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博士学位論文

ニホンライチョウ雛の死亡率低減に資する卵質検査

Assessment of egg quality parameters as a possible clue to chick mortality in captive Japanese rock ptarmigan *Lagopus muta japonica* chicks

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1) Japanese rock ptarmigan displays high levels of polyunsaturated fatty acids in egg yolk compared to chicken or quail (2022). Vik, A., Tsuchida, S., Kobayashi, A., Akiba, Y., Harafuji, M. and Ushida, K. *J Vet Med Sci.* 84(9): 1221-1224.

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2) Comparison of egg antibody levels between wild and captive Japanese rock ptarmigans (2023). Vik, A., Tsuchida, S., Suzuki-Hashido, N., Kobayashi, A., Akiba, Y., Harafuji, M. and Ushida, K. *Jpn J Zoo Wild Med.* 28(2): 95-101.

Chapter 1

General Introduction

1-1 Captive breeding and conservation of endangered species

Globally we are seeing a rapid decline in biodiversity which most critically affects endangered species. There are multiple reasons for this and the problems are only expected to increase in the future especially due to the effects of global warming. Endangered animals often include many which have significant impacts not only as a part of the ecosystem of which they live in, but also economically and culturally to humans.

Captive breeding of endangered species is a frequently used mean to save species from extinction in the wild by increasing population number in a place safe from the external factors that triggered the decline in the first place (Ralls and Ballou 1986). However these artificial environments are often very different to those which the animals have initially adapted their lifestyle to, and breeding protocols cannot often reflect a perfect scenario to accommodate this problem. This can cause issues related to the health and viability, and also propagation of captive individuals.

One such species is the Japanese rock ptarmigan *Lagopus muta japonica* (Figure 1-1), an endangered alpine bird species that is currently undergoing captive breeding in order to save it from extinction in the wild (Akiba 2020).

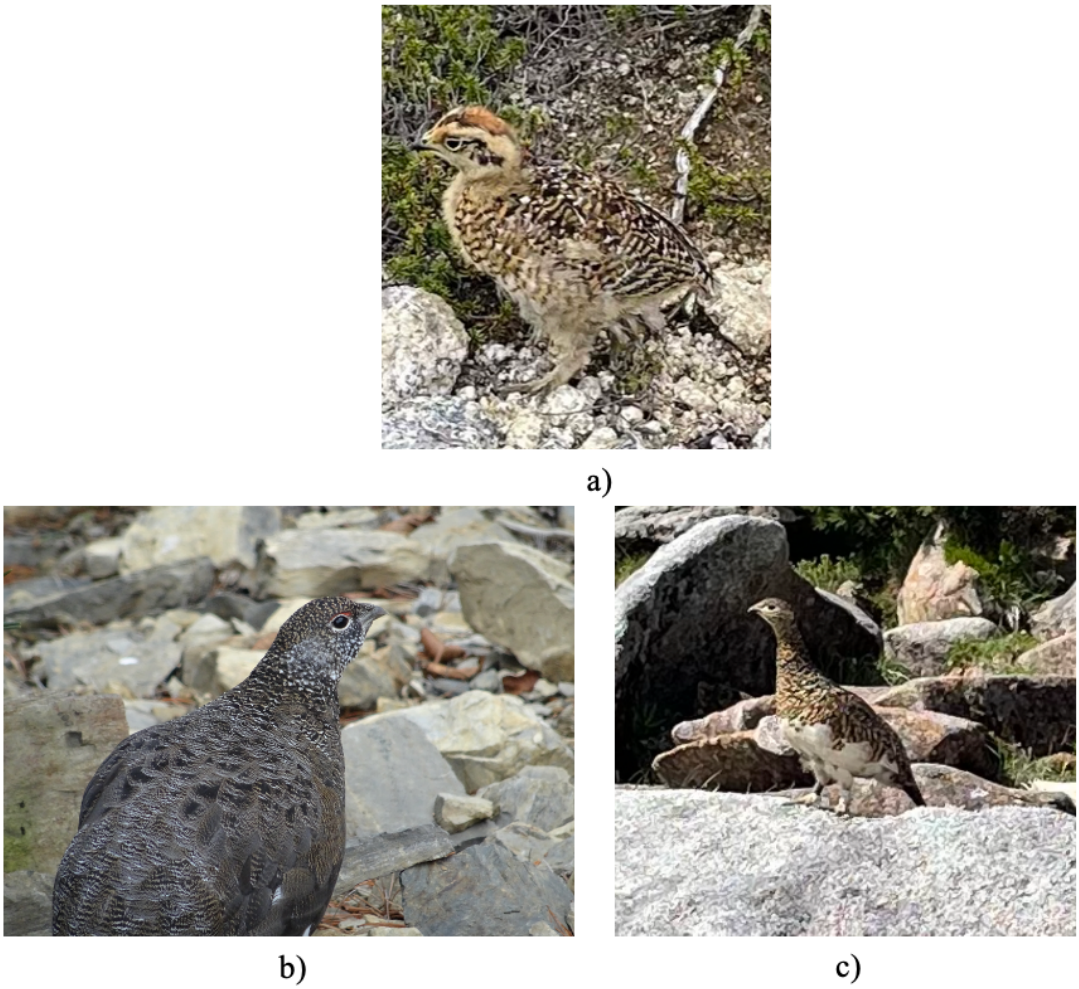


Figure 1-1. Japanese rock ptarmigan *Lagopus muta japonica*.
a) Chick Chuo Alps. b) Female Southern Alps. c) Female Chuo Alps.

The captive conservation work has seen its fair share of issues related to the survival and health of individuals, especially chicks, and this is potentially hampering conservation efforts in the long term. Although the reasons for this are likely multifaceted, when it comes to the chicks' survival, one clue can lie with the condition of the "start package" the chicks are supplied with as they meet life for the first time – the egg. The Japanese rock ptarmigan *Lagopus muta japonica* is an alpine gallinaceous bird species endemic to the Japanese island of Honshu. A relic of the latest ice age, the habitat of these species has severely diminished to

a few isolated mountainous areas as the ice retracted. It can now be found in the Japanese Alps in the Chubu region, with minor populations further north in the Kubiki mountains (Figure 1-2).



Figure 1-2. Distribution of Japanese rock ptarmigan *Lagopus muta japonica*. Red = inhabiting, black = extinct. Modified and adapted from Asahi Shimbun Digital.

Having both ecological and cultural significance for the Japanese people, it was designated a special natural monument and protected species by the Japanese Ministry of the Environment (MOE) in 1955 (MOE 2012).

Population numbers of this species have severely decreased through recent decades, from an estimated 3000 individuals in the early 1980s (Haneda et al. 1985) to under 2000 at present. The ptarmigan is now classified as endangered (EN) in the MOE's red list. Reasons for the decline in the wild are many, and include loss of habitat, predation and pollution mainly due to increased mountain tourism, all which have had an increased impact due to global warming (Kobayashi and Nakamura 2013; Nakamura 2007).

In effort to save the species from extinction in the wild, the Japanese government launched a national conservation program consisting of *in situ* conservation in the wild (Figure 1-3 and Figure 1-4), governed by MOE, as well as *ex situ* captive breeding in zoos across Japan. The main goal of the latter is to secure founders that could produce viable progeny in zoos, that could later be released to the wild again at a later point to increase total population numbers.

There are several issues that must be overcome for this final goal to happen, and one of these is to reduce the high chick mortality in zoos. Captive breeding of Japanese rock ptarmigan started in 2016 after being green-lit by the MOE after breeding trials using another subspecies – the Svalbard rock ptarmigan *Lagopus muta hyperborea* – had allowed the zoos to develop successful breeding protocols. This work is undertaken by member zoos of the Japanese Association of Zoos and Aquariums (JAZA), and as of today captive breeding of Japanese rock ptarmigan is taking place in 10 locations across the nation. Founders were initially secured from eggs taken at Mt Norikura in the Northern Alps (Figure 1-3), and after some initial failed attempts the captive population has been steadily growing. Despite captive individuals in most cases being able to produce viable progeny, the health and survival of chicks has been a major obstacle to increase the population. If the *ex situ* conservation is to succeed in the long term, it is of utmost importance to uncover possible causes behind these hindrances.

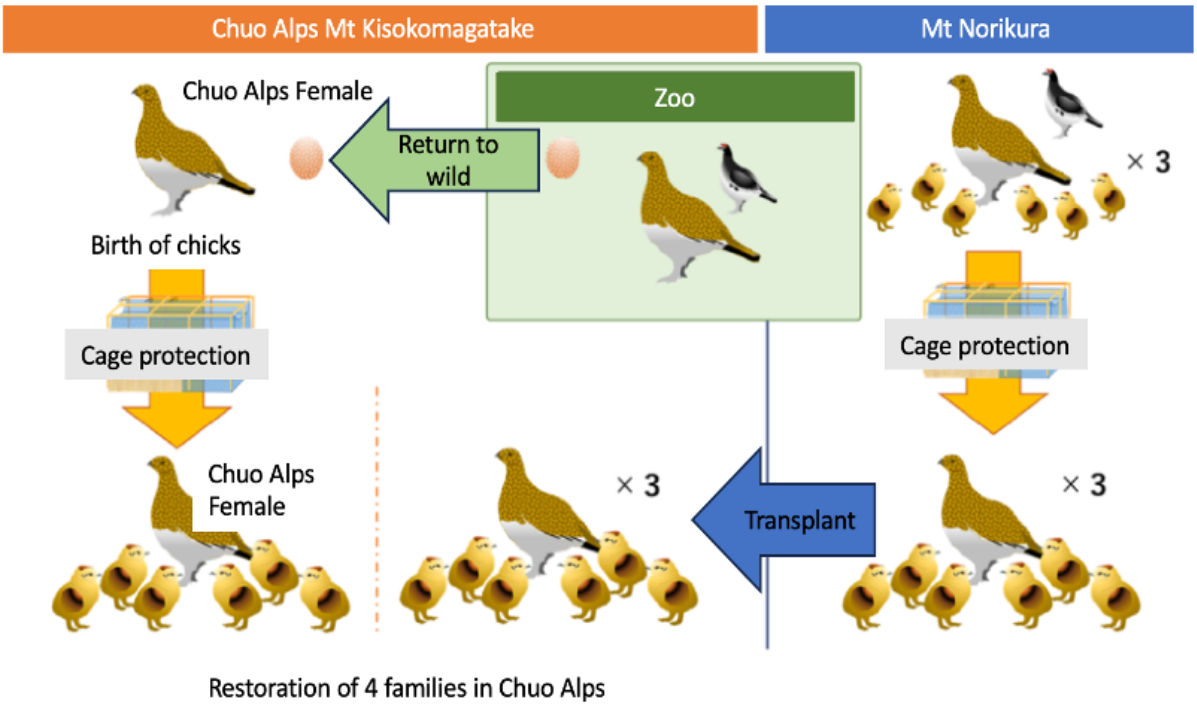


Figure 1-3. History of the *in situ* (cage protection) and *ex situ* (captive breeding) of Japanese rock ptarmigan since the start in 2016. Founders were secured from Mt Norikura for captive breeding in zoos. In 2018 a solitary female was discovered in Mt Kisokomagatake Chuo Apls. The population in Chuo Alps was revived by an egg exchange (pre-fertilized eggs from zoos) to be incubated by the solitary female, and by transplantation of 3 families from neighboring Mt Norikura in 2020. The cage protection of ptarmigan families at Mt Kisokomagatake has been ongoing since 2021. Modified and adapted from hnbirdlabo.org.

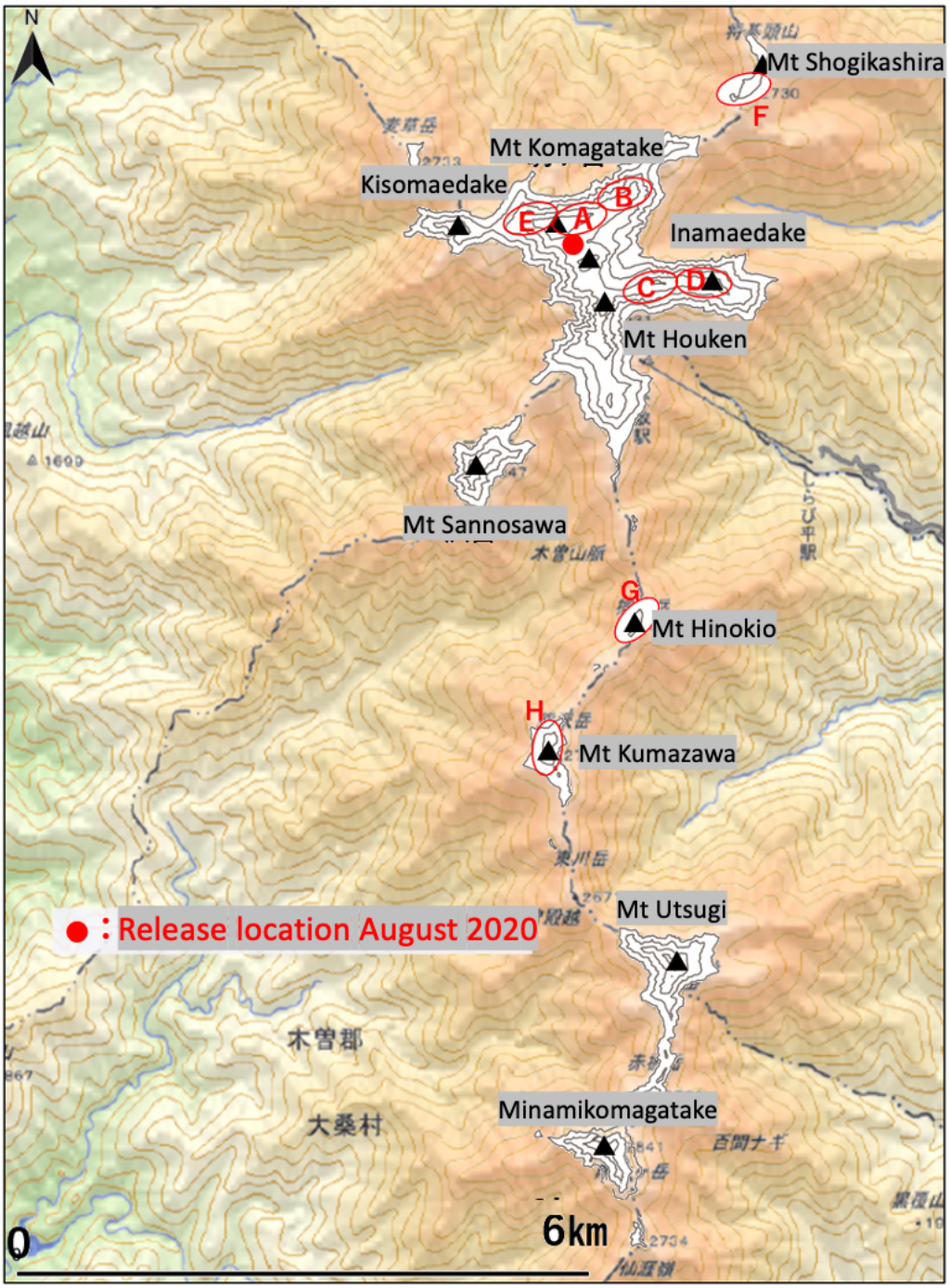


Figure 1-4. Map of Mt Kisokomagatake, Chuo Alps, where cage protection has been carried out since 2021. After alone female was discovered here in 2018, 3 families from neighboring Mt Norikura was released after cage protection in 2020. The red circles A-H marks the various established territories in the area. Modified and adapted from hnbirdlabo.org.

