# *Avian reproductive physiology*

**Report from a workshop at Hiiumaa/Dagö, Estonia, December 10-12, 2006**

CRU Report 20

Mats Björklund and Mare Lõhmus (*editors*)

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## Contents

## Foreword

As a means to promote interactions among researchers working with all aspects of reproductive biology in birds we organised a workshop held at the island of Hiiumaa/Dagö, Estonia 1-2 December 2006. The participants were from the Centre of Reproductive Biology in Uppsala at Uppsala University and Swedish University of Agricultural Sciences in Uppsala, Tartu University, Estonia and University of Daugavpils, Latvia. The participants had a background from ecology, endocrinology, parasitology, and poultry science. The cooperation workshop was financially supported by "Nya Visbyprogrammet" at the Swedish Institute, Stockholm.

The workshop gained considerable interest at the island of Dagö. The organisers (Björklund and Löhmus) were interviewed by a journalist for an article in local and national newspapers. The county governor of Dagö was informed by the journalist about our meeting and expressed great satisfaction that we had chosen Dagö for the workshop.

The workshop was held at Tuuru local educational centre (http://www.tuuru.edu.ee/) in Kärdla city centre. This center has been renovated recently with support from the European Union and provided a conference room of excellent standard. Accommodation and board were provided by Padu Hotell, Kärdla. The hotel has a high standard and is an ideal choice for a group size like the present.

The organisers want to thank all participants for their help in making this a very rewarding workshop and we hope the content of this report as well as the ongoing discussion between the participating scientists will spur to further interaction.

Uppsala December, 2006

Mats Björklund Mare Lõhmus

## Background and aims of the workshop

Evolutionary biologists have had an interest in reproduction for a very long time. This interest has spanned from an understanding of the relationship and trade-off between the number and size of offspring, and the number of reproductive events, as well as the decisions concerning whether to breed once or many times. Furthermore, great interest has been devoted to the understanding of the vast variation among species in reproductive behaviour. These studies have to a very large extent concerned ultimate question, i.e. the question why various solutions to the trade-offs has evolved, or why some species are polygynous while others are monogamous.

This approach has been very successful, but at the expense of the deeper understanding of the mechanisms in terms of, for example, physiology. These are the proximate 'how'-questions. This neglect has become apparent in the last decades when many researchers have realised that an insight into the immune system is necessary for the understanding of reproductive effort in most animals. This in turn has led ecologists to a renewed interest in the role of parasites. In addition, the role of proximate cues such as hormones play a role that cannot be neglected. On the other hand, the proximate, mechanistic, questions have had a very prominent role in applied science there the goal has mainly been to understand what factors can be improved to maximise economical gain.

Thus, scientists working in these two fields of reproductive behaviour have over the years gained a substantial amount of knowledge in their fields, whereas the interaction has been scarce. This is unfortunate as each field is most likely to benefit from taking part of the knowledge from the other field. The purpose of this workshop was to bring together scientists from a broad area of research to share ideas and results. The group of people ranged from empirical and theoretical ecologists from Uppsala University, applied poultry scientists from the Swedish University of Agricultural Sciences, and ecophysiologists from Tartu (Estonia) and Daugavpils University (Latvia). In addition, one of the organisers (Löhmus) has a strong background in bird endocrinology. A second purpose of this workshop was to strengthen and develop scientific collaboration with the Baltic states in general, and the University of Tartu in particular.

## List of participants Workshop Avian reproductive physiology Dagö 1-2 December 2006

Prof. Mats Björklund, Dept. Animal Ecology, Uppsala University, Sweden - organiser

Dr. Mare Lõhmus, DFO/UBC Centre of Aquaculture and Environmental Research, Vancouver, Canada - organiser

Prof. Raivo Mänd, Inst. Zoology and Hydrobiology, Univ of Tartu, Estonia

Ass. Prof. Lena Holm, Dept. of Anatomy and Physiology, Swedish University of Agricultural Sciences, Sweden

Senior Researcher Peeter Hõrak, Inst. Zoology and Hydrobiology, University of Tartu, Estonia

Senior Researcher Indrikis Krams, Dept. Sciences, University of Daugavpils, Latvia

