=0.7%), and Chlamydiae (5.8 ±SE=1.2%). The relative abundance of Firmicutes changed significantly with age (p=0.04) as they increased from 7d to 13d (Fig 3), whereas other phyla did not show significant changes between 7 and 13 days (Table 1).

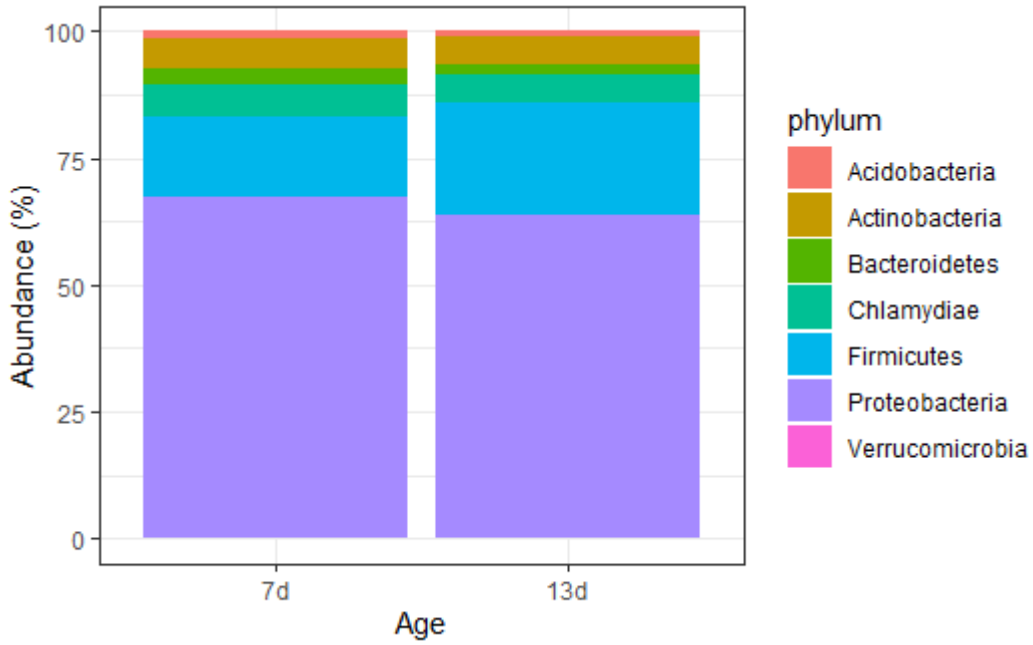

Figure 3. The average relative abundances of main bacterial phyla in 7- and 13-day old nestlings. Sample sizes: 7d n=57 nests/99 nestlings, 13d n=56 nests/91 nestlings.

Table 1. The results of linear mixed model showing the effects of different fixed and random factors on the abundance of seven main bacterial phyla found in guts of pied flycatcher nestlings. Fixed effects include cold treatment, cross-fostering (cross-fostered or not), the age of nestling, and the number of chicks in rearing nest at day 2 (brood size). The interaction of cold treatment and age was included as fixed effect as well. Random effects include individual, nest of origin (the nest chick was born in), and nest of rearing (the nest since day 2). The subscripts of F-values represent the numerator and denominator degrees of freedom. The bolded p-value refers to statistical significance at the 0.05 level.

	Acidobacteria		Actinobacteria		Bacteroidetes		Chlamydiae		Firmicutes		Proteobacteria		Verrucomicrobia	
Fixed effects	F-value	P-value	F-value	P-value	F-value	P-value	F-value	P-value	F-value	P-value	F-value	P-value	F-value	P-value
Cold treatment	$0.183_{1,49}$	0.671	$0.353_{1,184}$	0.553	$0.951_{1,52}$	0.334	$0.236_{1,33}$	0.630	$0.265_{1,45}$	0.609	$0.353_{1,44}$	0.555	$1.485_{1,83}$	0.227
Cross-fostering	$0.253_{1,175}$	0.616	$0.231_{1,184}$	0.631	$0.090_{1,173}$	0.765	$2.275_{1,59}$	0.137	$0.058_{1,38}$	0.810	$1.671_{1,166}$	0.198	$0.017_{1,83}$	0.896
Age	$0.128_{1,138}$	0.721	$0.433_{1,184}$	0.511	$0.449_{1,139}$	0.504	$0.089_{1,109}$	0.766	$4.355_{1,84}$	**0.040**	$0.952_{1,119}$	0.331	$0.553_{1,63}$	0.460
Brood size (d2)	$0.005_{1,52}$	0.942	$0.884_{1,184}$	0.348	$0.001_{1,54}$	0.975	$0.002_{1,56}$	0.961	$2.068_{1,48}$	0.157	$2.711_{1,53}$	0.106	$0.233_{1,85}$	0.631
Cold*Age	$0.025_{1,138}$	0.874	$0.427_{1,184}$	0.514	$3.534_{1,139}$	0.062	$5.539_{1,109}$	**0.020**	$0.058_{1,84}$	0.810	$1.359_{1,119}$	0.246	$0.804_{1,63}$	0.373
Random effects	Variance		Variance		Variance		Variance		Variance		Variance		Variance	
Individual	0.000		0.00		0.000		8.589		52.08		5.856e-07		0.959	
Nest of origin	0.000		0.00		0.000		4.770		158.49		56.84		0.000	
Nest of rearing	0.937		0.00		7.934		27.629		117.73		147.40		0.000	
Residual	7.419		89.74		43.526		215.466		385.14		685.40		0.299	

There was no impact of the age on three alpha diversity indices: Shannon (7d: mean 1.84 ±SE=0.11, 13d: 1.82 ±SE=0.10), observed richness (7d: 25.68 ±SE=3.06, 13d: 23.34 ±SE=2.87), and Pielou evenness (7d: 0.66 ±SE=0.03, 13d: 0.67 ±SE=0.02) (Table 2).

