
**Testosterone, sexual ornamentation and
immune function in the Black-headed Gull
*Larus ridibundus***

An experimental test of the
immunocompetence handicap hypothesis

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2. ABSTRACT

Yet there are just a few *experimental* studies addressing the immunocompetence handicap hypothesis of Folstad and Karter (1992). We implanted juvenile black-headed gulls *Larus ridibundus* with testosterone releasing tubes to experimentally test this hypothesis. The effect of testosterone on immune function, secondary sexual traits and body mass were examined. To determine immune function both the primary and the secondary antibody response after immunisation with sheep red blood cells were measured. Also the volume percentage of white blood cells, the sedimentation rate and the number of foot infections were measured. Secondary sexual traits we quantified were the brown mask, the colour of the legs and the bill and the behavioural displays that the gulls performed. As predicted by the immunocompetence handicap hypothesis, testosterone had a positive effect on mask development, bill colour brightness and display rate. However contrary to the hypothesis' predictions neither primary, nor secondary antibody response, nor any other measure of immunocompetence was affected by the testosterone treatment. Testosterone did not affect body mass either. We suggest that the testosterone treated gulls might have compensated for their greater display rates and sexual ornament sizes by having a higher food intake. We also investigated the relation between immunocompetence and secondary sexual traits and between mate choice, and immunocompetence and secondary sexual traits. We found no consistent association between immune function and secondary sexual traits. Our results on mate choice were not conclusive due to low sample size. We conclude that our results do not support the immunocompetence handicap hypothesis.

3. INTRODUCTION

Sexual Selection and the Handicap Hypothesis

Numerous species show elaborate sexual ornaments and displays, such as the antlers of deer, the tail plumes of the peacock, and the vocalisations and ritualised postures of many bird species. These secondary sexual characters or sexual ornaments have probably evolved under sexual selection (Darwin 1871).

Sexual selection is the selection that arises when individuals of one sex gain an advantage over others of the same sex in obtaining mates. There are two scenarios in which this could happen. One is when individuals of the same sex directly compete with each other over mates. This direct competition creates a selective pressure favouring an increase in fighting ability in the competing sex. The selection in this scenario is called *intrasexual* selection. In the second, *intersexual* selection scenario, individuals of the competing sex compete in a more *indirect* way with each other by being attractive to the other sex. The selective pressure is then posed by the mate choice of the other sex. In this scenario the development of traits that are attractive to the other sex is favoured in the competing sex.

One of the mechanisms, by which intersexual selection could work, is stated by the 'handicap hypothesis' (Zahavi 1975, Zahavi 1977). The handicap hypothesis assumes that sexual ornaments are indicators of the quality of the bearer. The expression of these traits would be costly (i.e. reduces survival). Individuals of high quality would be better able to pay the costs of developing or maintaining such a handicapping trait. Thus individuals selecting for mates that show well-developed sexual ornaments select for quality. It would be profitable for the *selecting* sex to choose mates with such a quality marker, as long as the handicap it imposes on a mate (and its offspring), results in a fitness loss that is smaller than the extra fitness gained by securing a better mate. For the *selected* sex it is profitable to express these traits, as long as the fitness loss as a result of having a handicap, is smaller than the fitness gain by being more attractive to the other sex.

Grafen (1990) demonstrated in a theoretical model that the handicap principle could indeed work. To be honest and evolutionary stable a signal of quality should be both relatively and absolutely more expensive to low-quality individuals than to high-quality individuals. Cheating, by having well-developed quality markers without being a high quality individual, would then be impossible.



Figure 1. Examples of animals having sexual ornaments, from the left to the right, the Peacock, the Red Deer, and the Black-headed Gull, the subject of this study.

The Immunocompetence Handicap Hypothesis

A major question is what kind of quality animals signal about, by their sexual ornaments. Hamilton and Zuk (1982) suggested that animals signal about heritable parasite resistance. If an individual is well able to cope with parasites, because of genetically determined resistance, it will mostly have good health and vigour. Such a healthy individual will be better able to pay the costs of having large ornaments than an individual that has lower parasite resistance, which will be less healthy. As a result individuals with better parasite resistance will have bigger ornaments. Genetic variation in parasite resistance would be maintained despite directional selection, because of the particular ability of parasites to co-evolve with their hosts.

Folstad and Karter (1992) proposed a mechanistic model for a trade-off between immune function and sexual ornaments. They based their model on the evidence (mainly found in the literature on mammals) for an immuno-suppressive effect of testosterone (review by Grossman 1985) and the fact that testosterone promotes the expression of secondary sexual characters in many species (see Folstad and Karter 1992 for references). The basic idea of their 'immunocompetence handicap hypothesis' is that animals need to produce androgens (e.g. testosterone) to develop sexual ornaments. However at the same time these hormones suppress immune function. Therefore only individuals with low parasite burden or with good resistance against disease can afford to have high testosterone levels. Thus only these individuals will show great expression of sexual ornaments.

In their original paper, Folstad and Karter attributed an obligatory immunosuppressive effect to testosterone. Wedekind and Folstad (1994) argued that this scenario would not be evolutionary stable. Cheaters could emerge, in which one of the connections between the immune system, sex hormones, and sexual ornamentation had been eliminated. These cheaters would be able to produce strong signals without having high testosterone levels, or to have high testosterone levels without suppressing immune function.

Wedekind and Folstad suggested that the immunosuppression would be in the animal's own interest. Animals only have a limited amount of resources (e.g. energy, metabolites) which have to be allocated to the different physiological systems. Resources, needed for the development or maintenance of sexual ornaments, have to be drawn from somewhere else in the body, for instance from the immune system. Testosterone and other hormones play a role in regulating this distribution of resources. Cheating would be impossible, in case the immunosuppressive effect of testosterone is just the result of the regulatory role of testosterone in the adaptive allocation of limited resources.

Evidence for the Immunocompetence Handicap Hypothesis

Until now, most studies on the immunocompetence hypothesis concern correlative data (e.g. Skarstein and Folstad 1996, Saino and Møller 1994, Seutin 1994, Weatherhead *et al.* 1993). In these studies, parasite load or indicators of immune function, the expression of sexual ornaments, and testosterone levels have been related to each other.

The difficulty with such correlative studies is that it is hard to predict what kind of relationship is to be expected between these different variables (Shykoff and Widmer 1996). The outcomes of the different studies are inconsistent and sometimes difficult to interpret. For example, Saino and Møller (1994) found a *negative* correlation between tail length and the intensity of infestation by some ectoparasite species. On the contrary Skarstein and Folstad (1996) found a *positive* correlation between colour brightness and the intensity of infestation by several parasite species in Arctic charr. Seutin found *no* relationship between plumage colour and blood parasites in redpolls. Also Weatherhead *et al.* (1993) did *not* find any clear relationship between several secondary sexual characters, several parasite species, and testosterone levels in red-winged blackbirds.

Until now only few experimental studies were done to test the predictions of the immunocompetence handicap hypothesis (Norris *et al.* in prep., Saino *et al.* 1995, Salvador 1996). One of these experimental studies was carried out in black-headed gulls (Ros *et al.* 1997).

Testing the Immunocompetence Handicap Hypothesis in the Black-headed Gull

The Black-headed Gull is very suitable to test the predictions of the immunocompetence handicap hypothesis. The Black-headed Gull is a monomorphic species of which males and females show similar display behaviour and virtually the same nuptial plumage. Testosterone plays an important role in the expression of several sexual ornaments of the Black-headed Gull, both in males and females. In the first place testosterone treatment enhances the frequency at which these birds perform their very conspicuous behavioural displays (Groothuis and Meeuwissen 1992). Furthermore the development of the nuptial plumage with the brown mask is dependent on testosterone, and testosterone influences the colour of the bill and the legs (Van Oordt and Jung 1933). The Black-headed Gull is a long-living species that breeds in dense colonies in which the risk of infection is high (Pavlásek 1993, Literák *et al.* 1992). Thus resistance against disease may be an important trait, on which selection is acting in this species.

It may seem odd to test predictions of sexual selection theory in a monomorphic species. However a monomorphic species can be as suitable for this as any dimorphic species. The sexual selection mechanism is in principle the same in a monomorphic species as in a dimorphic species. The only difference is that in a monomorphic species individuals of both sexes may be competing for mates, whereas in a dimorphic species one sex is competing. When individuals of both sexes compete for mates and are choosy when selecting their mates, intersexual selection will pose similar selective pressures on both males and females. This will lead to the development of sexual ornaments in both males and females, such as in the monomorphic Black-headed Gull.

The Experiment of Ros *et al.*

Ros *et al.* treated juvenile black-headed gulls with testosterone releasing implants to test the main premise of the immunocompetence handicap hypothesis. This premise states that testosterone has a negative effect on immune function, while having a positive effect on ornamentation. In contradiction with the immunocompetence handicap hypothesis, these authors found no effect of testosterone on the primary antibody response after a challenge with sheep red blood cells. The primary response is the antibody response elicited by the presentation of a novel antigen to the immune system.

Nevertheless they did find a positive correlation between display rate and antibody response. This indicates that display rate is indeed an honest signal for the quality of the immune system. However, there could be an alternative explanation for this finding. The gulls were housed in aviaries in small groups together, which is an unnatural situation. Social hierarchies may have evolved in these groups, from which it was impossible to escape for the individuals. Possibly low-ranking gulls suffer from great social stress in this situation. It is known that social stress can lead to chronically elevated basal corticosterone levels resulting in suppression of immune function (review by Bohus and Koolhaas 1993). Stress caused immunosuppression in low-ranking individuals would lead to a positive correlation between social status and immune function. Also display rate is probably positively correlated with social status (Ros *et al.* 1997). Hence immunosuppression in low-ranking individuals induced by social stress could be the alternative explanation for the observed positive correlation between display rate and immune function.

