# PARENTALLY-DERIVED BABY FOOD:
# CROP MILK IN CAPTIVE-REARED DOVES IMPACTS GROWTH AND MICROBIOME COMPOSITION

by

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Many animals have evolved parental care strategies to invest in their offspring and consequently improve their chances of survival and future reproductive success. Some produce few young that they invest in heavily with the production and provisioning of milk. Milk is a nutritious substance produced from the body of a parent and fed to offspring, pre- or post-parity. It occurs in many diverse animal taxa and provides a variety of nutritional benefits. This thesis combines a comprehensive literature review and an experimental study to examine how milk production arose and how it contributes to offspring fitness.

In Chapter 1, I define "milk" and review its occurrence in diverse animal taxa. After describing and comparing the types of milk seen in mammals, birds, amphibians, teleost fish, cartilaginous fish, echinoderms, arachnids, hymenopterans, dipterids, cockroaches, isopods, earwigs, and mollusks, I investigate two main topics. First, I explore how and why some animal lineages evolved the ability to produce milk. Certain ecological factors likely predispose animals to milk production, whether directly or indirectly. These include unpredictable food availability, inaccessibility of parental diet, high predation risk, and extreme environmental conditions. I then investigate what factors determine where milk production evolves on the parent's body. It is

likely that milk production evolved in structures that originally served secretory functions that were later exapted to serve an additional nutritive function.

In Chapter 2, I focus on crop milk and its effect on the growth and gut microbiome compositions of captive-reared ring-necked doves (*Streptopelia risoria*). Young pigeons and doves (members of the family Columbidae) are fed crop milk, a nutritious substance synthesized in the crops of their parents. When hand-rearing columbids, specialized formulas are used that mimic the nutritional composition of crop milk. However, the success rates of chicks fed with these formulas is low, raising the possibility that essential microorganisms present in crop milk, but missing from the formulas, are responsible. I performed an experiment to determine how crop milk affects the growth and gut microbiome composition of captive-reared doves. Ring-necked dove chicks were raised on three different diets: 1) natural crop milk diet, raised by parents, 2) formula diet, raised by hand and 3) formula diet plus inoculations of crop milk, raised by hand. My results revealed that parentally-delivered crop milk improved chick growth rate and resulted in a richer and more diverse microbiome composition at earlier life stages. Inoculating hand-raised chicks with small amounts of crop milk resulted in an earlier onset of rapid growth when compared to hand-raised chicks that did not receive inoculations. This suggests that crop milk-associated microorganisms present in the inoculation may have shortened the initial "stalling" period that occurs before grow rate sharply increases in formula-raised chicks. Shortening this period of time could be important as this was when mortality in formula-raised chicks was highest. Once this inoculation method is refined, it could be used to improve success rates of traditional formula diets.

Overall, this thesis provides insight into the various forms and mechanisms of milk. By reviewing what is known about milk production in diverse animal taxa, it has highlighted

interesting trends and identified areas where more research is needed. By presenting the results of an experiment comparing growth rates and microbiomes of doves raised with and without crop milk, it has underscored the nutritional potency of this substance and provided potential avenues to improve columbid husbandry practices.

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A Thesis

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# CHAPTER 1: THE *OTHER* MILKS: DIVERSE ANIMAL PARENTS SYNTHESIZE FOOD FOR OFFSPRING


## Abstract

Milk, a nutritious substance produced from the body of a parent and fed to offspring, is a remarkable evolutionary adaptation that contributes to offspring fitness. Milk production (also known as histophagy) is one of five matrotrophic modes, the others being placentotrophy, oophagy, embryophagy, and histotrophy. The production of ingestible milk is one of the more elaborate forms of parental care as it requires specialized secreting structures. These structures can range from simple secretory surfaces to elaborate glands with dramatically increased surface area. To investigate this phenomenon, I reviewed literature on the occurrence of milk-production across the animal kingdom and examined trends. I first considered how and why some animal lineages evolved the ability to produce milk. Certain ecological factors that likely determine in which animals milk production occurs, whether directly or indirectly, include unpredictable food availability, inaccessibility of parental diet, high predation risk, and extreme environmental conditions. I then considered what factors determine where milk production evolves on the parent's body. It is likely that milk production evolved in structures that originally served secretory functions that were later exapted to serve an additional nutritive function.


## Introduction

Animals across the tree of life have evolved diverse ways to nourish their developing offspring and many terms exist to describe them (Table 1.1, Table 1.2). One of the simplest forms is lecithotrophy where embryos receive nutrition solely from yolk supplied by mothers

that is accumulated prior to ovulation. This style of nutrient provisioning is commonly associated with oviparity (Blackburn 1999; Ostrovsky et al. 2016). Matrotrophy, more commonly associated with viviparity and/or brooding, is any type of additional nutrient provisioning that an offspring receives at any stage of development, pre- or post-parity. This supplementary provisioning can take different forms including placentotrophy, histotrophy, histophagy, oophagy, and embryophagy (Ostrovsky et al. 2016). The focus of this review will be on histophagy, the transfer of energetic resources from parent to offspring via ingestion of nutritive secretions or parental tissues. This nutritional mode, colloquially described as 'milk', is characteristically ingested instead of absorbed, supplied to offspring in later stages of development, and is vital to their growth and development (Oftedal 2012).

**Table 1.1** Etymological roots used to describe reproduction and parental care.

| | | | | | |
|---|---|---|---|---|---|
| histo- | "tissue" | matro- | "mother" | -trophy | "to nourish" |
| adeno- | "gland" | derma- | "skin" | -phagy | "to eat" |
| lecitho- } | | vivi- | "alive" | -parity | "to birth" |
| vitella- } | | ovo- | "egg" | | |

**Table 1.2** Common terms used to describe reproduction and parental care.

| | |
|---|---|
| Viviparity | A reproductive mode in which eggs are fertilized internally and retained within the mother's body until being released at an advanced state |
| Oviparity | A reproductive mode in which females lay eggs that develop and hatch outside of the body |
| Ovoviviparity | An outdated term previously used to describe a reproductive mode with yolk-nutrition and live birth. Its use causes more confusion than clarity. |
| Lecithotrophy | A developmental mode in which an embryo is exclusively provisioned with nutrients from yolk |
| Matrotrophy | A developmental mode in which maternal nutrition other than yolk is used to provision offspring at any point before nutritional independence |
| Aplacental | A form of matrotrophy where the embryo receives extra-vitelline nutrition prior to parturition without a connection between parental and embryonic tissue |
| Adenotrophy | A developmental mode in which nutrition is derived from intra-uterine glands, often associated with aplacental viviparous insects |
| Histotrophy | Absorption of nutrients directly through embryonic epithelium from surrounding nutritional fluid in the parent's body cavity |
| Histophagy | Ingestion of secretions from parental glands or eating of parental epithelial tissue by an offspring |
| Brooding | Incubation of an embryo on the body surface or inside its infoldings |
| Hypertrophy | The increase in size of a tissue caused by the enlargement of its cells |

To be considered a milk here within, I am considering only secretions or tissues that possess the following five characteristics: 1) non-yolk nutrition, 2) synthesis by parent as opposed to being collected, 3) ingestion by young instead of absorption, 4) vital to growth and development of young, and 5) does not result in death of the parent. Following this definition, milk production is known to exist in at least some of the members of the following taxa: mammals, birds (pigeons, flamingos, & penguins), amphibians (caecilians & salamanders), teleost fish (discus fish), cartilaginous fish (sharks & rays), echinoderms (starfish & sea cucumbers), arachnids (spiders & pseudoscorpions), hymenopterans (bees & wasps), dipterans, cockroaches, isopods, earwigs, & mollusks (snails, clams, & mussels).

Excluded from this definition are animals that provision with trophic eggs (as seen in amphibians including members of Oophaga, as well as many fish and insects), animals that provision with collected food that has been partially digested (as seen in the burying beetle *Nicrophorus vespilloides*, as well as many groups of birds including members of Procellariiformes), animals that provision nutrients directly through embryonic epithelium (as seen in male pipefish and seahorses including *Hippocampus abdominalis*), or animals that are killed and consumed by their young (as seen in spiders including *Amaurobius fero*x and *Stegodyphus lineatus*).

I will first provide a general overview of milks that meet all five characteristics and then discuss two of the fascinating evolutionary questions that we can ask and begin to answer by comparing these diverse substances. Firstly, how and why did some animal lineages evolve the ability to produce milk? Secondly, what determines where milk production evolves on the parent's body? It is my hope that this review highlights the many unknowns surrounding these substances and consequently inspires future research.

## Adaptations of milk-producing taxa

### *Mammals*

Named for the presence of mammary glands in females, the mammals are best known for their milk-producing abilities. Members of class Mammalia are viviparous, with the only exceptions being the oviparous monotremes (comprised of the platypus, *Ornithorhynchus anatinus,* and echidnas within the family Tachyglossidae). All mammals, without exception, produce milk for their developing young. Epithelial alveolar cells within lobules secrete a liquid containing water, protein, lipids and antibodies that is collected by a series of ducts and delivered to young via nipples or milk patches in the case of monotremes (Oftedal 2002a; Skiebiel et al. 2013).

### *Birds*

All birds are oviparous, and several groups have evolved the ability to produce milk that is fed to their young. Males and females of all species of pigeons and flamingos, and additionally male Emperor penguins, produce milk in the crop, an enlarged segment of the avian esophagus where food is stored and softened before being ground and digested in the gizzard. This highly nutritious, fat and protein-rich substance is then regurgitated into the mouths of their chicks.

In pigeons and doves (Columbidae), prolactin activates specialized epithelial cells in the parent's crop to sequester fat and protein. These whole cells are then compressed into small rice-shaped pellets that are regularly sloughed from the lining of the crop through rhythmic contractions of the parent's gular muscles (Gillespie et al. 2011). Chicks insert their beaks into the open mouth of the parent and ingest these nutritive packages of cells.

In flamingos, a thin fluid containing fat, protein, red and white blood cells, and carotenoids is secreted by glands lining the upper part of the esophagus. This crop milk is frequently regurgitated and dribbled into the open mouth of the chick (Ward et al. 2001).

The constituents of the crop milk produced by male emperor penguins are poorly known, but a small amount is produced by male parents to sustain a single chick for about five days after hatching (Prévost and Vilter 1963).

*Amphibians*

Most amphibians are oviparous with some groups having evolved viviparity. While milk production is only known to occur in a single anuran and a single salamander, the entire order containing caecilians (Gymnophiona) is thought to produce a type of milk that they use to provision their offspring.

The only known salamander to provision its young with milk is the viviparous alpine salamander (*Salamandra atra*). Once the young have depleted their yolk reserves, they feed on specialized epithelial cells of their mother's oviduct lining, specifically in the anterior region of the oviduct (Greven 1984).

The only known anuran to produce milk is the viviparous western Nimba toad (*Nimbaphrynoides occidentalis*). In this species, the young hatch within the oviduct and are provisioned with a nutritive secretion from the anterior end of the oviduct (Xavier 1977; Viler and Lugand 1959).

All caecilians (order Gymnophiona) have likely evolved the ability to produce a type of milk with which to provision their young. About 25% of all caecilian species are oviparous and 75% are viviparous. It is known that in at least three species of oviparous caecilians

*(Microcaecilia dermatophaga*, *Siphonops annulatus, Boulengerula taitanus)*, brooding females develop a fatty outer layer of skin that is periodically peeled off and consumed by their young (Kupfer et al. 2006; Wilkinson et al. 2008; Wilkinson et al. 2013). Because the caecilian species that have been observed to have maternal dermatophagy are distantly related, it has been hypothesized that maternal dermatophagy is ancestral and occurs in most if not all oviparous caecilians (Wilkinson et al. 2008). In viviparous species, the developing fetal young periodically consume their mother's hypertrophied oviduct lining; an example of this seen in *Dermophis mexicanus* (Wake 1993; Gower et al. 2008).

*Teleost fish*

Most teleost fish are oviparous. However, a few groups have evolved viviparity (Blackburn 2015). Members of the family Cichlidae are oviparous and at least thirty species within this taxon feed their newly hatched young with an epidermal mucous secreted from their flanks (as seen in the Midas cichlid, *Amphilophus citrinellus*, and the red discus fish, *Symphysodon discus*) (Noakes 1979; Hildemann 1959). This mucous-based milk is produced by both parents and contains antibodies, protein, and growth hormone, as well as calcium and sodium ions (Buckley et al. 2010; Whittington and Wilson 2013). The young fish nip at the sides of their parents' bodies and consume the secreted mucous along with epidermal cells (Schütz and Barlow 1997).

*Cartilaginous fish*

Members of the subclass Elasmobranchii include the sharks and rays. Species within this subclass display a wide variety of reproductive strategies with oviparous and viviparous groups.

Many phylogenetic uncertainties remain within this taxonomic group, making it challenging to characterize the evolution of their milk-producing abilities (Blackburn 2015).

Within the sharks, certain viviparous species of carpet shark (Orectolobiformes), ground shark (Caracharhiniformes), dogfish sharks (Squaliformes) and mackerel sharks (Lamniformes) are known to produce a nutritive secretion that is ingested by the young in utero (Musick 2010; Furumitsu et al. 2019; Blackburn 2015). While most of these milks are simple mucoid secretions (often referred to as "limited histotroph"), the milk produced by certain species including the white shark (*Carcharodon carcharias*) is a more complex, lipid-rich secretion (often referred to as "lipid histotroph"). In this species, the uterine walls are densely covered in secretory lamellae that deliver milk to the offspring in utero (Satoh and Sowersby 2016)

In the rays, certain viviparous species within the electric rays (Torpediniformes), shovelnose rays (Rhinopristiformes), skates (Rajiformes), and stingrays (Myliobatiformes) produce milk to provision their young in utero. Similar to milks seen in the sharks, some of the milks produce by rays are described as being limited histotroph while others are described as lipid histotroph. All stingrays produce a thick, lipid-rich milk for their developing young (as seen in the red stingray, *Hemitrygon akajei*) that is secreted by filamentous extensions of the uterine lining called trophonemata (Furumitsu et al. 2019; Blackburn 2015; Musick et al. 2005).

*Echinoderms*

A variety of echinoderms are viviparous, but only a few have been confirmed to produce milk for developing young. Some viviparous sea cucumbers retain developing young in the ovaries where they provide them with nutritive mucous (seen in *Oneirophanta mutabilis affinis*) and others retain developing young within the body cavity and provide them with ingestible

mucous and fluids (as in *Neoamphicyclus lividus, Synaptula hydriformis, and Leptosynapta clarki)* (Hansen 1968; Hansen 1975; Sewell and Chia 1994; Hickman 1978, Frick 1998).

The sea stars known to produce milk for young do so either within aboral brood chambers (seen in *Pteraster militaris*), within bursae (seen in *Ophioderma wahlbergii* and *Amphipholis squamata*), or within the ovary (seen in *Ophionotius hexactis*). Mucous and other fluids are secreted and ingested by the young (McClary and Mladenov 1990; Turner and Dearborn 1979; Landschoff and Griffiths 2015; Byrne 1991; Hendler and Tran 2001).

*Arachnids*

Within the arachnids, most spiders are oviparous, whereas most scorpions and pseudoscorpions are viviparous. One known species of jumping spider, *Toxeus magnus*, provisions its newly hatched young with milk. The milk contains proteins, fats, and sugars and is secreted from glands within the epigastric furrow, a region near the opening of the oviduct. Milk droplets are deposited by the mother during first week, then the young spiders drink the substance directly from their mother's epigastric furrow (Chen et al. 2018).