1-2 General biology of the Japanese rock ptarmigan

The rock ptarmigan *Lagopus muta* has a circumpolar distribution throughout alpine and tundra areas in Eurasia and North America (Figure 1-5), with 23 to 30 subspecies recognized to exist worldwide (Johnsgard 1983; del Hoyo et al. 1994). The Japanese subspecies *Lagopus muta japonica* is endemic to the Chubu mountain region on the Japanese island of Honshu (Figure 1-2).

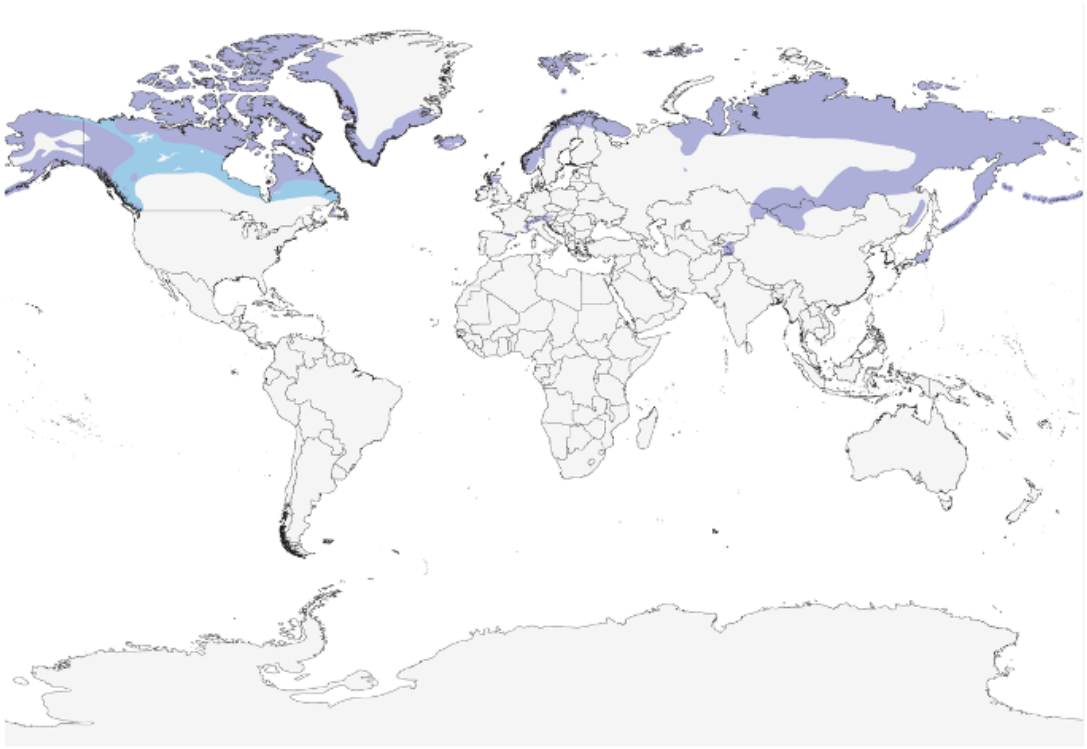


Figure 1-5. Worldwide distribution of rock ptarmigan *Lagopus muta*.
Dark blue = year-round, Light blue = non-breeding. Adapted from birdsoftworld.org.

Although it shares a lot of similarities with its Arctic subspecies from individuals whom started the captive breeding program - the Svalbard rock ptarmigan *Lagopus muta hyperborea* (Figure 1-6) - it also has some opposing parallels. Whereas the Svalbard rock ptarmigan is the world's most northern inhabiting subspecies, the Japanese rock ptarmigan

comprise the world's southernmost populations. Another difference is the size of the birds, where the Arctic relative comes in as the largest of all subspecies and the Japanese the smallest. The topography of their respective habitats is also fairly different. Inhabiting the high mountains at an altitude between just under 2,000m to as high as just above 3,000m (Nakamura 2007) the Japanese rock ptarmigan can be found in the high alpine areas for most time of the year. In winter however they tend to descend to lower altitudes towards the coniferous forests. This entails a more steeper migration incline compared to rock ptarmigan species inhabiting tundra areas like that of the Svalbard rock ptarmigan (Figure 1-6).

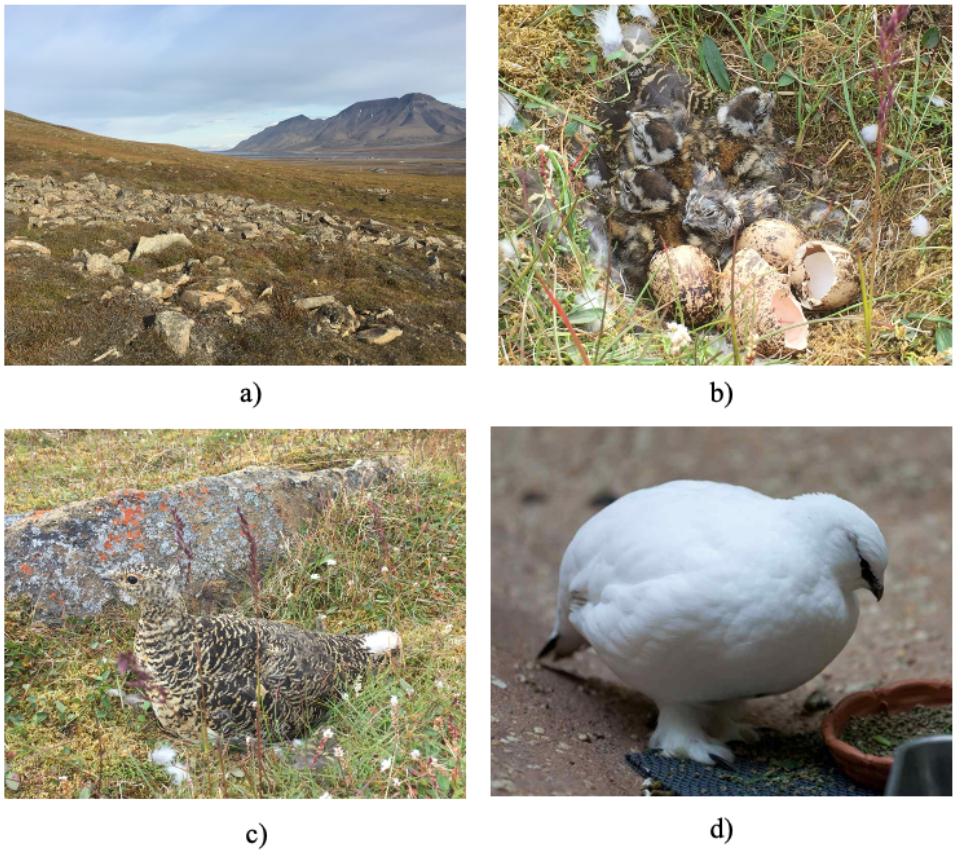


Figure 1-6. Svalbard rock ptarmigan *Lagopus muta hyperborea*.

The largest subspecies of rock ptarmigan and the species that started the breeding trials prior to the current captive breeding of Japanese rock ptarmigan in Japanese zoos. a) Rock ptarmigan habitat Spitsbergen, Svalbard. b) Newly hatched Svalbard rock ptarmigan chicks in nest. c) Svalbard rock ptarmigan hen. d) Svalbard rock ptarmigan hen in winter plumage under captivity, Ueno Zoo, Japan

Like other rock ptarmigan subspecies the Japanese rock ptarmigan undergoes seasonal changes in molting pattern from brown in the spring/summer to white in the winter, with males and females differing in timing and peculiarities of summer pattern due to the breeding process and its energetic cost. Males establish territories in early spring prior to snowmelt, and mating occurs late May-early June. Egg laying takes place early- to mid June, with the female laying her eggs sequentially over consecutive days until she starts full incubation under the layers of creeping pine *Pinus pumila* (Figure 1-7). This plant is a characteristic

feature of the preferred habitat of the Japanese rock ptarmigan, serving as both nesting place, hiding place and shelter as well as for food.



Figure 1-7. Creeping pine *Pinus pumila* – also known as Japanese stone pine or Siberian dwarf pine. The Japanese rock ptarmigan is dependent upon this plant species for nesting, cover and even food.

Japanese rock ptarmigan hens can lay anything between 2 to 8 eggs in the wild, the most frequent number being 6 to 7 eggs (Sawa, Takeuchi and Nakamura 2011; Nakamura 2007). In case of failed first attempts, second egg laying are also possible albeit with fewer eggs. Eggs hatch late June early July depending on the area, and the chicks almost immediately get out of the nest following the hen closely while feeding on wild plants and insects learning from their mother the art of feeding and recognizing dangers of possible predators that might be lurking nearby. In autumn the when the chicks are fully grown juveniles they leave their

family to pass the winter in the mountains. In the wild, the most challenging time for survival for the chicks is the time after hatching. During early to mid-summer the mountains experience prolonged periods of heavy rain combined with harsh winds, and with the inability to efficiently thermoregulate chicks often succumb to the low temperatures. In addition, predators like fox *Vulpes vulpes japonica*, marten *Martes melampus*, stoat *Mustela erminea*, the common kestrel *Falco tinnunculus*, long-billed crow *Corvus macrorhynchos* and even Japanese macaques *Macaca fuscata*, as well as other birds of prey, often see their chance to get an easy meal out of the small ptarmigan chicks.

Significantly reducing these dangers are the main reason the *in situ* conservation efforts by way of cage protection during the summer months have been so effective in increasing wild population numbers of these species. It also allow the chicks to have normal contact with the mother hen and learn the ways to handle challenges brought upon them in the wild, unlike chicks hatched under captive conditions whereby chicks hatch from eggs in artificial incubators and are completely separated from the hen.

The diet of the Japanese rock ptarmigan is extremely varied and changes throughout the seasons (Figure 1-8) and is a consequence of both seasonal availability as well as physiological needs related to life history events, as evidenced by studies observing the number of pecks at various times of the year (Kobayashi and Nakamura, 2011). The seasonal preferences and food intake vary slightly between male and female individuals as well as chicks, which is likely related to their energetic needs at their various stages in life. For males, the highest food intake occurs during early spring coinciding with the establishment of territories. For females, the most critical period is that during egg-laying and incubation. For chicks, it will be during the first months after hatching as they are developing fast.