Ros *et al.* could not demonstrate an immunosuppressive effect of testosterone in the Black-headed Gull. Therefore their results contradict one of the major premises of the immunocompetence handicap hypothesis. However, there is still a possibility that these authors might not have detected an immunosuppressive effect of testosterone, while it actually was there. There could be several reasons for this:

- 1) The dose at which testosterone was administered was too low.
- 2) The duration of testosterone treatment, 16 days, was too short.
- 3) Testosterone had no effect on primary antibody response to SRBC, but may have affected other aspects of immune function that were not measured, such as the secondary response,

in which the immunological memory plays an important role, or the number of white blood cells.

This Study

To be sure that an actual immunosuppressive effect of testosterone in the Black-headed gull was not overlooked we did a more extensive experiment in which we again treated juvenile black-headed gulls with testosterone. We introduced several modifications to the original experimental set-up of Ros *et al.* to be better able to detect a possible immunosuppressive effect of testosterone:

- 1) We administered a 1.5 × higher dose to the animals.
- 2) The testosterone treatment was sustained for a much longer period, 8 weeks until the end of the experiment.
- 3) We investigated both the primary and the secondary response to SRBC. Furthermore we examined several other parameters related to immune function: sedimentation rate, hematocrit, the number of white blood cells and the occurrence of certain wart-like infections under the feet of the gulls.

Furthermore we examined the effect of testosterone on the display behaviour like Ros *et al.* did. We also investigated the relation between sexual ornaments and testosterone by monitoring the effect of testosterone on the brown mask, and the bill and leg colour.

Another aim of this study was to test whether individuals that are more immunocompetent are preferred as mate, which is prediction of the immunocompetence handicap hypothesis. We tested this by housing one female and two males in one cage together, thus allowing the female to choose between two possible mates. By this set-up we were able to investigate the role of display rate and other secondary sexual characters, like the stage of the brown mask, and the bill and leg colour, in mate choice in relation to the immunocompetence handicap hypothesis.

To find out whether corticosterone can indeed cause impairment of immune function in testosterone treated black-headed gulls, we treated one experimental group with both corticosterone and testosterone.

4. METHODS

Experimental Animals

Black-headed gulls were captured, just before fledging, in colonies along the shore of Groningen, The Netherlands. The birds were housed in two large outdoor aviaries (9.0 × 4.3 × 2.0 m). One month before the experiment started, the birds were placed in six groups of six animals (size aviaries: 3.0 × 4.3 × 2.0 m), matched for weight (males: mean = 286 g, SD = 25 g, n = 23; females: mean = 256 g, SD = 18, n = 12) and sex. We determined the sex of the birds by the length of the head plus bill. In fledged birds, this length is greater for males than for females (discriminant value: 8.1 cm) and has a reliability of 95% (Coulson *et al.* 1983; Koopman 1990). Before and during the experiment food (dry pellets) and water, both for drinking and bathing, were available *ad libitum*. The water was refreshed continuously. Occasionally mashed hard-boiled eggs were fed. All birds were individually marked by colour rings. At the start of the experiment the gulls were 9 months old.

Treatment Groups

At the start of the experiment the 6 groups were split and divided over 12 aviaries (1.5 × 4.3 × 2.0 m), each containing three birds. In 11 aviaries two males and one female were housed together, to enable the females to choose between two possible mates. In one aviary three males were housed together, because we had only 11 females. Unfortunately, many gulls died in the course of the experiment and we had to move animals to maintain a minimum group size (see appendix 1 of Zwiggelaar 1996). The aviaries were separated by closed partitions to prevent social interactions between aviaries.

We planned to implant 15 of the birds with a silastic tube filled with testosterone (T), 9 birds with both a testosterone filled tube and a crystalline pellet containing corticosterone (TB) and 12 birds would be implanted with an empty tube as a control (C). However, one T-animal died before implantation and two controls were implanted with a testosterone tube by mistake. Ten days later one of these animals was implanted with a corticosterone pellet as well to let it replace a TB-bird that had died after implantation. So eventually we had 15 birds implanted with testosterone, 10 birds implanted with both testosterone and corticosterone and 10 control animals (table 1). The testosterone implanted and control birds were implanted with a cholesterol pellet as well as a control for the corticosterone pellet.

Table 1. Overview of sample sizes. T: testosterone, TB: testosterone +corticosterone, C: control.

Treatment	Planned sample size	Actual sample size just after implantation	Sample size 10 days after implantation
T	15	16	15
TB	9	9	10
C	12	10	10

All gulls in one aviary had the same treatment. We did not place gulls with different treatments in one aviary together because testosterone treated gulls are usually dominant over controls. This could cloud a possible immunosuppressive effect of the testosterone treatment, because the subdominant control-birds may also have a suppressed immune function caused by the stress of having low social status.

Time Schedule of the Experiment

Day 0. Just before implantation we took a blood sample to determine pre-immunisation number of white blood cells, hematocrit and antibody responsiveness to SRBC. Furthermore we determined the size and number of the wart-like foot infections that the gulls had, as another indicator of the immunocompetence of the birds. Finally, we determined the stage of the brown mask and the bill and leg colour (figure 2).

Day 9. The gulls were caught to determine the stage of the brown mask and the bill and leg colour. From here on I will consider the estimates made on day 9 as representative for the mask stage and bill colour on day 10.

Day 10. Ten days after implantation we immunised all gulls for the first time with a 2 % sheep red blood cells (SRBC) solution. We changed the corticosterone pellets from 25 % to 80 % in the TB-animals (see under *Hormonal Implants*).

By day 10 there was a very clear difference between the display rates of testosterone treated and control animals. The testosterone treated animals were displaying at much higher rates (Zwiggelaar 1996). Nevertheless display rates were lower than we expected. Therefore we replaced the closed partitions between aviaries by open ones (wire netting) to allow social stimulation from neighbouring cages.

Day 16. Six days after the first immunisation when the peak in antibody levels arises we took a blood sample, to measure the antibody response against SRBC (later referred to as 'response 1'). Size and number of foot infections, stage of the mask and the bill and leg colour were determined.

Day 45. We challenged the gulls 32 days after the first immunisation for a second time with a 2 % SRBC solution. In chickens four weeks is sufficient to develop an immunological memory for a novel antigen (Munns and Lamont 1991). Therefore we assumed that the gulls to have built up their immunological memory by the time we gave the second challenge. Mask stage and the bill and leg colour were determined.

Day 51. Six days after the second challenge a blood sample was drawn to measure the antibody response (response 2). The birds were challenged for the third time, with a 10 % SRBC solution. A higher SRBC dose was given to find out whether a higher dose would elicit a higher response. Size and number of foot infections, stage of the mask, and the bill and leg colour were determined.

Day 57. Six days after the third challenge we took blood samples to measure the third antibody response (response 3). Size and number of foot infections, stage of the mask, and the bill and leg colour were determined.

Day 87. Thirty days later the gulls were caught for the last time to determine size and number of foot infections, stage of the mask and bill and leg colour.

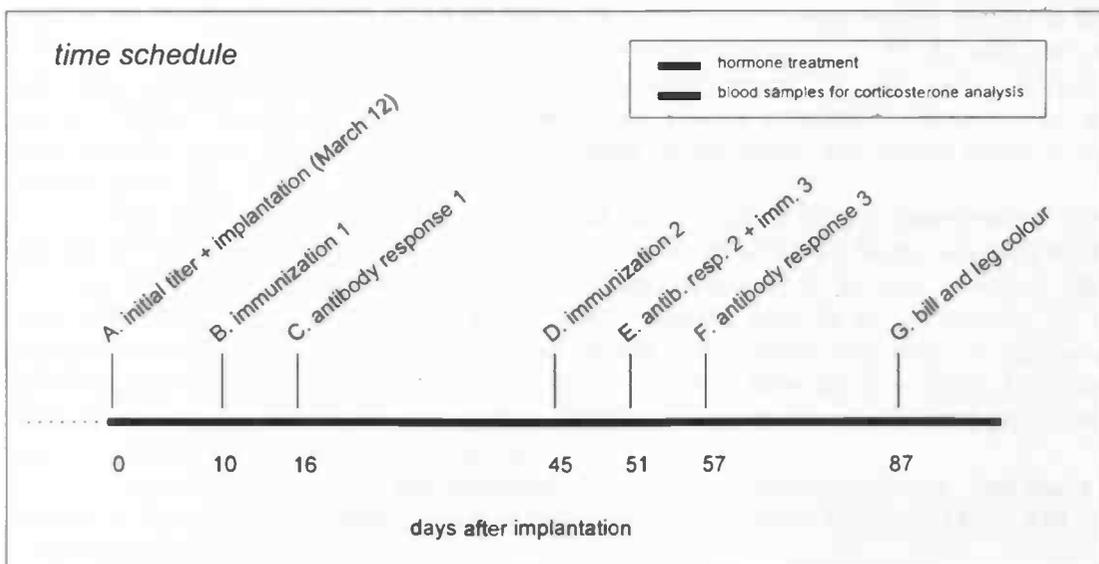


Figure 2. Time schedule of the experiment.