In certain viviparous scorpions, milk-provisioning of young occurs within the ovariuterine tubules (as seen in the black rock scorpion, *Urodacus manicatus)* (Mathew 1968). In all pseudoscorpions, young are retained in a brood sac where they are provisioned with milk. The young employ a specialized organ that pumps milk into their embryonic gut (Weygoldt 1969).

*Hymenopterans*

Bees, wasps, and ants (order Hymenoptera) are oviparous with many species exhibiting maternal care (or in some cases, "sororal care" as it is the sisters who raise their mother's

offspring). All possess a specialized cephalic gland called the hypopharyngeal gland which secretes digestive enzymes. In certain highly eusocial bees and wasps, this gland has been modified to produce a protein-rich substance that is fed to developing larvae (Spradbery 1973; Costa and Cruz-Landim 2000). This substance is most well-known in the western honeybee (*Apis mellifera*) where it is referred to as "royal jelly". Other known hymenopterans that likely feed their broods with secretions from hypopharyngeal glands include members of the genera *Bombus, Vespula, Dolichovesoula*, *Melipona,* and *Scaptotrigona* (Lauer 1975; Michener 1974; Takenaka et al. 1990; Silva-de-Moraes et al., 1996). Very little is known about the origins of these secretions, and it has been recommended that the hypopharyngeal glands in more basal groups of hymenopterans also be studied (Costa and Cruz-Landim 2000).

*Dipterans*

Flies (order Diptera) are a diverse group. The majority of species within this order are oviparous, but certain groups like the Hippoboscoidea and the Mesembrinellinae are viviparous. The superfamily Hippoboscoidea is composed of the parasitic tsetse flies, louse flies, and bat flies. These species provision their young with uterine milk secreted from modified accessory reproductive glands, a characteristic referred to as "adenotrophic viviparity". The milk contains proteins, lipids, and amino acids and larvae ingest it using straw-like mouth parts (Meier et al. 1999; Benoit et al. 2015). The blow flies (subfamily Mesembrinellinae) secrete a milk from their spermathecae that is fed to their developing young (Meier et al. 1999; Guimarães 1977).

*Cockroaches*

Cockroaches (order Blattodea) display a range of oviparous, brooding, and viviparous reproductive modes (Roth 1989). Most species are lecithotrophic, with the exception being the Pacific beetle cockroach (*Diploptera punctata*). The eggs of this species are transferred to the maternal brood sac where, once the young hatch, they increase in size dramatically while provisioned with a highly nutritious milk. This milk is secreted by glandular cells within the lining of the brood sac and is rich in protein and carbohydrates (Stay and Coop 1973; Youngsteadt et al. 2005).

*Isopods*

While most isopods are oviparous, several groups brood their young in brood sacs referred to as "marsupiums" (as seen in the Antarctic giant isopod, *Glyptonotus antarcticus,* common rough woodlouse, *Porcellio scaber*, and common woodlouse, *Oniscus asellus)*. In these groups, the young consume milk that is secreted from microvillar cotyledons within the marsupium (Hoese and Janssen 1989; Janssen and Hoese 1993; Akahira 1956; Ostrovsky et al. 2016).

*Earwigs*

Milk-production is known to occur in one species of earwig (order Dermaptera), *Arixenia esau*. In this viviparous earwig, specialized epithelial cells in the terminal ovarian follicle and uterus are consumed by the embryonic young (Tworzydlo et al. 2013).

*Mollusks*

Some gastropods feed young within brood pouches located outside of the body cavity (as seen in freshwater snails in the family Thiaridae), or within the oviduct (as seen in the violet seasnail, *Janthina janthina*). Within these structures, glands lining the interior surfaces secrete a mucous that nourishes the developing young (Strong and Glaubrecht 2007; Lalli and Gilmer 1989).

Bivalves known to produce food for their young (the swan mussel, *Anodonta cygnea,* and some freshwater clams in the family Sphaeriidae) do so within brood pouches located between gill folds. Mucous and other particles are shed from the interior surfaces of these pouches that are then ingested by offspring (Wood 1974; Beekey et al. 2000; Korniushin and Glaubrecht 2003).

*Other groups*

Less well understood forms of ingestible nutrition are provided to young in viviparous species of goblet worms (Kamptozoa), velvet worms (Onychophoran), planarian worms (Tricladida), bristle worms (Polychaeta), shipworms (Teredinid), and nematodes (Nematoda) (Ostrovsky et al. 2016). The production of milk in these diverse groups requires more study before meaningful comparisons can be made to other histophagous taxa.

**Why did some animal lineages evolve the ability to produce milk?**

Investigating the environmental pressures that may have led certain groups to evolve milk provisioning compels us to draw parallels between diverse organisms. Milk production requires food ingested by the parent to be transformed into energy reserves and stored in the body. This

process is costly in terms of time and energy, as biochemical reactions are never completely efficient (Dall and Boyd 2004). It is therefore necessary that an evolutionary pressure exists for milk production to evolve and be maintained. In the presence of parental care, evolutionary pressures that likely favored the origin and persistence of milk production are unpredictable/seasonal changes in food availability and the inability of young to gain adequate nutrition from the parental diet (Clutton-Brock 2019). A strong association between viviparity and intrauterine milk-production, exemplified by certain amphibians, cartilaginous fish, echinoderms, arachnids, and dipterans, complicates the investigation of other selective pressures favoring milk production. It may be that in these cases, milk-production is linked to strong selection for viviparity under environmental pressures. Selection pressures that likely favor viviparity in these groups are high predation and/or parasitism risk and extreme temperatures or other environmental conditions unfavorable to development (Clutton-Brock 2019). Rapid development facilitated by highly nutritious milk would be beneficial under these scenarios.

Unpredictable food availability can be especially detrimental to young organisms. Developing young have high metabolic needs and lack energy stores with which to tolerate extended periods of time without feeding (Dall and Boyd 2004). During times of patchy access to food, modeling has shown that early mammals that could store energy from previous foraging success and translate it into a reliable source of food for their young likely had higher reproductive success than those that solely collect food from the environment (Dall and Boyd 2004). It is likely that pigeons and doves gained similar reproductive advantages through the development of crop milk. By not having to rely on seasonally available insect prey to feed their young, pigeons and doves are able to extend their breeding seasons longer than any other temperate birds (Billerman and Lovette 2020).

The inability of young to access or process the diet of the parents is another strong selective pressure that favors the production of milk. Young discus fish (*Symphysodon aequifasciatus*) feed exclusively off the mucous secreted by their parents' lateral surfaces for three weeks before they can process the diet of the parent. Young can starve when separated from their parents if an appropriate alternative food is not accessible (Satoh and Sowersby 2021). Similarly, young *Toxeus magnus* spiders are completely dependent on their mother's milk for the first twenty days after hatching. Experimental blocking of the maternal milk gland showed that the young spiders cannot survive in the absence of this secretion (Chen et al. 2018). Another striking example of this is found in flamingos (order Phœnicopteriformes). Adult flamingos are sustained on a diet of small crustaceans and algae that they filter from water using a specialize bent beak with comb-like sieves. Young flamingo chicks, however, cannot access this food as their beaks have not yet developed this specialized structure (Allen 1956). The crop milk produced by both male and female flamingos sustains a single chick for up to six weeks while this specialized beak develops (Rooth 1965; Ward et al. 2001).

High predation pressure has likely selected for viviparity and intra-uterine milk production in a number of sharks that live in the tropics including carpet sharks, ground sharks, and dogfish sharks (Musik 2010). The adenotrophic viviparity seen in tsetse flies and other members of Hippoboscoidea is theorized to have been driven by high rates of wasp parasitism in the offspring (Benoit et al. 2015).

Extreme temperature and environmental conditions have also likely selected for milk production, whether directly or indirectly, in several species. Examples of this may be seen in Antarctic isopods and alpine salamanders. It has been suggested that the brooding and milk-provisioning behaviors seen in the Antarctic giant isopod (*Glyptonotus antarcticus*) are

adaptations to living in frigid Antarctic waters (Janssen and Hoese 1993). Similarly, the viviparous alpine salamander (*Salamandra atra*) may have evolved milk-provisioning as an adaptation to living at high altitudes (Greven 1984).

**Why is milk produced where it is on the body?**

Considering the physiological site of milk production on the parent's body also provides interesting insight into the evolutionary origins of these substances. It is likely that these body structures evolved for another purpose but have subsequently been exapted to allow for the transfer of energy from parent to offspring. (Oftedal 2002a; Capuco and Akers 2009). Considering the anatomy of the animal taxon in conjunction with its evolutionary origins and ecology can potentially be useful in helping to explain why we see milk secretion where we do: on the ventral surface of mammals, in the crops of birds, on the skin of caecilians, in the uterus of sharks and rays, within the slime coating of discus fish, in the cephalic glands of bees, etc. (Figure 1.1).

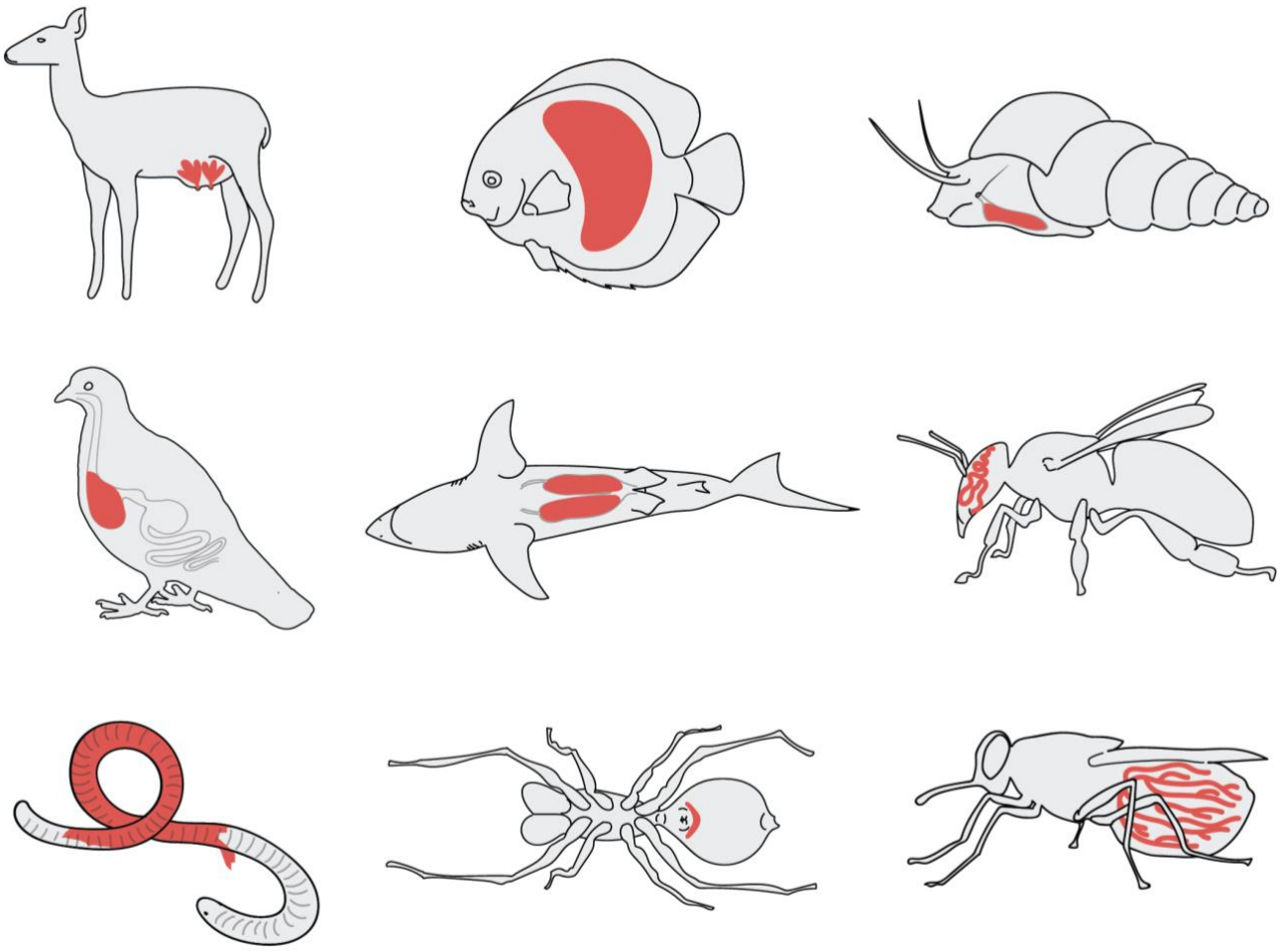

**Figure 1.1** Representatives of the diverse animal taxa that provision their young with milk. Physiological location of milk production shown in red. First column from top to bottom: white-tailed deer (*Odocoileus virginianus*), rock dove (*Columba livia*), oviparous caecilian species (*Microcaecilia dermatophaga*). Second column from top to bottom: red discus fish (*Symphysodon discus*), great white shark (*Carcharodon carcharias*), jumping spider species (*Toxeus magnus*). Third column from top to bottom: pagoda snail species (*Mieniplotia scabra*), western honeybee (*Apis mellifera*), tsetse fly species (*Glossina morsitans*).

The origin of mammalian milk has been a subject of interest for many researchers starting with Charles Darwin (Darwin 1872; Oftedal 2002b). Diverse hypotheses have been developed with the most compelling being based on the theory that proto-mammalian synapsids laid parchment-shelled eggs that lost water easily. It is proposed that watery secretions from the skin evolved to rehydrate these eggs during incubation and that this liquid was taken up through the egg's permeable shell (Oftedal 2002a). This secretory area of skin on a synapsid's ventral surface could have analogous to the brood patches of modern-day birds (Oftedal 2002a), highly vascularized, with a smooth skin surface for efficient transfer. This simple secretion could have become more complex and nutrient-rich once oviparous synapsids evolved to be viviparous.

No salient theories exist for the origin of avian crop milk. The avian crop is thought to have evolved along with the gizzard as a seed-eating adaptation in early cretaceous birds (Zheng et al. 2011). In the absence of teeth, seeds were softened by mucous in the crop before passing along to the gizzard to be ground. The crop also allowed these birds to quickly gather and store food that could be later processed or provisioned in a safer location. The crop continues to serve these original functions in modern birds. In certain groups of birds (pigeons, flamingos, and male Emperor penguins), the crop has developed the additional function of crop milk-production (Kierończyk et al. 2016). Because the crop has long been used to store collected food that could be regurgitated for chicks and secrete digestive mucous, it is plausible that the ability to produce crop milk arose through natural selection acting on a beneficial mutation that supplemented regurgitated food with additional nutrients from the parent.

The skin of amphibians serves many purposes including respiration, water transport, and pathogen defense (Varga et al. 2019). This thin skin is regularly shed as amphibians grow and the shed skin is almost always consumed by the animal to recycle the nutrients within. Without

another reliable food source for offspring, this skin-shedding process has taken on an additional function in caecilian mothers (order Apoda). About 25% of all caecilians are oviparous and in at least three species *(Microcaecilia dermatophaga*, *Siphonops annulatus, Boulengerula taitanus)*, brooding females develop a fatty outer layer of skin that is periodically peeled off and consumed by their young (Kupfer et al. 2006; Wilkinson et al. 2008; Wilkinson et al. 2013). This nutritious outer layer of skin then regrows and is ready to be peeled again in a matter of days. Remarkably, these caecilian species with maternal dermatophagy are distantly related, having diverged with the earliest phylogenetic split of the order. It has therefore been hypothesized that maternal dermatophagy is ancestral and occurs in most if not all oviparous caecilians (Wilkinson et al. 2008). The remaining 75% of caecilians are viviparous and instead of feeding on the external skin of the mother, the developing fetal young periodically consume their mother's oviduct lining (Gower et al. 2008).