Table 2. The results of linear mixed model showing the effects of different fixed and random factors on three alpha diversity indices (Shannon index, observed richness, and Pielou evenness). The subscripts of F-values represent the numerator and denominator degrees of freedom.

	Shannon index (H')		Observed richness		Pielou evenness (J)	
Fixed effects	F-value	P-value	F-value	P-value	F-value	P-value
Cold treatment	$0.150_{1,55}$	0.700	$0.593_{1,51}$	0.445	$1.953_{1,47}$	0.169
Cross-fostering	$0.666_{1,172}$	0.416	$0.001e\text{-}01_{1,179}$	0.993	$0.535_{1,173}$	0.466
Age	$0.019_{1,141}$	0.891	$0.290_{1,142}$	0.591	$0.071_{1,137}$	0.790
Brood size (d2)	$1.313_{1,57}$	0.257	$2.842_{1,54}$	0.098	$0.036_{1,49}$	0.851
Cold*Age	$0.003_{1,141}$	0.955	$0.097_{1,142}$	0.756	$0.092_{1,137}$	0.762
Random effects	Variance		Variance		Variance	
Individual	0.000		0.000		0.000	
Nest of origin	0.000		1.375e-07		3.274e-11	
Nest of rearing	0.193		44.08		0.003	
Residual	0.919		799.50		0.054	

The two age groups did not differ in microbial beta diversity either, that is, they did not have differences in their microbiota assemblages (Adonis: $F_{1,188}$ =1.278, p= 0.162) (Fig 3).

3.2 Contribution of genetic and environmental variation on gut microbiota

The cross-fostering, that is, the transfer of nestlings between nests did not influence the abundance of main bacterial phyla, and neither did the number of nestlings in the rearing nest at day 2 ("brood size d2") (Table 1). Individual explained some variation of abundance in Firmicutes, and to some extent in Chlamydiae and Verrucomicrobia as well, whereas in other phyla individual did not explain the variation. The nest chick was born in (nest of origin) explained more of the variation than the nest chick was raised in only in Firmicutes. On the contrary, the nest of rearing, where nestlings grew up since day 2, explained more of the variation than nest of origin for Proteobacteria, Chlamydiae, and Bacteroidetes as well as slightly for Acidobacteria. However, the high estimates of residuals compared to random effects refer to significant amounts of unexplained variation in nestling gut microbiota (Table 1).

The cross-fostering did not have an impact on alpha diversity of gut microbiota communities of pied flycatcher nestlings, neither had the brood size at day 2 (Table 2). Individual did not explain the variation in any three alpha diversity indices (Table 2).

Also, the nest of origin did not influence alpha diversity of nestlings' microbiota. Only the nest of rearing had a minimal impact on alpha diversity: it explained slightly the variation for Shannon index and observed richness (Table 2). Like in the linear mixed model for main bacterial phyla, the residuals were also high for alpha diversity indices in relation to random effects (Table 2).

3.3 Temperature decrease and microbiota

The cooling treatment reduced the nest box temperature approximately by 1.3 ºC, resulting in significant difference in nest box temperatures between control and treatment groups ($F_{1,57}$= 7.640, p=0.008). The average temperature (marginal means ±SE) in a treatment group was 19.3 ±0.31 ºC and in a control group 20.6 ±0.32 ºC during the 7-day temperature manipulation. However, the variation in daily nest box temperatures was remarkable, which can be observed in a significant range between minimum and maximum nest box temperatures (Fig 4).

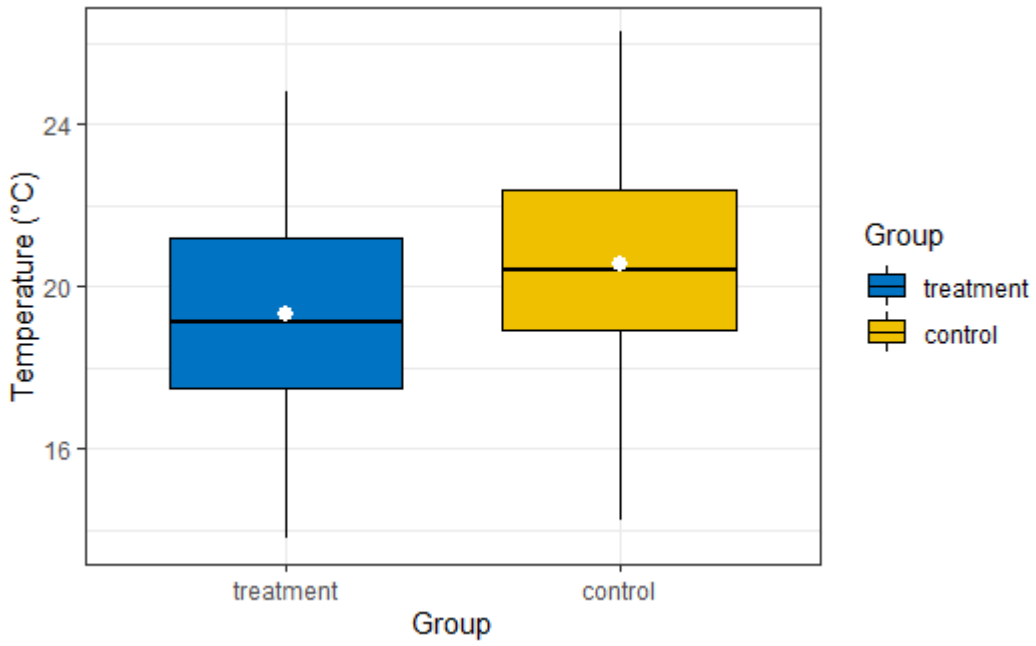

Figure 4. Nest box temperatures (ºC) in treatment and control groups. Median is represented by the black crossbar, the mean by the white dot and 25 % and 75 % quartiles by the lower and upper box. Whiskers show the range between minimum and maximum value. Sample sizes: treatment n=30 nest boxes, 7 measurements/nest box, control n=29 nest boxes, 7 measurements/nest box.