Dr. Vallo Tilgar, Inst. Zoology and Hydrobiology, University of Tartu, Estonia

Dr. Lauri Saks, Inst. Zoology and Hydrobiology, University of Tartu, Estonia

PhD-student Alexandra Hermansson, Dept of Anatomy and Physiology, Swedish University of Agricultural Sciences, Sweden

PhD-student Mårten Hjernqvist, Dept Animal Ecology, Uppsala University, Sweden

PhD-student Ulvi Karu, Inst. Zoology and Hydrobiology, University of Tartu, Estonia

PhD-student Priit Kilgas, Inst. Zoology and Hydrobiology, University of Tartu, Estonia

PhD-student Tatjana Krama, Inst. Zoology and Hydrobiology, University of Tartu, Estonia

PhD-student Pauli Saag, Inst. Zoology and Hydrobiology, University of Tartu, Estonia

PhD-student Joanna Sendecka, Dept Animal Ecology, Uppsala University, Sweden

Msc-student Elise Sonn, Inst. Zoology and Hydrobiology, University of Tartu, Estonia

## Program for the workshop Avian reproductive physiology 1-2 December 2006

| | | |
|---|---|---|
| **Thursday 30 nov** | **Arrival** to Dagö, get-together | |
| **Friday 1 dec** | 09:30 | Mats Björklund<br>*Introduction and presentation of CRU* |
| | 09:45 | Peeter Hõrak<br>*Antioxidants for animal ecologists: towards an understanding of immunity?* |
| | 10:30 | Lauri Saks<br>*Antioxidant protection, carotenoids, and the cost of immune challenge in greenfinches* |
| | 11:00 | Priit Kilgas<br>*Hematocrit in great tit (Parus major) nestlings in relation to sex, growth and immune function* |
| | 11:30 | Ulvi Karu<br>*Intestinal parasites (coccidia) in birds - their reproduction and impact on reproductive performance of birds* |
| | 12:00 | Lunch and excursion |
| | 15:30 | Coffee |
| | 16:00 | Mare Löhmus<br>*Leptin in reproduction - what can we do about it?* |
| | 17:00 | Mats Björklund<br>*Allocation models and life-history decisions in birds* |
| | 18:30 | Dinner and discussions |

| | | |
|---|---|---|
| **Saturday 2 dec** | 09:30 | Raivo Mänd<br>*Looking for mechanisms behind habitat-related variation in reproductive performance of great tits* |
| | 10:00 | Lena Holm & Alexandra Hermansson<br>*Effects of embryonic exposure to oestrogen on the structure and function of avian reproductive organs* |
| | 10:30 | Joanna Sendecka<br>*What comes out from the flycatcher's egg?* |
| | 11:00 | Mårten Hjernqvist<br>*Avian sex allocation* |
| | 12:00 | Lunch & excursion |
| | 15:30 | Coffee |
| | 16:00 | Pauli Saag<br>*Feather degrading bacteria as a potential selective agent in birds* |
| | 16:30 | Indrikis Krams, Tatjana Krama<br>*Overnight weight loss in wintering great tits: implications of social dominance* |
| | 17:00 | Vallo Tilgar<br>*Alkaline phosphatase as an indicator of skeletal development in wild birds* |
| | 18:30 | Dinner and discussions |
| **Sunday 3 dec** | **Departure** | |

# Leptin and reproduction - what can we do about it?

*Dr. Mare Lõhmus*
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In most environments, food availability typically varies both in time and space. Consequently, organisms need to store excess energy when food is abundant and utilize the storage during periods of food shortage. One would also think that animals must have a mechanism that indicates when time can be devoted for other activities than foraging, such as reproduction. This mechanism should be linked to the regulation of energy transfer between storage and use.

Hypothalamus is a structure in the lower part of the brain that was many years ago identified as an important site in the regulation of body weight. Until quite recently it was not known what kind of messenger was informing the hypothalamus about the amount of fat in the body, but there were some cues suggesting that a substance that originated from adipose tissue, and was circulating in the blood, was the agent. The hormone leptin was first described 1994 and has been a “popular” hormone ever since. The worldwide interest in this hormone was mostly due to its capability to regulate the body weight, activity, and appetite. Leptin is primarily produced by fat cells and is found in the circulation in a free form or bound to leptin-binding proteins. Circulating leptin concentrations are highly correlated with fat mass. The most known function of leptin is to inform the central system about the amount of body fat but leptin receptors are found in many kinds of organs and most tissues can be directly influenced by leptin.