A special type of cephalic gland called the hypopharyngeal gland is present only in the bees, wasps, and ants (Costa and Cruz-Landim 2000). In ants, this gland is thought to secrete enzymes that serve digestive or chemical signaling functions (Bussador Do Amaral and Caetano 2005). In honeybees, the hypopharyngeal gland of worker bees secretes a highly nutritious substance called royal jelly which is fed to bee larvae for their first three days (Ahmad et al. 2021). It is plausible that this cephalic gland originally served a more basic digestive function before exaptation to this nutritive function in bees.

Milk production seems to arise in physiological structures that have existing exocrine functions (avian crops secrete mucous that soften seeds, fish skin secretes slime that protects against pathogens, hymenopteran head glands secrete enzymes and pheromones that serve communicative functions, etc.). As these structures are already adapted for secreting substances,

they are likely sites to develop a nutritional component to the secretion. The transport of nutrients across a membrane is significantly more efficient in structures with increased surface area (Tomita et al. 2017; Shennan and Peaker 2000), and this is what we see in most cases where animals have evolved the ability to produce milk.

**Conclusion**

The ability to produce milk is clearly not unique to mammals. A staggeringly diverse range of animals have evolved the ability to provision their young with food synthesized from their bodies. Considering anatomy, developmental constraints, and ecology can help us understand the evolutionary origins of different milk producing structures and where milk production arose. By comparing the diverse forms of milk that exist across the tree of life, it will be possible in the future to address many other questions including: Are similar biochemical processes (ex. prolactin stimulation) employed to produce these milks? What phylogenetic patterns exist in milk formulations among animal groups? What ecological, physiological, and developmental factors facilitate the transition from oviparity to matrotrophic viviparity? Milk production is a fascinating area of study that is rich in potential new discoveries.

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# CHAPTER 2: IMPACTS OF CROP MILK ON CAPTIVE-REARED DOVE GROWTH AND MICROBIOME COMPOSITION


## Abstract

Threatened species that are difficult to raise in captivity cause challenges for conservation efforts aimed at bolstering their population numbers. Aviculturists acknowledge that birds within the family Columbidae (pigeons and doves) are notoriously difficult to hand-raise. The reason lies in the fact that parents feed their newly hatched offspring a milk-like substance that they synthesize in their crop, an enlargement of the esophagus. Specialized diets have been formulated to mimic the nutritional composition of crop milk, but the use of these formulas when hand-raising columbids has had mixed results. Could it be that certain essential microorganisms present in crop milk are missing from these formulas? To determine how crop milk effects the growth and gut microbiome composition of captive-reared doves, I performed an experiment where ring-necked dove (*Streptopelia risoria*) chicks were raised on three different diets: 1) natural crop milk diet, raised by parents, 2) formula diet, raised by hand and 3) formula diet plus inoculations of crop milk, raised by hand. I measured growth rates of chicks in the different groups and sequenced chick gut microbiomes at different life stages: nestling (days 1-14), fledgling (days 15-40), and subadult (days 41-250). My results showed that parent-raised chicks grew faster and were heavier later in life when compared with hand-raised chicks. Hand-raised chicks that received crop milk inoculations showed an earlier onset of rapid growth when compared with formula-raised chicks that did not receive an inoculation. This effect suggests that the crop milk-associated microorganisms present in the inoculation may have shortened the initial "stalling" period that occurs before grow rate sharply increases in formula-raised chicks. Shortening this period of time could be important as this was when mortality in formula-raised

chicks was highest. With further refining of this inoculation technique, it has the potential to improve columbid husbandry protocols when raising chicks with formula.

## Introduction

All animals have evolved in the presence of microorganisms (McFall-Ngai et al. 2013). Certain microorganisms have colonized the external and internal surfaces of animal bodies and formed site-specific microbial assemblages, also known as microbiomes. The associations between animals and their microbiomes can provide mutualistic benefits to both parties. Within a host, microorganisms can receive a reliable habitat, a source of nutrition, and potential for transmission to new hosts. The animal host in turn can receive certain beneficial functions from microorganisms such as providing access to otherwise inaccessible nutrients, protecting against pathogens, tailoring the immune system, and influencing development and physiology (Sommer and Bäckhed 2013, Waite and Taylor 2015, Visconti et al. 2019, Al Nabhani and Eberl 2020). Not all bacterial taxa are beneficial to the host; some can be neutral or pathogenic. Because an animal's health can depend on the composition of their microbiomes, they have evolved ways in which to shape the structure of their associated microbial communities. This includes filtering via host physiology and immune system, as well as facilitating vertical transmission of microorganisms to offspring (Mallot and Amato 2021), a process that is investigated in this study.

Developing embryos were previously thought to exist within a sterile environment, but recent work has shown that the guts of embryonic humans harbor microbial communities (Willis et al. 2019) as do the guts of chicken embryos (Lee et al. 2019). While young animals are not a complete microbial "blank slate" before birth or hatching, their microbial communities are

simple with low species richness (Lozupone et al. 2012, Rodriguez et al. 2015). At the point of birth or hatching, young are exposed to a complex assortment of microbes from their immediate surroundings. Through phenomena referred to as priority effects, the order that microbial taxa arrive at a site can influence the ability of subsequent microbes to colonize. Priority effects can significantly alter the successional trajectories of the entire host-associated microbiome, making it important that certain microbial communities are introduced to the neonate microbiome at an early point (Debray et al. 2021). Vertical transmission, or the pathway of transmission where a symbiotic organism is transferred directly from a host parent to their offspring, allows animals a degree of control over which microbial communities a neonate is first exposed to (Mallot and Amato 2021).

In animals with parental care, associated behavior such as incubating, brooding, and provisioning brings parent and offspring into close contact and provides opportunities for vertical transmission of microbes to occur. Milk provisioning provides a particularly efficient pathway for microorganisms to pass directly from the body of the mother to the gut of the neonate (Zivkovic et al 2011; Mallot and Amato 2021). While milk production is typically associated with mammals, a wide variety of non-mammalian animals produce functionally similar substances. Defined as any extra-vitelline substance that is synthesized by a parent on which an offspring depends (Oftedal 2012), milk is produced by some birds, amphibians, teleost fish, cartilaginous fish, arachnids, hymenopterans, and dipterans, among others (Chapter 1).

In this study, I investigated the milk produced by parents of the domesticated ring-necked dove (*Streptopelia risorii*). Pigeons and doves belong to the family Columbidae, a group of 348 species within 49 genera. All members of the family have altricial young that they provision with crop milk. This milk production has shaped much of their life history. The ability for a parent to

transform any food they eat into an acceptable form for their young allows pigeons and doves to be generalists. This in turn enables them to extend their breeding season longer than any other temperate bird and to occupy a global range with species living in habitats as diverse as deserts, tropical rainforests, and dense urban areas (Winkler et al. 2020).

Both male and female parents produce milk in the crop, a muscular pouch near the throat that functions to soften and store food prior to digestion. Prolactin activates specialized epithelial cells to enlarge with fat and protein; these whole cells are then sloughed off and regurgitated to feed offspring (Horseman and Buntin 1995). The crop milk in columbids is not a liquid, but rather a thick curd-like substance comprised of small packages of cells shaped like grains of rice. The amount of crop milk a single bird can produce is limited. Therefore, columbids are almost always restricted to having broods of no more than two chicks (Westmoreland and Best 1987). Parents begin to produce crop milk around two days before their first chick hatches. They will not eat during this time to avoid contaminating the crop milk with food particles that would be indigestible to the chick (Ding et al. 2020; Winkler et al. 2020). The composition of crop milk shifts over the course of its secretion with different proteins, fats, and minerals present at different stages (Duerr 2007). It is the only food the chicks consume for the first three days of their lives, then a gradually increasing amount of seeds is incorporated as the chicks grow. From day twelve to twenty-four, crop milk production tapers off and chicks are fed regurgitated seeds until they become nutritionally independent between day thirty to forty (Maslanka et al 2009).

Crop milk and mammalian milk are produced by different biological processes, but they have many compositional and functional similarities. Human breast milk contains 87-88% water, 7% carbohydrates, 1% protein, 3.8% fat, and 0.2% minerals (Butts 2018), whereas rock dove crop milk contains 75-77% water, 1% carbohydrates, 11-13% protein, 5-7% fat, and 1.2-1.8%

minerals (Baskett et al. 1993; Shetty et al. 1994). In both cases, production is regulated by the hormone prolactin. Breast milk and crop milk both serve nutritional and immunological functions. Additionally, they both contain microorganisms and prebiotics that have been shown to alter the gut microbiome of neonates (Shetty et al. 1990; Schwartz et al. 2012; Gillespie et al. 2012). In a study where rock dove crop milk was fed experimentally to young domestic chickens, the chicks fed crop milk had increased body mass and a more diverse microbiome when compared with a control group. The unique microbial species found in the crop of these milk-fed individuals were *Veillonella criceti, V. caviae, V. magna, V. ratti, Enterococcus columbae, and Sutterella stercoricanis* (Gillespie et al. 2012). Studies have similarly shown differences in the gut microbiomes of human infants raised with breast milk when compared with those raised with only formula. Those raised with breast milk exhibited higher levels of *Lactobacillus* and *Bifidobacterium* (Schwartz et al. 2012; Ma et al. 2020; Selma-Royo et al. 2021).

Due to difficulty in raising doves and pigeons by hand with formula, no study so far has investigated the differences between crop milk-fed columbids and formula-fed columbids. One study (mentioned above) used chickens to investigate the benefits of rock dove crop milk (Gillespie et al. 2012), but this does not address the importance of the evolved relationships between hosts and their associated microbiomes. While it is still challenging to raise columbids by hand, animal husbandry techniques now exist that allow doves and pigeons to be successfully hand-raised from egg to adulthood. Artificial formulas have been developed that mimic the nutritional composition of crop milk. While their use tends to result in higher chick mortality when compared with parent-raised chicks, their success rates can be as high as 75% when used properly (D. Foote, pers. comm).

For many, the word 'pigeon' invokes images of invasive, city-dwelling rock doves that congregate in enormous flocks. Yet there are confamilial species around the world that are of conservation concern. About 34% of columbid species have declining populations and are at risk as a result of anthropogenic effects that include habitat loss, hunting, and climate change (Winkler et al. 2020). Current efforts to conserve threatened columbid species by captive-breeding programs include programs for the endangered pink pigeon (*Nesoenas mayeri*) by the Mauritian Wildlife Foundation, the critically endangered Socorro dove (*Zenaida graysoni*) by the Island Endemic Foundation, the endangered Mariana fruit dove (*Ptilinopus roseicappilla*) by the Pacific Bird Conservation, and the critically-endangered Negros bleeding-heart dove (*Gallicolumba keayi*) by the Bristol Zoological Society. Learning more about the effects of crop milk and formula on the growth rates and microbiomes of columbids could potentially impact conservation work being done to bolster population numbers of these threatened species.

Here I present for the first time, an experimental study to compare the developmental and microbial differences between parent-raised and formula-raised columbids. Domesticated ring-necked doves (*Streptopelia risorii*) were selected as research subjects because they are docile and relatively small, requiring less space than pigeons. Ring-necked doves, hereafter referred to as "doves", were raised within three treatment groups: 1. Parent-raised, fed a natural diet of crop milk and crop-softened seeds (P), 2. Hand-raised, fed a diet of formula (F), and 3. Hand-raised, fed a diet of formula and received an inoculation of crop milk (Fi) (Figure 2.1). Doves raised by parents served as a baseline and represent the "natural" captive state against which to measure hand-raised doves.

Two related questions were explored. First, does gut microbial composition vary between parent-reared and hand-reared chicks, and is this correlated with growth rate? I predicted that the

gut microbiome of hand-raised chicks (F & Fi) would differ in composition from the gut microbiome of parent-reared chicks (P) and that this compositional difference would be correlated with slower growth rates. Second, can crop milk be used to enhance traditional formula diets and promote the health of hand-raised chicks? I predicted that raising chicks with formula supplemented with collected crop milk (Fi) would result in a gut microbiome more similar to that of parent-raised chicks (P) and that this shift in microbial composition would be correlated with improved growth rates when compared with the group fed only formula (F).

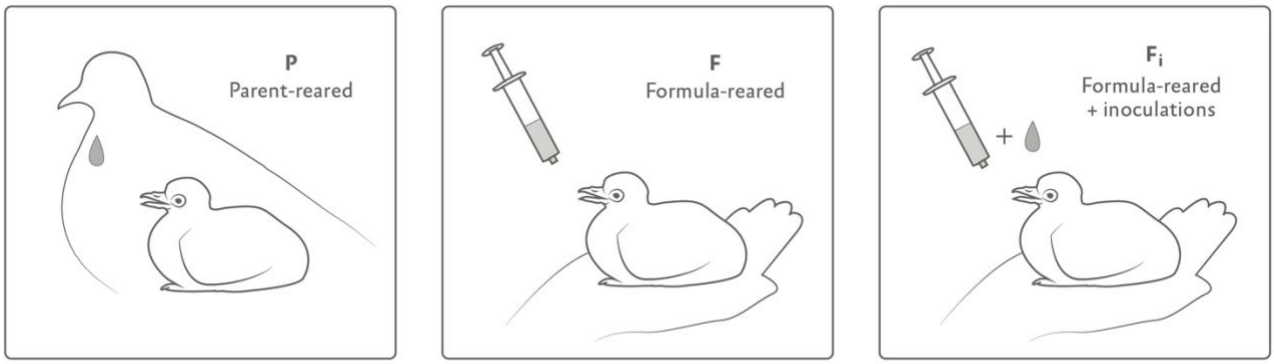


**Figure 2.1.** The three treatment groups. From left to right, P = parent-reared, F = formula-reared, Fi = formula-reared plus inoculation. Crop milk is represented with a teardrop symbol and formula is represented with a syringe. An outline of a dove represents chicks being raised by a parent; an outline of a hand represents chicks being raised by a human.

## Methods

*Study site and breeding pairs*

All animal use was approved by ECU's Animal Care and Use Committee (AUP #D359). From May through December 2020, ten pairs of captive doves owned by and housed at Sylvan Heights Bird Park (SHBP), in Scotland Neck, North Carolina, U.S.A., were used to breed thirty-eight chicks for this study. The twenty breeding doves came from two sources: thirteen came from the existing collection at SHBP where they had been used as brooders. Another seven were bred in Ithaca, NY. Sex was determined by observing *bow-coo* behavior in males. Existing male and female pairs were maintained, and remaining individuals were paired with a bird of the opposite sex from the other population. Pairs were housed individually in wire metal cages within a semi-outdoor barn located in an off-exhibit area of the park.

*Egg monitoring and treatment assignment*

Nests were checked daily and newly laid eggs were recorded, given a unique ID number, and labeled with pencil. Eggs were candled after 5-7 days of incubation to determine egg viability, at which point non-viable eggs were discarded and viable eggs were assigned to one of three treatment groups (Figure 2.1). Treatment group assignment was determined by the order the eggs were laid (e.g. first viable egg assigned to P, second F, third $F_i$, fourth P, fifth F…). This system helped to avoid laying-order bias in group assignment and ensured equivalent sample sizes. Based on the clutch size of 2, this also ensured mixed genotypes and laying order among groups. All eggs were incubated by parents for an average of fourteen days until a pip star was observed. Eggs assigned to the hand-raised treatment groups (F & Fi) were removed from the

nest and placed in an incubator that had been cleaned with a 10% bleach solution and set to 95°F with humidity over 50%.