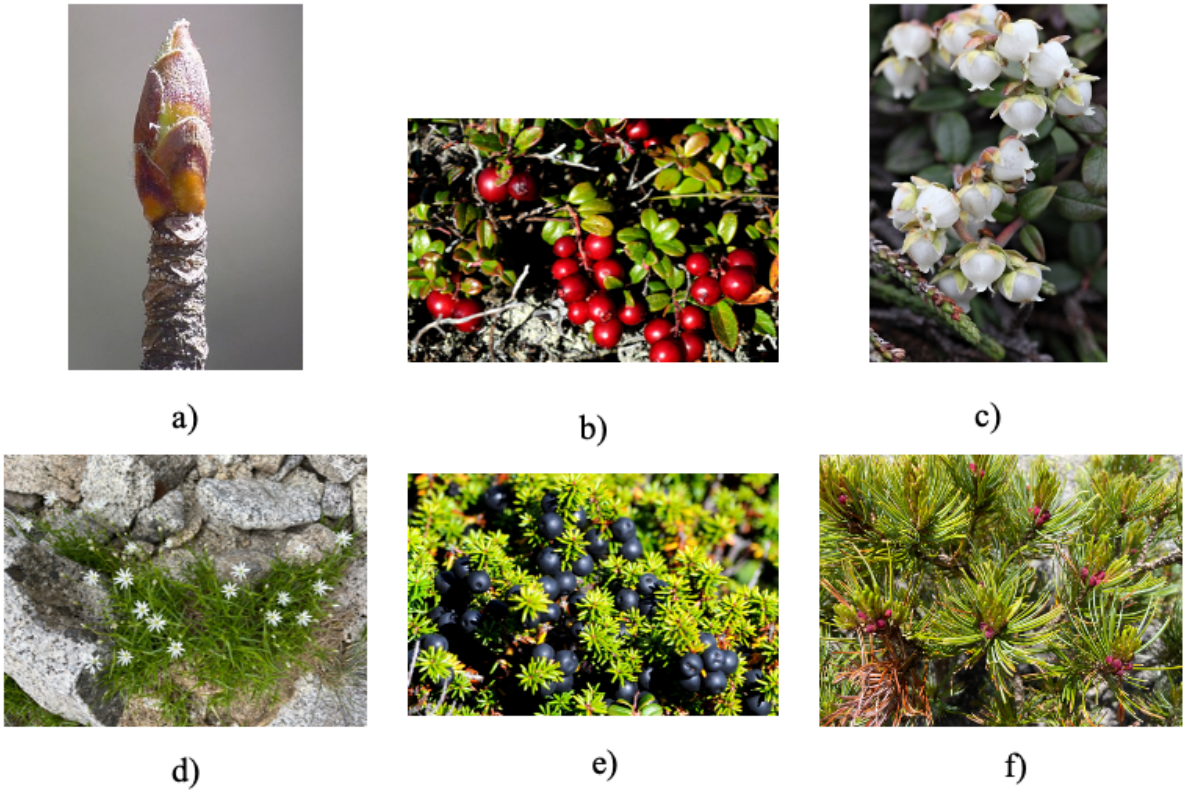


Figure 1-8. A few of the many wild plants comprising the diet of wild Japanese rock ptarmigan.

a) Winter bud of *Betula ermanii* (credit: shizuka.sakura.ne.jp)

b) *Vaccinium vitis-idaea* (credit: hnbirdlabo.org)

c) *Arcterica nana* (credit: wildplants.sakura.ne.jp)

d) *Stellaria nipponica*

e) *Empetrum nigrum* (credit: hnbirdlabo.org)

f) *Pinus pumila*

In winter from late November until snowmelt in late April, the time during which food is most scarce and the birds have descended to the subalpine zone below the tree line, winter buds of *Betula* and *Alnus* dominate the birds' diet. Mid-spring around late April when the snow starts to melt the diet changes to leaves and branches of evergreen dwarf shrubs mainly belonging to the *Ericaceae* and *Orchidaceae* families, a preference that continues throughout the early summer months when flowers of these plants also account for a larger proportion of

the diet. During this period insects are also frequently eaten, and this constitute an important addition to the diet especially for newly hatched chicks. Mid-summer around July leaves and flowers of herbaceous plants are eaten, and late summer-early autumn their fruits and seeds constitute a larger proportion of the feed ingested. Late autumn around October/November the diet changes to primarily the seeds of *Pinus pumila*. The highly varied diet of Japanese rock ptarmigan constitute plants that are hard to digest and a unique feature of this species is their pair of large ceca (Figure 1-9) in which they efficiently ferment cellulose from the high fibrous plant feed, and where populations of symbiotic microorganisms efficiently ferment and detoxify plant metabolites found in the many plant species they survive on (Tsuchida et al. 2017; Ueda et al. 2018).

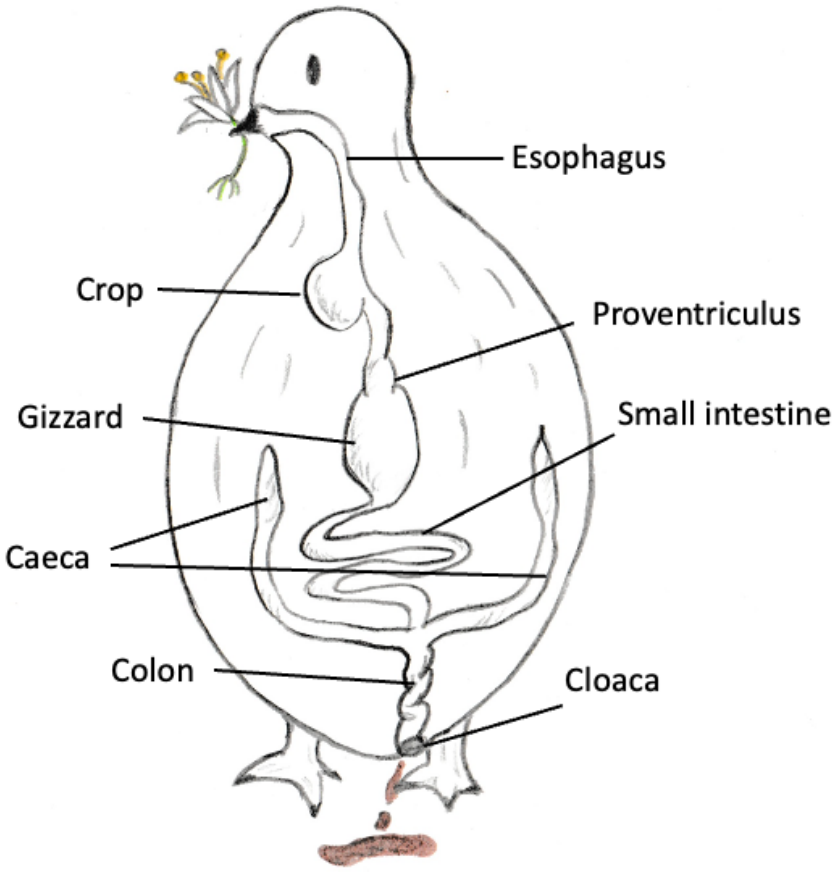


Figure 1-9. Digestive system of rock ptarmigan.
Especially prominent are the pair of long caeca where the gut microbiota efficiently ferment and detoxify secondary metabolites from the many wild plants that comprise their feed.

Indeed the rock ptarmigan has adapted very well to utilize the often limited food resources their wild environment subjects them to especially during winter, and this is reflected in their physiologic traits. The extreme change in diet these birds must re-adjust to in captivity coupled with the use of antibiotics have had implications on their microbial composition and thus their ability to digest and efficiently utilize nutrients in their wild food, something that has already been confirmed by comparative studies on wild and captive birds

cecal microbiota (Ushida et al. 2016). In the wild chicks acquire beneficial microbial strains by consuming the hen's cecal feces (Kobayashi et al. 2019), enabling them to safely feed on wild plants. Among the wild-type microbiota are also several health-conferring and possibly immune-stimulating strains, which likely have positive effect on the health of newly hatched chicks. In captivity chicks have no way of acquiring these strains as they are separated from the hen before hatching takes place. Studies comparing wild and captive birds have demonstrated a significant shift in gut microbiota composition, whereby captive individuals have bacterial species more similar to their surrounding environment. Lacking the stimuli from the beneficial bacteria found in wild birds' gut they are also far more prone to infectious disease in captivity where they encounter many opportunistic pathogens. As far as infectious disease in the wild is concerned, the main pathogen is the *Eimeria* parasite (Matsubayashi et al. 2018a), from which two species have been identified in Japanese rock ptarmigan (Matsubayashi et al. 2018b). Apart from this some blood parasites like *Leucocytozoon* spp. have been observed as well (Murata et al. 2007; Hagihara et al. 2004). As the impact of mountain tourism and pollution has been and is continuing to increase, one might see other pathogens emerge in wild individuals in the future. Although as of now, the pressure from pathogenic infections is not nearly as great as that which is seen in captive institutions like zoos, which we will get to later.

1-3 Egg quality

Development of a healthy embryo and hatchling depends heavily on the condition of the egg laid by the hen and is a key factor in the success of captive breeding programs like that of the Japanese rock ptarmigan. As such egg quality is one important parameter which can in turn influence breeding protocols. Ultimately the hen must deposit all nutrients and energy into the egg before laying, and the amounts and forms of nutrients must meet the need of the developing embryo (Figure 1-10, Figure 1-11). This in turn is a direct reflection of the

condition of the hen and in terms of nutrients the feed of the hen (Vieira 2007). Any gross alterations or inadequacies in diet composition could eventually affect the success of producing a healthy embryo and chick. The diet must for example include sufficient levels of calcium and phosphorous which are both essential for eggshell formation – which protects the chick during embryonic development, as well as chick skeleton formation and maintenance (Taylor 1970). Inadequate levels of these nutrients can cause skeletal disorders, hampered chick development and even premature death in captive chicks – something that is also experienced in captive rock ptarmigan. In terms of the egg, the major source of energy is the egg yolk lipids for which the chick will survive on during embryonic development and first few days after hatching before it can feed on its own.

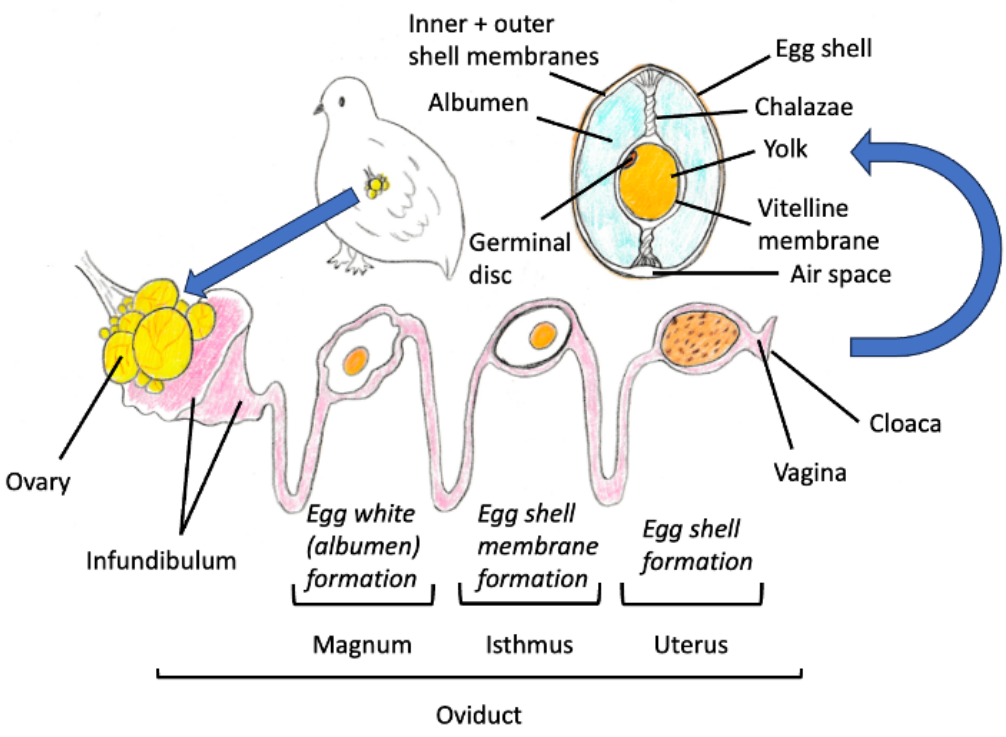


Figure 1-10. Development of the egg throughout the hen's reproductive system. Developing ova (yolks) mature and enter the oviduct – termed ovulation – via the infundibulum. If the egg is fertilized, this occurs in the infundibulum when the mature ovum enters. In the various parts of the oviduct, albumen (egg white), egg shell membrane and egg shell formation takes place sequentially until the egg enters the vagina for oviposition (turning the egg large side first) for egg laying.

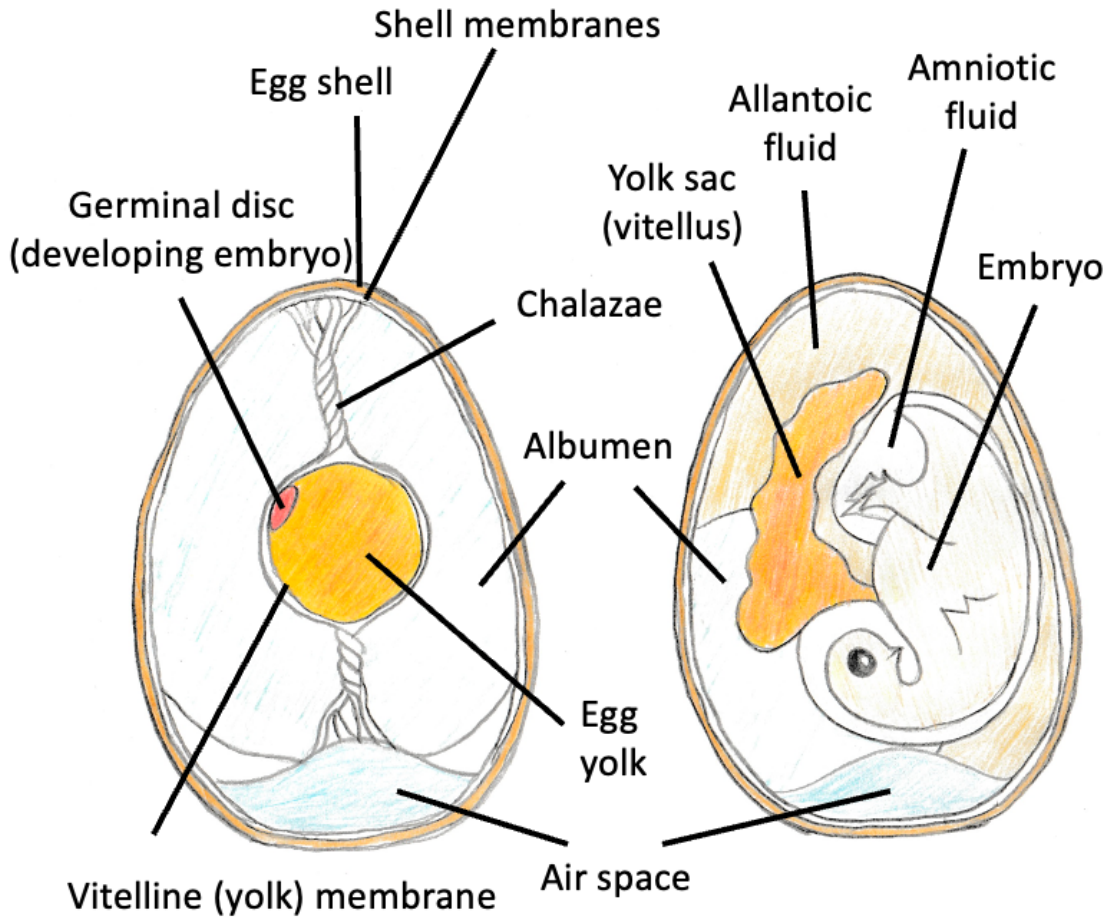


Figure 1-11. Embryonic development of chick. The chick embryo develops from the germinal disc on the yolk (vitellus). Surrounding amniotic fluid protects the embryo. Allantoic fluid aids in gas exchange and excretion of waste products from the embryo, as well as delivering protein from albumen and absorbing calcium from the shell required for the structural needs of the developing chick. The yolk sac is slowly absorbed by the chick and provides it with vital nutrients as well as maternal antibodies.