We weighed the birds every time they were caught. On day 28 and 38 the stage of the brown mask was scored with use of a binocular. We continued monitoring mask development for 7

months after the last blood sample had been taken (see appendix G for an overview of the parameters we measured on the different days).

Between day 16 and day 45 we caught all birds once to take a blood sample to determine corticosterone plasma levels. This was done as quickly as possible, most of these samples were taken within 5 minutes after catching the bird (mean: 3.45 min.). All samples were taken between 16:05 h and 17:15 h to minimise variation in corticosterone levels caused by circadian fluctuations. During this whole period the weather was nice and stable, therefore extra variation caused by varying weather circumstances will be minimal.

Hormonal Implants

Testosterone implants: Silastic tubes were filled with crystalline testosterone (Sigma, St. Louis, internal diameter 1 mm, external diameter 3 mm, length 24 mm sealed on both sides with 2 mm of silicone glue).

Ros *et al.* treated juvenile Black-headed gulls with similar silastic tubes of which a length of 10 mm was filled with testosterone. In their study mean plasma level of testosterone-birds was 2.42 ng/ml, with individual levels ranging from 0.56-5.16 ng/ml. At the start of the breeding season the mean testosterone level of adult males is about 4.5 ng/ml (Groothuis and Meeuwissen 1992) with individual levels that may be above 10 ng/ml (Groothuis pers. com.). By administering a double testosterone dose compared to the dose administered by Ros *et al.* we expected our birds to have a mean plasma level of about 5 ng/ml, while individual levels would still be within the physiological range. Similar, but empty tubes were implanted as a control.

Corticosterone implants: Corticosterone was administered in the form of solid crystalline pellets, which consisted of a mixture of cholesterol and corticosterone. It was possible to change the dose given to the animals by changing the ratio of these two substances. Pellets consisting of 100 % cholesterol served as a control.

We had no prior experience with the administering of corticosterone to black-headed gulls. We could not find any literature about the magnitude of natural corticosterone levels in black-headed gulls or about the administering of this hormone to this species. Four adult black-headed gulls that we had handled for half an hour showed plasma corticosterone levels ranging from 40 to 93 ng/ml (mean 75 ng/ml SD 12.0 ng/ml) as revealed by High Pressure Liquid Chromatography (HPLC). We extrapolated the effect of corticosterone pellets in rats and decided to treat the gulls with 25 % pellets. A pellet consisting for 25 % of corticosterone leads to an average corticosterone level of 51 ng/ml in adult rats of 250-300 g after one week (Meyer 1979). We expected the 25 % pellets to give a substantial elevation of the corticosterone levels, but not to raise corticosterone levels above the values found in the handled gulls.

One week after implantation we analysed blood samples of two experimental birds with the 25 % pellets and of two pilot animals, one implanted with a 25 % and one with an 80 % pellet. This analysis showed corticosterone plasma levels of 0, 10 and 13 ng/ml after treatment with 25 % pellets, and 30 ng/ml after treatment with 80 %. Apparently 25 % implants induced relatively low corticosterone plasma levels, which may even be similar to baseline levels in several bird species (e.g. Smith *et al.* 1994; Beletsky *et al.* 1989; Le Maho 1992). Therefore we changed the 25 % pellets for 80 % pellets in the experimental gulls when we gave the first SRBC challenge on day 10.

The hormonal treatment was sustained after the third antibody response. This made it possible to investigate long-term effects of testosterone on immune function. These will not be reported in this paper.

Blood Samples

Blood samples of 1 ml were drawn from the brachial wing vein with a heparinised needle and syringe. Two blood smears were made for cell counts. Two capillary tubes were filled from each sample for estimation of the percentage white blood cells, hematocrit, and sedimentation

rate. The rest of the sample was stored in a 1.5-ml microtube on ice until centrifugation. The samples were centrifuged at 2600 rpm for 10 min. Plasma was removed and stored at -20°C until further analysis. Part of the plasma was used in the hemagglutination assay. The rest was stored for hormone assays, to determine corticosterone and testosterone levels (with use of Radio Immune Assays (RIA), in collaboration with Hubert Schwabl, has still to be done).

Immunisation and Hemagglutination Assay

Sheep red blood cells (SRBC) stored in an Alsever's solution were used as an antigen to challenge the immune system of the gulls. The erythrocytes were three times washed in phosphate buffered saline (PBS) and re-suspended in PBS. After the last centrifugation the erythrocytes were re-suspended at a 2 % (challenge 1 and 2) or 10 % (challenge 3) concentration in PBS.

The birds were immunised by injecting 0.5 ml of the SRBC suspension intra peritoneal. As the first and the second challenge a 2 % suspension and as the third challenge a 10 % suspension was injected.

Six days after each challenge, post immunisation blood samples were drawn to determine the antibody response against the SRBC challenge. We choose this six-day interval, because we expected the peak in antibody levels to be six days after challenge. We based this expectation on a pilot experiment. In this pilot experiment the antibody response peak was after six days, both for the primary and the secondary response (see Zwiggelaar 1996 for details, appendix A). Also in literature the peak of response is reported to be six days after immunisation in chickens, pheasants and quails (Aitken and Parry 1974). On the day of implantation blood samples were drawn to determine pre-immunisation antibody responsiveness to SRBC.

The antibody response to the SRBC challenge was determined by the following procedure. Plasma was heated at 56°C for 30 min to prevent lysis of sheep red blood cells by complement. Thereafter, a dilution series of the plasma was made on U-shaped micro-titer plates. The dilution series we used for determining of the first response was: $2^1, 2^2, 2^3, \dots$, with all wells containing 50 μl of diluted plasma. After making this dilution series, 50 μl of 2 % SRBC in PBS was added to each well. The dilution series we used for determining the second and third responses were: $2^{0.5}, 2^1, 2^{1.5}, \dots$, with all wells containing 35 μl of diluted plasma. After making this dilution series 35 μl 2 % SRBC in PBS was added to each well. The micro-titer plates were incubated at 37°C for 1 h after adding SRBC. Titers were scored after one night as the highest dilution step showing hemagglutination and represented as integers on a \log_2 scale. By subtracting the pre-immunisation titer from the titer found after immunisation we corrected for cross-reaction with SRBC. To these corrected values I will refer as the antibody *responses*, whereas I refer to the uncorrected values as the antibody *titers*.

We used two different methods to determine the hemagglutination titers: the 'rough method' and the 'raindrop method'. The basic difference between these two methods is that in 'raindrop method' the titers were scored by tilting the plates, whereas in the 'rough method' titers were scored without tilting the plates (see Zwiggelaar 1996 for details). Zwiggelaar chose to use the 'raindrop method' for the data analysis, because we only had a reliable estimate of the pre-immunisation antibody response by this method. We had only a preliminary estimate made just after the incubation of the plates of the pre-immunisation responsiveness as measured by the 'rough method'.

Sedimentation Rate, Hematocrit and Percentage White Blood Cells

To measure sedimentation rate, heparinised capillary tubes filled with blood were put in vertical position for about 5 h. Sedimentation rate was expressed as (*the length of the part of the liquid column not occupied by blood cells / total length of the liquid column*) / *time in vertical position* (Saino and Møller 1996). Then the capillaries were centrifuged for 10 min at 13000 rpm. Hematocrit was expressed as *length of column containing red blood cells / total column length*, after centrifugation. Volume percentage of white blood cells was expressed as

length of column containing white blood cells / length of total column after, centrifugation. The blood smears have still to be analysed.

Foot Infections

The diameter and number of the wart-like infections under the feet of the gulls, probably caused by a virus, were determined. The diameter of all foot infections was summed per bird. This total size and the total number of the foot infections showed a close correlation (appendix C). In further analyses the total size was used as a measure for the intensity of foot infections.

Sexual Ornaments

To measure the effect of testosterone on the development of sexual ornaments the colour of the bill and leg were scored using the Munsell Book of Color (Kollmorgen Corporation, Baltimore). This is a system containing numerous curtain cards with reference colours arranged according to a scale based on three components that determine the colour: chroma (=degree of saturation), value (=relative lightness or darkness) and hue (=tint).

Bill colour was determined at the upper mandible, just above the nostrils. Unfortunately, we faced the problem that many gulls had this spot of their bill injured in the wire-netting during the experiment. These injuries resulted in the development of scar tissue with a deviating colour. Because of this, the colour measured *above* the nostrils was no longer representative of overall bill colour (appendix D). Therefore we started measuring the colour at the side of the upper mandible *below* the nostrils, a spot that was never injured. However, we did this only from response 2 onwards and we do not have an estimate of the colour of this spot of the bill at the beginning of the experiment. Leg colour was measured at the left lower leg at the outer side.

Stage of the development of the brown mask was scored on a scale ranging from 1, the adult winter plumage, to 6, the fully developed mask of adult during breeding season (figure 3, Groothuis 1992).



Figure 3. The different developmental stages of the brown mask.

Behavioural Observations

Observations were made during the whole course of the experiment. The observations were carried out from the windows of an adjacent building. The distance between the aviaries and this building was about 5 – 10 m. When needed a binocular was used to read the colour rings. The observations were recorded on tape. Data were collected on the frequencies of the oblique, forward, choking and various sexual displays, as well as on the occurrence of aggressive behaviour. See Groothuis (1989) for a description of the different displays (figure 4). Two observers carried out the observations, mostly in the morning, during 20-min periods

in which two adjacent aviaries were observed at the same time by one observer. Each observation day both observers watched all cages to minimise the effect of inter observer variation. The different cages were observed in the same sequence, but each successive day the observations were started at another aviary to minimise time of day effects.