The cooling treatment did not influence different metrics of alpha diversity (Shannon, observed richness, Pielou evenness) (Table 2) but there was a remarkable inter-individual variation in Shannon indices (Fig 5).

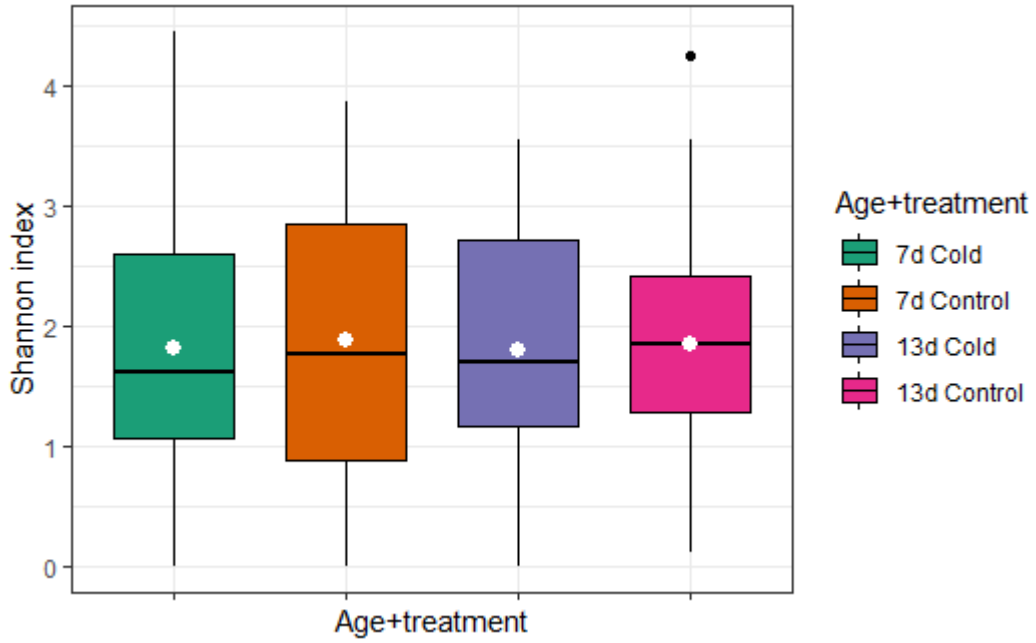

Figure 5. Shannon indices of four different age+treatment groups. 7d nestlings have been sampled prior to cold treatment and 13d nestlings after cold treatment. Median is represented by the black crossbar, the mean by the white dot and 25 % and 75 % quartiles by the lower and upper box. Whiskers show the range between minimum and maximum value and black dot represents an outlier. Sample sizes: 7d cold n=28 nests/51 nestlings, 7d control n=29/48, 13d cold n=30/47, 13d control n=26/44.

The treatment did not have an impact on the relative abundances of six bacterial phyla out of seven (Fig 6, Table 1, see cold*age interaction), but cold treatment had a significant interaction effect with age (p=0.02) on Chlamydiae bacteria (Table 1). This suggested differences between some of the age+treatment groups, but statistical differences were not detected between any groups in pairwise comparisons, which is due to the opposite responses with age in the two treatment groups: Chlamydiae had higher relative abundance in 7d nestlings from cold treatment (EMM=8.73 ±SE=2.44) than in older nestlings from the same group (EMM=3.00 ±SE=2.52), while the pattern within the control group was opposite (7d: EMM=2.33 ±SE=2.46, 13d: EMM=6.77 ±SE=2.56). The cooling treatment and age had nearly significant interaction effect on Bacteroidetes as well (p=0.062), but there were no differences between groups in pairwise comparison.

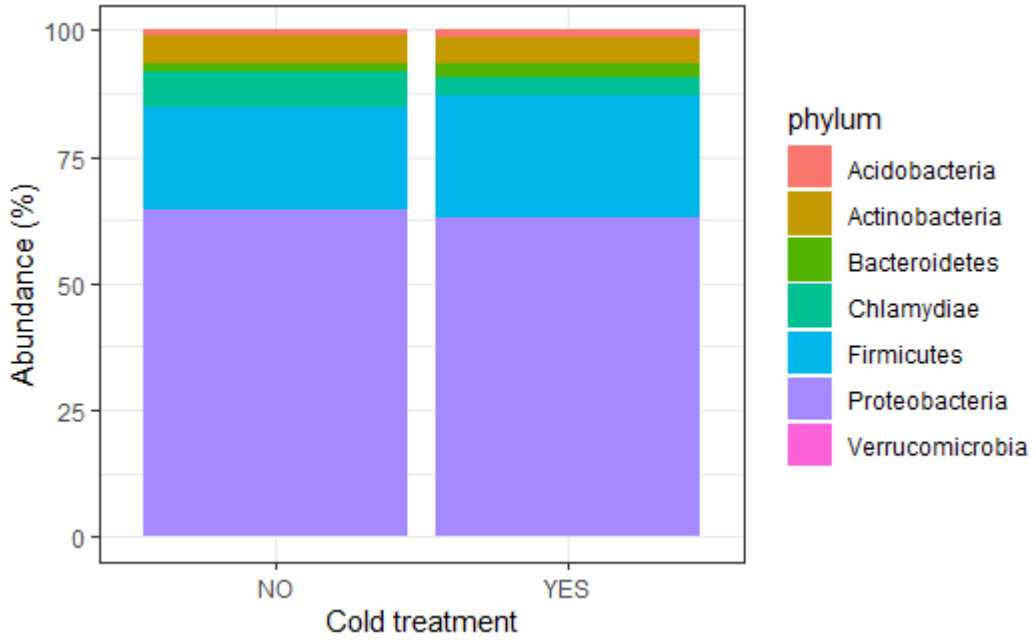

Figure 6. The average relative abundances of main bacterial phyla in control (NO) and treatment (YES) groups of 13 days old pied flycatcher nestlings. Sample sizes: control n=26 nests/44 nestlings, treatment n=30 nests/47 nestlings.