In addition to provide energy, certain fatty acids (FA) especially the long chain polyunsaturated fatty acids (PUFA) (Figure 1-12) – linoleic acid (LA, 18:2 n6) and alpha-linolenic acid (ALA, 18:3 n3), as well as their respective derivatives arachidonic acid (AA, 20:4 n6) and docosahexaenoic acid (DHA, 22:6 n3) - are essential in embryonic development as vital structural components of phospholipid membranes affecting neurological and cardiac

development (Noble and Cocchi 1990). In addition they also serve important immunological functions (Wang et al. 2000).

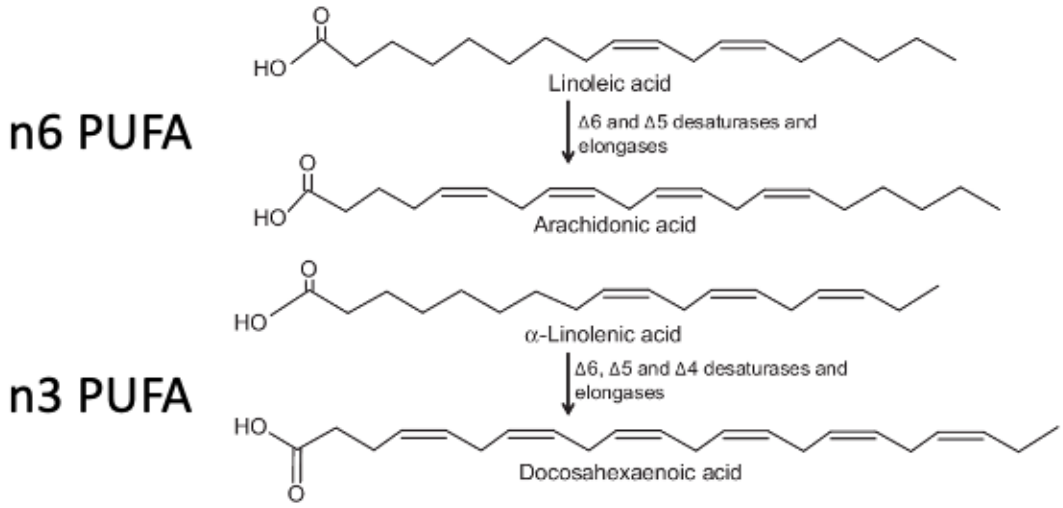


Figure 1-12. Chemical structure of PUFA. Linoleic acid (LA, 18:2 n6) and alpha-linolenic acid (ALA, 18:3 n3) are considered "essential fatty acids" as they cannot be synthesized *de-novo* by higher animals. Their derivatives are arachidonic acid (AA, 20:4 n6) and docosahexaenoic acid (DHA, 22:6 n3), respectively. Modified and adapted from Elsherbiny, Amara and Godbout 2013.

The amounts and ratios of various FA the hen can deposit into the yolk is often influenced by the diet, and the original FA signature of egg yolks in various species is often closely related to their dietary modes in the wild. In terms of wild animals in captivity, their diet is most often strikingly dissimilar to their natural one, and for many animals including several domesticated avian species, this has affected the egg yolk FA signatures (Surai and Speake 2008). Compared to the varied diet in the wild mentioned above, in captivity Japanese rock ptarmigans are provided a diet more similar to commercial poultry species, and this might in turn have had an effect on the FA composition in their egg yolks. This could possibly have implications on the status of their progeny, and might hold a clue to the many health complications encountered in captivity.

In addition to diet, immune status of chicks is another important point when it comes to chick morbidity and mortality in zoos. The chick is fully dependent on maternally transferred immunity before and during the first few weeks post-hatch until it can produce antibodies by itself, and in birds this is provided via the egg. Avian antibody isotypes includes IgA, IgM and IgY (IgM) (Rose, Orlans and Buttress 1974) (Figure 1-13).

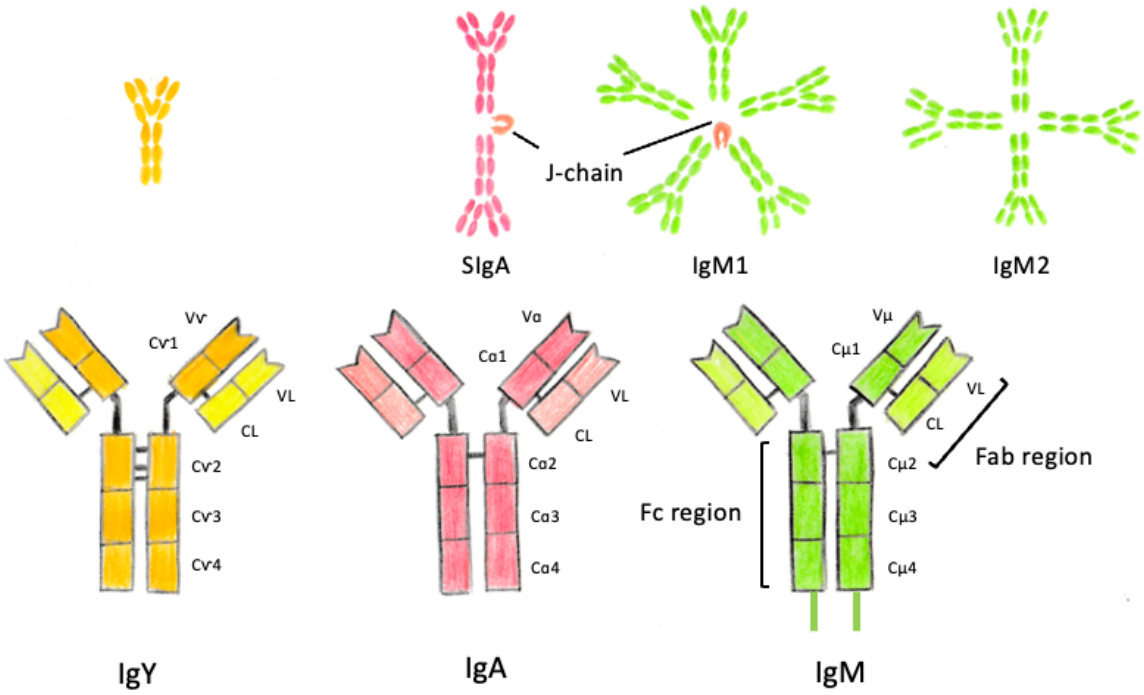


Figure 1-13. Avian immunoglobulin isotypes. IgY is the predominant antibody in birds, found in serum and in egg yolk. IgA is found in serum, mucosal secretions and egg white. The secretional type is dimeric, known as SIgA. IgM is found in serum, mucosal secretions and egg white, occuring as either a pentamer (IgM1) or tetramer (IgM2). The monomeric type is membrane bound serving as a B-cell receptor for antigens.

Immunoglobulin Y (IgY) is incorporated from the mother hen's blood circulation into the egg yolk prior to ovulation (Figure 1-14). From the oviduct mucosa smaller amounts of IgA and IgM is deposited into the egg white. These antibodies will serve as the primary form of defense for the chick until its own immune system is fully developed, and the amount of

antibodies available, particularly that of IgY, could potentially influence their likelihood of survival (Grindstaff 2010).

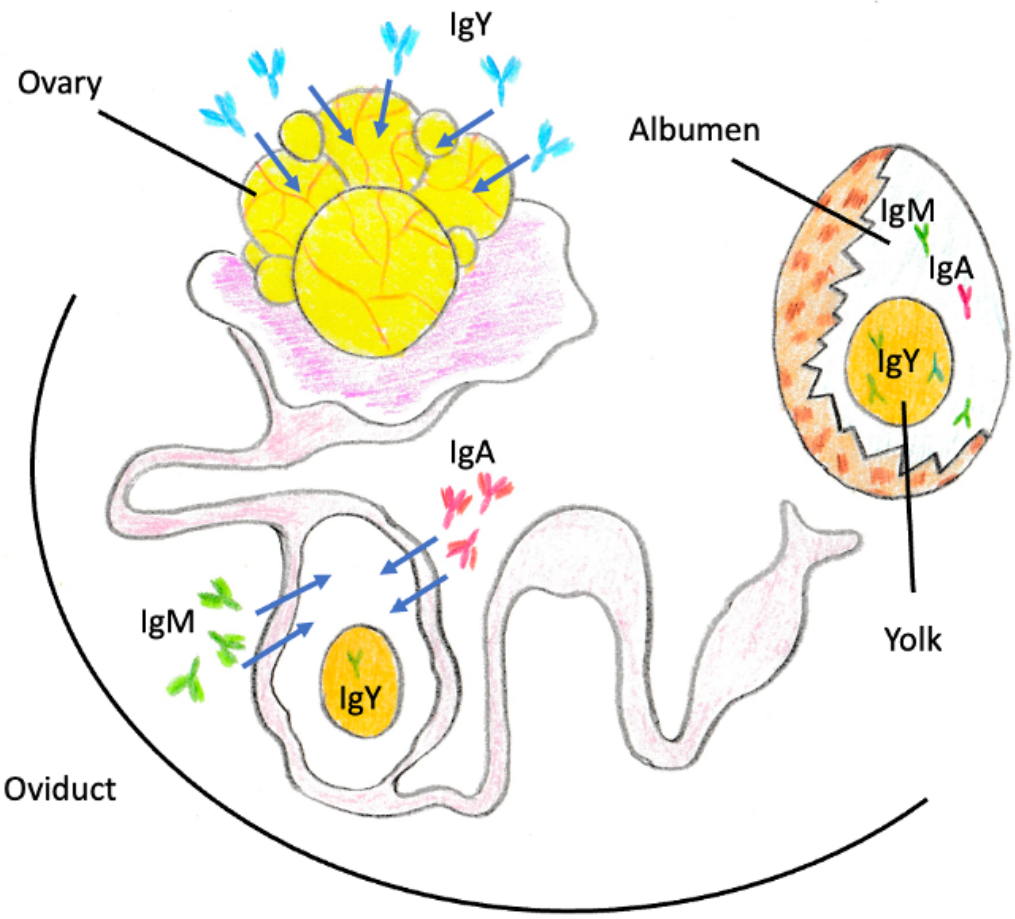


Figure 1-14. Transfer of maternal immunoglobulins during egg development. IgY is transferred into the developing ovaries prior to ovulation. Through the oviduct mucosa, IgA and IgM are transferred into the albumen (egg white). In the laid egg, IgY is found in the yolk with smaller amounts of IgA and IgM in the albumen. The levels of antibodies in the egg is a direct reflection of the hen's serum levels of antibodies.

Chicks in zoos faces a much higher load of pathogens, many of which includes opportunistic species, compared to those born in the wild. The high chick mortality in captivity might indicate insufficient antibody levels to handle the pathogenic stressors subjected upon them. Egg antibody levels could therefore be another egg quality factor holding information to this pressing issue.

1-4 Aim of this study

The goal of this study was to look into two egg quality parameters that could hold clues to the high chick mortality of captive Japanese rock ptarmigan chicks (Figure 1-15). Chapter 2 seeks to study the effect of diet upon FA composition in rock ptarmigan egg yolk, and whether the provision of a commercial-type diet has altered this since the initiation of domestication in this species. Chapter 3 looks into the antibody levels of rock ptarmigan eggs by measuring egg yolk IgY and egg white IgA and IgM, and whether the levels in captivity are any indication of the ability for chicks to mount an effective immune response to infection. Given the status of the Japanese rock ptarmigan as a protective endangered species, the study used unfertilized captive rock ptarmigan eggs. In addition, thanks to an egg exchange experiment conducted by MOE in 2020 at Mt Kisokomagatake, Chuo Alps (Figure 1-3 and Figure 1-4), we were able to secure samples from six unfertilized eggs laid by a lone wild female individual. This allowed us to compare the captive and wild situation of the aforementioned egg quality parameters in a non-invasive fashion, possibly enabling us to shed light on some of the reasons behind the high chick mortality in zoos. All analyses in the study were approved by MOE as part of the National Conservation Program for Rock Ptarmigans.



Figure 1-15. Hens, unfertilized eggs and processing method for egg yolk of the Japanese rock ptarmigan. a) Eggs laid in the wild at hen's nest (7 June 2020). b) Wild solitary hen at Kisokomagatake 2020. c) Eggs laid in captivity. d) Hen (N99) in captivity. e) Separation of egg yolk from egg white.

Chapter 2

Japanese rock ptarmigan displays high levels of polyunsaturated fatty acids in egg yolk compared to chicken or quail

2-1 Introduction

In terms of egg quality and its importance for development and survival of the chick, nutritional components are one of the major things to consider. As such, the egg yolk fatty acid (FA) composition is a vital factor. Egg yolk lipids serve as one of the major nutritional components of which the chick will survive on both during embryonic development and the initial few days post-hatch before it is capable of feeding on its own. Among the many FAs, especially the long chain polyunsaturated fatty acids (PUFAs) are of critical importance. Linoleic acid (LA, 18:2 n6) and alpha-linolenic acid (ALA, 18:3 n3), and their respective derivatives arachidonic acid (AA, 20:4 n6) and docosahexaenoic acid (DHA, 22:6 n3) constitute major components of the phospholipid layers of cell membranes, making them vital for neurological and cardiac development (Noble and Cocchi 1990). In addition to this these FAs also have immunological significant roles (Wang et al. 2000).