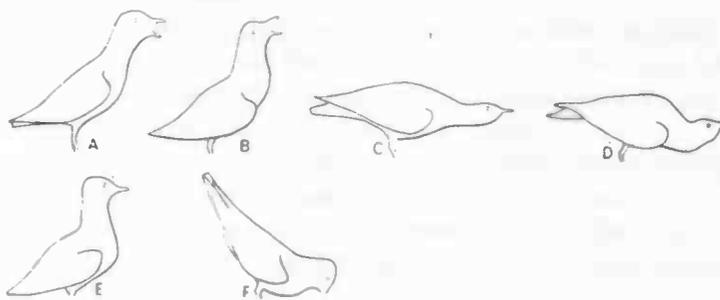


Figure 4. Some of the displays performed by the Black-headed Gull. A. oblique, B. extreme oblique, C. forward, D. extreme forward, E. upright, F. choking

Data Analysis

Two of the control and three of the testosterone implanted gulls died before the end of the experiment (see Zwiggelaar 1996 for the effect of corticosterone on survival). Only gulls that survived until the end of the experiment, after the third antibody response, were included in the analyses, unless otherwise stated. Twelve testosterone treated and 8 control animals survived until the end of the experiment. Only results concerning the testosterone treatment will be shown. See Zwiggelaar (1996) for the analysis of the data on the corticosterone treatment.

Before applying parametrical statistics the behavioural data were Poisson ($\sqrt{(X + 1)}$) transformed (Ros *et al.* 1996). Data on hematocrit, volume % white blood cells and sedimentation rate were arcsin(\sqrt{X}) transformed (Zar 1984). The mask scores, ranging from 1 to 6, were transformed to percentages of the surface coloured brown in respect to the brown surface of a fully developed mask of an adult black-headed gull. (table 2). An index was made of the three Munsell components (chroma, value, hue) of bill and leg colour. This was done by normalising all data on one component and substituting these standardised values in the next formula:

$$\text{Colour Index} = (X - Y - Z)$$

$$X = \text{chroma}, Y = \text{value}, Z = \text{hue}$$

A high index value indicates a colour that is more saturated, darker and redder, and which is more similar to the colour of the legs and bill of an adult black-headed gull during the breeding season. A low index value indicates a colour that is less saturated, lighter and more yellow, which is more similar to the dull colour of the legs and bill of a juvenile black-headed gull during winter.

All significance values stated are two-tailed *P*-values. Alpha is set to 0.05. Graphs represent means and standard errors, unless otherwise stated.

Table 2. Transformation from mask score to percentage brown surface.

Mask score	1	2	3	4	5	6
% brown surface	2 %	5 %	10 %	30 %	70 %	100 %

5. RESULTS

5.1. The Effect of Testosterone on Immune Function

Summary of Zwiggelaar's Results

The effect of testosterone on several measures of immune function: antibody responsiveness, the percentage of white blood cells and the occurrence of foot infections, has been analysed by Zwiggelaar. An extensive description of the results of these analyses can be found in her graduate thesis (Zwiggelaar 1996). To summarise these results:

- 1) No effect of testosterone on antibody response against SRBC was found (appendix A).
- 2) The percentage white blood cells was not affected by testosterone.
- 3) There was no effect of testosterone on the occurrence of foot infections.

In conclusion, Zwiggelaar's results did not indicate any immuno-suppressive effect of testosterone.

Antibody Response: the 'Rough' Method

Zwiggelaar analysed the effect of testosterone on the antibody response as measured by the 'raindrop' method. Figure 5 shows the effect of testosterone on antibody response as measured by the 'rough' method. There was no difference in antibody response between testosterone treated and control animals (repeated measures MANOVA, dependent variable with 3 levels: antibody response 1, 2 and 3, factors: treatment and sex, treatment: $F_{1,15}=1.17$, $P=0.297$). No sex difference, or interaction of sex with treatment was found (sex: $F_{1,15}=1.13$, $P=0.305$, sex \times treatment: $F_{1,15}=1.42$, $P=0.252$). As expected, the secondary responses were higher than the primary response ($F_{2,30}=8.55$, $P=0.001$).

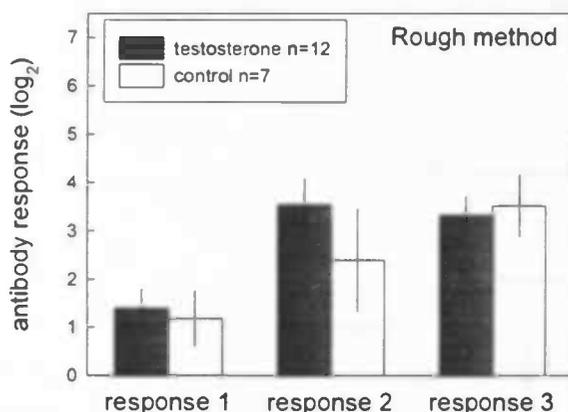


Figure 5. Antibody response against SRBC in testosterone treated and control animals as measured by the 'rough' method. One control animal is missing, because we were not able to take a blood sample from this individual to measure the second response.

It could be argued that responses 2 and 3 were principally different from response 1, because response 1 was a primary antibody response, whereas responses 2 and 3 were secondary responses. Primary and secondary antibody responses depend on different mechanisms. The immunological memory plays an important role in the secondary response, but not in the primary response. Because of this difference I believe that it is legitimate to apply a repeated measures MANOVA over only response 2 and 3. This statistical test did not indicate an effect of testosterone either (repeated measures MANOVA, dependent variable with 2 levels: antibody response 2 and 3, factors: treatment and sex, treatment: $F_{1,15}=1.61$, $P=0.224$, sex: $F_{1,15}=0.017$, $P=0.898$, sex \times treatment: $F_{1,15}=0.741$, $P=0.403$). There was no time effect either

(time: $F_{1,15}=2.478$, $P=0.136$). It is remarkable that the third challenge with 10 % SRBC did not elicit a higher response than the second challenge with only 2 % SRBC.

The 'Raindrop' versus the 'Rough' Method

Zwiggelaar used the 'raindrop'-estimates of antibody response in her analyses. To make easy comparisons possible between Zwiggelaar's results and the results in this paper I also used the raindrop-estimate in further analyses. Based on the following two observations, I do not expect that using the rough-estimate would have led to other conclusions. Firstly, the above rough- results on the effect of testosterone on antibody response are qualitatively very similar to Zwiggelaar's raindrop-results. Secondly, the rough and raindrop-estimates of antibody response were highly correlated (appendix A).

Sedimentation Rate

There were no differences between sedimentation rates of testosterone treated and control birds (figure 6, repeated measures MANOVA, dependent variable with three levels: sedimentation rate on day 45, 51 and 57, factors: treatment and sex, treatment: $F_{1,14}=0.25$, $P=0.624$). There was no sex effect and no interaction of sex with treatment (sex: $F_{1,14}=1.35$, $P=0.266$, treatment \times sex: $F_{1,14}=0.04$, $P=0.850$). There was a tendency of a time effect ($F_{2,28}=3.16$, $P=0.058$). Multiple comparisons with paired t -tests showed a significant difference between day 45 and day 51, but not between day 45 and day 57 or between day 51 and day 57 (table 3).

The decrease in sedimentation rate on day 51 after the second challenge on day 45 could be related to the antibody response that was elicited. This hypothesis is supported by the significant correlation of the within individual differences between day 45 and day 51 in sedimentation rate and antibody response (table 3). Within individual differences in sedimentation rate and antibody response between day 45 and day 57, and between day 51 and day 57, did not show a significant correlation (table 3). This is consistent with the fact that sedimentation rates were not significantly different between these days.

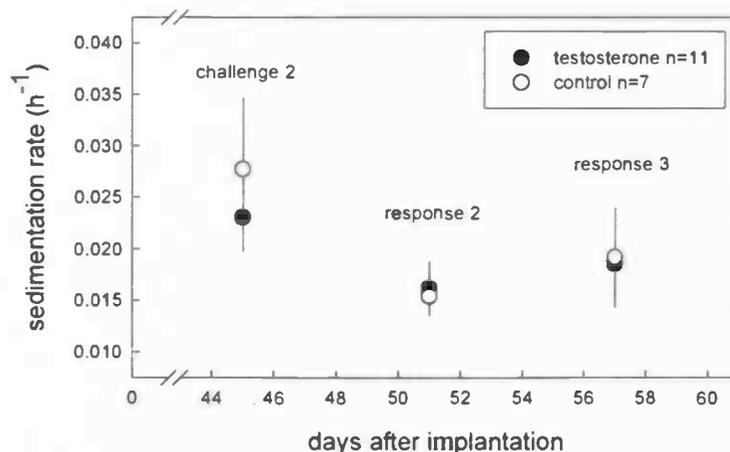


Figure 6. Sedimentation rates in testosterone treated and control birds.

Table 3. *The relation between sedimentation rate and antibody response.*

	Difference in mean sedimentation rate between days			Correlations of within individual differences in sedimentation rate and antibody titer		
	<i>t</i>	<i>df</i>	<i>P</i>	<i>R</i>	<i>N</i>	<i>P</i>
Day 45 – day 51	2.689	17	0.016	0.489	18	0.039
Day 45 – day 57	-1.528	17	0.145	0.167	18	0.509
Day 51 – day 57	-0.746	17	0.492	0.087	18	0.701

Conclusion

The finding that there was neither an effect of testosterone on the 'rough' estimate of antibody response, nor on the sedimentation rate underline the conclusion, already made by Zwiggelaar (1996), that testosterone did not have an immunosuppressive effect in the black-headed gulls.