*Crop milk collection*

Crop milk was collected from parents using a novel, minimally invasive method. A soft silicone tube (exterior diameter 3.5 mm, interior diameter 1.5 mm) with tip trimmed at a diagonal was attached to a syringe and inserted through the mouth and into the crop of a lactating parent. A small amount of warm, distilled water was introduced into the crop which was then externally palpated to try to loosen the crop milk cells. The plunger of the syringe was gently pulled up to draw crop milk into the syringe. Crop milk was collected from recently fed parent-raised chicks using a similar method without adding water to the crop. Crop milk collection was timed to be within the first five days of secretion because after this point, seeds foraged by the parent begin to be included with the crop milk fed to chicks. The collected amounts averaged between 0.25-0.5 mL. Collected crop milk was transferred to a sterile collection tube and either frozen at -20°C until DNA extraction or refrigerated at 4°C for no longer than 24 hours before being fed to a chick (see below for inoculation procedure).

*Raising chicks by hand*

Once chicks assigned to hand-raised treatment groups had hatched, they were fed according to a husbandry protocol that was modified from one developed by Mick Regas and Virgil Bates of the Florida Avian Conservancy (Table 2.1). Syringes used were Exel Luer lock tip syringes in 3-mL and 20-mL sizes. Feeding needles used were Cadence Science curved, stainless steel feeding needles in 1.5-inch 20G and 3-inch 16G sizes. Formulas used were

Roudybush Squab diet, Kaytee Exact Handfeeding Formula, and Harrison's High Potency Fine Pellets. Formula was mixed fresh for each feeding using heated, distilled water between 38-40°C and care was taken to clean and dry syringes and feeding needles between chicks. To feed chicks, a syringe with feeding needle attached was loaded with formula and gently inserted into the mouth, down the esophagus, and into the crop. At each feeding, the crops of the chicks were substantially filled but not to the point of being tight. Feeding frequency generally enabled crops to empty completely between feedings. If crops showed signs of being slow to empty, chicks were given warm, distilled water or watered-down formula until the crop had emptied.

Hand-raised chicks within the inoculation treatment group (Fi) received two crop milk inoculations each, one on Day 1 and one on Day 3. In each case, crop milk inoculate was sourced from the chick's biological parents because the secretion was appropriately timed. Formula was prepared with 0.1 mL crop milk mixed-in to achieve a ratio of 1 part crop milk to 20 parts formula. This ratio, used in other inoculation studies (Rovira 1963), was chosen to ensure that any effect seen from the added crop milk would be a result of the microorganisms as opposed to other nutritional components of the crop milk. This mixture was then fed to chicks using the standard procedure described above.

Chicks in all treatment groups were weighed daily and tarsus length was measured once per week. Body condition was calculated by dividing weight by tarsus length. Starting at one week of age, fecal samples were collected weekly by holding a disposable plastic petri dish underneath a chick until defecation. Larger chicks that did not tolerate being held were placed into a small cage with a disposable tray at the bottom of the cage until defecation. Samples were transferred to 1.7 mL microcentrifuge tubes, labeled with date, chick identification number, chick age, sample type, and treatment. Samples were then frozen at -20°C until DNA extraction.

**Table 2.1.** Husbandry protocol for raising columbids modified from one developed by Mick Regas and Virgil Bates of the Florida Avian Conservancy. Formulas used were Roudybush Squab diet, Kaytee Exact Handfeeding Formula, and Harrison's High Potency Fine Pellets. Heated, distilled water was used to mix formula to 38-40°C then fed to chicks via syringe and a sterilized, ball-tipped animal feeding needle. Housing temperature specifies temperature of incubator or enclosure.

| Age | Diet | Feedings/Day | Housing Temp. (ºC) |
|---|---|---|---|
| Day 0 | 2 or 3 drops water | 1 | 32 - 35 |
| Days 1-2 | 1 part Roudybush, 8 parts water | 8 | 32 - 35 |
| Days 3-6 | ½ part Kaytee Exact, ½ Roudybush, 6 parts water | 7 | 30 - 32 |
| Days 7-14 | 1 part Kaytee Exact, 4 parts water | 6 | 27 - 30 |
| Days 15-23 | 1 part Kaytee Exact, 3 parts water | 5 | 24 - 27 |
| Days 24+ | 1 part Kaytee Exact, 3 parts water + Harrison's pellets softened with water | 4 | 21 - 24 |

*DNA extraction, library preparation, and sequencing*

DNA was extracted from fecal and crop milk samples using a DNeasy PowerLyzer PowerSoil Kit (Qiagen). The kit protocol was followed except for the following changes which were made to increase DNA yield. After the sample and the PowerBead Solution had been added to the PowerBead tube, it was vortexed briefly and then incubated on a heat block set to 55°C for 30 minutes instead of preceding immediately to the bead-beating step. Solution C1 was also placed on the heat block before being added to the PowerBead tube to redissolve precipitate. We increased the time of the first vortexing step from 10 minutes to 20 minutes and increased the first 10,000g centrifugation step from 30 seconds to 3 minutes to fully pellet soil. Additionally, we decreased the amount of the final elution buffer (Solution C6) added to the MB spin column from 100 μL to 50 μL to concentrate the DNA.

DNA concentration was measured with a NanoDrop 2000 Spectrophotometer (ThermoFisher) and samples with concentrations higher than 10 ng/μL were diluted to that concentration with molecular grade water prior to running PCR. The V4-V5 section of bacterial 16s subunit of ribosomal RNA was targeted and amplified using PCR. Each reaction was run in triplicate and contained 34.75 μL of molecular grade water, 5 μL $MgCl_2$, 5 μL 10x buffer, 1 μL dNTPs, 1 μL 806 RB, 1 μL barcoded primer, 0.25 μL Amplitaq Gold (Applied Biosystems), and 2 μL template DNA. All samples were run through the same heating cycles in a thermocycler (Eppenforf 6331 Nexus Gradient Flexlid), first heating to 94°C for three minutes followed by 30 cycles of 94°C for 45 seconds, 50°C for 30 seconds, and 72°C for 90 seconds. After this cycling, samples were held at 72°C for 10 minutes before a final hold of 4°C until they were removed from the machine and stored at -20°C to await library preparation.

PCR products were visualized by agarose gel electrophoresis to confirm that amplification was successful. Triplicate PCR products were then pooled and cleaned using Quantabio sparQ PureMag Beads (Beverly, MA, USA) to remove fragments shorter than 200 base pairs. Cleaned DNA was eluted into 20 μL of Tris-EDTA Buffer (10 mM Tris pH 8.0). DNA concentrations were then measured using Invitrogen Quant-iT dsDNA Broad Range Assay Kit and Qubit fluorometer (Eugene, OR, USA). Based on Qubit result, samples were diluted to 5 ng/μL using molecular grade water. Barcoded samples were combined into a single 1.7 mL microcentrifuge tube at equimolar concentrations and sent to Indiana University's Center for Genomics and Bioinformatics for a 16S rRNA amplicon sequencing run on Illumina MiSeq V2 500 platform with 20% PhiX spike-in.

*Analysis of weight gain and body condition*

R version 4.1.2 (R Core Team 2021) and ggplot2 package (Wickham 2009) were used to generate a scatterplot of chick weight over time. To fit a growth curve for each treatment group to the scatterplot, non-linear least squares were used to generate maximum likelihood estimates. Maximum likelihood estimation functions were then written to estimate the parameters for Gompertz growth curve models, and these were then visualized using ggplot. Differences in the growth curves of treatment groups were evaluated by comparing the age at inflection point, or the point on the growth curve when the curve changes concavity (representing the steepest period of growth). An ANOVA was run on the differences between age at inflection point to determine statistical significance. Box plots of chick weight and body condition after 150 days of age were generated using ggplot. An ANOVA was run to determine statistical significance between treatment groups.

*Analysis of microbiome data*

The mothur MiSeq standard operating procedure was followed to process sequences (Schlos et al. 2009). Paired fastq files for each sample were given a sample ID and combined into contigs using mothur version 1.46.1. Duplicate sequences were merged. Sequences longer than 275 base pairs and sequences with ambiguous bases were removed. Remaining sequences were aligned to the SILVA bacterial reference database (Quast et al. 2013). Chimeras, or the combinations of two distinct sequences, were removed using the VSEARCH algorithm and non-bacterial sequences were removed (Rognes et al. 2016). Sequences were clustered into operational taxonomic units (OTUs) based on 97% similarity. OTUs with less than two occurrences across all samples were removed.

Bar graphs showing relative abundance of microbial OTUs present in sequences samples were generated using MicrobiomeAnalyst (Dhariwal et al. 2017). Diversity metrics were examined using R. Separate analysis was performed on each distinct life stage: nestling (days 0-14), fledgling (days 15-40), and subadult (41-250). Within each of these life stages, I investigated alpha diversity by calculating OTU richness, Shannon diversity, and Pielou's evenness. I tested statistical significance in alpha diversity measurements by running separate ANOVAs for each life stage. Within each life stage, I investigated beta diversity by calculating Bray-Curtis distances between samples. I visualized beta diversity within each life stage using ordination methods with the *vegan* package in R and applied 95% confidence intervals around my treatment groups (Oksanen et al. 2017). I then tested statistical significance in beta diversity measurements by running separate PERMANOVAs for each life stage.

To determine how much variation in chick weight is explained by the microbiome, I conducted a distance-based partial least-squared regression analysis on each life stage using the

*dbplsr* function in the *dbstats* and *pls* packages (Boj et. al 2017, Mevik et al. 2019). I used a Bray-Curtis dissimilarity matrix to run this analysis.

To identify bacterial taxa that were representative of the different treatment groups within the three life stages, I ran indicator species analysis using the *indval* function within the *labdsv* package (Roberts 2019)

**Results**

*Influence of treatment on chick mortality*

No statistical difference in chick mortality was seen between the three treatment groups ($p$=0.517) (Table 2.2). Mortality of chicks in the parent-raised group (P) as 2/14, mortality of chicks in the formula-raised group (F) was 4/12, and mortality of chicks in formula-raised group that received inoculations was 3/12.

*Influence of treatment on dove weight gain and body condition*

Chicks within different treatment groups had temporal differences in onset of rapid weight gain, shown in the differences between age at growth curve inflection (F-Fi $p$=0.014, Fi-P $p$<0.001) (Figure 2.2). The shapes of growth curves in all three treatment groups were similar, but the peak rate of growth did not occur at the same time. The overall shape of the growth curves shows an initial brief period of slow growth followed by a longer period of rapid growth that then slows dramatically and begins to plateau. The steepest period of growth was initiated earlier in the parentally-fed (P) chicks when compared to both formula-raised treatment groups.

**Table 2.2.** Rearing success and mortality rates of chicks in the three treatment groups.

$\chi^2 = 1.32$, 2 df, $n = 38$, $p = 0.517$.

| Treatment | # Total | # Survived | # Died | % Mortality | Age at death |
|---|---|---|---|---|---|
| Parent-raised (P) | 14 | 12 | 2 | 14.29% | 4 days<br>22 days |
| Formula-raised (F) | 12 | 8 | 4 | 33.33% | 2 days<br>4 days<br>6 days<br>9 days |
| Formula-raised + inoculation (Fi) | 12 | 9 | 3 | 25.00% | 4 days<br>5 days<br>6 days |

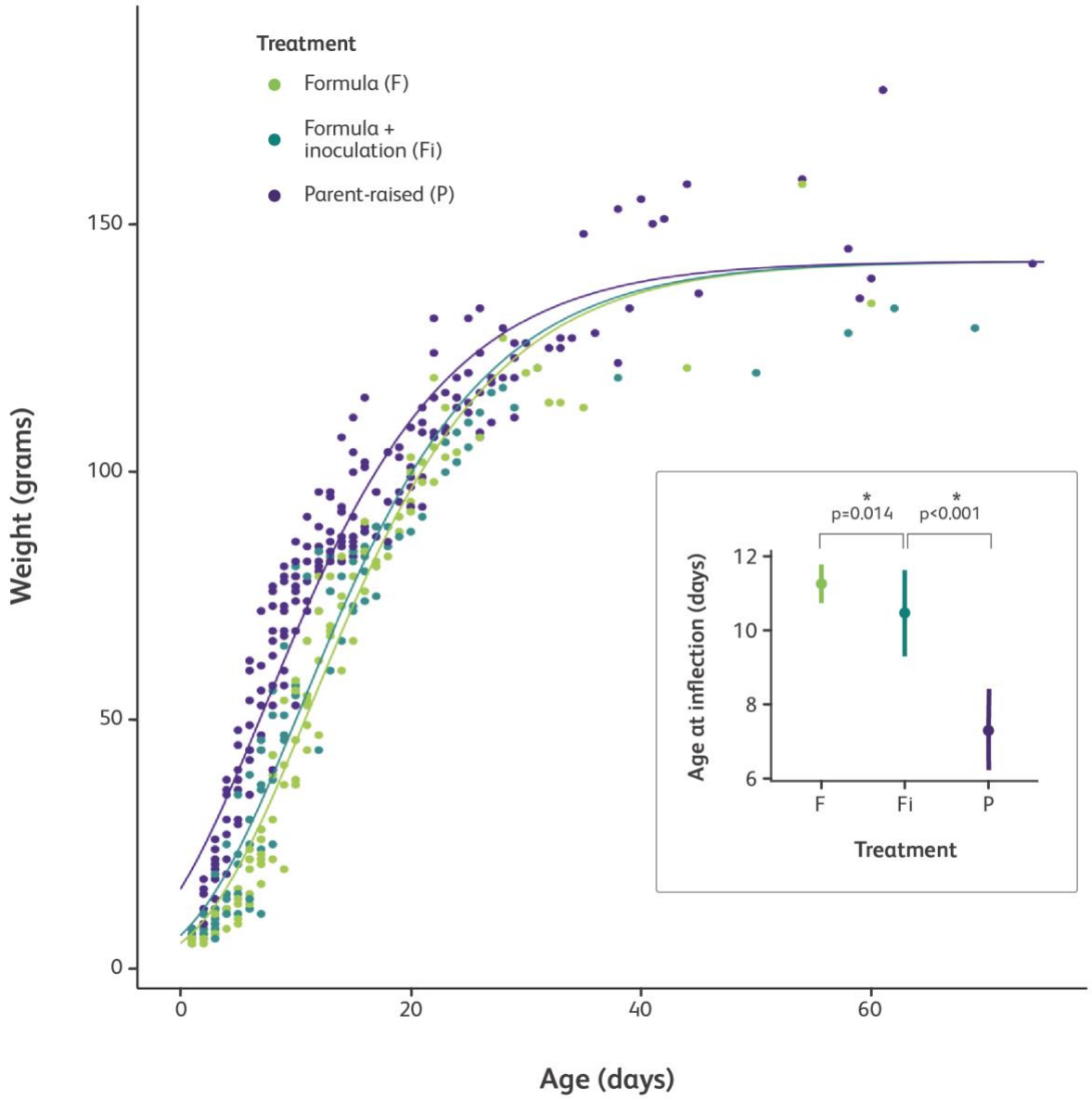


**Figure 2.2.** Gompertz growth curves of weight for chicks in different treatment groups ($n_F$ = 10, $n_{Fi}$ = 11, $n_P$ = 14). Parent-raised chicks (P) reached point of growth curve inflection before formula-raised + inoculation chicks (Fi) and formula-raised chicks (F) ($p < 0.001$). Formula-raised chicks (F) reached this point of growth curve inflection slightly after formula-raised + inoculation chicks (Fi) ($p = 0.014$). Asterisk represents significance at α-level of 0.05.

The steep period of growth began earlier in the inoculated formula-raised (Fi) chicks when compared to formula only (F) chicks. At around 50 days post-hatch, growth plateaued and no differences were observed between treatment groups.