Temperature decrease did not cause differences in microbial compositions between treatment and control group (Adonis: $F=0.832_{1,89}$, p=0.708) (Fig 7).

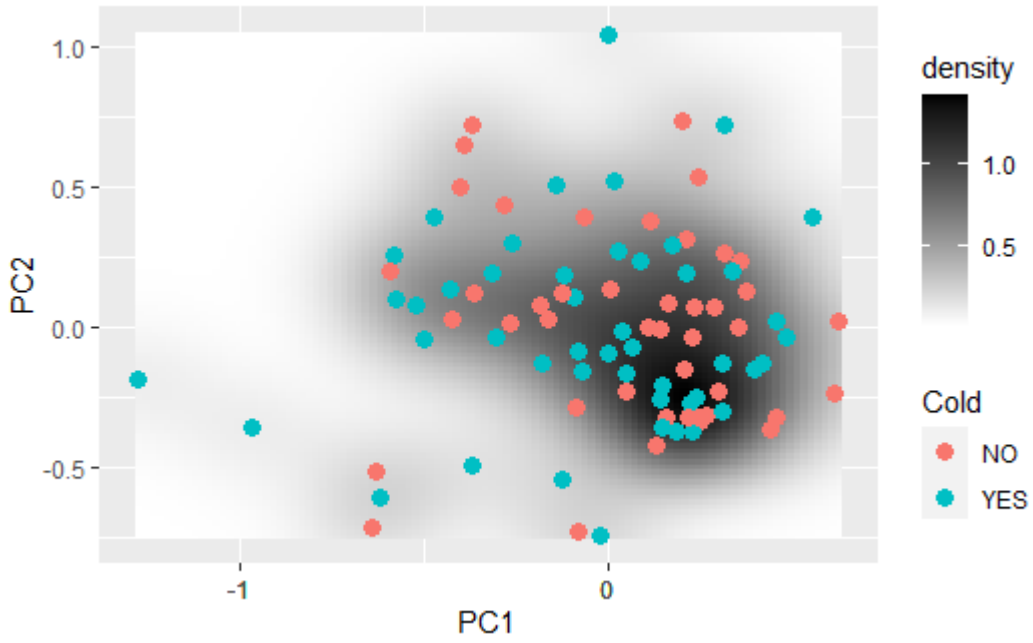

Figure 7. Principal coordinate analysis (PCoA) based on Bray-Curtis dissimilarity between cold treatment (YES) and control (NO) groups. The analysis includes only 13 days old pied flycatcher nestlings. Sample sizes: treatment n=30 nests/47 nestlings, control n=26 nests/44 nestlings.

4 DISCUSSION

4.1 The gut microbiota composition of pied flycatcher and other birds

The most common bacterial phyla in pied flycatcher nestlings' gut were Proteobacteria (relative abundance: 65,6%) and Firmicutes (18,8%), followed by Actinobacteria (5,8%) and Chlamydiae (5,8%). The nestling gut microbiota contained the small proportions of phyla Bacteroidetes, Acidobacteria, and Verrucomicrobia as well. The other studies conducted on wild birds have also reported the predominance of Proteobacteria and Firmicutes in avian gut: these phyla were most dominant in house sparrows (*Passer domesticus*) (Kohl et al. 2019), in Swainson's thrushes (*Catharus ustulatus*), and in gray catbirds (*Dumetella carolinensis*) (Lewis et al. 2016) as well as in bunch of Neotropical bird species (Hird et al. 2015). Also tits, which generally use very similar habitats to pied flycatchers, have shown to harbor mostly Proteobacteria and Firmicutes in their guts, even though the abundance ratios of these two phyla seem to vary. For example, two studies conducted on great tits reported the dominance of Firmicutes, Proteobacteria being second abundant phylum (Maraci et al. 2022; Bodawatta et al. 2022), while another study on great tits indicated the opposite result, as Proteobacteria were the most dominant group comprising over 50 % of the microbiota composition on average (Davidson et al. 2020).

However, Teyssier et al. (2018) indicated that the gut microbiota of great tit nestlings was dominated by Firmicutes and Actinobacteria, while the relative abundance of Proteobacteria remained significantly low. The high abundance of Actinobacteria was also reported in the blue tit study, where Actinobacteria were the most abundant bacterial phylum (Dion-Phenix et al. 2021). Another interesting point is that Chlamydiae bacteria were among the four most abundant phyla in my study, even though Actinobacteria and Bacteroidetes are usually reported to be the next most common bacterial phyla after Proteobacteria and Firmicutes in birds (Waite & Taylor 2014; Hird et al. 2015; Grond et al. 2018). Nevertheless, Bacteroidetes were the fifth abundant phylum in this study. It is noteworthy that some of the differences between these avian studies may be due to different sample types, as some of the studies have used fecal samples instead of cloacal samples. Overall, the same bacterial phyla found in pied flycatcher nestlings are, to a large extent, found in other wild bird species as well, and the most dominant phyla in this study (Proteobacteria and Firmicutes) have been reported to dominate several wild bird guts.

4.2 Age effects on gut microbiota

The statistically significant impact of age on relative abundance of different bacterial phyla was detected only in Firmicutes as the relative abundance of Firmicutes increased from age 7d to 13d. The increase of Firmicutes is consistent with my prediction and the finding of Teyssier et al. (2018), whose study showed that the proportion of Firmicutes increased with age (from 8d to 15 days) in great tit nestlings. They also reported a significant decrease in Proteobacteria from age 8d to 15d. These bacterial phyla showed similar age-related changes in house sparrows as well: the relative abundance of Firmicutes increased remarkably from 6d to 9d, while simultaneously Proteobacteria decreased significantly (Kohl et al. 2019). Based on the previous results (Teyssier et al. 2018; Kohl et al. 2019) I predicted to see a decrease in Proteobacteria in my data as well, and there was, in fact, a slight decrease with age, but the decrease was not statistically significant.