5.2. The Effect of Testosterone on Sexual Ornamentation and Display

Display Rate

Zwiggelaar showed that testosterone treatment had a very strong positive effect on display rate (Zwiggelaar 1996).

Mask Development

In the course of the experiment the animals moulted from their winter plumage, without the brown mask, to the nuptial plumage with the brown mask. This is reflected in a significant increase of the percentage brown coloured surface of the mask, both in testosterone treated and control birds (figure 7, Wilcoxon-signed-rank, stage on day 57 – stage on day 0: $Z=-3.9244$, $P=0.0001$). The testosterone treated birds showed a significantly steeper increase of the percentage brown coloured mask surface (Mann-Whitney-U on within individual differences between day 0 and day 57: $U=22.5$, $P=0.0480$).

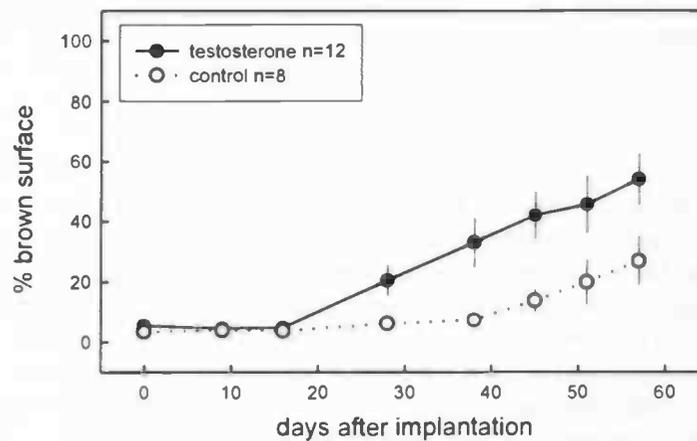


Figure 7. Mask development in testosterone treated and control animals.

After the last blood sample had been taken, the stage of the brown mask was determined every month until the gulls had completed their second moult (figure 8). It deserves attention that both the testosterone and the control group did not reach the adult level of mask development during the summer season. This phenomenon has been found before in juvenile black-headed gulls (Groothuis 1992). At the end of the summer the control animals changed their nuptial plumage to the winter plumage, without the brown mask. In contrast the testosterone treated birds showed a further development of the brown mask, to the stage of the fully developed mask of an adult bird in the breeding season.

An interesting detail is that the graph suggests that the start of the post-nuptial moult had been delayed in the testosterone treated birds. On day 140 the control animals had already completely moulted their brown mask, while the testosterone treated birds still showed the same stage of mask development as on day 87. On day 175 the testosterone treated birds showed a change in the stage of the brown mask for the first time. It has also been found in other species that testosterone treatment can cause a delay in the onset of the post-nuptial moult (e.g. Nolan *et al.* 1992, Schlessner *et al.* 1985).

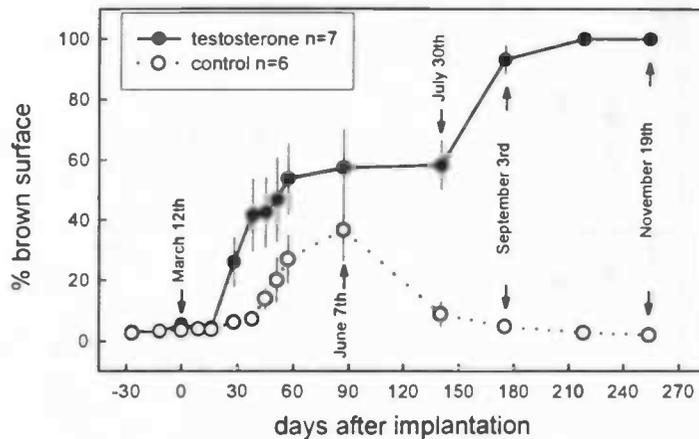


Figure 8. The effect of testosterone on mask development after the second moult. After the last blood sample two more testosterone treated and two more control animals died. Three testosterone treated gulls escaped. All these individuals were left from the figure.

Bill Colour above the Nostrils

There was no effect of testosterone on the colour of the upper mandible above the nostrils. Neither chroma, nor value, nor hue was affected by the testosterone treatment. The within individual differences in the bill colour components between day 0 and day 57 were compared between both treatments to test this (figure 9A-D, Mann-Whitney-U, chroma: $U=57$, $P=0.47$, value: $U=41.5$, $P=0.599$, hue: $U=60$, $P=0.327$, index: $U=52$, $P=0.757$).

In the course of the experiment the gulls of both experimental groups developed darker and redder bills (figure 9B-C, Wilcoxon-signed-rank-test, day 0 – day 57, value: $Z=3.962$, $P=0.000$, hue: $Z=-2.944$, $P=0.003$). There was no change in saturation (figure 9A, $Z=0.000$, $P=1.000$). Also the index, in which all three colour components are integrated, showed a significant rise during the season (figure 9D, $Z=3.734$, $P=0.000$). The change in bill colour in the control birds has probably been caused by increased endogenous testosterone production, which normally occurs in spring. The development of the brown mask and the performance of behavioural displays in control birds are dependent on testosterone (Groothuis and Meeuwissen 1989). The development of the mask and the increase in display rates in the control birds in the course of the season indicate that the gulls had a higher endogenous testosterone production later in the season. Apparently just small elevations of testosterone levels had already a strong effect on the colour of the bill, on which our testosterone treatment did not superimpose an additional effect.

The bill colour of the females was more saturated than the bill colour of the males on day 0 (Mann-Whitney-U, $n=11$, $n=24$, chroma: $U=187.5$, $P=0.031$). There were no differences between the sexes in the other bill colour components on day 0 ($n=11$, $n=24$, value: $U=139$, $P=0.791$, hue: $U=140.5$, $P=0.743$, index: $U=165$, $P=0.230$). I tested for differences between the sexes on day 0 only, because it is possible to pool the three experimental categories for this day. Furthermore the N value is maximal on day 0, because no animals had died yet.

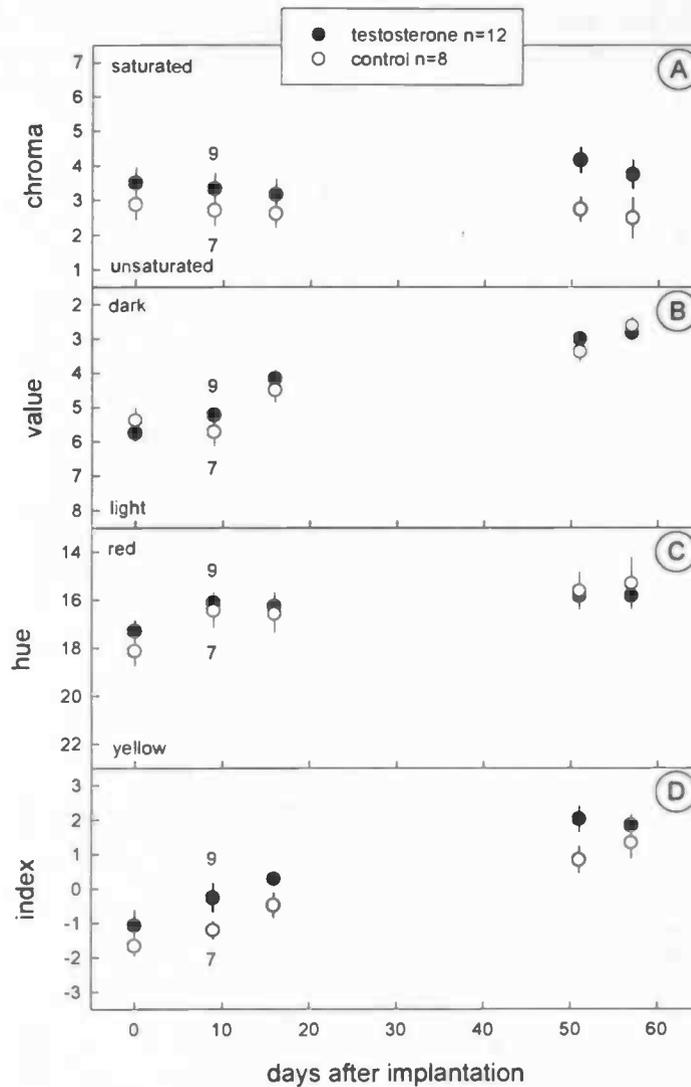


Figure 9. The effect of testosterone on three components of bill colour, measured above the nostrils. A: chroma (saturation), B: value (darkness), C: hue (tint). Graph D shows an index in which all components are integrated. The numbers in the graph indicate deviating sample sizes. The reason for these missing individuals is that it was impossible to make proper estimates of the bill colour of these gulls, because their bills were severely injured on this day.

Bill Colour below the Nostrils

The bill colour as determined below nostrils is probably a more reliable estimate for overall bill colour than the bill colour estimate determined above the nostrils (see appendix D). The below-nostrils-estimate indicates an effect of testosterone on bill colour. Testosterone treated birds tended to have a more saturated bill colour (figure 10A, Mann-Whitney-U, day 51: $U=68$, $P=0.100$, day 57: $U=68.5$, $P=0.096$). Furthermore testosterone treated birds had significantly darker bills (figure 10B, day 51: $U=22.5$, $P=0.015$, day 57: $U=26.5$, $P=0.050$). There was no effect of testosterone treatment on the hue of the bills (figure 10C, day 51: $U=36$, $P=0.314$, day 57: $U=40$, $P=0.456$). The difference between the two experimental groups was also reflected in the colour index (figure 10D, day 51: $U=80$, $P=0.013$, day 57: $U=75$, $P=0.036$).