Effects of treatment on older chicks (over 150 days of age) were investigated by measuring weight and body condition ($n$ = 19) (Figure 2.3). When I compared the effects of treatment on chick weight, a significant difference was observed between formula-raised and parent-raised chicks ($p < 0.05$) (Figure 2.3). Parent-raised chicks achieved heavier weights on average than hand-raised birds. Chicks were not sexed, but as the sex differences in adult dove weights were slight, chick sex was not expected to have influenced these results. No significant difference was observed between the mean weights of F and Fi chicks over 150 days of age. There were no significant differences in body condition between any of the treatment groups, but there was a trend showing differences between P and Fi chick body condition ($p = 0.107$).

*Influence of treatment on dove gut microbiomes at different life stages*

I sequenced 122 samples, including 108 fecal samples and 14 crop milk samples. More than 100,000 reads were generated per sample. One fecal sample was removed from analysis due to low OTU abundance. I performed analyses on samples separated into three life stages: 1) Nestling stage, days 0-14, chicks were nest-bound and fed by parents, 2) Fledgling stage, days 15-40, chicks had left nest but continued to be fed by parents, and 3) Subadult stage, days 41-250, chicks were fully weaned and eating on their own. The results of my analysis are presented sequentially according to these three life stages.

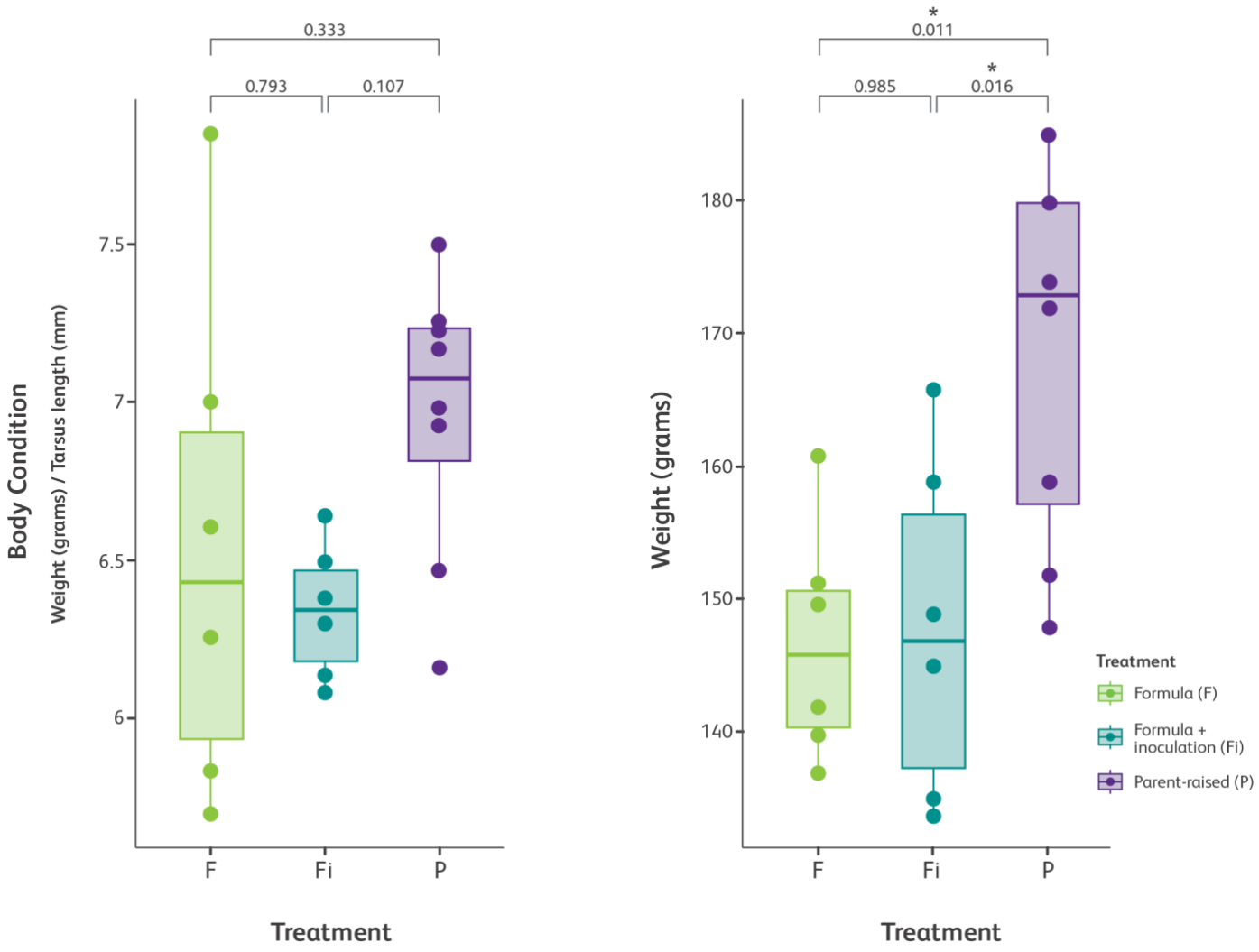


**Figure 2.3.** Body condition and weight over 150 days of age ($n_F = 6$, $n_{Fi} = 6$, $n_P = 8$). Differences in body condition were compared between F-P ($p = 0.333$), Fi-P ($p = 0.107$), and F-Fi ($p = 0.794$). Differences in weight were compared between F-P ($p = 0.011$), Fi-P ($p = 0.016$), and F-Fi ($p = 0.985$). Asterisk represents significance at α-level of 0.05.

*Nestling stage (Days 0-14)*

Relative abundance analysis showed that Firmicutes were the most dominant phyla within the gut microbiomes of nestling doves. Firmicutes made up an average of 97.25% in F, 92.01% in Fi, and 67.14% in P. Parent-raised nestlings had greater percentages of Actinobacteria, Proteobacteria, and Acidobacteria than hand-raised chicks (Figure 2.4). Actinobacteria made up an average of 0.18% in F, 2.23% in Fi, and 20% in P. Proteobacteria made up an average of 2% in F, 2.81% in Fi, and 6.84% in P. Acidobacteria made up an average of 0.29% in F, 1.43% in Fi, and 2.73% in P.

OTU richness in nestling fecal samples showed that samples contained less than 1,000 OTUs. No significant difference in OTU count was observed at this stage (Figure 2.5). Community evenness at the nestling stage showed that most samples had a Pielou's evenness value less than 0.6. Analyzing evenness across nestling treatment groups, P chicks showed a significantly higher evenness than F and Fi chicks (Figure 2.6). Nestling fecal samples also showed low community diversity with most samples falling below 3 on the Shannon Diversity Index. P chicks showed significantly higher community diversity than F chicks (Figure 2.7).

Principal coordinate analysis showed that formula-raised nestlings (F & Fi) had similar bacterial community compositions to one another, while parent-raised nestlings had significantly distinct bacterial communities (Figure 2.8). Only one OTU, a species of *Lactobacillus*, was identified as an indicator species associated with F nestling fecal samples which was a species of Lactobacillus. By contrast, many indicator species were identified for P nestlings, several of which belong to the orders Clostridiales, Actinomycetales, Bifidobacteriales, and Lactobacillales (Table 2.3).

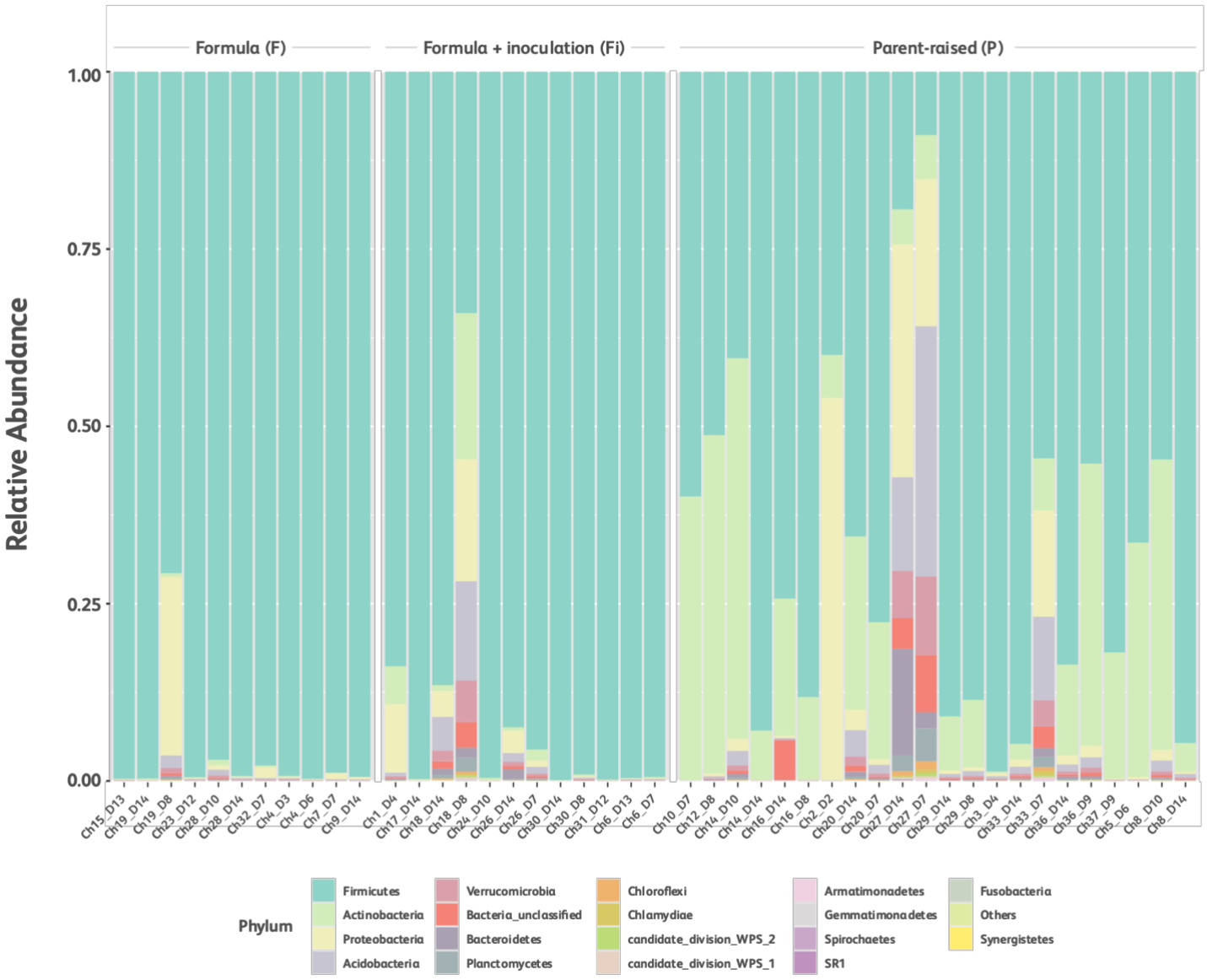


**Figure 2.4.** Relative abundance of bacterial phyla in each fecal sample from nestlings (days 0-14) across all treatment groups. Firmicutes made up an average of 97.25% in F, 92.01% in Fi, and 67.14% in P. Actinobacteria made up an average of 0.18% in F, 2.23% in Fi, and 20% in P. Proteobacteria made up an average of 2% in F, 2.81% in Fi, and 6.84% in P. Acidobacteria made up an average of 0.29% in F, 1.43% in Fi, and 2.73% in P ($n_F$ = 11, $n_{Fi}$ = 12, $n_P$ = 22).

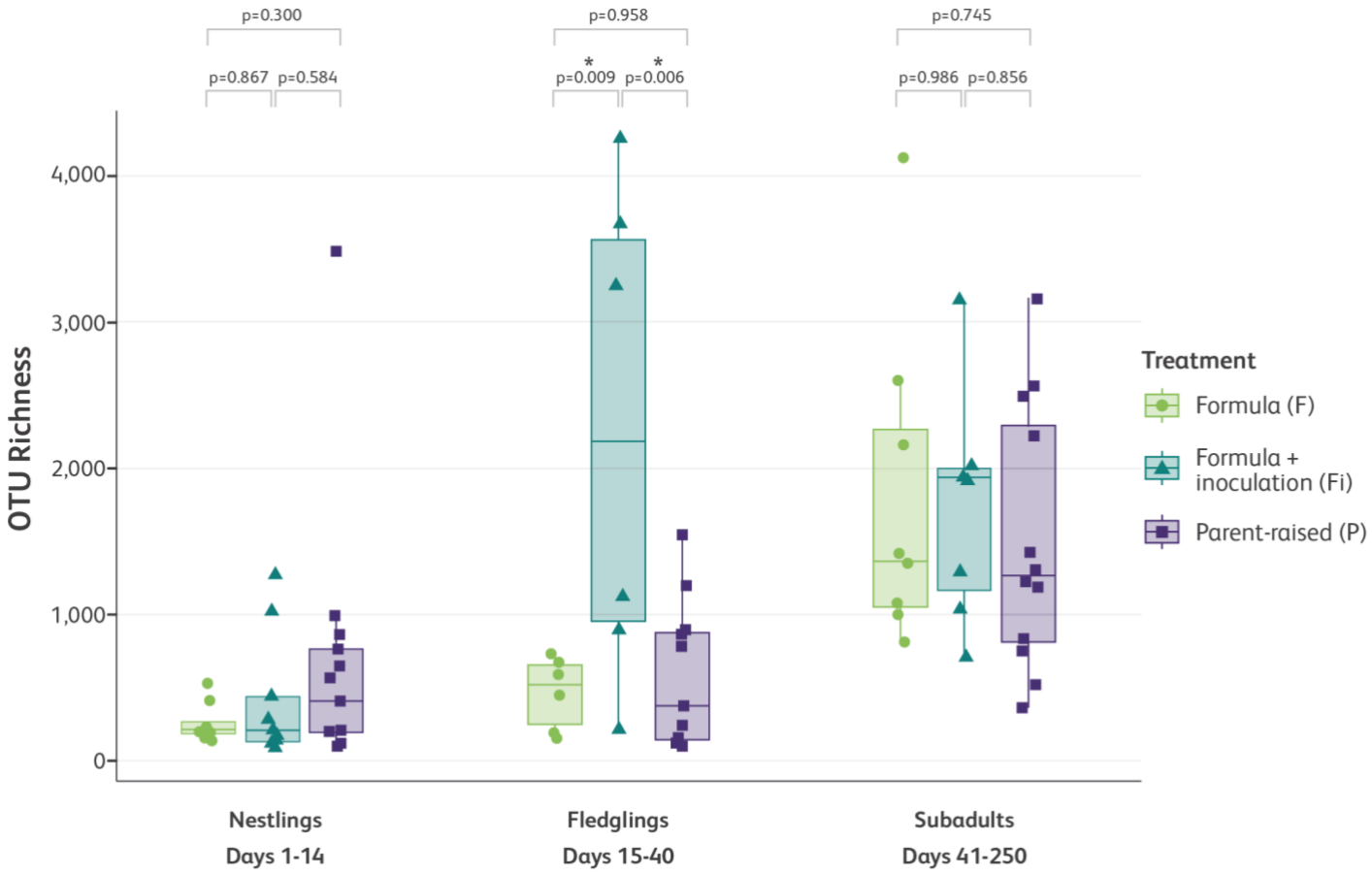


**Figure 2.5.** OTU richness of fecal samples from experimental chicks at three different life stages: nestling (days 1-14), fledgling (days 15-40), and subadult (days 41-250). Sample sizes for nestlings were as follows: $n_F = 8$, $n_{Fi} = 10$, $n_P = 13$. Sample sizes for fledgling were as follows: $n_F = 6$, $n_{Fi} = 6$, $n_P = 12$. Sample sizes for subadults were as follows: $n_F = 8$, $n_{Fi} = 7$, $n_P = 12$. Asterisk represents significance at $\alpha$-level of 0.05.