Birds like mammals are not able to synthesize LA or ALA *de-novo*, thus these FAs must be supplied via the diet and are thus known as “essential fatty acids” (Figure 1-10). It is important that the ratios of the different PUFAs meet a specific ratio-requirement in order to meet the needs of the developing embryo. In various species, egg yolk FA composition is often related to their dietary mode in the wild. As pointed out earlier, the Japanese rock ptarmigan has adapted to a varying diet that changes along with the seasons in the alpine environment. Classified primarily as a herbivorous species feeding on leaves, buds, flowers, fruits, seeds and even stalks of various plants, they also supplement their diet with insects

during spring and summer. These birds have by observation been confirmed to utilize 75 kinds of plants (Kobayashi and Nakamura 2011; Nakamura 2007), and several new plant taxa have been added to this list later thanks to DNA barcoding and -metabarcoding studies on fecal samples from wild individuals (Fujii et al. 2022; Fujii et al. 2019). On the other hand, in captivity the ptarmigan is sustained on a diet regime consisting largely of commercial formula feed supplemented with various leafy vegetables like Japanese mustard spinach, cabbage and celery, as well as berries and mealworms (Figure 2-1).



Figure 2-1. Feed provided to Japanese rock ptarmigan under captive conditions. Clockwise from top right Japanese mustard spinach, a mix of berries and commercial pellet formula feed mixed with mealworm.

The contrasting limited diet provided to this species in captivity might have potentially altered the natural FA composition in the birds' egg yolks, as can be seen in other bird species bred in captivity on sub-optimal diet regimes compared to their wild counterparts (Morrow, Koutsos and Toepfer 2019), and this is not only limited to herbivorous avian species but those with other feeding modes too (Surai et al. 2001).

Wild herbivorous diets are often higher in n3-PUFA compared to the n6-PUFA rich formula feed (Crawford 1968). Due to this, domestication often sees a shift in the n6:n3 PUFA ratio from a lower to a higher one (Table 2-1), a finding observed in other herbivorous birds like the pheasant, partridge, duck, ostrich, rhea and prairie chicken (Surai and Speake 2008; Morrow, Koutsos and Toepfer 2019; Navarro et al. 2001; Noble et al. 1996).

Table 2-1 Comparison of Yolk Fatty Acic Compositions of Wild and Captive Birds*

	Pheasant		**Partridge**		**Duck**	
	Wild	Captive	Wild	Captive	Wild	Captive
n6:n3	0.4	6.8**	3.6	27.2**	1.9	10.7**

*Table modified and adapted from Surai and Speake 2008.
**Significantly different from wild ($p < 0.05$).

Imbalance between egg yolk FAs could potentially influence survivability of the newly hatched chick and how efficiently it can cope with the many health-related challenges seen in captivity. Given the major health concerns and high mortality related to the captive breeding of Japanese rock ptarmigan previously mentioned, a possible altered egg yolk FA signature brought about by the captive diet might hold some clues to the elevated chick mortality seen in captivity, and whether this is brought on by a change in diet or not.

2-2 Materials and Methods

This study used egg yolk from 17 captive and 6 wild unfertilized rock ptarmigan eggs. Information regarding the individual hens, including age, ovulation status and diet, can be found in Table 2-2.

Table 2-2 Information regarding the Japanese rock ptarmigan individuals used in the current study

Egg ID	Egg collection date	Hen ID	Hen's origin*	Age of hen at egg collection	Generation** after first founders born in zoos (2015)	Ovulation status	Basal Feed***
T-1	2021.05.21	N99	TMFPZ	1 year (hatched at 2020.08.04)	4th generation	1st ovulation	Formula feed (RM-4) and a vitamin supplement (Minevitn), Japanese mustard spinach
T-2	2021.05.24					2nd ovulation	
T-3	2021.05.29					3rd ovulation	
T-4	2021.06.01					4th ovulation	
T-5	2021.06.04					5th ovulation	
T-6	2021.06.07					6th ovulation	
T-7	2021.06.12					7th ovulation	
T-8	2021.06.15					8th ovulation	
T-9	2021.06.18					9th ovulation	
T-10	2021.06.24	N103	TMFPZ	1 year (hatched at 2020.08.04)	4th generation	1st ovulation	
N-1	2021.05.09	N49 or N97	N49:TMFPZ N97:NAK	N49: 3 years (hatched at 2018.06.13) N97: 1 year(hatched at 2020.07.18)	N49: 2nd generation N97: 3rd generation	unrecorded	Formula feed (Bunny Selection Maintenance and ZPC), Japanese mustard spinach, Lingonberry fruits, mealworms
N-2	2021.05.27					unrecorded	
N-3	2021.05.31					unrecorded	
N-4	2021.05.13	N93	NAK	1 year (hatched at 2020.07.15-16)	3rd generation	1st ovulation	
N-5	2021.05.14					2nd ovulation	
N-6	2021.06.23	N89	NAK	1 year (hatched at 2020.07.15)	3rd generation	1st ovulation	
N-7	2021.06.27					2nd ovulation	
W-1	2020.06.07	Wild solitary Mt Kisokomagatake	wild: estimated at Japanese Northern Alps	before 2016 (estimated)	-	undefined, but 2017-laid eggs were discovered on site in 2018	Alpine vegetation, insects
W-2							
W-3							
W-4							
W-5							
W-6							

* TMPF, Toyama Municipal Family Park Zoo; NAK, Nasu Animal Kingdom Zoo

**Generation is numbered from first chicks, assigned as the 1st domesticated generation, which were hatched from eggs laid in zoos by first founders born in 2015.

***Formula feed was offered 40 g/d. RM-4 (Funabashi farm Co. Ltd., Funabashi, Chiba), Bunny selection Maintenance (Yeaster Co. Ltd., Tatsuno, Hyogo) and ZPC (Oriental Yeast Co.Ltd, Tokyo).

Adapted and modified from Table 1 in Vik *et al.* 2022.

The wild eggs laid by a solitary hen at Mt Kisokomagatake were recovered by MOE during an egg exchange experiment in June 2020. Eggs were then sent for egg extraction at Nippon Veterinary and Life Science University. These samples arrived frozen in microtubes at our lab at Chubu University, whereby they were stored at -20˚C until further use. Captive eggs arrived at our lab at Chubu University from Toyama Municipal Family Park (n = 10) and Nasu Animal Kingdom (n = 7) early summer 2021, and extraction was carried out promptly upon arrival. Eggs were carefully cracked open, yolk and white were divided into separate petri dishes, taking care to minimize contamination between the two. 1 mL syringes were used to carefully puncture the yolks and approximately 0.5 mL aliquots (3-6 per egg) were drawn and put into microtubes. Similarly, egg white samples were drawn into separate microtubes the same way for later experiments (see Chapter 3). All samples were immediately frozen at -20˚C until start of analyses.

In order to study the effect of a commercial diet akin to that provided to captive individuals on yolk FA composition, egg yolk samples from chicken (n = 1) and quail (n = 1) – both purchased at a local supermarket – were used as controls in this study. Extraction was done immediately after purchase and using the same procedure as for the ptarmigan eggs, and samples were stored frozen (-20˚C). Yolk samples were thawed and accurately re-weighed into new microtubes before being sent for FA compositional analysis. In addition, samples of the formula feed provided in zoos (RM-4 and Bunny Selection Maintenance) were sent for the same type of analysis. The hydrolyzation, methylation and GC-MS analyses were undertaken by Dojin Glocal Co., Ltd. (Mashikimachi, Kumamoto, Japan). Initially hydrolyzation and methylation was briefly carried out by a FA methylation kit for glycerides (Nakalai Tesque, Kyoto, Japan). Methylated FA were then analyzed by GC8890/MSD5977B GC-MS (Agilent Technologies, Waldbronn, Germany). Separation was achieved by a VF-WAXms column (30 m x 0.25 mm x 0.25 μm) (Agilent Technologies) with He as carrier gas

(1 mL/min). Program for oven temperature was as follows: initially 60˚C held for 4 min, then ramped at 10˚C/min to 120˚C before finally being ramped at 3˚C/min to 240˚C and held for 10 min. Temperature of the splitless injector was 250˚C. MS analyses were carried out in scan mode. Proportions of individual FAs, given as relative abundance (% of total FAs) were calculated for each yolk or formula feed sample. FAs detectable in at least one egg yolk/feed sample were included in the analysis. Individual FAs were pooled into total saturated fatty acid (SFA), total monounsaturated fatty acid (MUFA) and total polyunsaturated fatty acid (PUFA). PUFA was again split into n6 and n3 PUFA, and the n6/n3-ratio was subsequently calculated for all samples. Welch's *t*-test for unequal variances as applied to compare captive and wild ptarmigans. Differences were considered significant at $p < 0.05$. Data processing and statistical analyses were all carried out using R version 4.1.2 (R Core Team, 2021).

2-3 Result and Discussion

FA-ratios from Japanese rock ptarmigan – both captive and wild specimens - were different from the commercial control species (Table 2-3). Whereas the SFA profiles were similar between all three species, the rock ptarmigan egg yolk samples displayed much higher PUFA levels (twice as much as poultry species) compared to MUFA (two thirds of poultry species). The situation was the opposite for the chicken and quail samples. Levels of both n6 and n3 PUFA were higher in ptarmigan yolk samples, most evident by the increased amount of n3 PUFA. Consequently, the ptarmigan also displayed a much lower n6/n3-ratio as that seen in commercial controls. In addition to all this, there were also significant differences between captive and wild rock ptarmigan for most FA parameters (Table 2-3).

Table 2-3 Fatty acid composition (relative abundance in %) of egg yolk from captive and wild Japanese rock ptarmigan and commercially-bred birds.

Fatty Acid	Captive Rock Ptarmigan (n=17)	Wild Rock Ptarmigan (n=6)	Welch's *t*-test	Chicken (n=1)	Quail (n=1)	Reference Value for Chicken (n=5) ***	Reference Value for Quail (n=5) ***
Myristic acid (C14:0)	0.35±0.02	0.30±0.00	**	0.3	0.4	0.359 ± 0.016	0.636±0.021
Pentadecanoic acid (C15:0)	0.11±0.01	0.10±0.00	NS	ND	ND	0.071± 0.012	0.061±0.006
Palmitic acid (C16:0)	27.56±0.21	25.55±0.27	**	25.9	25.5	23.836 ± 0.189	23.875±0.359
Margaric acid (C17:0)	0.39±0.01	0.40±0.03	NS	0.1	0.1	0.212 ± 0.05	0.21±0.013
Stearic acid (C18:0)	9.42±0.20	9.43±0.13	NS	9.8	8.6	10.536 ± 0.469	11.14±0.071
Total SFA	**37.84±0.36**	**35.78±0.40**	**	**36.1**	**24.6**	**35.015 ± 0.423**	**35.936±0.433**
Palmitoleic acid (C16:1)	3.18±0.11	2.65±0.06	**	2.4	4	4.234 ± 0.157	5.137±0.186
Heptadecenoic acid (C17:1)	ND	0.05±0.05	-	ND	ND	0.237±0.026	0.236±0.023
Oleic acid (C18:1n-9)	29.07±0.74	35.43±1.14	**	47	44.8	43.521±1.324	42.28±0.232
Eicosenoic acid (C20:1n9)	ND	ND	-	0.2	0	0.380±0.041	ND
Total MUFA	**32.25±0.80**	**38.13±1.12**	**	**49.6**	**48.8**	**48.371±1.125**	**47.854±0.452**
Linoleic acid (C18:2n6)	23.43±0.97	17.32±0.76	**	11.5	13.6	11.672±0.532	11.618±0.099
γ-Linolenic acid (C18:3n6)	0.14±0.01	0.10±0.00	**	0.1	0.1	0.195±0.025	0.293±0.013
Eicosadienoic acid (C20:2n6)	0.01±0.01	ND	NS	ND	ND	0.241±0.032	ND
Dihomo-γ-Linolenic acid (C20:3n6)	0.14±0.02	0.07±0.02	*	ND	ND	0.228±0.029	ND
Arachidonic acid (C20:4n6)	0.88±0.04	0.60±0.03	**	1.7	1.7	2.401±0.035	2.546±0.007
Total n6-PUFA	**24.61±1.01**	**18.08±0.75**	**	**13.3**	**15.4**	**15.483±0.618**	**15.278±0.100**
α-Linolenic acid (C18:3n3)	4.12±0.37	6.47±0.68	*	0.2	0.2	0.326±0.057	0.410±0.027
Eicosapentaenoic acid (C20:5n3)	0.26±0.02	0.32±0.02	NS	ND	ND	ND	ND
Docosahexaenoic acid (C22:6n3)	0.87±0.03	1.22±0.04	**	0.8	ND	0.578±0.010	0.522±0.026
Total n3-PUFA	**5.26±0.40**	**8.00±0.68**	**	**1**	**1**	**0.903±0.064**	**0.932±0.053**
Total PUFA	**29.86±0.87**	**26.08±1.40**	*	**14.3**	**16.4**	**16.614±0.710**	**16.210±0.055**
n6/n3-ratio	**5.17±0.46**	**2.30±0.10**	**	**13.3**	**15.4**	**17.165±0.560**	**16.59±1.133**

SFA: saturated fatty acid, MUFA: monounsaturated fatty acid, PUFA: polyunsaturated fatty acid.
Values for rock ptarmigan are given as mean ± SEM. ND = not detected. NS = not significant.
Welch's *t*-test was applied to compare the composition between captive and wild.
**: $P < 0.01$, *: $P < 0.05$, NS: $P > 0.05$.
***: Data modified and adapted from Wang *et al.* 2014.
Table adapted and modified from Table 2 Vik et al. 2022.