Age-related changes in microbiota are supposed to stem from competition between bacterial taxa as well as diet shift: during nestlings' early development bacterial groups compete for available resources, which may result in the exclusion of some bacterial taxa (van Dongen et al. 2013). In addition, some bird species go through a diet shift during the nestling stage (from insects to seeds, for example), which exposes nestlings to new microbes and contributes to changes in microbial community structure (Kohl et al. 2019). Pied flycatchers are insectivorous immediately after hatching and hence their diet does not change as radically throughout the nestling period, even though prey type provided to nestlings has been shown to vary depending on the nestling age (Samplonius et al. 2016). Young chicks usually consume smaller and softer prey items, while older nestlings are provisioned with larger and harder prey (Wiebe & Slagsvold 2014).

Surprisingly, age did not influence the diversity of pied flycatcher nestlings' gut microbiota. Previous studies have, however, revealed quite contrasting results concerning changes in alpha diversity with age in birds. In black-legged kittiwakes (*Rissa tridactyla*) more OTUs (operational taxonomic units) were identified in older chicks than younger chicks (van Dongen et al. 2013), whereas the microbiota of great tits underwent a significant reduction in microbial diversity between the ages of 8 and 15 days (Teyssier et al. 2018). Similar trend was detected in the chicks of arctic shorebird species, whose gut microbiota increased exponentially in diversity during the first three days after hatching but started to decrease right after that (Grond et al. 2017). It is noteworthy that

the establishment of bacterial communities continues after the nestling period, as studies have shown that adults and nestlings differ in their microbiota diversity, adults often harboring more diverse microbial communities than nestlings (Kreisinger et al. 2017; Kohl et al. 2019). However, the patterns of gut microbiota development during the post-fledging period and adulthood are controversial as well since some studies have reported no differences in microbiota alpha diversity between different post-fledging age groups (Davidson et al. 2020; Drobniak et al. 2022).

Age did not have an impact on the beta diversity of gut microbiota either. In other words, gut microbial communities did not differ between younger and older pied flycatcher nestlings overall, even though there was a difference in Firmicutes. A similar observation was made by Kreisinger et al. (2017) in their barn swallow study, as they did not detect significant age effects on microbial community membership and structure within the nestling stage. Yet, changes in microbial community compositions during the nestling stage were almost significant in house sparrows (Kohl et al. 2019). Although Kreisinger et al. (2017) did not find differences during the nestling stage of barn swallows, they documented significant compositional differences in microbiota between adults and nestlings. Quite similar results were reported in a house sparrow study (Kohl et al. 2019), where adults and nestlings had significantly different gut microbial communities. Considering all these studies reporting highly variable results of changes in alpha and beta diversity with age, it seems that the development of gut microbiota diversity and composition is not always as linear as commonly suggested. In other words, some of these quite recent studies provide new insights into age-related changes in microbiota that contradict the conventional understanding that microbiota diversity increases during early development and becomes more stable at adulthood (Kohl 2012; van Dongen et al. 2013; Kohl et al. 2019).

4.3 Environmental and genetic effects on gut microbiota

The transfer of pied flycatcher nestlings between nests, cross-fostering, did not have an impact on gut microbial composition or diversity. Such a transfer has a potential to cause stress: nestlings might experience stress during the transfer or stress could be induced by more intense competition in a new nest, for example. Since stress is known to lead to physiological changes (Bakaloudis et al. 2020), it could cause alterations in gut microbiota as well. However, the brood size of rearing nest, which could at least partly

reflect the amount of competition and stress, was not associated with the gut microbiota composition or diversity.

Cross-fostering resulted in an experimental setup that enabled the investigation of the roles of genetic background and rearing environment in shaping nestlings' gut microbiota. I predicted that rearing environment has more significant impacts on the composition of gut microbiota than genetic factors. The rearing nest explained the microbial composition in Chlamydiae and Proteobacteria, and slightly in Firmicutes, Bacteroidetes as well as in Acidobacteria, while the effect of rearing nest was literally zero or near zero in other bacterial phyla. Yet, the effect of rearing nest on gut microbiota was very small, which was somewhat unexpected result since previous microbiota studies have shown quite clearly that foster siblings living in the same nest share more similar gut microbiota than biological siblings inhabiting different nests (Lucas & Heeb 2005; Teyssier et al. 2018). The effect of nest environment on microbial communities has been explained by parental effects, shift of bacteria between nestlings, and nest composition (Lucas & Heeb 2005; Teyssier et al. 2018). The nest of origin, which represented a genetic factor (and prenatal maternal effects) in this study, was surprisingly more important modifier of gut microbiota in Firmicutes than rearing nest. However, only in Firmicutes the nest of origin explained more the variation than the rearing nest. In phylum Chlamydiae the significance of original nest in explaining the variation was only minimal. Nevertheless, it seems that there are some phylum-related differences, whether genetic background (nest of origin) or environment (rearing nest) explains more the variation in microbial communities.

All in all, the rearing nest seems to explain more the variation in gut microbiota than the original nest, although its role as an explaining factor was quite small. Why the significance of both rearing and original nest in shaping nestling microbiota remained minor could be at least partially explained by the possibilities of extra-pair copulations. Pied flycatchers have been reported to exhibit extra-pair matings (Gelter & Tegelström 1992; Lubjuhn et al. 2000; Lehtonen et al. 2009; Canal et al. 2011; Moreno et al. 2015), which can lead to extra-pair paternity (EPP) and broods, whose nestlings do not share a same father. This uncertainty of relationships between nestlings makes the investigation of the roles of genetics and environment more challenging since molecular tools were not used in this study to verify full siblings. However, the frequency of cuckoldry and extra-pair paternity among pied flycatchers seems to vary between populations as some studies have reported quite low EPP frequencies (3–5% of nestlings) (Lubjuhn et al. 2000; Lehtonen et al. 2009), while also relatively high levels (20–24 %) of EPP have been

reported (Gelter & Tegelström 1992; Canal et al. 2011). The extra-pair paternity in pied flycatchers was observed to be low in our study area in 2005–2006 (Lehtonen et al. 2009). Nevertheless, EPP frequencies have been shown to vary within a population between years (Johnsen & Lifjeld 2003), so it is possible that EPP has been more frequent in our study year (2019) than in 2005 and 2006.