It might be that these differences were already present before the gulls were implanted. However, this is not likely, because on day 0 there were no differences in the bill colour measured above the nostrils between both groups (Mann-Whitney-U, chroma: $U=58.5$,

$P=0.378$, value: $U=56$, $P=0.507$, hue: $U=35.5$, $P=0.285$, index $U=61.5$, $P=0.293$). On day 0 the colour *above* the nostrils probably still correlated with the colour *below* the nostrils, because most birds still had sound bills then (appendix D).

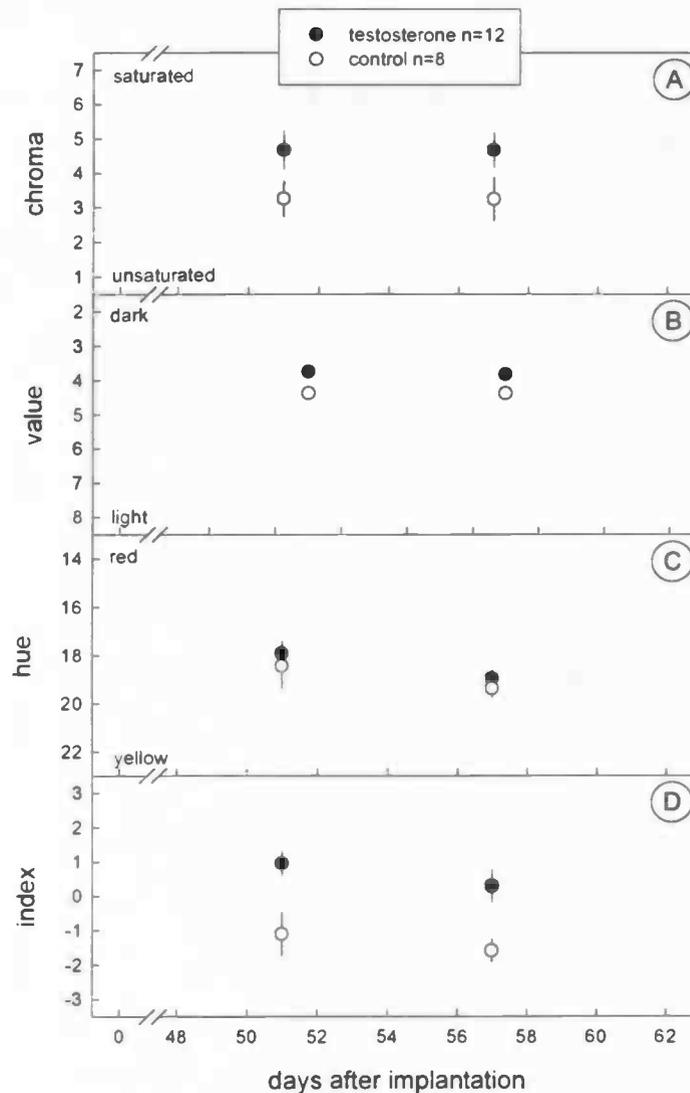


Figure 10. The effect of testosterone on three components of the bill colour as determined below the nostrils. A: chroma (saturation) B: value (darkness) C: hue (tint). Graph D shows an index in which all components are integrated.

Leg Colour

Testosterone treatment did not have an effect on leg colour. I tested this by comparing the within individual differences in leg colour between day 0 and day 57 between both treatments (figure 11A – D, Mann-Whitney-U, chroma: $U=48$, $P=1.000$, value: $U=41$, $P=0.540$, hue: $U=68.5$, $P=0.098$, index: $U=48$, $P=1.000$).

During the season the colour of the legs became darker and redder (figure 11B – C, Wilcoxon-signed-rank, day 0 – day 57, value: $Z=-3.581$, $P=0.000$, hue: $Z=-2.125$, $P=0.034$). The saturation did not change over time (figure 10A, $Z=-0.272$, $P=0.785$). The changes were also reflected in the rise of the colour index (figure 11D, $Z=2.841$, $P=0.004$). Apparently, endogenous produced testosterone had an effect on leg colour as well.

On day 0 the legs of the males were redder than the legs of the females (Mann-Whitney-U, $n=11$, $n=24$, hue: $U=199.5$, $P=0.010$). There were no differences between the sexes in the other leg colour components on day 0 ($n=11$, $n=24$, chroma: $U=159$, $P=0.082$, value: $U=181$, $P=0.070$, index: $U=94.5$, $P=0.179$). Again I tested for differences between the sexes on day 0 only, because for that day it is possible to pool all males and females of the three experimental categories and the N value is maximal.

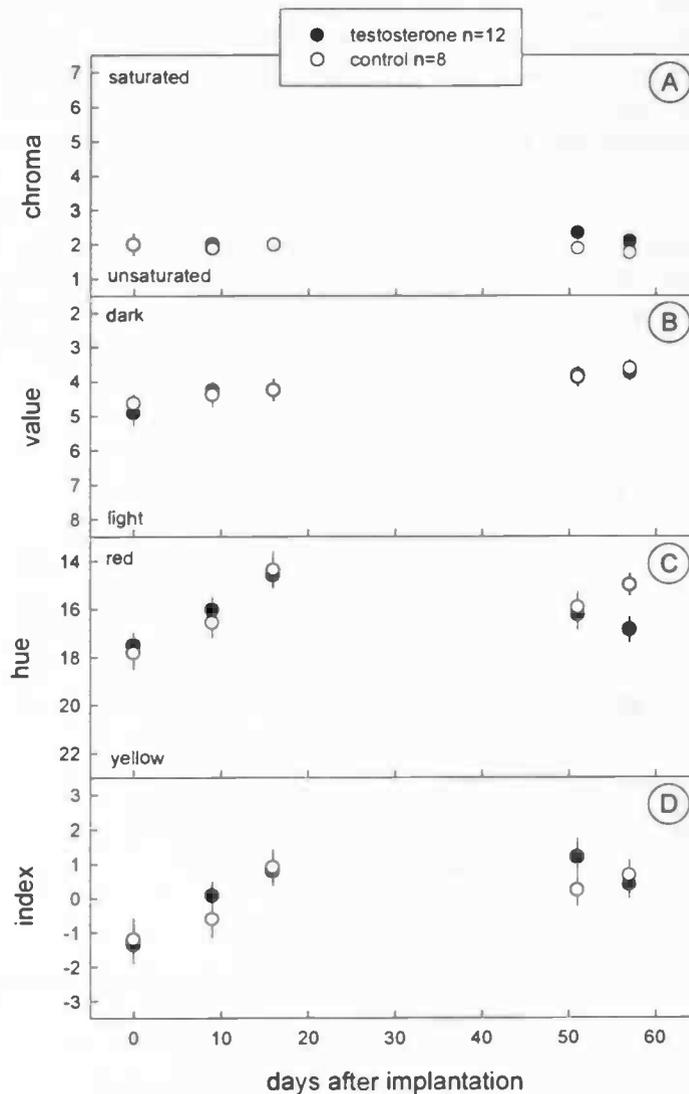


Figure 11. The effect of testosterone on three components of leg colour. A: chroma (saturation) B: value (darkness) C: hue (tint). Graph D shows an index in which all components are combined.

Conclusion

Testosterone had a positive effect on several secondary sexual traits in the Black-headed Gull. Testosterone treated birds clearly show a faster development of the brown mask. Although we had difficulties with measuring bill colour changes, because of damaged bills, we have indications that testosterone treated gulls develop darker and redder bills. There was no effect of testosterone on leg colour.

5.3. The Effect of Testosterone on Body Mass and Condition

Body Mass

Considering the lack of an immunosuppressive effect of testosterone on the one hand, and the clear positive effect of testosterone on several secondary sexual characters on the other hand, the question rises how the testosterone treated animals did compensate for the extra resources put into these traits. It could well be that the testosterone treated animals developed these traits at the expense of a lower body mass. However, this was not the case (figure 12). In contrast with the results of Ros *et al.* we did not find an effect of testosterone on body mass. Testosterone treated animals even seemed to loose less weight than controls, but this difference was not significant (MANOVA with factors sex and treatment on within individual differences in weight between day 0 and day 57: treatment: $F_{1,16}=2.054$, $P=0.171$, sex: $F_{1,16}=0.130$, $P=0.724$, treatment \times sex: $F_{1,16}=0.508$, $P=0.486$).

There was a decrease of body mass in time, which is a normal phenomenon during the breeding season in free-living black-headed gulls (paired t -test, day 0- day 57: $T=8.460$, $df=19$, $P=0.000$). There is a clear sex difference in body mass, males are heavier than females (t -test, day 0: $T=2.669$, $df=18$, $P=0.016$).