Captive individuals exhibited a more similar composition to that of the commercial species. Indeed yellow corn and soybean meal that constitutes the main proportion of the formula feed provided to commercially bred chicken and quail, is naturally higher in n6-PUFAs compared to the herbs of which the rock ptarmigan feeds on in the wild. This is mainly due to the inclusion of rice bran and fish meal. As such the composition of the diet provided to rock ptarmigan in captivity is in fact closer to commercial feeds than their natural diet (Table 2-4), and this might reflect in the different FA composition in captive individuals compared to wild ones.

Table 2-4 Fatty acid composition (relative abundance in %) of formula feed for captive Japanese rock ptarmigans.

Fatty Acid	Bunny selection Maintenance	RM-4
Hexanoic acid (C6:0)	0.0	0.1
Decanoic acid (C10:0)	0.0	0.0
Dodecanoic acid (C12:0)	0.2	0.1
Myristic acid (C14:0)	0.5	0.4
Pentadecanoic acid (C15:0)	0.2	0.2
Palmitic acid (C16:0)	19.7	17.3
Margaric acid (C17:0)	0.2	0.2
Stearic acid (C18:0)	2.5	4.3
Arachidic acid (C20:0)	0.5	0.5
Heneicosanoic acid (C21:0)	0.0	0.1
Behenic acid (C22:0)	0.6	0.5
Tricosylic acid (C23:0)	0.1	0.2
Lignoceric acid (C24:0)	0.5	0.4
Total SFA	25.0	24.3
Palmitoleic acid (C16:1)	0.6	0.2
Heptadecenoic acid (C17:1)	0.0	0.1
Oleic acid (C18:1n9)	18.6	17.2
Eicosenoic acid (C20:1n9)	0.6	0.5
Total MUFA	19.8	18.0
Linoleic acid (C18:2n6)	44.5	49.5
Total n6-PUFA	44.5	49.5
α-Linolenic acid (C18:3n3)	10.3	8.2
Eicosapentaenoic acid (C20:5n3)	0.0	0.1
Docosahexaenoic acid (C22:6n3)	0.5	0.2
Total n3-PUFA	10.8	8.5
Total PUFA	55.3	58.0
n6/n3-ratio	4.1	5.8

Modified and adapted from Supplementary Table 2 in Vik *et al.* 2022.

Despite most of the differences between captive and wild individuals being significant, they are much smaller in comparison to the other poultry species. This is also evident when comparing changes in FA composition between battery-cage chickens and free-

range chickens (Table 2-5, adapted from a study by Wang et al. 2014). Here the intraspecific effect of diet is much more prominent than the differences seen between captive and wild rock ptarmigan (Table 2-3), especially for the amounts of n3 PUFA and thus the n6/n3-rato which is almost 50% lower in free-range chickens. At present, there seem to be no urgent issue as to whether the current feed in captivity has altered the FA composition in rock ptarmigan egg yolk.

Table 2-5 Reference values of fatty acid composition (relative abundance in %) of egg yolk in commercially-bred birds*

Fatty Acid	Reference Value for Chicken (n=5)	Reference value for free-range Chicken (n=5)	Reference Value for Quail (n=5)
Myristic acid (C14:0)	0.359 ± 0.016	0.470 ± 0.010	0.636±0.021
Pentadecanoic acid (C15:0)	0.071± 0.012	0.359 ± 0.016	0.061±0.006
Palmitic acid (C16:0)	23.836 ± 0.189	25.278 ± 0.052	23.875±0.359
Margaric acid (C17:0)	0.212 ± 0.05	0.230 ± 0.026	0.21±0.013
Stearic acid (C18:0)	10.536 ± 0.469	11.570±0.469	11.14±0.071
Total SFA	35.015 ± 0.423	37.619 ± 0.072	35.936±0.433
Palmitoleic acid (C16:1)	4.234 ± 0.157	3.537 ± 0.033	5.137±0.186
Heptadecenoic acid (C17:1)	0.237±0.026	0.151 ± 0.020	0.236±0.023
Oleic acid (C18:1n9)	43.521±1.324	36.870 ± 0.153	42.28±0.232
Eicosenoic acid (C20:1n9)	0.380±0.041	0.514 ± 0.011	ND
Total MUFA	48.371±1.125	41.078 ± 0.123	47.854±0.452
Linoleic acid (C18:2n6)	11.672±0.532	14.806 ± 0.226	11.618±0.099
γ-Linolenic acid (C18:3n6)	0.195±0.025	0.196 ± 0.016	0.293±0.013
Eicosadienoic acid (C20:2n6)	0.241±0.032	0.327 ± 0.014	ND
Dihomo-γ-Linolenic acid (C20:3n6)	0.228±0.029	0.272 ± 0.032	ND
Arachidonic acid (C20:4n6)	2.401±0.035	2.892 ± 0.137	2.546±0.007
Total n6-PUFA	15.483±0.618	19.310 ± 0.135	15.278±0.100
α-Linolenic acid (C18:3n3)	0.326±0.057	0.858 ± 0.041	0.410±0.027
Eicosapentaenoic acid (C20:5n3)	ND	ND	ND
Docosahexaenoic acid (C22:6n3)	0.578±0.010	1.136 ± 0.014	0.522±0.026
Total n3-PUFA	0.903±0.064	1.993 ± 0.051	0.932±0.053
Total PUFA	16.614±0.710	21.304 ± 0.096	16.210±0.055
n6/n3-ratio	17.165±0.560	9.693 ± 0.310	16.59±1.133

*Table modified and adapted from Wang *et al.* 2014, used as Supplementary Table 1 in Vik *et al.* 2022.

Values expressed as mean ± SD.

SFA: saturated fatty acid, MUFA: monounsaturated fatty acid, PUFA: polyunsaturated fatty acid.

ND = not detected.

In addition to diet, phylogeny has been indicated to influence egg yolk FA composition. This can be seen in the hen's enzymatic ability to convert LA and ALA to AA and DHA, respectively. Some other bird species are able to maintain fairly stable FA compositions of their egg yolk despite having different feeding modes (Speake, Surai and Bortolotti 2002), and perhaps this is also the case for the Japanese rock ptarmigan. The wild diet of the rock ptarmigan is not completely fixed due to seasonal availability, thus it seems plausible that having a somewhat balanced FA ratio is genetically attributable.

The alpine environment – also subject to extreme seasonal fluctuations in temperature – might be an additional contributor to the egg yolk PUFA content. During the egg-laying season the rock ptarmigan hen will lay her eggs every other day but not go ahead with the actual incubation until all her eggs have been laid. A higher PUFA content will increase the fluidity of the egg yolk, enabling it to pass the sub-zero temperatures that the eggs might be exposed to pre-incubation, and thus survive damage from freezing. In order to more accurately determine whether yolk FA profiles of this species is due to genetic factors or diet, and whether the captive diet has indeed had any altering effect on these parameters, the nutritional composition of the many wild dietary sources needs to be further assessed like that of the formula feed (Table 2-4). It might also be probable that the timeframe since domestication of this bird species has as of yet been too short to modify more significant changes to their yolk FA composition. Since its initiation in 2015, the hens used in this study comprise the 3rd and 4th generation born in zoos (Table 2-2), and have all been hatched using artificial incubators. The status of the progeny could potentially be influenced in case a gradual change over time were to ensue. As a consequence of this, the situation should ideally be monitored over time.

Chapter 3

Comparison of egg antibody levels between wild and captive Japanese rock ptarmigans

3-1 Introduction

In addition to dietary deficiencies possibly resulting in developmental issues, another main issue in captivity is the significantly increased infection pressure compared to the species' wild environment. In their natural environment, Japanese rock ptarmigan chicks die mainly from predation, inability to thermoregulate efficiently during the rainy season (Kobayashi and Nakamura 2013) or from infections from the *Eimeria* spp. parasite (Matsubayashi et al. 2021). Despite the captive environment being free from any of these dangers, the substantially increased pathogenic load inside the captive breeding facilities poses an equally big challenge to the survival and propagation of this species. 23 of 59 chicks born in zoos between breeding seasons in 2017 to 2019 died during the 1-month post-hatch period (Table 3-1). In many cases the exact causes of death remain unestablished, however in those cases where such information exists infection by opportunistic pathogens have been listed as a contributing factor (Akiba 2020; Sato 1986).

Table 3-1 Mortality rates in captive chicks born in zoos between breeding seasons 2017 to 2019*

Breeding Season	Egg-laying Females Captivity	Number of Eggs Laid Average Number og Eggs Laid	Eggs Hatched	Upper : Hatchlings Lower : Hatching Rate	Upper: Number of Deceased Individuals Lower : Mortality Rate			
					7 Days Old	30 Days Old	100 Days Old	1 Year Old
Eggs Derived From Wild	—	—	22	21	1	1	6	7
2015～2016				95%	5%	5%	29%	33%
Captive Breeding	3	60	58	22	8	10	10	10
2017		20.0		38%	36%	45%	45%	45%
Captive Breeding	3	31	29	12	3	3	4	5
2018		10.3		41%	25%	25%	33%	42%
Captive Breeding	5	73	73	25	6	10	10	10
2019		14.6		34%	24%	40%	40%	40%

*Data provided by the Japanese Association of Zoos and Aquariums (JAZA).

As mentioned in chapter 1, chicks receive maternal antibodies from the mother hen via the egg in the form of egg yolk IgY (Brambell 1969) and smaller amounts of egg IgA and IgM in egg white (Rose, Orlans and Buttress 1974) (Figure 1-12). Egg yolk IgY is a direct reflection of the serum levels of IgY in the hen (Hamal et al. 2006), and by measuring IgY from unfertilized captive and wild rock ptarmigan eggs, as well as egg white IgA and IgM, we were able to study whether the levels of maternal antibodies were insufficient for chicks to successfully mount an effective immune response in the face of infection.

3-2 Materials and Methods

This study only used four of the captive egg samples from Toyama (n = 2) and Nasu (n =2), and three of the wild egg samples used in the FA analysis (see Chapter 2). Information regarding the individuals producing the eggs used can be found in Table 3-2. Extraction procedures for the egg yolk and white samples can be found in Chapter 2-2 Materials and Methods.

Table 3-2 Information regarding the Japanese rock ptarmigan individuals used in the current study

Egg ID	Egg collection date	Hen	Hen's origin*	Age of hen at egg collection	Generation** after first founders born in zoos (2015)	Ovulation status	Basal Feed***
T-1	2021.06.04	N99	TMFPZ	1 year (hatched on 2020.08.04)	4th generation	5th ovulation	Formula feed (RM-4) and a vitamin supplement (Minevitn), Japanese mustard spinach
T-2	2021.06.12	N99	TMFPZ			7th ovulation	
N-1	2021.05.11	N49 or N97	N49:TMFPZ	3 years (hatched on 2018.06.13)	N49: 2nd generation N97: 3rd generation	unrecorded	Formula feed (Bunny selection Maintenance and ZPC) and Japanese mustard spinach, Lingonberry fruits and mealworms
			N97: NAK	1 year (hatched on 2020.07.18)		unrecorded	
N-2	2021.05.31	N49 or N97	N49:TMFPZ	3 years (hatched on 2018.06.13)		unrecorded	
			N97: NAK	1 year (hatched on 2020.07.18)		unrecorded	
W-1							
W-2	2020.06.07	Wild solitary Mt Kisokomagatake	wild: estimated at Japanese Northern alps	2015-2016 (estimated)	-	undefined, but 2017-laid eggs were discovered on site in 2018	Alpine vegetation, insects
W-3							

* TMPF, Toyama Municipal Family Park Zoo; NAK, Nasu Animal Kingdom Zoo
**Generation is numbered from first chicks, assigned as the 1st domesticated generation, which were hatched from eggs laid in zoos by first founders born in 2015.
***Formula feed was offered 40 g/d. RM-4 (Funabashi farm Co. Ltd., Funabashi, Chiba), Bunny selection Maintenance (Yeaster Co. Ltd., Tatsuno, Hyogo) and ZPC (Oriental Yeast Co.Ltd, Tokyo).
Adapted and modified from Table 1 in Vik *et al.* 2023.

Egg yolk and white samples from one chicken egg was used as a positive control to check validity for the Chicken IgY (yolk) and IgM/IgA (white) assays. These samples were again extracted using the same protocol as for all other eggs used in this and the former study (Chapter 2). Egg yolk IgY levels (mg IgY/g egg yolk) from extracted ptarmigan and chicken egg yolk samples were determined using a commercially available Immunoglobulin Y (IgY) Chicken SimpleStep ELISA® kit (ab189577, Abcam, Cambridge, UK) following the manufacturers' instruction. All samples were analyzed in duplicate utilizing a standard

(0.16~10 ng/mL). Prior to dilution, 1X Cell Extraction Buffer PTR from the kit was mixed directly into the egg yolk in a 2:1 ratio. After homogenization and centrifugation, the lysate was collected and pre-diluted in the same buffer in a ratio 1:1000. Pre-determined working dilutions of the samples (10^{-4} for ptarmigan, 10^{-7} for chicken control) were applied to the assay. Samples were incubated in the dark with tetra methyl benzidine (TMB) for 10 min on a plate shaker set to 400 rpm. The reaction was terminated by adding the stop solution followed by 1 min on the plate shaker.

Likewise, IgA and IgM levels from pre-extracted egg white samples were commercially available IgA Chicken ELISA Kit (ab157691, Abcam) and IgM Chicken ELISA Kit (ab157692, Abcam), following the manufacturers' instructions. The samples were analyzed in duplicate for each kit, utilizing a standard (12.5~400 ng/mL) for IgA and sample dilution of 1:20, and (3.125~200 ng/mL) with a sample working dilution of 1:30 for IgM, respectively. For both egg white assays, the dilution factors were similar for ptarmigan and chicken control samples. Egg white samples were directly diluted in 1X Diluent Solution from the kits. The protocol was similar for both assays, with samples being incubated in the dark with TMB for 10 min, and the reaction stopped by adding the stop solution.