4.4 Effects of temperature drop on gut microbiota

The cooling effect on nest box temperatures remained quite small, as the gained temperature drop was on average 1,3 degrees Celsius and the daily nest box temperatures varied significantly. The conclusion is that freezer blocks were not effective enough to buffer against the heat during the midday as the nest box temperatures tended to rise in the daytime. This weak buffering capacity was a bit surprising given that a 2 degrees temperature drop was managed to achieve in a pilot test before the study settings in Ruissalo. Since the cooling effect did not last the whole day, we could have changed the new freezer blocks again in the afternoon to try to improve the cooling effect, but it would have caused too much disturbance and stress for the nestlings and parents for one day. Additionally, the exchange of blocks twice a day would have extended our workdays significantly and required a greater number of the blocks. However, the aim of this study was to examine the impact of minor temperature changes, and thus the temperature manipulation can be considered successful, even though the goal of 2 degrees drop remained unreached.

I expected that cooling would impact the most abundant bacterial phyla of birds (Firmicutes, Proteobacteria, Bacteroidetes, and Actinobacteria) but temperature-related changes were not observed in the diversity or composition of nestling gut microbiota. Since cooling treatment as a main effect combines the data of 7d and 13d nestlings in linear mixed model, and temperature effects cannot be seen in 7d nestlings, only the interaction of treatment and age shows the possible temperature-related effects. It seemed first that temperature had a significant interaction effect with age on Chlamydiae but surprisingly, there were no differences between any of the four age/treatment groups, even though the interaction effect suggested so. However, the lack of statistical differences between groups was explained by opposite responses in the two treatment groups: Chlamydiae relative abundance decreased from 7d (pre-treatment) to 13d (post-treatment) in the cooling treatment group while the opposite pattern was observed within the control group. It remains uncertain where these differences between treatment groups

result from. It is likely that there has been random variation between the groups prior to cooling treatment due to small sample sizes. Despite the assumption that randomly chosen groups do not differ from each other at the beginning of study it seems that there have been some differences, even though not statistically significant.

Studies on other animals have indicated temperature-related changes in gut microbiota (Chevalier et al. 2015; Wen et al. 2020). For example, cold exposure led to notable changes in the mouse gut microbiota: it increased the relative abundance of Firmicutes, decreased the proportion of Bacteroidetes, and nearly depleted Verrucomicrobia (Chevalier et al. 2015). Another mouse study (Wen et al. 2020) showed that Actinobacteria and Saccharibacteria (formerly TM7) decreased in low ambient temperature. In addition, temperature and diet-induced obesity had an interaction effect on gut microbiota diversity and composition, as Firmicutes increased and Bacteroidetes decreased in obese and lean mice at different temperatures in relation to control mice (Wen et al. 2020). In these cold exposure experiments the cooling treatments were quite radical: Wen et al. (2020) exposed mice either to 4 ºC or 23 ºC, meaning that temperature difference was almost 20 ºC between two temperature groups. Pretty similar conditions were carried out by Chevalier et al. (2015), as control mice were kept at room temperature, while cold-exposed individuals were kept at 6 ºC.

Since newly hatched altricial nestlings resembles ectotherms in terms of thermoregulation, it is reasonable to consider how temperature impacts the ectotherm microbiota: Kohl & Yahn (2016) found out that tadpoles reared at cool temperature (18 ºC) had more Firmicutes and Proteobacteria in their guts than tadpoles exposed to warm temperature (28 ºC). The diversity of gut microbiomes did not differ significantly between these two temperature groups. Fontaine et al. (2018), in turn, exposed salamanders to three different temperatures (10, 15, and 20 ºC), and they showed that temperature impacted 25 bacterial genera: 14 of those genera decreased significantly in abundance with increasing temperature, while six genera increased at 15 ºC but decreased at 20 ºC. Contrary to tadpoles, salamanders showed differences in microbial diversity, as all used alpha diversity metrics were lowest at high temperature (Fontaine et al. 2018). Given the mentioned mouse and ectotherm studies, where temperature significantly affected gut microbiome, temperature differences were much larger in those studies than in this study. Thus, the lack of effect of the cooling treatment on pied flycatcher microbiota could be explained by the small temperature drop that did not cause detectable effects on microbiota structure.

In addition to the magnitude of temperature differences, I can also speculate about an exposure time: a longer cool exposure may have increased the probability to see temperature-related changes in nestling microbiota. A newly published meta-analysis covering the microbiomes of 43 terrestrial and aquatic species revealed that sudden long and static temperature shifts caused most pronounced diversity loss in microbiome under both warming and cooling exposure (Li et al. 2022). If the cooling treatment had been started on very young nestlings (less than 7d old), there could have been more likely detectable temperature-induced changes in microbiota as younger nestlings have very limited thermoregulation capacity compared to older nestlings (Dawson & Evans 1960; Rodriguez & Barba 2016a). Nevertheless, the brooding behavior of the female had to be taken into consideration. Since pied flycatcher broods its offspring until the age of 7 days (Moreno et al. 1995), the cooling experiment could not be started on nestlings younger than 7d, as the heat produced by a female would have disturbed and buffered the cooling effect. However, the results from laboratory studies have to be interpreted with caution, as laboratory environment alters the microbiome (Eichmiller et al. 2016; Bowerman et al. 2021), and therefore experiments conducted under laboratory conditions do not necessarily tell the whole truth about temperature effects on microbiota. Since this study was conducted in natural habitats of pied flycatcher nestlings, this study is more relevant for an investigation of effects of temperature variation on animal microbiota than the ones performed in laboratory.