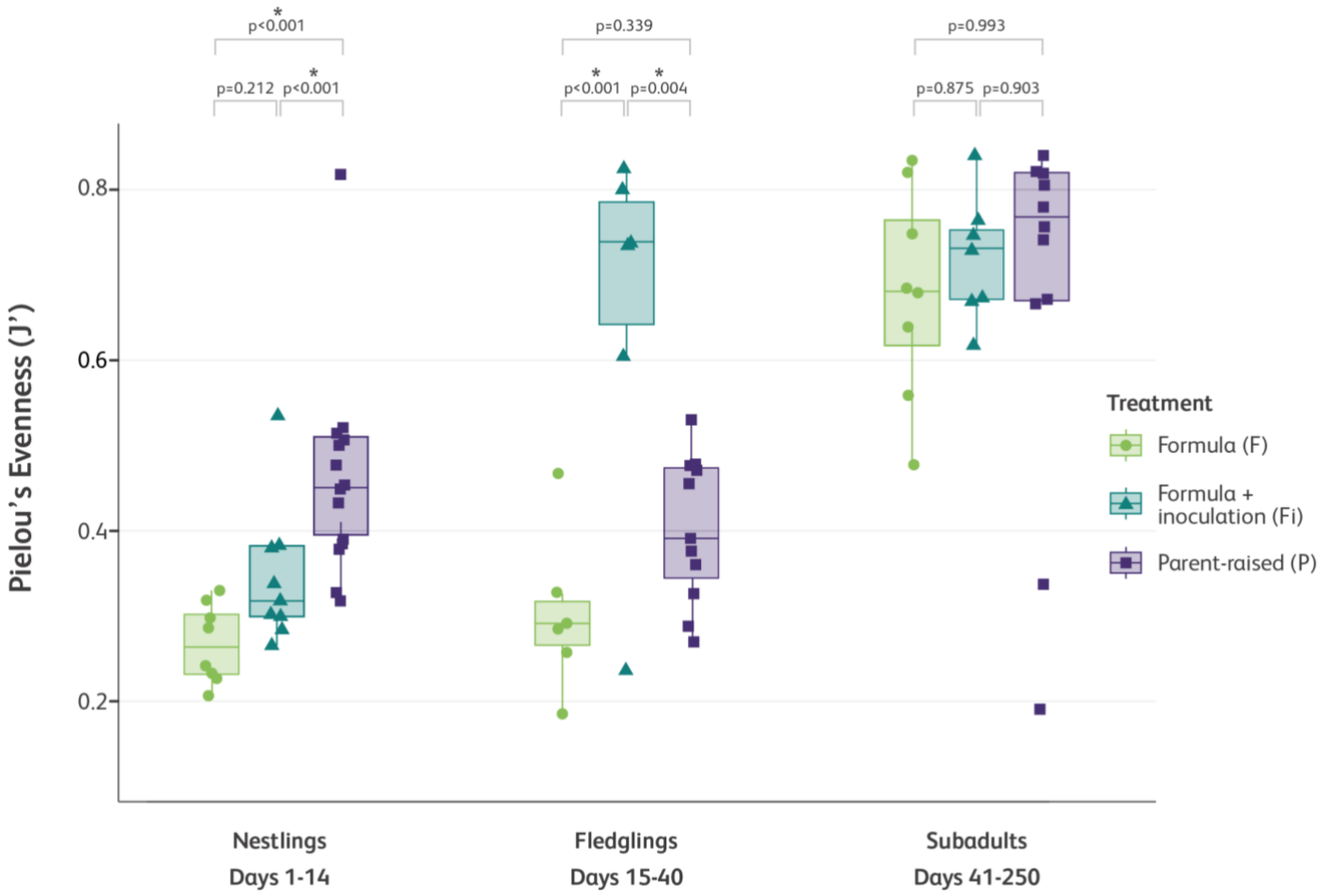


**Figure 2.6.** Community evenness of fecal samples from experimental chicks at three different life stages: nestling (days 1-14), fledgling (days 15-40), and subadult (days 41-250). Sample sizes for nestlings were as follows: $n_F = 8$, $n_{Fi} = 10$, $n_P = 13$. Sample sizes for fledgling were as follows: $n_F = 6$, $n_{Fi} = 6$, $n_P = 12$. Sample sizes for subadults were as follows: $n_F = 8$, $n_{Fi} = 7$, $n_P = 12$. Asterisk represents significance at $\alpha$-level of 0.05.

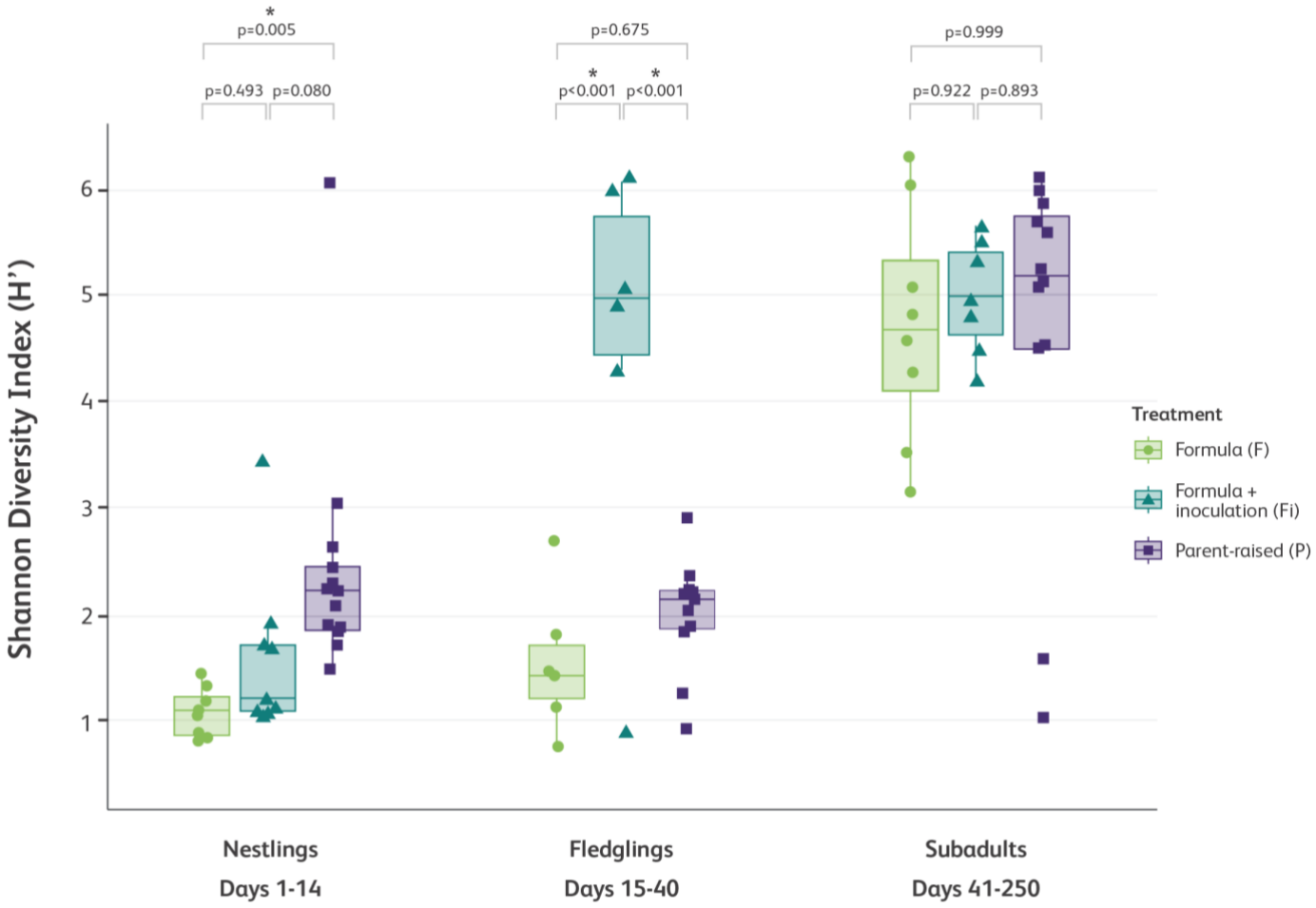


**Figure 2.7.** Community diversity of fecal samples from experimental chicks at three different life stages: nestling (days 1-14), fledgling (days 15-40), and subadult (days 41-250). Sample sizes for nestlings were as follows: $n_F = 8$, $n_{Fi} = 10$, $n_P = 13$. Sample sizes for fledgling were as follows: $n_F = 6$, $n_{Fi} = 6$, $n_P = 12$. Sample sizes for subadults are as follows: $n_F = 8$, $n_{Fi} = 7$, $n_P = 12$. Asterisk represents significance at $\alpha$-level of 0.05.

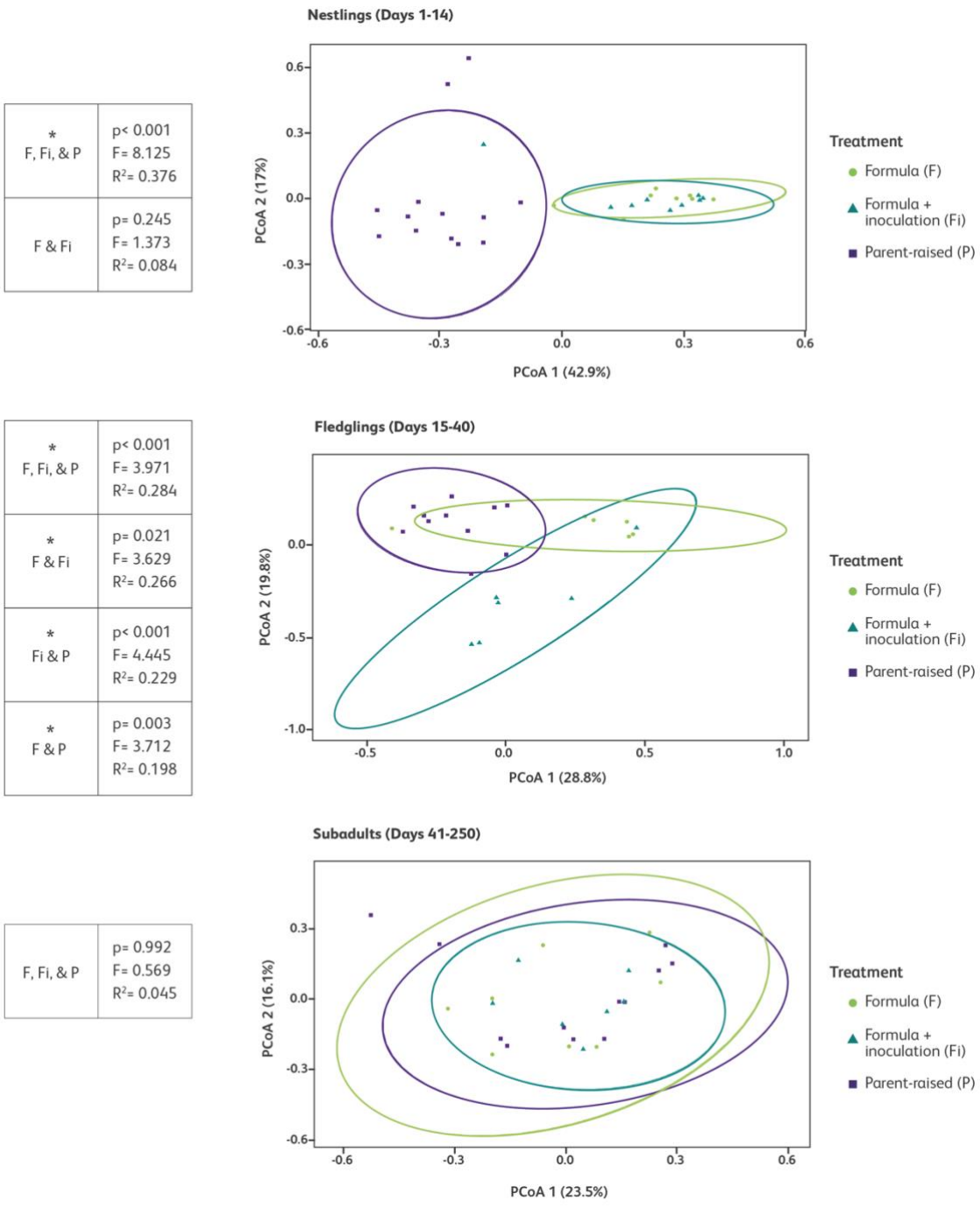


**Figure 2.8.** PCoA plots of community composition values for fecal samples and 95% confidence ellipses based on treatment at three different life stages: nestling (days 1-14), fledgling (days 15-40), and subadult (days 41-250). Sample sizes for nestlings were as follows: $n_F = 8$, $n_{Fi} = 10$, $n_P = 13$. Sample sizes for fledgling are as follows: $n_F = 6$, $n_{Fi} = 6$, $n_P = 12$. Sample sizes for subadults are as follows: $n_F = 8$, $n_{Fi} = 7$, $n_P = 12$.

**Table 2.3.** Indicator OTUs for nestling fecal samples (days 0-14). Green shaded row is an indicator for treatment F (chicks raised with formula). White rows are indicators of treatment P (chicks raised by parents). "UC" stands for "Unclassified".

| OTU | IndVal | Prob | Phylum/ Class/ Order/ Family/ Genus |
|---|---|---|---|
| 001 | 0.445 | 0.041 | Firmicutes/ Bacilli/ Lactobacillales/ Lactobacillaceae/ Lactobacillus |
| 012 | 0.94 | 0.017 | Firmicutes/ Clostridia/ Clostridiales/ Clostridiaceae 1/ Clostridium sensu stricto |
| 011 | 0.93 | 0.006 | Firmicutes/ Clostridia/ Clostridiales/ Peptostreptococcaceae/ Romboutsia |
| 004 | 0.92 | 0.001 | Actinobacteria/ Actinobacteria/ Bifidobacteriales/ Bifidobacteriaceae/ Aeriscardovia |
| 018 | 0.852 | 0.001 | Firmicutes/ Clostridia/ Clostridiales/ Clostridiaceae 1/ Clostridium sensu stricto |
| 033 | 0.832 | 0.002 | Firmicutes/ Bacilli/ Bacillales/ Planococcaceae/ Lysinibacillus |
| 009 | 0.819 | 0.001 | Actinobacteria/ Actinobacteria/ Actinomycetales/ Corynebacteriaceae/ Corynebacterium |
| 006 | 0.802 | 0.001 | Firmicutes/ Bacilli/ Lactobacillales/ Lactobacillaceae/ Lactobacillus |
| 025 | 0.78 | 0.021 | Actinobacteria/ Actinobacteria/ Actinomycetales/ Corynebacteriaceae/ Corynebacterium |
| 044 | 0.775 | 0.005 | Firmicutes/ Bacilli/ Bacillales/ Planococcaceae/ Kurthia |
| 023 | 0.726 | 0.02 | Firmicutes/ Erysipelotrichia/ Erysipelotrichales/ Erysipelotrichaceae/ Turicibacter |
| 010 | 0.709 | 0.012 | Actinobacteria/ Actinobacteria/ Bifidobacteriales/ Bifidobacteriaceae/ Bifidobacteriaceae UC |
| 003 | 0.693 | 0.002 | Firmicutes/ Bacilli/ Lactobacillales/ Lactobacillaceae/ Lactobacillus |
| 040 | 0.681 | 0.002 | Firmicutes/ Clostridia/ Clostridiales/ Peptostreptococcaceae/ Terrisporobacter |
| 107 | 0.531 | 0.044 | Firmicutes/ Clostridia/ Clostridiales/ Ruminococcaceae/ Faecalibacterium |
| 054 | 0.522 | 0.006 | Actinobacteria/ Actinobacteria/ Coriobacteriales/ Coriobacteriaceae/ Coriobacteriaceae UC |
| 050 | 0.471 | 0.043 | Actinobacteria/ Actinobacteria/ Actinomycetales/ Actinomycetaceae/ Actinomyces |

Distance-based partial least squared regression shows how much variation in nestling weight is explained by the composition of their gut microbiomes (Table 2.4). At the nestling stage, the percent of variation in weight attributable to bacterial community composition is relatively low compared to later life stages described below.