Absorbance for all assays (IgY, IgA and IgM) was immediately measured at 450 nm on an iMark microplate reader (Bio-Rad Laboratories, Hercules, CA, USA) using the Microplate Manager® Software. Blank-adjusted data values were exported to an Excel-file, and from this data a standard (4 parameter logistic) curve was generated for the relation between concentrations of the standard values and the absorbance values read from the plate using the drc-package (v3.0.1; Ritz et al. 2015) in R version 4.1.2 (R Core Team, 2021). In order to determine differences in egg yolk IgY and egg white IgM and IgA levels between captive and wild eggs, a non-parametric approach was applied in the form of a Wilcoxon

rank-sum test using the rstatix package (v.0.7.0; Kassambara 2021) in R. Differences were considered significant at $p < 0.05$.

3-3 Result and Discussion

Median egg yolk IgY levels differed between captive and wild eggs (Table 3-3).

Table 3-3 Egg antibody levels in unfertilized eggs of Japanese rock ptarmigans

Antibody	Yolk IgY (mg/g yolk)	White IgA (μg/g white)	White IgM (μg/g white)
Working Dilution	1:10,000	1:20	1:30
Captive (n = 4)	0.58 (0.09)	0.0 (0.29)**	11.1 (2.07)
Wild (n = 3)*	0.33 (0.06)	0.78 (0.58)	12.2 (0.21)
Chicken (n = 1, control)	38.44***	195.53	261.6

Values for ptarmigan are given as median and interquartile ranges in parentheses.
*All samples originated from one single individual.
**Only one sample gave detectible results in the ELISA assay.
***Dilution factor for chicken samples was 10^{-7}.
Adapted and modified from Table 2 in Vik *et al.* 2023.

The median IgY concentration for captive rock ptarmigan was 0.58 mg/g yolk (interquartile range IQR = 0.09), whereas for wild ptarmigan it was 0.33 mg/g yolk (IQR = 0.06). None of these differences were significant at $p < 0.05$ ($p = 0.057$) according to the Wilcoxon test.

Regarding the egg white IgA and IgM, the situation was the opposite in that levels for both antibodies were slightly higher in the wild samples compared to the captive. Median IgA concentration was 0.0 μg/g white (IQR = 0.29) for captive and 0.78 μg/g white (IQR = 0.58) for wild, respectively. Median IgM concentration was 11.1 μg/g white (IQR = 2.07) for captive and 12.2 μg/g white (IQR = 0.21) for wild, respectively. Again none of these differences for either antibodies at $p < 0.05$ ($p = 0.199$ for IgA, $p = 0.229$ for IgM).

The limitation of the sample size in this study is evident, and in case of the wild samples all came from the same laying hen. Given the status of the Japanese rock ptarmigan

as a protected endangered species it is usually not feasible to secure wild unfertilized eggs, thus the constraints due to this reflected in this case. However this has presented us with an unique opportunity to compare captive and wild specimens in a non-invasive manner, one which could possibly provide some insights into one of the major issues related to the captive conservation efforts.

Results from this study should also be viewed in the context that this was a trial experiment to test whether or not commercially available ELISA IgY, IgA and IgM kits (in this case tailored for chicken, which was the closest avian species available) could produce detectable readings for rock ptarmigan samples. As evident by the much higher readings for the chicken control samples, these kits are indeed much more sensitive for this species that it is specifically tailored to, and such was to be expected. Comparing the kits used here, the IgY ELISA kit (utilizing polyclonal antibodies) displayed the highest sensitivity for the ptarmigan samples, whereas the IgA and IgM kits (both using monoclonal antibodies) were considerably less sensitive for this task. It is also worth mentioning that in case of the IgA measurements, which also gave the lowest readings, only one of the four captive samples rendered detectable results on the standard curve. All kits did however function accurately for both chicken and ptarmigan samples as evidenced by decreasing absorbance by higher dilution factor on all ELISA assays.

This raises the question whether the low readings of ptarmigan samples, especially for egg white, are due to species sensitivity of the kits used or whether the amounts of antibodies in rock ptarmigan eggs are in fact this low. The low affinity of the IgA and IgM kit towards ptarmigan antibodies compared to chicken might be due to low sequence homology between their heavy chain region which the kits' monoclonal antibodies target. To establish whether this was the case here, amino acid sequence homology between the heavy chain constant regions of ptarmigan and chicken IgA and IgM were obtained by conducting NCBI-BLAST

search against the Japanese rock ptarmigan genome sequence (accession number GCA004320205) using the chicken sequences for IgA (S40610) and IgM (X01613) as query sequences. Next, pairwise sequence alignments were performed by MAFFT (v 7.511) (Figure 3-1) and their homology score calculated. Numbers came in at 67% and 71% sequence homology, respectively. Despite these numbers not being the highest, they are still within the range where cross-reactivity can be expected to occur (Bublin and Breiteneder 2019), indicating that the kits measuring IgA and IgM might not be that effective for detecting egg white immunoglobulins in this particular species. Regardless of this, all in all the trends in the relative amounts of IgY in egg yolk and IgA and IgM in egg white are similar between the two species, in the order of yolk IgY >>> white IgM > white IgA.

a)

```
GallusIgA  LSDPTQEDPERRVLLVCLVEGLPSAGAAIQWLQDNEEMTPAPESDESGCSDCTESGVTQW
LagopusIgA LSDPTQDDPEGRVLLVCLVEGSDLSNINIQWLEGGEEVS--PEREDVSCSTCTDEKSKQW
           ******:*** **********   :.  ****:..**::  ** :: .** **:.  .**

GallusIgA  SRVNVTRKSWEGGAQFGCRVTHGALKEPVTATVSTDCDATPQLQVSLLPPTLEELLVSHN
LagopusIgA SRINVTRKAWDAGNVFGCRVTNEKTQEEVTATISGLCN-VPSLDVTLNPPTLEELFISHN
           **:*****:*:.*  ******:   :* ****:*  *: .*.*:*:* *******::***

GallusIgA  ATVTCVVSNAAAADGVSVSWSRSSGGGLDVSQTEDRQADGRYTVRSFLRVCAEEWNGGET
LagopusIgA ATVTCVVSNVEDPENLSITWTRSSGAELDVSQTEDRQTDGLYTVSSSLRVCVEEWNGGET
           *********.  .:.:*::*:****. **********:** *** * ****.********

GallusIgA  FGCSVR--EEGVVVAEESIRKETDTPLHAPSVYVFPPPAEELSLQETATLTCMASSFLPS
LagopusIgA FECTVKVDEEEVT---KSIQKDLGATTQPPSVYIFPPSAEELTLQESATLTCLATSFLPS
           * *:*:  ** *.   :**:*: .:. :.****:***.****:***:*****:*:*****

GallusIgA  SILLTWTQQNQPISPQNYLIFGPEKDGDFYSLYSKLKVSVEDWQRGDVFGCVVGHDGIPL
LagopusIgA SILLTWTHQNQPVSTQNYLNFGPVKDGDSYSLYSKLVIPVSDWQNGDVFGCVVGHDGIPL
           *******:****:*.**** *** **** ******* :.*.***.***************

GallusIgA  NFIHKRIDKNAGKASHVNVSVVLSDADVTCY
LagopusIgA NFIHKSIDKSAGKASHVNVSVVLSDSDVTCY
           ***** ***.***************:*****
```

b)

```
GallusIgM  TPNGIPLFVTMHPPSREDFEGPFRNASILCQTRGRRRPTEVTWYKNGSPVAAAATTATTV
LagopusIgM PSTPTPLLLTIHPPSRDDFEGPFRNASILCQTRGRRRPGAVTWYKNGISIPGSVVTVVKA
             .  **::*:*****:**********************  ******* .:..:..*....

GallusIgM  GPEVVAESRISVTESEWDTGATFSCVVEGEMRNTSKRMECGLEPVVQQDIAIRVITPSFV
LagopusIgM GAETVAESRVGVTESEWDSGVTFSCLVEGEMRNTSKRMECGCESPTRP-ITVRVVPPSFV
           *.*.*****:.*******:*.****:*************** *. .:  *::**:.****

GallusIgM  DIFISKSATLTCRVSNMVNADGLEVSWWKEKGGKLETALGKRVLQSNGLYTVDGVATVCA
LagopusIgM DIFTSKSAKLTCRVSNMGNVESLDVSWWKETGGKLETAVGQRVLQSNGLYAVEAVASVCA
           *** ****.******** *.:.*:******.*******:*:*********:*:.**:***

GallusIgM  SEWDGGDGYVCKVNHPDLLFPMEEKMRKTKASNARPPSVYVFPPPTEQLNGNQRLSVTCM
LagopusIgM DEWERGEDYICKVNHHELLFPVEERLKKTEG-NARPPKVYVFPPPRGQLTDRQSLSITCL
           .**: *:.*:***** :****:**:::**:. *****.*******  **...* **:**:

GallusIgM  AQGFNPPHLFVRWMRNGEPLPQSQSVTSAPMAENPENESYVAYSVLGVGAEEWGAGNVYT
LagopusIgM AQGFNPPQFFIRWLRNGEPLPQSHSLTSSPMAERPENESYVAYSVLRVGAEEWGAGNVYT
           *******::*:**:*********:*:**:****.************ *************

GallusIgM  CLVGHEALPLQLAQKSVDRAS
LagopusIgM CVVGHEALELQLVQKSVDKAS
           *:****** ***.*****:**
```

Figure 3-1. Pairwise amino acid sequence aligment (here shown in CLUSTAL W format) for a) IgA and b) IgM in chicken (*Gallus gallus*) and Japanese rock ptarmigan (*Lagopus muta japonica*).

Former studies in separate avian species have indicated several factors, including age of hen, dietary differences, pathogen exposure and for species like chicken between-strain differences to affect egg yolk IgY levels. The results from the current study remain too inconclusive to tell whether any of these factors are the case for the Japanese rock ptarmigan, although it is interesting that the wild yolk samples displayed seemingly much lower levels of IgY compared to captive samples (Table 3-3). There are some possible clues as to why this might be the case.

The most credible is perhaps the increased exposure to pathogens in captivity compared to the birds' wild environment having lead to an increased IgY production in exposed hens and thus the yolk IgY levels. Previous studies have demonstrated that IgY levels can be connected to pathogen load (Gates et al. 2021; Wattrang et al. 2021), a finding further supported by the numerous immunization trials to produce antigen-specific IgY for analytical, diagnostic and therapeutical purposes (Carlander et al. 2000; Sugita-Konishi et al. 1996). By incorporating immunostimulants of bacterial origin in the vaccination process, one can further increase the amount of antigen-specific IgY which can ultimately account for up to 10% of total egg yolk IgY (Lévesque, Martinez and Fairbrother 2007; Schade and Hlinak 1996). Compared to the captive environment, the wild environment poses much fewer risks when it comes to pathogens, and the lack of pathogenic stimuli might be why wild ptarmigan eggs contain less IgY. In the wild the main pathogenic threat is the *Eimeria* parasites (Matsubayashi et al. 2021), for which cell-mediated immune responses are important (Kim, Chaudhari and Lillehoj 2019) even though some studies demonstrate that offspring can receive Eimeria-specific antibody protection from the hen (Smith et al. 1994). Future studies should ideally target Eimeria-specific egg yolk and egg white antibodies to see whether this could be the case for rock ptarmigan. Despite Eimeria-infection is relatively universal in wild

individuals, it is completely absent from the captive environment due to the zoo's strict hygiene control.

Another factor to consider is the age of the hens, as other studies have shown older hens to deposit less IgY into their yolks compared to younger birds (Barua, Furusawa and Yoshimura 2000). The wild hen at Mt Komagatake was over twice the age to that of her captive counterparts, which might explain her eggs' lower IgY levels. Other studies have suggested the opposite though (Pauly et al. 2009), and this might suggest it depends on the species in question.

Due to the inconclusive conclusions from various studies and the fluctuating IgY levels in egg yolk, the outcomes seem to depend on the strains of birds used as well as the extraction method (Pauly et al. 2009). In this case, the kits used – primarily tailored for chicken, as evidenced by the much higher read at a much higher dilution factor for the chicken control sample (10^{-7}) compared to the ptarmigan samples (10^{-5}) – might not reflect the true situation in terms of IgY levels in rock ptarmigan egg yolk, indicating it is yet a case of species-specificity. Similar is the situation for IgM and IgA, although the importance of egg white antibodies as a form of initial immunological defense for the chick post-hatch is debatable given their minor concentrations in egg white compared to yolk IgY in both species.

Methodological limitations notwithstanding, the high mortality rate of chicks in captivity is likely not attributed to insufficient egg antibody levels as a first form of immunological defense, given the differences here are too small to be significant. In order to fully understand the full picture related to immunological defense in ptarmigan chicks and possible prospective for conservative efforts in captivity, future studies should preferably extract and purify rock ptarmigan antibodies, especially IgY, in order to determine the

potential of rock ptarmigan IgY as a defense form against common pathogens encountered in captivity.

Chapter 4

General Discussion and Conclusion

4-1 General discussion

This thesis encompasses the two separate studies comparing egg yolk FA composition and egg antibody levels in captive and wild Japanese rock ptarmigan eggs.

In chapter 2 concerning the FA composition of rock ptarmigan egg yolk captive and wild rock ptarmigan eggs displayed significantly differing composition for most fatty acids, including the ratio of n6 and n3 PUFA. In this case, captive rock ptarmigans have a FA composition more similar to commercial poultry species (Chapter 2, Table 2-3). The difference between captive and wild ptarmigan is much smaller however when comparing against the two commercial poultry species, and this is further corroborated by the changes seen in free-range and battery cage poultry species (Chapter 2, Table 2-5).