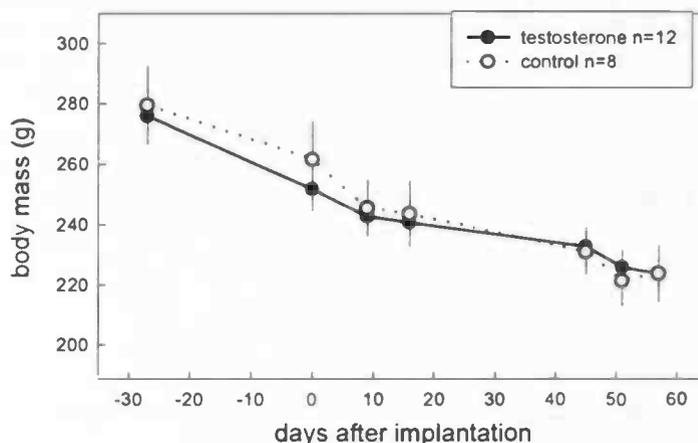


Figure 12. Testosterone and body mass

Condition

Condition, which is body mass corrected for structural size, calculated by the formula $body\ mass/(tarsus\ length)^3$, gives the same picture of the effects of testosterone as body mass. It seems that the testosterone treated gulls showed a smaller decrease in condition than controls, but this difference was not significant (figure 13, MANOVA with factors sex and treatment on within individual differences in condition between day 0 and day 57, treatment: $F_{1,16}=2.825$, $P=0.112$, sex: $F_{1,16}=2.375$, $P=0.143$, treatment \times sex: $F_{1,16}=1.627$, $P=0.220$).

It is remarkable that females have a higher condition than males, which means that females were relatively heavier than males (t -test, day 0: $T=-2.740$, $df=18$, $P=0.013$).

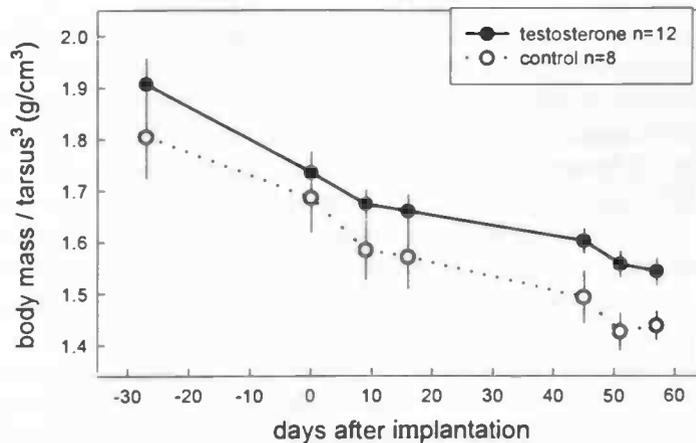


Figure 13. Condition and testosterone.

The Association between Body Mass Change and Display Rate

During the first two weeks of testosterone treatment, Ros *et al.* found a positive correlation in the testosterone treated gulls between body mass loss and display rate. This is an indication that display behaviour is energetically costly. However, we find the opposite result. Body mass loss in the testosterone group is negatively correlated with display rate (sum of all forward and oblique displays) during the first two weeks of the experiment (figure 14, $r=0.633$, $N=12$, $P=0.027$).

There was no correlation between display rate and body mass change over the whole experimental period (day 0 – day 57, testosterone birds: $r=0.302$, $N=12$, $P=0.340$, controls: $r=-0.181$, $N=8$, $P=0.668$, birds of all treatments together, including the testosterone + corticosterone treatment: $r=0.131$, $N=22$, $P=0.563$).

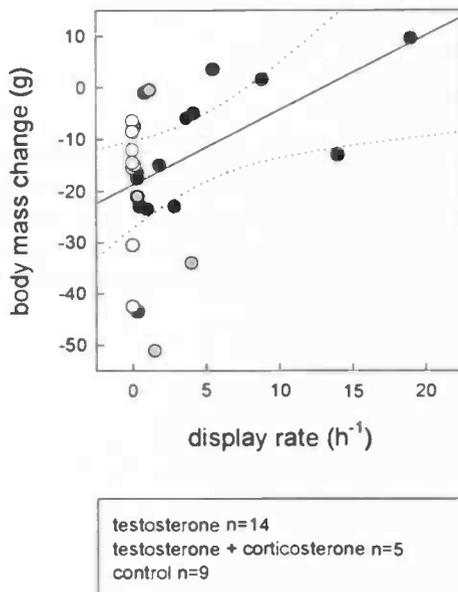


Figure 14. Correlations between individual display rates (oblique + forward), and change in body mass during the first two weeks after implantation. A body mass change that is negative indicates loss of body mass. Plotted is the linear regression line through the testosterone treated birds only, with 95 % confidence intervals.

Our Experiment versus the Experiment of Ros *et al.*

Testosterone had no effect on body mass in our experiment, whereas it had a negative effect in the experiment of Ros *et al.*. Furthermore we found a positive association between display rate and body mass change during the first two weeks after implantation, whereas Ros *et al.* found a negative one. To understand this discrepancies I compared the body mass of the gulls in our experiment with the body mass of the gulls in the experiment of Ros *et al.* In figure 15 our data and the data of Ros *et al.* on body mass are plotted together.

At implantation (day 0), the birds of Ros *et al.* were much heavier than ours (ANOVA with factors: experiment (Ros *et al.*'s or ours) and sex, experiment: $F_{1,52}=15.147$, $P=0.000$, sex: $F_{1,52}=22.158$, $P=0.000$, experiment \times sex: $F_{1,52}=0.355$, $P=0.554$). This difference could not be explained by the seasonal fluctuating of body mass, because both experiments were carried out at exactly the same time of the year.

The body mass change after two weeks of treatment differed significantly between the experiments (ANOVA with factors treatment, sex and experiment: interaction treatment \times experiment: $F_{1,31}=7.168$, $P=0.012$). In the experiment of Ros *et al.* only the testosterone treated animals loose body mass, whereas in our experiment both the testosterone treated and control animals seem to loose body mass.

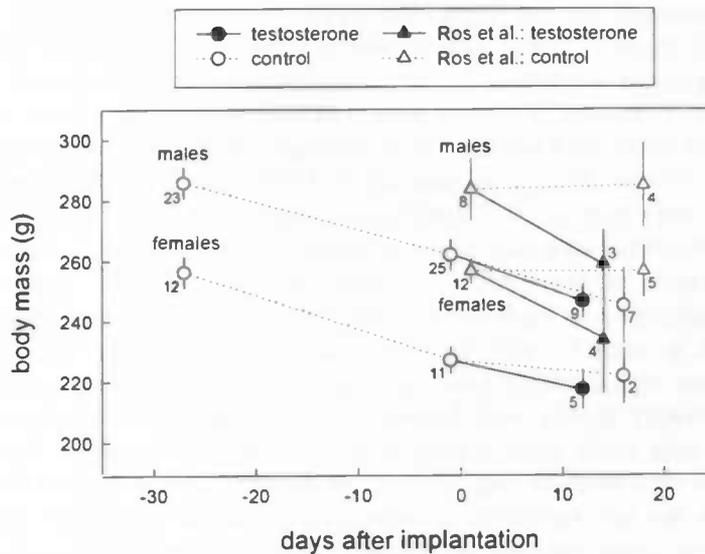


Figure 15. Body mass of the gulls in the experiment of Ros *et al.* compared to body mass of the gulls in our experiment. Circles refer to our experiment, triangles to the experiment of Ros *et al.* Filled circles and triangles represent average body mass of testosterone treated gulls, open circles and triangles represent body mass of the gulls before treatment or of control animals. Mean male and female body mass are plotted separately. Numbers are N values.

It is possible that differences in the experimental set-up have caused the different mean body mass on day 0, the different effect of testosterone on body mass, and the different associations between display rate and body mass in the two experiments. The following differences in the execution of the experiment of Ros *et al.* and ours may have been important:

- 1) Our gulls were caught and handled one month before day 0 (on day -29), whereas the gulls of Ros *et al.* were not.
- 2) Our birds were re-housed, both on day -29 (from 2 large aviaries to six smaller ones) and on day 0 (the six aviaries were split up in 12), whereas the gulls of Ros *et al.* were not re-housed.
- 3) Our birds were longer handled every time that they were caught, because we did more extensive measurements than Ros *et al.*, for example the determination of mask stage, and of bill and leg colour.

Re-housing is probably a stressful event for black-headed gulls. This is for instance indicated by a decrease in display rate after re-housing (Westerveld *et al.* 1997). Also catching and handling probably cause a lot of stress in black-headed gulls.

The stress of re-housing on day -29 could be the main reason for the lower body mass in our animals on day 0. This view is supported by the fact that the mean body mass of our gulls, when they were caught for the first time on day -29 is virtually the same as the mean body mass of Ros *et al.*'s gulls on day 0, when his animals were caught for the first time (ANOVA with factors experiment and sex, experiment: $F_{1,52}=20.151$, $P=0.843$). Only *after* that our gulls had been caught and re-housed on day -29 the body mass difference arose.

The significantly different effect of testosterone on body mass in the two experiments could be caused by the fact that our animals suffered from more intense stress, because these were caught more often and handled longer times. In our experiment the negative effect of testosterone on body mass was possibly masked by the continual stress-caused decrease in body mass in both treatment groups. It is possible that there is a threshold body mass, under which the gulls do not allow their body mass to drop. If such a threshold exists, the testosterone treated gulls in our experiment may have adjusted their energy expenditure, to reduce their weight loss. Perhaps the birds lowered their energy expenditure by decreasing their display rate. In that case animals with a greater body mass loss, which were in bad condition, would have displayed at lower frequencies than animals with relatively little loss of body mass. This could also explain the opposite associations that we and Ros *et al.* found between body mass change and display rate during the first two weeks after implantation.