Leptin is released from adipose tissue into the bloodstream and from the blood the signal is transmitted to the central nervous system informing the brain about the amount of peripheral energy in an organism. Leptin binds to its receptor in hypothalamus leading to an activation of neural pathways that mediate the decrease in food intake and the activation of sympathetic nervous system. Activation of the sympathetic nervous system leads to an increase in energy expenditure by increasing physical activity. By increasing activity and decreasing foraging the fat mass in an organism will reduce. The direct benefits of functioning leptin system have been investigated mostly by creating stems of mice that are lacking the leptin gene. These leptin defective mice are known in the literature as the ob/ob mice, and they are (like all leptin deficient animals including humans) extremely fat and have problems with heat production. Additionally these animals are infertile indicating that a threshold level of leptin is obligatory for a sustained central stimulation of gonads.

The association between fertility and fat is an ancient concept. Twenty thousand years old artefacts from France and Austria that are thought to represent fertility goddesses depict women who would be considered obese by today standards. Also it was discovered already in 50's and 60's that puberty is somehow linked to body weight and more specifically to fat storage. Later in the70's it was shown that the loss or restoration of menstrual cycles in young girls had something to do with minimum weight. In addition, it was documented that normal girls become relatively fatter from the period of early puberty to later reproductive maturity. From these findings the “critical weight” hypothesis was generated. The hypothesis states that a functional reproductive system requires a minimum amount of stored adipose mass and that a metabolic signal may be responsible for the initiation of reproduction.

Organisms that can not produce leptin or have defective receptors, are morbidly fat and infertile and they also have atrophic reproductive organs. It has now been shown that

treatment with leptin can recover the reproductive system in the ob/ob mouse by leading to growth and restored function of the reproductive organs and secretion of reproductive hormones. In an evolutionary context the connection between leptin and reproductive system is quite natural – it is critical for animals to coordinate reproduction with periods of sufficient nutrient supply. Since fat is the most labile component of body weight, a system that informs the reproductive system about energy stored in the adipose mass would be essential for the maintenance of reproduction. It is therefore likely that leptin has evolved as a signal that mediates the status of the food supply and body fat stores to the hypothalamic-pituitary axis in order to regulate release of reproductive hormones.

One other early finding in leptin's scientific history was that the serum leptin level is higher in women than in men. The difference between sexes is relatively high –leptin release from adipose tissue to the blood is 2-3 times higher in females than in males. That could, however, be explained in two different ways. The most straightforward explanation would be that, in general women have greater amount of body fat compared to men of equivalent body mass. However, in healthy lean men and women that are closely matched for body fat mass and for fat distribution, leptin is still greater in women than in men. So it is more likely that the differences in leptin synthesis between men and women lay in differences in secretion of gonadal steroids. It has been shown that estrogens stimulate and testosterone reduces leptin secretion.

Experiments on rats have shown that leptin is able to stimulate the secretion of gonadotropin releasing hormones (GnRH) from hypothalamus and the release of follicle stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary. So leptin has been shown to have direct stimulatory effects on the hypothalamus-pituitary-gonadal (HPG) axis on both the hypothalamic and the pituitary level. It is not entirely clear if the GnRH-secreting neurons itself express leptin receptors or if leptin's effect there is mediated by some neuropeptide messenger. In the pituitary, leptin directly stimulates LH, and to a lesser extend FSH release. A decrease in leptin levels can also be induced by fasting and fasting in turn can to some extent destroy the reproductive function. In fasting animals, both males and females, the gonadotropin pulse frequency will decrease, but administration of leptin has been shown to totally or partially reverse the inhibitory effects of starvation. Leptin administration can also reverse fasting-induced anestrus (the period of non-reproduction) in some species, and the delay of puberty in food restricted mice and rats.