*Fledgling stage (Days 15-40)*

Relative abundance analysis showed that Firmicutes continued to be the most dominant phyla in fledgling fecal samples. Firmicutes made up an average of 90.02% in F, 43.61% in Fi, and 84.42% in P. A dramatic increase in compositional complexity was seen in Fi fledglings. Proteobacteria, Acidobacteria and Verrucomicrobia increased in relative abundance at this stage. Proteobacteria made up an average of 1.09% in F, 17.32% in Fi, and 1.82% in P. Acidobacteria made up an average of 1.08% in F, 15.88% in Fi, and 1.11% in P. Verrucomicrobia made up an average of 0.37% in F, 6.33% in Fi, and 0.45% in P (Figure 2.9)

Formula-raised fledglings that received inoculations (Fi) showed a sharp increase in OTU richness, community evenness, and community diversity after leaving the nest that was not seen in the other treatment groups. No significant differences were identified in alpha diversity metrics between F and P fledglings, but Fi fledglings were significantly different in all comparisons of alpha diversity (Figures 2.5, 2.6, 2.7).

**Table 2.4.** Summary of distance-based partial least squares regression showing how much variation in chick weight is explained by each additional component of a bacterial community Bray-Curtis distance matrix. gvar = total weighted geometric variability; crit = value of criterion defined in method.

Nestlings

| Components | Comp 1 | Comp 2 | Comp 3 | Comp 4 | Comp 5 | Comp 6 |
|---|---|---|---|---|---|---|
| $R^2$ | 5.734 | 20.57 | 27.67 | 37.37 | 49.065 | 57.965 |
| adj$R^2$ | 3.542 | 16.79 | 22.38 | 31.1 | 42.534 | 51.327 |
| gvar | 56.422 | 69.65 | 84.79 | 88.88 | 91.386 | 92.448 |
| crit | 13.168 | 11.62 | 11.09 | 10.08 | 8.609 | 7.474 |

Fledglings

| Components | Comp 1 | Comp 2 | Comp 3 | Comp 4 | Comp 5 | Comp 6 |
|---|---|---|---|---|---|---|
| $R^2$ | 42.228 | 63.52 | 67.44 | 72.792 | 77.555 | 82.925 |
| adj$R^2$ | 39.917 | 60.48 | 63.19 | 67.845 | 72.211 | 77.803 |
| gvar | 38.856 | 48.73 | 67.35 | 75.582 | 84.021 | 87.598 |
| crit | 6.077 | 4.15 | 4.02 | 3.657 | 3.297 | 2.753 |

Subadults

| Components | Comp 1 | Comp 2 | Comp 3 | Comp 4 | Comp 5 | Comp 6 |
|---|---|---|---|---|---|---|
| $R^2$ | 10.836 | 20.27 | 36.78 | 49.74 | 71.339 | 80.43 |
| adj$R^2$ | 7.533 | 14.14 | 29.19 | 41.36 | 65.109 | 75.09 |
| gvar | 49.866 | 76.37 | 84.95 | 89.17 | 90.244 | 91.65 |
| crit | 14.385 | 13.83 | 11.83 | 10.17 | 6.294 | 4.68 |

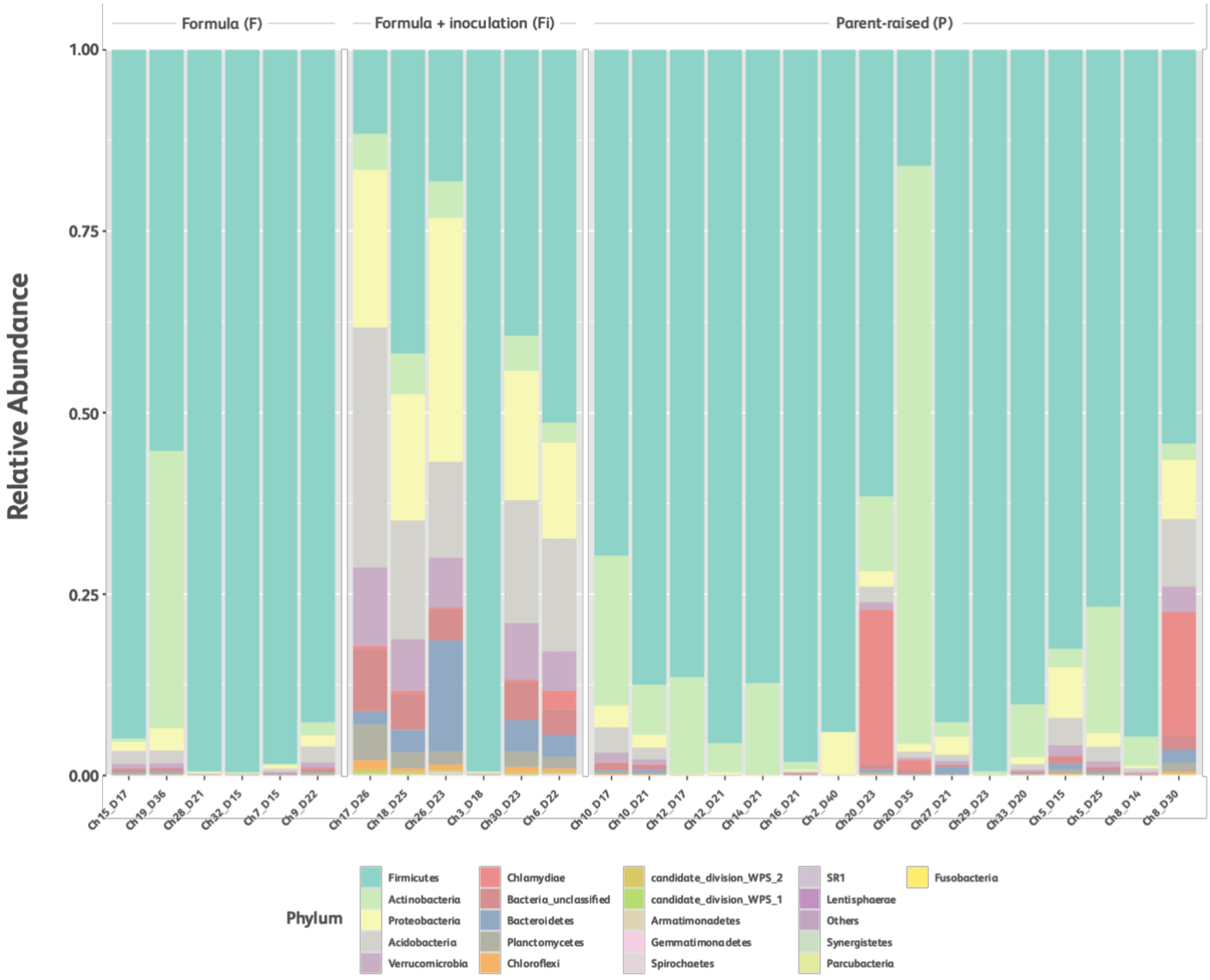


**Figure 2.9.** Relative abundance of bacterial phyla in each fecal sample from fledglings (days 15-40) across all treatment groups. Firmicutes made up an average of 90.02% in F, 43.61% in Fi, and 84.42% in P. Actinobacteria made up an average of 6.82% in F, 3.9% in Fi, and 9.48% in P. Proteobacteria made up an average of 1.09% in F, 17.32% in Fi, and 1.82% in P. Acidobacteria made up an average of 1.08% in F, 15.88% in Fi, and 1.11% in P. Verrucomicrobia made up an average of 0.37% in F, 6.33% in Fi, and 0.45% in P. Chlamydiae made up an average of 0.02% in F, 0.685% in Fi, and 1.89% in P ($n_F = 6$, $n_{Fi} = 6$, $n_P = 16$).

Beta diversity analysis of fledglings show significant differences between all treatment groups. Ordination plots show some overlap in community composition between Fi fledglings and both P fledglings and F fledglings (Figure 2.8). Indicator species analysis identified three Lactobacillus OTUs as indicators of F fledglings. Indicators associated with Fi fledglings included many unclassified Acidobacteria and Spartobacteria OTUs. Indicator OTUs of parent-raised fledglings included several Lactobacillus species (Table 2.5).

Distance-based partial least squared regression shows how much variation in fledgling weight is explained by the composition of their gut microbiomes (Table 2.4). At the fledgling stage, the percent of variation in weight attributable to bacterial community composition reached its highest point.

*Subadult stage (Days 41-250)*

Relative abundance graphs of microorganisms present in subadult fecal samples showed that all treatment groups had more complex communities than were seen in earlier life stages. Firmicutes still made up the largest percentage of any phyla, but on average, they accounted for less than half the bacteria in any of the treatment groups: 36.91% in F, 31.71% in Fi, and 33.08% in P. Actinobacteria made up an average of 15.42% in F, 15.03% in Fi, and 14.52% in P. Proteobacteria made up an average of 14.92% in F, 16.67% in Fi, and 16.71% in P. Acidobacteria made up an average of 15.29% in F, 18.2% in Fi, and 16.37% in P. Verrucomicrobia made up an average of 6.48% in F, 6.82% in Fi, and 6.46% in P (Figure 2.10).

**Table 2.5.** Indicator OTUs for fledgling fecal samples (days 15-40). Green shaded rows are indicators of treatment F (chicks raised with formula), blue shaded rows are indicators of treatment Fi (chicks raised with formula + inoculation), and white rows are indicators of treatment P (chicks raised by parents). "UC" stands for "Unclassified"

| OTU | IndVal | Prob | Phylum/ Class/ Order/ Family/ Genus |
|---|---|---|---|
| 015 | 0.715 | 0.005 | Firmicutes/ Bacilli/ Lactobacillales/ Lactobacillaceae/ Lactobacillus |
| 002 | 0.661 | 0.05 | Firmicutes/ Bacilli/ Lactobacillales/ Lactobacillaceae/ Lactobacillus |
| 001 | 0.513 | 0.043 | Firmicutes/ Bacilli/ Lactobacillales/ Lactobacillaceae/ Lactobacillus |
| 057 | 0.928 | 0.002 | Proteobacteria/ Epsilonproteobacteria/ Campylobacterales/ Campylobacteraceae/ Arcobacter |
| 027 | 0.911 | 0.001 | Firmicutes/ Bacilli/ Lactobacillales/ Streptococcaceae/ Streptococcus |
| 048 | 0.891 | 0.002 | Proteobacteria/ Alphaproteobacteria/ Rhizobiales/ Bradyrhizobiaceae/ Bradyrhizobium |
| 088 | 0.891 | 0.001 | Acidobacteria/ Acidobacteria Gp2/ Gp2/ Gp2 UC/ Gp2 UC |
| 114 | 0.89 | 0.001 | Verrucomicrobia/ Spartobacteria/ Spartobacteria_UC/ Spartobacteria_UC/ Spartobacteria_UC |
| 061 | 0.888 | 0.001 | Actinobacteria/ Actinobacteria/ Actinomycetales/ Thermomonosporaceae/ Actinoallomurus |
| 067 | 0.886 | 0.001 | Acidobacteria/ Acidobacteria Gp6/ Gp6 UC/ Gp6 UC/ Gp6 UC |
| 035 | 0.876 | 0.002 | Acidobacteria/ Acidobacteria Gp2/ Gp2 UC/ Gp2 UC/ Gp2 UC |
| 069 | 0.872 | 0.002 | Acidobacteria/ Acidobacteria Gp2/ Gp2 UC/ Gp2 UC/ Gp2 UC |
| 036 | 0.872 | 0.002 | Verrucomicrobia/ Spartobacteria/ Spartobacteria UC/ Spartobacteria UC/ Spartobacteria UC |
| 082 | 0.871 | 0.001 | Acidobacteria/ Acidobacteria Gp3/ Gp3 UC/ Gp3 UC/ Gp3 UC |
| 022 | 0.871 | 0.001 | Proteobacteria/ Alphaproteobacteria/ Rhizobiales/ Rhizobiales UC/ Rhizobiales UC |
| 030 | 0.867 | 0.002 | Verrucomicrobia/ Spartobacteria/ Spartobacteria UC/ Spartobacteria UC/ Spartobacteria UC |
| 062 | 0.861 | 0.001 | Acidobacteria/ Acidobacteria Gp3/ Gp3 UC/ Gp3 UC/ Gp3 UC |
| 026 | 0.859 | 0.004 | Acidobacteria/ Acidobacteria Gp2/ Gp2 UC/ Gp2 UC/ Gp2 UC |
| 032 | 0.856 | 0.002 | Proteobacteria/ Alphaproteobacteria/ Rhizobiales/ Roseiarcaceae/ Roseiarcus |
| 011 | 0.987 | 0.013 | Firmicutes/ Clostridia/ Clostridiales/ Peptostreptococcaceae/ Romboutsia |
| 014 | 0.866 | 0.001 | Firmicutes/ Bacilli/ Lactobacillales/ Lactobacillaceae/ Lactobacillus |
| 017 | 0.859 | 0.002 | Firmicutes/ Bacilli/ Bacillales/ Staphylococcaceae/ Staphylococcus |
| 004 | 0.824 | 0.002 | Actinobacteria/ Actinobacteria/ Bifidobacteriales/ Bifidobacteriaceae/ Aeriscardovia |
| 003 | 0.807 | 0.006 | Firmicutes/ Bacilli/ Lactobacillales/ Lactobacillaceae/ Lactobacillus |
| 006 | 0.751 | 0.009 | Firmicutes/ Bacilli/ Lactobacillales/ Lactobacillaceae/ Lactobacillus |
| 008 | 0.743 | 0.002 | Firmicutes/ Bacilli/ Lactobacillales/ Lactobacillaceae/ Lactobacillus |
| 081 | 0.733 | 0.003 | Bacteria UC/ Bacteria UC/ Bacteria UC/ Bacteria UC/ Bacteria UC |
| 007 | 0.698 | 0.028 | Firmicutes/ Bacilli/ Lactobacillales/ Lactobacillaceae/ Lactobacillus |


**Figure 2.10.** Relative abundance of bacterial phyla in each fecal sample from subadults (days 41-250) across all treatment groups. Firmicutes made up an average of 36.91% in F, 31.71% in Fi, and 33.08% in P. Actinobacteria made up an average of 15.42% in F, 15.03 in Fi, and 14.52% in P. Proteobacteria made up an average of 14.92% in F, 16.67% in Fi, and 16.71% in P. Acidobacteria made up an average of 15.29% in F, 18.2% in Fi, and 16.37% in P. Verrucomicrobia made up an average of 6.48% in F, 6.82% in Fi, and 6.46% in P ($n_F = 9$, $n_{Fi} = 8$, $n_P = 12$).

Alpha diversity metrics of bacterial communities associated with subadult fecal samples showed that OTU richness, community evenness, and community diversity were consistently high across the three treatment groups. None of the alpha diversity measurements in this life stage were significantly different among treatment groups. However, community evenness and community diversity both revealed significant differences among median values with P subadult medians higher than Fi subadults, and Fi subadults higher than F subadults (Figures 2.6, 2.7)

Beta diversity at the subadult stage showed no statistical difference between treatment groups. Ordination plots revealed that the three treatment groups overlapped substantially in community composition (Figure 2.8). Indicator species identified for Fi subadults included several unclassified Acidobacteria OTUs. A single indicator species, Acidobacteria Gp13, was identified for F subadults (Table 2.6).