Temperature has been shown to cause multiple physiological responses through altered microbiota composition. For example, Chevalier et al. (2015) indicated that once the gut microbiota of cold-exposed mice, “cold microbiota”, was transplanted to germ-free mice, this cold microbiota increased insulin sensitivity and the browning of white fat, promoting fat loss. They also observed that the cold microbiota alone was capable of increasing the intestinal absorptive surface and hence energy uptake (Chevalier et al. 2015). Fontaine et al. (2018) also investigated the role of microbiota in energy homeostasis, and they suggested that the gut microbiota of salamanders might mediate the linkage between ambient temperature and digestive performance. Despite the fact that the temperature manipulation did not cause statistically significant differences in nestlings’ gut microbiota in this study, even a minor temperature drop can be biologically significant and cause physiological changes, although not necessarily via gut microbiota. These changes in host physiology may concern hormonal functions (Lynn & Kern 2014; Shang et al. 2021) as well as host growth (Rodriguez & Barba 2016b; Andreasson et al. 2018), which were

discussed in more detail in the introduction part. However, it is also possible that temperature-driven effects on gut microbiota come with a delay, especially if the temperature manipulation during early development has long-term effects on host physiological functions, which in turn might impact the later gut microbiota composition. Such a case reminds us of the bidirectional nature of host-microbiota interactions, where both influence each other.

4.5 Other factors explaining nestling microbiota and future prospects

One reason why temperature change and none of the other chosen predictors clearly explained variation in nestlings' gut microbiota might result from the overriding effects of other uncontrolled factors that shape avian microbiota. Diet is suggested to be an extremely important modifier of gut microbiota in birds (Hird et al. 2014; Colston & Jackson 2016; Lewis et al. 2017). The diet of pied flycatcher nestling is mainly composed of caterpillars, spiders, and flying insects (Lundberg & Alatalo 1992; Siikamäki et al. 1998), while also ants and other Hymenoptera have been shown to be important food sources for nestlings (Silverin & Andersson 1984). However, the proportions of these prey items in nestling diet vary depending on the time of breeding season and habitat (Burger et al. 2012). For example, caterpillar abundance is known to have strong seasonal variation, as it tends to peak towards the end of May and the beginning of June (Eeva et al. 1997). This variation in different prey abundance across the breeding season provides an interesting study question for future research on the role of hatching date on nestling gut microbiota. Moreover, this study area in Ruissalo consists of different habitat types from oak forests to pine-dominated forests mixed with spruce and birch (Ruiz et al. 2016), so it is justified to assume that these different forest types provide different food sources with varying proportions to pied flycatchers. Such a diverse study area raises an intriguing question for upcoming microbiome studies whether habitat could explain the differences in available prey type and thus variation in nestling gut microbiota. Habitat-specific differences in gut microbiota were found in blue tits (Drobniak et al. 2022), suggesting that similar habitat-related effects might be found in pied flycatchers as well. The investigation of effects of habitat on available prey would require the examination of two somewhat same-aged nestlings in different habitats to minimize the chance that the effect of seasonal variation would be detected on prey supply and thus on microbiota.

In addition to food consumed by chicks, nestling sex was not determined in this study, leaving room for speculation about its role in shaping nestling gut microbiota. Only little

is known about the sex-related differences in wild bird gut microbiota and none of the studies investigating this topic is conducted on pied flycatchers. Some avian studies have indicated sex-differences in gut microbiota (Su et al. 2014; Escallon et al. 2019), while others have not found differences between sexes (Kreisinger et al. 2015; Whittaker et al. 2016). Sex-related differences in gut microbiota have been supposed to result from hormonal functions (Valeri & Endres 2021). Evidence suggests that testosterone influences microbial community structure (Escallon et al. 2017; 2019): it can directly affect immune functions, which enables the invasion of new bacteria, or it can indirectly change male's mating behavior by increasing extra-pair copulations and hence the risk for new bacteria (Escallon 2019). However, studies investigating sex-differences in microbiota structure in birds have performed on adults and juveniles, and therefore knowledge on sex-related differences during nestling period is lacking.

The results indicated that both inter- and intra-individual variation in gut microbiota community structure and diversity were high in this study. Also previous studies investigating avian gut microbiota have reported high inter-individual variability in adult birds (Hird et al. 2014; Kreisinger et al. 2015; Lewis et al. 2016) as well as in nestlings (Lucas & Heeb 2005; Goossens et al. 2021). This pronounced variation in microbial assemblages between individuals is supposed to reflect several environmental and physiological factors that shape individual microbial communities (Lewis et al. 2016). However, a significant intra-individual variation in pied flycatcher nestlings was surprising, as in many bacterial phyla a value for individual was zero or almost zero (the linear mixed model rounded the value to 0 if it was very close to 0). This means that individual did not explain the variation in microbiota practically at all, that is, the gut microbiota of the same individual changed completely from 7d to 13d. Extremely high intra-individual variation may be explained by the characteristics of early life microbiota, which is suggested to be highly dynamic and unstable (Koenig et al. 2011; Schloss et al. 2012; van Dongen et al. 2013; Teyssier et al. 2018), as many transient bacterial species colonize the gut (van Dongen et al. 2013).

Nevertheless, I have to consider the fact that cloacal samples were taken by nine people, which increases the chance for some sampler-related differences in sample quality. Despite the careful sampling sample quality could not be guaranteed, as the cloacal samples contained only very small amounts of DNA. Thus, it is possible that some bacterial groups were not represented in both samples of the same individual because it is, at least to some extent, coincidental how good samples you manage to get with swabs.

In the future, fecal samples might be a better option for avian microbiota studies, as they include much more DNA due to a larger amount of sample material, which would reduce the variation resulted from sampler and sample size. Collecting fecal samples can be considered more ethical option as well because it does not include any invasive procedures unlike swabbing. Moreover, cloacal swabs might be problematic with very young and small nestlings, if their cloaca is too narrow for swabbing due to which cloacal sampling cannot be regarded as a safe method for these individuals. Even though cloacal swabbing requires intrusion into internal body cavity, the method is regarded as non-invasive and is widely used in microbiota studies nowadays (Videvall et al. 2018; Bodawatta et al. 2020). Cloacal sampling is reliable, easy to perform (Videvall et al. 2018), and it takes only a few seconds, which shortens the handling time of the animal (Miller 2006), and thus presumably reduces the amount of stress animal experiences during handling. Given the advantages of cloacal swabs, it is not self-evident which of the two sampling methods is more ethical, suggesting that study-specific characteristics need to be considered when choosing a proper sample type.