Leptin has also been found to exert endocrine and/or direct paracrine effects on the gonadal organs. The connection between leptin and the ovary has been supported in several studies. It is not yet known if leptin is produced by ovaries but what we know for sure is that there are many leptin receptors in the ovary (both in granulosa and in thecal cells). This suggests that leptin at least exerts a direct action on the ovary and that leptin has a functional role in follicle growth and/or maturation. It is also interesting to know that follicular fluid contains leptin in concentrations comparable to that in blood. In other words, follicular fluid leptin levels correlate with body fat mass as do blood levels. Leptin is directly influencing the follicular growth. Factors having have major importance on egg cell quality are vascularity and metabolic state of the developing follicle. Under-vascularized follicles result in poorer quality embryos and lower implantation and pregnancy rates. Leptin seems to promote blood vessel formation, increase blood flow and facilitate oxygen delivery to the follicle.

Surprisingly, we find hundreds of studies that show a negative effect of leptin on ovarian function. Several of these suggest that high leptin levels decrease the production of sex steroids. Others say that leptin has a negative impact on ovarian response to gonadotropins. Inhibiting effects on follicle growth and ovulation are also a common conclusion of the effects of leptin in ovaries. So how can we explain this?. One explanation could be that when we raise the level of leptin in experimental conditions, the doses that are

used are too high compared to the normal natural leptin levels. If the negative effect of leptin on ovarian function is simply a question of dosage, this may explain the fertility problems that are very common in obese women.

Lifestyle-induced obesity is one of the most common causes to defective fertility in western society. High levels of leptin have a negative influence on follicle metabolism and endocrinology resulting in impaired follicular development and decreased implantation and pregnancy success. Obese females had fewer oocytes retrieved, a higher miscarriage rate, and lower live birth rate than normal weight women. Also severely obese men express several kinds of reproductive defects. Their testosterone levels are low, testicles are not responding to gonadotropins and the serum LH is also low. Not only extremely obese adolescents, but also individuals with low body fat mass, have delayed puberty and impaired development of the reproductive system. Eating disorders, including anorexia nervosa and bulimia nervosa, are associated with significantly lower serum leptin levels in both females and males. When leptin levels have dropped, some time later the LH/FSH pulsatility and reproductive function will also decline in both males and females. In humans with anorexia nervosa, this causes a reversion to the prepubertal state, which can be reversed by feeding. A threshold level of leptin for functioning LH secretion has been reported.

Because of the great differences between species in size, lifespan, and environment, there may be multiple cues timing puberty. In long-lived species, when energy availability is low and growth is retarded, puberty is delayed for months or even years until enough food is available. In small, short-lived species with high metabolism, the consequences of reduced nutrition and reduced growth are much more severe as they may not attain puberty before they die. Twenty years before the discovery of leptin, it was reported that there was a strong correlation between body fat and the time of first menstruation in humans. For several years scientist have been looking for a significant leptin increase that could work as a trigger for the initiation of puberty. For the most the data are rather conflicting. However it has been shown that the neurons in hypothalamus that regulate LH release have a peak in leptin sensitivity a short period before the first preovulatory release of LH.

The presence of leptin receptors in the placenta and in a variety of fetal tissues has led to the speculation that leptin plays a direct role in fetal growth and development. Also the circulating leptin levels are clearly elevated during normal pregnancy. This state of elevated maternal leptin can not be modified by a decreased food intake during pregnancy. In humans, the source of increased maternal leptin levels is the placenta, whereas in rodents, leptin seems to be over-secreted from adipose tissue. Leptin levels are the highest during the second and third trimester and return to normal within 24 hr of delivery. It has been reported that leptin levels in women suffering from spontaneous abortions during the first three months are 38 percent lower than in women who successfully maintain their pregnancy. Leptin is apparently needed for maintenance of pregnancy. Despite of the elevated levels of leptin, pregnancy is considered to be a leptin resistant state. Possibly, leptin resistance during pregnancy may be indirectly influencing fetal growth by sustaining maternal food intake and thus providing sustained energy sources for fetal growth. Potentially, the development of apparent leptin resistance in pregnancy could result from a fall in hypothalamic expression of Ob-Rb.

It is evident that if necessary energy reserves can not be made available for pregnancy, then there would be no need for the reproductive system to maintain its function and waste resources that can be utilized for other critical body functions. Thus, a factor that links the state of energy reserves in the adipose mass to the central control of reproduction would only allow firing of the reproductive system when energy reserves are adequate, thus shunting and saving energy resources for other functions that are necessary for survival.