Distance-based partial least squared regression shows how much variation in subadult weight is explained by the composition of their gut microbiomes (Table 2.4).At the subadult stage, the percent of variation in weight explained by the bacterial community composition was relatively low.

**Table 2.6.** Indicator OTUs for subadult (days 41-250) and breeding adult fecal samples. Blue shaded rows are indicators of treatment Fi (chicks raised with formula + inoculation), white row is indicator of treatment P (chicks raised by parents), and gray shaded rows are indicators of breeders. "UC" stands for "Unclassified".

| OTU | IndVal | Prob | Phylum/ Class/ Order/ Family/ Genus |
|---|---|---|---|
| 070 | 0.47 | 0.015 | Acidobacteria/ Acidobacteria Gp1/ Gp1/ Gp1 UC/ Gp1 UC |
| 071 | 0.445 | 0.014 | Proteobacteria/ Proteobacteria UC/ Proteobacteria UC/ Proteobacteria UC/ Proteobacteria UC |
| 094 | 0.428 | 0.023 | Acidobacteria/ Acidobacteria Gp3/ Gp3/ Gp3 UC/ Gp3 UC |
| 035 | 0.412 | 0.044 | Acidobacteria/ Acidobacteria Gp2/ Gp2/ Gp2 UC/ Gp2 UC |
| 098 | 0.41 | 0.024 | Acidobacteria/ Acidobacteria Gp1/ Gp1/ Gp1 UC/ Gp1 UC |
| 062 | 0.409 | 0.031 | Acidobacteria/ Acidobacteria Gp3/ Gp3/ Gp3 UC/ Gp3 UC |
| 056 | 0.404 | 0.026 | Acidobacteria/ Acidobacteria Gp1/ Gp1/ Gp1 UC/ Gp1 UC |
| 097 | 0.452 | 0.018 | Acidobacteria/ Acidobacteria Gp13/ Gp13/ Gp13 UC/ Gp13 UC |
| 040 | 0.99 | 0.001 | Firmicutes/ Clostridia/ Clostridiales/ Peptostreptococcaceae/ Terrisporobacter |
| 011 | 0.96 | 0.018 | Firmicutes/ Clostridia/ Clostridiales/ Peptostreptococcaceae/ Romboutsia |
| 006 | 0.606 | 0.044 | Firmicutes/ Bacilli/ Lactobacillales/ Lactobacillaceae/ Lactobacillus |

*Crop milk microbiomes*

Crop milk samples that were collected from the crops of parents versus from the crops of parentally-fed nestlings showed differences in relative abundance of bacterial orders. Lactobacillales made up an average of 56.43% in crop milk collected from the nestlings and 31.8% collected directly from parents. Bifidobacteriales made up an average of 32.19% in crop milk from nestlings and 13.41% from parents. Pasteurellales made up an average of 4.21% in crop milk from nestlings and 13.73% from parents. Actinomycetales made up an average of 1.86% in crop milk from nestlings and 14.08% from parents. Bacillales made up an average of 0.61% in crop milk from nestlings and 18.26% from parents (Figure 2.11). There were five Lactobacillus OTUs and well as an Aeriscardovia OTU that were identified as indicator OTUs for crop milk collected from nestlings (Table 2.7).

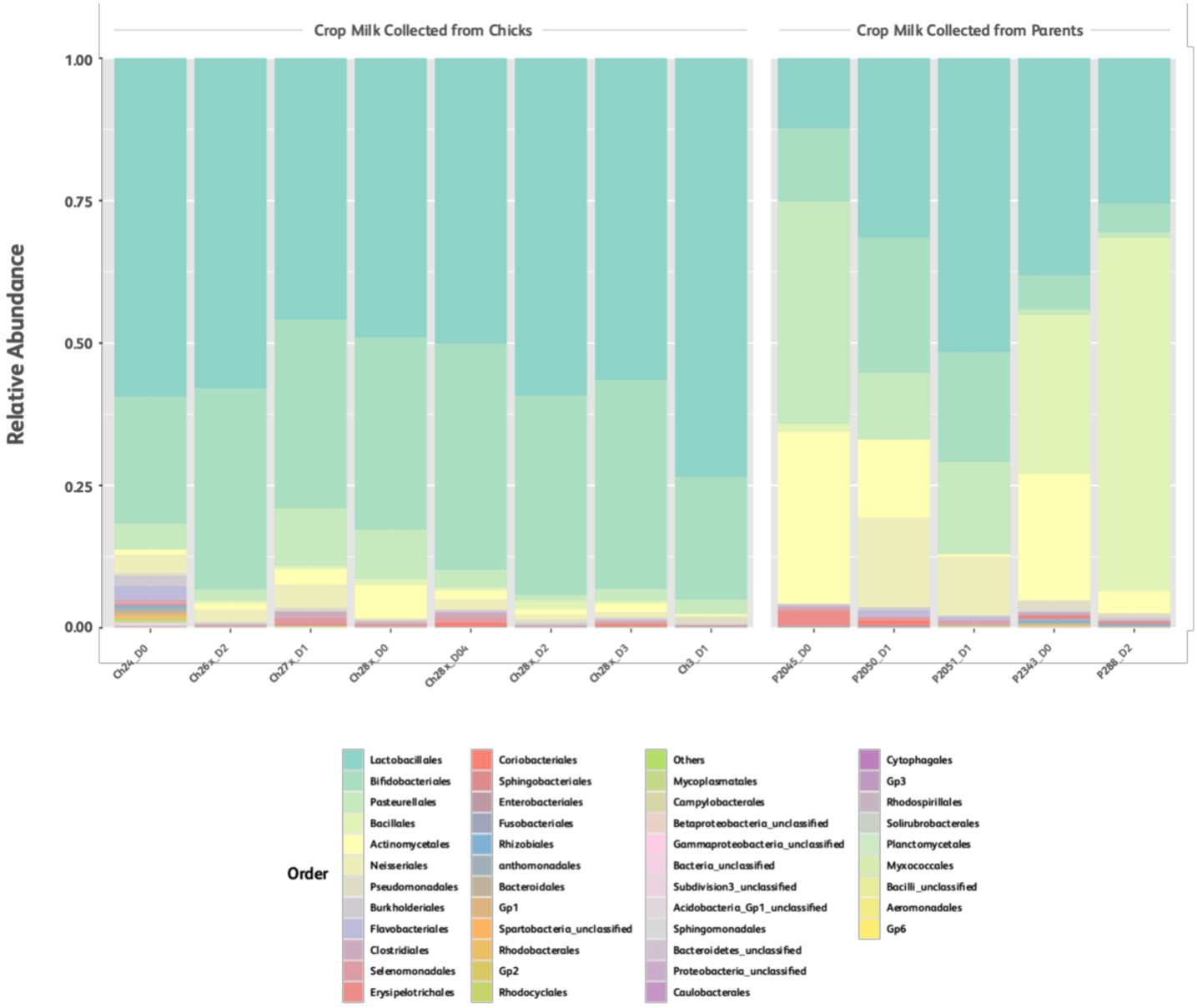


**Figure 2.11.** Relative abundance of bacterial orders in each crop milk sample, either collected from the crop of a parent or the crop of a nestling. Lactobacillales made up an average of 56.43% in crop milk from nestlings and 31.8% from parents. Bifidobacteriales made up an average of 32.19% in crop milk from nestlings and 13.41% from parents. Pasteurellales made up an average of 4.21% in crop milk from nestlings and 13.73% from parents. Actinomycetales made up an average of 1.86% in crop milk from nestlings and 14.08% from parents. Bacillales made up an average of 0.61% in crop milk from nestlings and 18.26% from parents.

**Table 2.7.** Indicator OTUs for crop milk samples collected from parents and nestlings. All OTUs listed are indicators of crop milk collected from the crops of nestlings.

| OTU | IndVal | Prob | Phylum/ Class/ Order/ Family/ Genus |
|---|---|---|---|
| 015 | 0.887 | 0.001 | Firmicutes/ Bacilli/ Lactobacillales/ Lactobacillaceae/ Lactobacillus |
| 004 | 0.828 | 0.001 | Actinobacteria/ Actinobacteria/ Bifidobacteriales/ Bifidobacteriaceae/ Aeriscardovia |
| 002 | 0.811 | 0.021 | Firmicutes/ Bacilli/ Lactobacillales/ Lactobacillaceae/ Lactobacillus |
| 008 | 0.763 | 0.001 | Firmicutes/ Bacilli/ Lactobacillales/ Lactobacillaceae/ Lactobacillus |
| 006 | 0.718 | 0.049 | Firmicutes/ Bacilli/ Lactobacillales/ Lactobacillaceae/ Lactobacillus |
| 014 | 0.714 | 0.039 | Firmicutes/ Bacilli/ Lactobacillales/ Lactobacillaceae/ Lactobacillus |

## Discussion

This is the first experimental study to sequence and compare the gut microbiomes of columbids successfully hand-raised with formula with parent-raised controls. Parent-raised chicks (P) showed earlier onset of rapid growth when compared with formula-raised chicks (F & Fi). Parent-raised chicks were also heavier than formula-raised chicks after 150 days of age. The gut microbiome composition of parent-raised chicks was distinct from the formula-raised chicks at the nestling (days 0-14) and fledgling stages (days 15-40) but was similar to formula-raised chicks at the subadult stage (days 41-250). Formula-raised chicks that received an inoculation (Fi) showed a slightly earlier onset of rapid growth when compared with formula-raised chicks that did not receive an inoculation (F). The gut microbiome composition of Fi chicks was distinct from F chicks at the fledgling stage (days 15-40), but not at the other life stages.

### *Hypothesis #1 (Parent-raised and formula-raised chicks)*

My first hypothesis was that the gut microbiome of chicks hand-raised with formula (F & Fi) would differ in composition from the gut microbiome of parent-reared chicks (P) and I predicted that this compositional difference would be correlated with slower growth rates. This prediction was supported by the results. However, because there are many factors that differ between the environments of parent-raised chicks and hand-raised chicks that cannot be controlled for, it is worth noting that the most salient comparisons I can make are between the two hand-raised treatment groups where the only difference is inoculation. Differences between the rearing environments of hand-raised and parent-raised chicks included nest type (natural vs. artificial), environment (semi-outdoor vs. indoor), and handling time. Because these differences exist, I cannot definitively state that the differences seen in the microbiome between formula-

raised chicks and parent-raised chicks were solely a result of their diet. However, I observed significant differences in the gut microbiomes of hand-raised and parent-raised chicks specifically at the nestling and fledgling stage, but not at the subadult stage. At this stage the birds were fully self-reliant and feeding on their own in communal housing. Thus, it made sense that their bacterial communities were more similar. The experiment demonstrated that raising doves with formula affected their gut-associated microbial communities at a young age, but there were no considerable, lasting impacts on the microbiome once the birds reached nutritional independence and were socially housed.

Hand-raising columbids with formula did, however, impact chick weight later in life. Formula-raised birds weighed less at the subadult stage when compared with parent-raised birds, an undesirable outcome. This difference in weight was only weakly correlated with microbial composition, therefore it is possible that other nutritional factors missing from formula are the cause of this difference.

*Hypothesis #2 (Formula-raised chicks with and without crop milk inoculation)*

My second hypothesis was that crop milk inoculation could be used to enhance traditional formula diets and promote the development of hand-raised chicks, resulting in higher rates of growth and survival. This prediction was only weakly supported by the results. One beneficial effect of my crop milk inoculations was observed in chick growth curves. In all three treatment groups, the shape of growth curves were similar but their mean inflection points did not occur at the same time. The steepest period of growth occurred earlier in the parent-raised chicks when compared to both formula-raised treatment groups, and this steep period of growth happened earlier in the lives of chicks inoculated with crop milk compared with the hand-reared

only group. The amount of crop milk provided to inoculated chicks was limited to ensure that any differences seen would be an effect of microorganisms and not other nutritive components. The observed differences suggest that the crop milk-associated microorganisms present in the inoculation may have shortened the initial "stalling" period that occurs before grow rate sharply increases in formula-raised chicks. Shortening this period of time could be important as this was when mortality in formula-raised chicks was highest.

Another effect of the crop milk inoculation was seen in the gut microbiomes of crop milk inoculated chicks at the fledgling stage. A substantial increase in OTU richness, community evenness, and community diversity was seen in Fi chicks at the fledgling stage (days 15-40). This could have been due to community coalescence, or the merging of two distinct microbial communities (Castledine et al. 2020). Priority effects could have also played a role in these results (Debray et al. 2021). By introducing the crop milk-associated microorganisms into the guts of formula-fed chicks, OTUs that did well in the presence of formula were likely selected for while other beneficial OTUs could not persist (Grond et al. 2019).

*Conclusions*

Even though inoculating hand-raised chicks with a small amount of crop milk did not result in weight gain similar to that of parent-raised birds, it did result in an earlier onset of rapid growth rate when compared with formula-raised chicks that did not receive an inoculation. It is likely that the microbiome associated with crop milk provides benefits to growing chicks but that certain conditions are required for the beneficial bacterial constituents to become established and persist. Factors such as low exposure and lack of essential prebiotics could have impacted the ability of beneficial microorganisms to successfully colonize the gut of formula-raised chicks

(Grond et al. 2019). Refinements to the inoculation method should focus on amount and scheduling of supplementation. It would be interesting to repeat this experiment and provide hand-raised groups with different amounts of supplemented crop milk (one group receiving a small amount, another receiving a large amount), or providing more frequent crop milk inoculations.

It is possible that the crop milk inoculation altered metrics of health besides weight, body condition, and survival that were not measured here. These might include immune system function, neurological processes, lifespan, reproductive success, and other health parameters of offspring. It would be interesting to explore these other metrics in future studies. For example, immune function could be tested in subadults by injecting subcutaneously a small amount of a suspension of sheep red blood cells, then collecting a blood sample 6 days later and measuring the magnitude of the antibody response (Grasman 2010)

By learning more about the effects of crop milk on the health of growing columbids, we can continue to improve husbandry practices associated with the captive breeding of threatened species of pigeons and doves. While the results of this study provide only weak support for recommending crop milk inoculations when raising columbids with formula, it has illuminated differences between columbids raised with and without crop milk and suggested further avenues to explore.

There are many nutritional, microbial and immune benefits of animal milks. By experimentally studying the milks produced by diverse animal parents, we can learn more about their compositions and functions. We are just beginning to scratch the surface of how vertical transmission from parent to offspring has given milk-producing species an adaptive advantage.

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Animal Care and Use Committee
003 Ed Warren Life Sciences Building | East Carolina University | Greenville NC 27354 - 4354
252-744-2436 office | 252-744-2355 fax

March 12, 2020,

Susan McRae, Ph.D.
Department of Biology
East Carolina University

Dear Dr. McRae:

Your Animal Use Protocol entitled, " Effects of Nutritional Supplements on Captive Bird Health and Microbiome Composition " (AUP #D359) was reviewed by this institution's Animal Care and Use Committee on 3/12/2020. The following action was taken by the Committee:

"Approved as submitted"

****Please contact Aaron Hinkle prior to any hazard use****

A copy of the protocols is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies. **Please ensure that all personnel associated with this protocol have access to this approved copy of the AUP/Amendment and are familiar with its contents.**

Sincerely yours,

Jamie DeWitt, Ph.D.
Vice Chair, Animal Care and Use Committee

JD/GD

enclosure