4.6 Conclusions

The main purpose of this study was to investigate the effects of ambient temperature on early avian microbiota, while the additional study questions concerned age effects and the relative significance of environment and genetics in shaping the gut microbiota. The gut microbiota of pied flycatcher nestlings was dominated by phyla Proteobacteria and Firmicutes, which have often been reported to be the most common bacterial phyla in wild bird guts. Contrary to expectations, age did not change the gut microbiota diversity and composition overall, even though the relative abundance of Firmicutes increased with age. Considering the cross-fostering experiment, the rearing nest explained more the variation in nestlings' gut microbiota than the original nest, like expected, but the impact of rearing nest was only minimal. Temperature decrease did not have an impact on nestlings' gut microbiota diversity and composition, which might be explained by many factors. For example, it is possible that temperature drop was too slight and/or the cooling period was too short to cause detectable changes in gut microbiota. In addition, the factors not controlled in this study, such as diet, might override the effects of the cooling experiment in shaping gut microbiota. Overall, the gut microbiota of nestlings exhibited extremely high inter- and intra-individual variation, which likely reflects the multiple environmental and physiological factors as well as the dynamic nature of early life

microbiota that shape gut microbiota. However, further research is needed to clarify the effects of ambient temperature on avian microbiota since knowledge on temperature-related effects on wild bird microbiota is still lacking. Given the ongoing climate change, it is critical to understand how different temperature changes impact the physiology and microbial assemblages of both endotherms and ectotherms, and how animals adjust their physiology to changing environment. It is also important to remember that these environment-host-microbiota interactions are complex and multidimensional, and therefore more studies are needed to clarify the unknown pathways by which environmental factors affect the host and its gut microbiota.

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7 APPENDICES

Appendix 1. DNA extraction protocol (ZymoBIOMICS DNA Microprep Kit version 1.3.1, applied with minor adjustments)

1. Cut the swab into a ZR BashingBead Lysis Tube. Add 750 µl ZymoBIOMICS Lysis Solution to the tube and cap tightly.
2. Secure in a bead beater fitted with a 2 ml tube holder assembly and process at a frequency of 28 Hz for 4 minutes (Note: processing time depends on sample input and bead beater).
3. Centrifuge the ZR BashingBead Lysis Tubes in a microcentrifuge at 10 000 x g for 1 minute.
4. Transfer 400 µl supernatant to a new 2 ml tube.
5. Add 1200 µl of ZymoBIOMICS DNA Binding Buffer to the supernatant.
6. Vortex and spin.
7. Transfer 800 µl of the mixture from step 5 to a Zymo-Spin IC-Z Column in a collection tube and centrifuge at 10 000 x g for 1 minute.
8. Discard the flow-through from the collection tube and repeat step 7.
9. Add 400 µl ZymoBiomics DNA Wash Buffer 1 to the Zymo-Spin IC-Z Column in a new collection tube and centrifuge at 10 000 x g for 1 minute. Discard the flow-through.
10. Add 700 µl ZymoBiomics DNA Wash Buffer 2 to the Zymo-Spin IC-Z Column in a collection tube and centrifuge at 10 000 x g for 1 minute. Discard the flow-through.
11. Add 200 µl ZymoBiomics DNA Wash Buffer 2 to the Zymo-Spin IC-Z Column and centrifuge at 10 000 x g for 1 minute.
12. Transfer the Zymo-Spin IC-Z Column to a clean 1.5 ml microcentrifuge tube and add 20 µl ZymoBIOMICS DNase/RNase Free Water directly to the column matrix and incubate at 70 ºC for 1 minute. Centrifuge at 10 000 x g for 1 minute to elute the DNA.

Appendix 2. Protocols for PCR1 & PCR2

PCR1:

PCR1 was conducted using heterogeneity primers, that is, different versions of forward (Bakt_341F) and reverse (Bakt_805R) primers. Two versions of each forward and reverse primer were used in one reaction. Replicate1-samples get versions 0 and 2, while replicate2-samples get 1 and 3.

1. Mix 12.6 µl of each four primer (10 µM), 630 µl of polymerase MyTaq HS Red Mix and 454 µl of AccuGENE water to make a master mix for 96 samples.
2. Pipet 11 µl master mix into each well.
3. Pipet 1.5 µl template DNA (sample) into each well, seal carefully and spin down.
4. Run the PCR1 program as follows:

Steps	Temperature	Time	Number of cycles
1. Initial denaturation	95 ˚C	3 min	1
2. Denaturation	95 ˚C	30 s	23 cycles of steps 2-4
3. Annealing	55 ˚C	30 s	
4. Extension	72 ˚C	30 s	
5. Final extension	72 ˚C	10 min	1
6. Cooling	20 ˚C	2 min	1

PCR2:

1. Mix 101 µl of AccuGENE water, 504 µl of MyTaq HS Red Mix and 101 µl of i7 reverse index adapters (5 µM) to make a master mix. Note: reverse index adapters are the same for each sample in one plate.
2. Pipet 6 µl of master mix into each well.
3. Pipet 1 µl of i5 forward index adapters (5 µM) individually into each well.
4. Dilute PCR1 product 1:4.
5. Pipet 2 µl of DNA, the diluted PCR product from PCR1, into each well.
6. Seal well and spin down.
7. Run the PCR2 as follows:

Steps	Temperature	Time	Number of cycles
1. Initial denaturation	95 ˚C	4 min	1
2. Denaturation	98 ˚C	20 s	14 cycles of steps 2-4
3. Annealing	60 ˚C	15 s	
4. Extension	72 ˚C	30 s	
5. Final extension	72 ˚C	3 min	1