# Antioxidants for animal ecologists: towards understanding immunity

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All animals are surrounded by pathogens and parasites. To survive the attacks from these pathogens we need a functional immune system. There is now substantial empirical evidence that the immune system is costly in terms of energy, which has effect on other aspects of the life of an individual. Individuals investing a large amount into the immune defence have been shown to pay a cost in terms of reduced reproductive effort. The cost aspect of the immune function is furthermore supported by the fact that there is a large variation among individuals in terms of immune function and that all individuals are not sustaining a maximum immunity at all times, even though this would be of obvious benefit. The most pertinent questions in this context are: what is optimal immunity, what makes immune activation costly, how to assess immunopathology in ecological model species and what is the role of oxidative stress and antioxidants in immunoecology?

The trade-off between immune functions and reproduction is well known. However, the immune system is very often treated as a black box by ecologists. This is unfortunate as there are more trade-offs to be considered. The immune system in vertebrates is traditionally divided into two parts: the adaptive immune system that produces antibodies as the means of long-term memory, and the non-specific (inflammatory) immune system. It is obvious that there must be a trade-off also between and within these two parts. Indeed, experiments using rats infected by a pathogen we were able to show that the two parts of the immune system are cross-regulated.

When we compare the two immune systems in terms of costs, it is clear that the non-specific part have a high nutritional and pathological cost of use. This suggests that if we are interested in understanding the trade-off between reproduction and immune-system in natural populations, the non-specific immune system is of particular interest. One of the ways for the non-specific mechanisms to handle pathogens is phagocytosis. This process relies on the usage of reactive oxygen, which in turn increases the production of free radicals. It is well-known that free radicals can cause many medical problems ranging from cellular injury and death, inactivation of critical enzymes, diabetes, cancer, cardiovascular diseases to mention a few. There are hundreds of chronic diseases associated with elevated levels of free radicals. The production of free radicals causes oxidative stress defined as a disturbance in the prooxidant antioxidant balance in the favour of the former.

As antioxidants per definition are the remedy for oxidative stress individuals that are relatively more successful in obtaining antioxidants should have a selective advantage in evolutionary context. Carotenoids are a group of antioxidants that can directly scavenge free radicals, Carotenoids have several immuno-stimulatory qualities – they have an effect on lymphocyte number and activity, stimulate production of cytokines, stimulate phagocytosis and bacteria-killing, and enhance antibody-production. It has been suggested that in many bird species the carotenoid colourations, such as the reds, greens and yellows of the plumage, have the dual function of reducing free radicals and signalling to potential partners of the individual's ability to handle infections ..

To evaluate the importance of carotenoids we performed an experiment using wild-caught greenfinches. We aimed to explain the relative importance of exogenous and endogenous antioxidants in the formation of antioxidant barriers and the prevention of

oxidative stress. We also intended to assess the costs of immune system activation in terms of effects of antioxidant barrier and induction of oxidative damages. To find the answer to these questions we experimentally supplemented the food with carotenoids and vitamin E, and induced immune challenge by using a powerful mitogen – phytohemaglutinin. As response variables we used plasma antioxidant potential, lipid peroxidation products.

The results showed that the supplementation of vitamin E had no effect. On the other hand, carotenoid supplementation increased plasma carotenoid levels, and increased immune response. This suggests that carotenoid supplement reduced lipid peroxidation, and immune activation increased antioxidant protection. In short, more damage - more protection.

The main conclusions from this and other studies are that the activation of immune system can cause oxidative stress which can be reduced by antioxidants such as carotenoids. Immune challenge induces a compensatory increase in plasma antioxidant protection. However, carotenoids did not contribute to plasma antioxidant protection. Uric acid – a not costly antioxidant that is obtained as a metabolic by-product seems to be a very important component in reduction of the oxidative stress.

## Concluding summary of the remaining talks

The importance and effect of variation in leptin production among individuals was analysed from a theoretical point of view (**Björklund**). Based on a very simple model of the relationship between fat levels and leptin levels it can be shown that the exact form of the function relating leptin levels and reproductive hormones matter. A linear relationship results in a distribution of reproductive output that differs considerably from the distribution when the relationship is more threshold-like. It also turns out that this can explain the differences among many species in terms of seasonal variation in reproductive output. Virtually nothing is known about the shape of this relationship in nature, or the mount of variation among individuals. This will be studied by **Lõhmus** in collaboration with **Björklund** and **Mänd** in a natural population of great tits (*Parus major*).