As mentioned for the Japanese rock ptarmigan egg yolk FA signatures are likely genetically determined, and at the time of study despite the formula feed provided, no acute change could be seen even after several generations under captive breeding. One would hope then that this have not had any detrimental effects on embryonic and post-natal development for offspring, which might exclude any reasons behind chick mortality. In fact the zoos adapted a new formula feed in 2021, however changes were not implemented until after the samples were taken. The new feed was initially developed to combat the issue of overweight birds in captivity partly attributed to the much higher nutritional content and higher digestibility of the formula feed. At this time captive birds were around 30-40 % heavier in captivity compared to the wild, and this caused several medical issues like skeletal disorders and lethargy, much like that seen in commercial broiler chicks that suffer from metabolic disorders and bone fractures. Although this issue has largely been solved after new feed

implementation, after our study, there is still a high chick mortality and seems to be largely related to the egg-laying process.

There might be a lack of adequate protein and lipids for the hen during egg-laying season in spring with the new feed. As previously mentioned, in the wild the hen of Japanese rock ptarmigan eats a large proportion of insects which are rich in these macronutrients. In captivity, the substitute the birds are provided are mealworm larvae. Looking back to the FA composition, mealworms are fairly high in the n6 PUFA series giving them a higher n6:n3-ratio compared to other insects like crickets (Kępińska-Pacelik et al. 2023) which may support the higher n3 ratio in egg yolk FA comparing to the mealworm. This might be one clue to the higher level of n3 PUFA seen in wild Japanese rock ptarmigan eggs compared to captive ones. Ptarmigan unlike laying hens breed and lay eggs during a specific season, thus the eggs used in our study should reflect the diet the hens subside on at the time of laying, in this case spring/summer when in the wild insects are favored by the hen. Looking at the FA amount in mg/g yolk, the differences between captive and wild individuals become even clearer (Table 4-1).

Table 4-1 Amount of FA (mg/g yolk) in egg yolk from captive and wild Japanese rock ptarmigan*

	Captive rock ptarmigan	Wild rock ptarmigan	Chicken	Quail
SFA	119.6	113.1	110.1	75.5
MUFA	101.2	120.5	152.3	149.8
PUFA	94.4	82.4	43.9	50.4
n6 PUFA	77.8	57.1	40.8	47.3
n3 PUFA	16.6	25.3	3.1	3.1
n6/n3	4.7	2.3	13.2	15.3
Total FA	316	316	307	307

SFA: saturated fatty acid, MUFA: monounsaturated fatty acid, PUFA: polyunsaturated fatty acid.
*Calculated based on average weight captive/wild Japanese rock ptarmigan eggs and % FA in yolk in Pheasant birds from Yamanaka and Furukawa 1975.

In our study, the weights of the captive yolks were not measured, so the calculations for captive individuals are based on yolk weights from unfertilized eggs recorded in other captive individuals. Average % of total FA in egg yolk from several species in the *Phasanidae*

family were used to calculate a rough estimate of what the weight of total FA in mg/g yolk would be in captive and wild ptarmigan egg yolk (based on results from Yamanaka and Furukawa, 1975). Although just a superficial estimation, it is fairly evident that there is a clear difference in wild and captive Japanese rock ptarmigan eggs especially when it comes to the amount of n3 and n6 PUFA, which again is reflected in the n6:n3-ratio. Whether an imbalance in the amounts of FAs is directly behind the issues of hatchability and chick mortality is difficult to confidently conclude by the current data, but it certainly can serve as guidance for future studies and improvement of breeding protocols and is too prominent to be overlooked. In terms of FA composition, the inclusion of n3 PUFA via the actual feed like that of insects might also prove more efficient in terms of bioavailability for the hen and thus the incorporation into the yolk, rather than provision via supplements alone (Visioli et al. 2003). This mainly due to the high susceptibility of oxidation of PUFAs due to their many double bonds. In terms of the amount of yolk FA available to the developing embryo by weight, more data using the actual yolk weight of the captive eggs used to measure FA composition should be undertaken to make a proper comparison in the future.

In terms of immune capacity, the study in chapter 3 on egg antibody levels did not give any indicative results that high chick mortality is attributable to insufficient immunoglobulin concentration in captive rock ptarmigan eggs. Contrary to prior belief, the captive eggs displayed higher yolk IgY levels compared to wild eggs, which is most likely due to higher pathogen exposure in captivity. While the opposite was the case for egg white antibodies (IgA and IgM), whereby wild eggs had higher levels, the much smaller relative amounts of these two antibodies in eggs compared to IgY leaves their importance as a form of first defense in chicks up for question. In fact there is very little research on this compared to that of IgY. In the case of our study, one cannot overlook the methodological limitations, which likely did not accurately reflect true egg antibody levels due to lower kit sensitivity for

rock ptarmigan compared to that of chicken, despite the relative amounts of the three antibodies being reflected correctly to that of chicken.

Since none of the differences in antibody levels between captive and wild individuals were statistically significant, there is likely no need for drastic concern that high chick mortality is due to short supply of egg antibodies in this species. However, IgY levels in captive eggs tended to be higher than those in wild ($p = 0.057$). This tendency may suggest higher microbial load under captive situations compared to wild conditions. Since pathogenic infection is one major cause of early death of chicks under captive breeding, such an increase in IgY levels might not be effective for chicks to survive in captivity. To get a full understanding of the picture surrounding rock ptarmigan antibodies, egg yolk IgY and alternatively IgA and IgM from egg white should preferentially be isolated and measured using conventional purification methods. Especially for IgY, it would be beneficial to see its potential against not only major pathogens in captive institutions, but also against the major parasite these birds encounter in the wild, *Eimeria* spp.

As mentioned in Chapter 2, high PUFA content, specifically that of the n3-series, of Japanese rock ptarmigan, might be beneficial living in the extreme alpine environment which is subject to high radiation and oxidative damage. In fact wild plants and especially leafy herbs similar to those these birds feed on have been found to have higher levels of n3 PUFA (Simopoulos 2004). In addition to antioxidants themselves, some FAs are also found to have antioxidating capabilities (Richard et al. 2008). In addition to maternal antibodies, certain PUFAs - both those of the n3 series as well as the n6 series - and their metabolites, have important immune-coordinating and immune-regulatory functions. These are often contradictory, with n3 PUFAs commonly known as anti-inflammatory whereas n6 PUFAs are typically considered pro-inflammatory (Calder 1996). It is this combinatory action of n3 PUFA as both anti-inflammatory and possibly anti-oxidizing that give them their

cardioprotective abilities (Endo and Arita, 2016). In other words it seems a lot of health-conferring and immune protecting benefits might be transferred to the rock ptarmigan straight from their diet. Coupled with the fact that several strains of beneficial gut bacteria have been identified in wild rock ptarmigan (Tsuchida et al. 2017; Ueda et al. 2018) this will likely have a good effect on the birds – and thus wild chicks overall immune capacity. One of the most successful interventions on increasing chick survival in captivity and overall bird health has in fact been the provision of probiotics. Developed from health-conferring strains isolated from the microbiota of wild individuals along with the supply of prebiotics, this have replaced the previously used antibiotics in zoos, which has greatly benefited the captive conservation efforts.

If attention could then be directed to the nutritional requirements of the hen and the developing embryo, it will likely benefit not only the egg production but also the robustness of newly hatched chicks to survive in the face of the many pathogenic threats they face in captivity.

4-2 Conclusion

The two aforementioned studies regarding the egg yolk FA composition and egg immunoglobulin levels as means to study egg quality in Japanese rock ptarmigans, and whether the high mortality of chicks in captivity can be attributed to any differences in these parameters for captive and wild individuals. The second study regarding egg antibodies seems to – at least at the time of study and given the insignificant differences – not being directly linked to the high chick mortality.

There seems however to be more concern linked to the dietary provisions in captivity which, despite some alterations, is still strikingly different to the wild conditions. A lot of this

is of course due to the logistics of being able to provide the birds alpine feed in captivity, but also likely due to the composition of the natural feed and the specific needs in macro- and micronutrients which will vary throughout the year and in terms of the hen and the chick – during the time of reproduction and egg-laying. As such an egg quality parameter like FA composition – especially in the relative amounts between essential FAs like linoleic acid and alpha-linolenic acid – and how much this relationship differs between the captive and wild situation, could prove helpful in influencing breeding protocols to increase the success of egg production and reduce chick mortality. Since there has been a significant shift in the n6:n3-ratio between captive and wild birds, it might be that this ratio is too unbalanced to meet the necessary requirements for healthy embryo and chick development. Over time as the generations develop in zoos from the initial founders, this could stray further from the native FA composition, escalating the current issues seen with high chick mortality. Given that this is likely linked to dietary insufficiencies there is a need for continued monitoring and research in the future to better understand the differences in the relationship between especially n3 and n6 PUFA between wild and captive eggs. found valuable in trials against pathogens targeting both captive and wild individuals of this species.

Complementary to this, FA composition of feed comprising the wild-type diet of the birds should also be undertaken, as this could influence further formula feed development to better meet the needs for the species in captivity. Future analyses on the wild plants these birds feed on in terms of FA and antioxidant composition could further help improve breeding protocols for these species in captivity, and will make the transition to reintroduction in the wild smoother.

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Summary

Captive breeding programs are an important tool used to save endangered species from extinction, however their success are often affected by high morbidity and mortality in captivity often attributed to the captive environment being vastly different from that of the wild the animals usually have adapted to. The Japanese rock ptarmigan *Lagopus muta japonica* is an endangered species that is currently undergoing a national conservation program to save the species from extinction in the wild. This consist of *in situ* cage protection in the wild, as well as *ex situ* captive breeding in zoos across Japan. The latter conservation effort is challenged by captive individuals in poor condition and a high chick mortality, making it difficult to secure new founders to increase the population numbers in the wild. Maternal health condition is often reflected in the progeny. For a mother hen to produce healthy and viable chicks, it is important she is able to transfer the necessary nutritional and immunological factors into the developing embryo – in other words, the egg. Egg quality parameters such as fatty acid (FA) composition and immunoglobulin concentrations measured as egg yolk IgY and egg white IgA and IgM levels can provide helpful clues to the health state of the captive individuals and inform the development of breeding protocols.

Chapter 2

Fatty acid (FA) composition of unfertilized wild and captive Japanese rock ptarmigan egg yolk was compared by FA compositional analysis to see if a change from wild to a captive diet have had any effect on the hen's ability to transfer a balanced ratio of essential FAs to the chick embryo, which could affect embryo development and survival in captivity. Wild egg yolk displayed a significantly higher level of n3 PUFA compared to the yolk of

captive individuals, and thus a lower n6/n3 ratio. Captive individuals had a FA composition more similar to that of other commercial poultry species provided commercial formula feed akin to that the birds are given under captive conditions, which is known to be higher in n6 PUFA compared to the feed of free-range birds living on wild plants. The difference in egg yolk FA composition between captive and wild Japanese rock ptarmigan, although significant, were much smaller relative to each other compared to the other two commercial poultry species (chicken and quail), indicating that Japanese rock ptarmigan egg yolk as a whole contain higher levels of PUFA – especially those of the n3-series – compared to the commercial species. The intraspecific difference being smaller than the interspecific indicates that in this particular species egg yolk FA composition is likely genetically determined rather than by diet alone, and as such the change in feed in captivity from the wild diet is likely not posing any acute danger to chick development at the time of study.

Chapter 3

In order to see if the high chick mortality in captivity could be attributed to an insufficient supply of maternal antibodies, egg antibodies in the form of egg yolk IgY and egg white IgA and IgM concentrations were measured from unfertilized wild and captive Japanese rock ptarmigan eggs using commercially available ELISA kits originally tailored for chicken. Contrary to prior suspicion, captive rock ptarmigan egg yolk had higher levels of IgY compared to that of wild individuals. The opposite was the case for egg white antibodies, with egg white IgA and IgM levels were higher in wild individuals compared to captive individuals. For all three types of antibodies none of these differences were significant, although there were evident limitations with the ELISA kits that were clearly more sensitive for chicken samples as evidenced by the much higher readings for the chicken control samples, which was further supported by immunoglobulin amino acid homology searches. Out of the three antibodies, the IgY kit gave the most sensitive readings. The higher levels in

captive egg yolk could likely be attributed to pathogenic stimuli which is much higher in the captive breeding environment compared to that in the wild. To get a clearer picture of the actual situation, other methods likely need to be adapted to specifically purify and measure rock ptarmigan egg antibodies. As of now, however, it seems like an insufficient supply of egg antibodies is likely not a major contributing factor to the high chick mortality in captivity, given the insignificance of the results.

Conclusion

Among the two studies comparing egg quality parameters between captive and wild Japanese rock ptarmigans, the difference in FA composition seen between eggs collected in the two environments is likely the most significant indicator to the high chick mortality seen in zoos. This again is likely connected to the diet, which has undergone many modifications over time with varying results. Especially is this prominent for the hen during egg production, as the nutritional needs during this critical period might be insufficient to produce viable and healthy progeny. Although it is a logistic challenge, being able to replicate the wild diet – which varies on a seasonal basis for this species and with their physiological needs at the time – will likely help the hatchability and survival rate. Recently attention has been raised to the substitute for the insects which is frequently consumed by the hens during egg-laying season, and to find a better alternative than the currently provided mealworms especially in terms of FA composition. It is clear that there has been a significant shift in FA composition of egg yolk in captive individuals which strays from the native situation, and this could very well affect the developmental needs of the embryo and hatched chick. It is therefore important that the situation is further assessed by monitoring the egg yolk FA composition in unfertilized captive eggs while adjusting the feed to improve breeding protocols so the conservation efforts can succeed in meeting their goals.

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