A very simple model of acquisition and allocation of resources (**Björklund**) show that the mean and variation in daily resource availability to a very large extent can explain the differences among populations. In populations where variation is high then in some years a large proportion can afford to lay a second clutch, while in others very few. In habitats where variation is low this is not true and in general most individuals do not breed twice. This model needs obviously to be connected with the leptin model (see above).

A well known problem in both poultry science and conservation biology is the decrease in eggshell quality with of eggs laid by older birds. This thinning of the eggshells is thought to be connected to DDT-compounds but the underlying mechanisms remain largely unknown. The problem is further complicated by the fact that the physiological processes involved in eggshell formation still remain to be clarified. In collaboration with Dept of Environmental Toxicology (Uppsala University) **Holm** and **Hermansson** showed that exposure to estrogenic pollutants during early embryonic development results in hens producing eggs with thinner shells as they reach adulthood. In these hens the localisation of carbonic anhydrase (CA), a crucial enzyme in eggshell formation, is altered. These are the first physiological clues to the mechanism behind eggshell thinning. In the roosters obtained from the same experiment the exposure led to reduced semen output, altered testicular morphology and cloacal deformations which may lead to impaired reproduction.

A large proportion of the workshop was related to the measurement and ecological correlates of health in birds. **Hõrak** summarised extensively the current knowledge concerning immune system, the role of antioxidants and caretonoids, both theoretically, and with experimental examples generated by him and his students (**Saks).** The reproductive output of the breeding is largely affected by physiological stress. Several factors, such as for example parasites ranging from coccidia to bacteria, affect consequences and levels of the stress, (**Karu, Kilgas, Saag**). In addition, factors such as social dominance affect nutritional status and survival during winter, which in turn affects condition during onset of breeding (**Krams & Krama**).

The relationship between breeding stress and habitat choice might be a great concern for conservation. **Mänd** showed that in modern managed coniferous forests there are almost no opportunities for great tits to breed, whereas deciduous forest provides such opportunities to a larger degree. By erecting nest-boxes in both habitats densities increased and more so in the deciduous forests, and females in the deciduous forests start to breed earlier and lay larger clutches than females in the coniferous forests. Females in the deciduous forests start to breed earlier and lay larger clutches than females in the coniferous forests. Despite that, fledgling success and adult survival is lower in the deciduous forest. Also physiological health,

measured by a number of criteria, such as hematocrit and albumin levels was better in birds breeding in coniferous forests. This suggests that birds are forced due to modern forestry to breed in suboptimal habitats with an increased physiological stress. How this affect the long-term survival of these populations, and also other species of birds is currently unknown. Apparently, there is room for research in the area of 'conservation physiology'.

Reproduction is a substantial energetic investment, and it is obvious that individuals are selected to maximise the gains from their investments. This can take many forms, for example, in differential investment in one sex of their offspring (**Hjernqvist**). It turns out that the sex ratio can be manipulated by females in an optimal way. For example in new areas a larger proportion of offspring can be males since producing males that can return and take a territory is beneficial at these locations. In older areas on the other hand, there a great competition for territories is already eminent, the production of female offspring is more advantageous. In addition, the egg contains a variety of hormones and antibodies that influence the growth and future reproductive success of the offspring (**Sendecka)**. For example, egg size has a positive effect on nestling survival, and the amount of lutein and lysozymes are positively rlelated to the size of the offspring, while the increasing concentrations of IgG has a negativeeffect on body mass in nestlings. The exact mechanisms involed and the ecological consequences are virtually unknown. Reproduction has not only gains, but also costs, for example in terms of impaired skeletal growth in offspring or decalcification in adults if conditions are bad. There are now attempts to find efficient methods to analyse this in natural populations (**Tilgar**).

In summary, the workshop pointed to a large number of interesting and promising areas of research where there is very little knowledge from natural populations. It was also apparent that the cumulated knowledge from poultry science has much to offer for ecologists, as well as the ecologists have much to offer poultry science.

## CRU Publication series

**Report 1**
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**Report 17**
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**Report 18**
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**Report 19**
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Proceedings from a symposium at the St Petersburg State Academy of Veterinary Medicine,Russia January 10-12, 2007, *by Renée Båge, Nina Fedosova, Kirill Plemyashov and Maria Stakheeva (editors)*

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