Another explanation for lack of a weight difference between testosterone treated and control birds in our experiment is that the testosterone treated gulls were differently affected by the same stressor. Testosterone treated birds may have exhibited for example a smaller decrease in feeding rate after being caught than control birds did.

Hematocrit and Testosterone

Hematocrit may be another indicator of general condition and healthiness. Diseased great black-backed gulls and herring gulls show lower hematocrit values (Averbeck 1992). In our gulls testosterone had no effect on hematocrit (figure 16, MANOVA with within individual differences in hematocrit between day 0 and day 57 and treatment and sex as factors, treatment: $F_{1,15}=0.1443$, $P=0.248$, sex: $F_{1,15}=1.235$, $P=0.284$, treatment \times sex: $F_{1,15}=1.380$, $P=0.258$). There was no difference between males and females (t -test, day 0: $T=-0.997$, $df=33$, $P=0.326$). In many bird species males have a higher hematocrit than females (Prinzinger and Misovic 1994). The lack of a significant difference between the hematocrit of males and females may be associated to the fact that the Black-headed Gull is a monomorphic species. Also in other gull species there is no sex difference (Averbeck 1992). During the experiment hematocrit significantly decreased (paired t -test, day 0 – day 57: $T=2.356$, $df=18$, $P=0.030$). In the Herring Gull it has been found that hematocrit shows seasonal variation, with lowest levels during the summer (Hüppop).

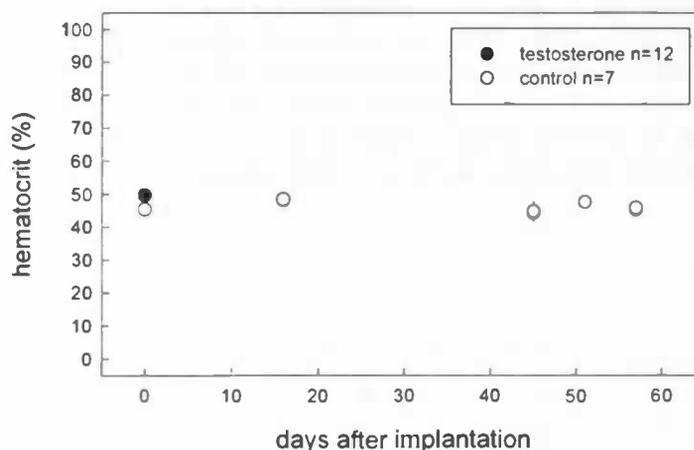


Figure 16. Hematocrit in testosterone treated and control gulls.

Conclusion

Testosterone treated birds did not compensate for a greater development of their sexual ornaments by lowering their body mass. This result is inconsistent with the result of Ros *et al.* This inconsistency could be explained by differences in the set-up between our experiment and the experiment of Ros *et al.* Testosterone did not affect hematocrit, which is another possible measure of general condition and healthiness.

5.4. Immune function, Sexual Ornamentation and Display

The results in sections 5.1 and 5.2 do not support the dual effect of testosterone: inducing the development of sexual ornaments, while simultaneously suppressing immune function. We found an effect of testosterone on sexual display, but not on immune function. Nevertheless it is still possible that the level of sexual display was a reliable signal of the immunocompetence of the signaller. In this section I will further investigate this possibility, but before that I will shortly discuss a peculiarity in respect to the secondary antibody responses that we found.

An Association Between Second and Third Antibody Response?

Figure 17 indicates that the magnitude of antibody response 3 depended on antibody response 2. Individuals that showed a high second response had a third response that was lower. Individuals that showed an intermediate second response had a third response of similar height. Individuals that had a relatively low second response had a higher third response. This observation is confirmed by a significant correlation between the height of response 2 and the difference between the second and the third response (figure 18, $R_s = -0.8025$ $N=22$ $P=0.000$).

A possible explanation for this is that individuals with a high second response had a relatively high clearance of the SRBC antigens that were injected as the third challenge. This was because in these individuals there were still large numbers of antibodies present when the third challenge was given. Perhaps as a result a lower amount of antigens triggered the immune system in these individuals. Therefore these individuals will have showed a lower subsequent response.

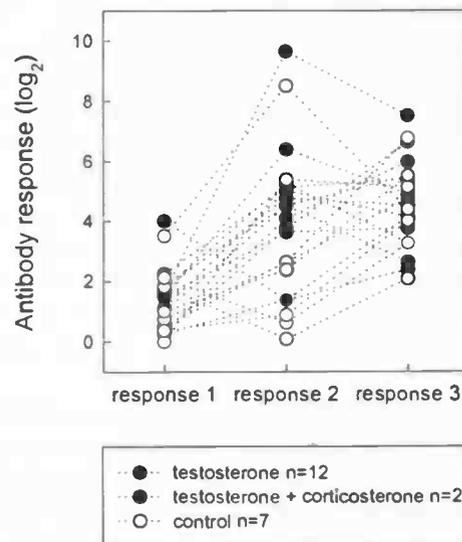


Figure 17. Antibody response against SRBC. Individuals are connected by dotted lines. Is there an association between response 2 and 3?

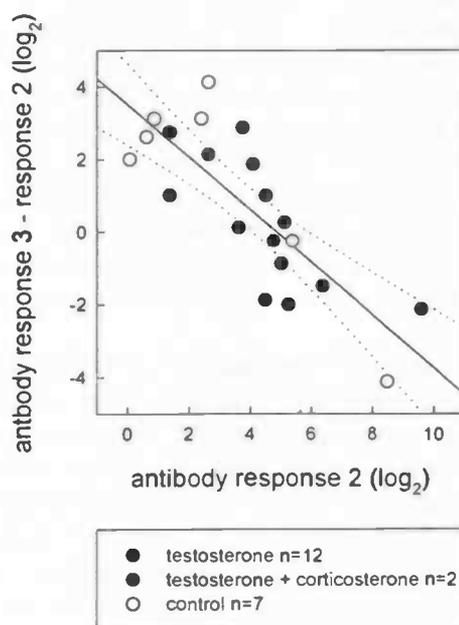


Figure 18. Correlation between response 2 and response 3-2. Linear regression line with 95 % confidence intervals is plotted.

This principle even has a medical application, in the prophylaxis of rhesus antagonism in Rh-negatif women being pregnant of an Rh-positive child. During the birth of the child the barrier between the blood streams of mother and child is less strong. Red blood cells of the child can enter the mother's blood stream. These cells can cause sensitisation to Rh-antigen of the mother's immune system. This would be a problem when the mother would become pregnant of an Rh-positif child again. Anti-rhesus-antibodies of the mother could damage the tissues of the foetus then. To prevent the mother's immune system of sensitisation against the Rh-antigens on the red blood cells, an amount of anti-rhesus-antibodies is injected in the bloodstream of the mother during or just after the delivery of the child. These antibodies eliminate the child's blood cells before these can trigger the mother's immune system (Souhami and Moscham 1994).

The dependence of the third response on the second response makes it hard to interpret the third response data in relation to sexual ornaments and display. This was an important reason for me to only use the second response in the analyses on the associations between sexual ornaments and display, and immune function. Furthermore the second response showed greater variation among individuals, which leaves more room for correlations between response height and the intensity of sexual ornaments and display. Leaving out the third response from the further analyses reduced the chance of a first order error, or if I would correct for that with Bonferoni, improved the statistical power.

Sexual Ornamentation, Display and Antibody Response

The first question to be answered was whether ornament size and display rate at the time of immunisation, or at the time of the elicited antibody response, were related to the height of the antibody response. This was investigated for both the primary response (response 1) and the secondary response (response 2). I tested whether there were significant correlations between the antibody responses and display rate, the bill and leg colour and the stage of the brown mask. I investigated these relationships both at the time of the challenges and responses. Also body mass and condition were included in these analyses. The analyses were done for testosterone treated and control animals separately, because the testosterone treatment may have interfered with the normally occurring associations between ornaments, display and antibody response.

The analyses did not show a clear and consistent relationship between antibody response, and sexual ornaments and display. A few correlations were significant, but non of them would remain significant after Bonferoni correction (First immunisation: testosterone animals: table 4, controls: table 5. Second immunisation: testosterone animals: table 6, controls: table 7). Most remarkable was the highly significant (without Bonferoni correction) positive correlation between the stage of the brown mask, both at the time of the second challenge and response, and the second antibody response in the control animals. This association indicates that the stage of the brown mask was an honest signal for immunocompetence in the gulls, at least for the secondary response.

Table 4. Correlations between sexual ornaments, display rate and body condition, and the primary antibody response against SRBC in testosterone treated black-headed gulls. Correlations with ornament size, display rate and body condition both at the time of challenge and response are depicted. NS: not significant. P-values are without Bonferoni correction.

		Time of challenge			Time of response		
		<i>r</i>	<i>N</i>	<i>P</i>	<i>r</i>	<i>N</i>	<i>P</i>
Stage of mask		-0.07	14	NS	-0.15	14	NS
Bill colour above nostrils	Chroma	0.08	10	NS	-0.07	14	NS
	Value	-0.23	10	NS	-0.01	14	NS
	Hue	0.08	10	NS	-0.07	14	NS
	Index	0.10	10	NS	-0.01	14	NS
Leg colour	Chroma	-	14	-	-	14	-
	Value	0.17	14	NS	0.19	14	NS
	Hue	0.24	14	NS	0.19	14	NS
	Index	-0.27	14	NS	-0.25	14	NS
Extreme display		-0.24	14	NS	-0.08	14	NS
Total display		-0.23	14	NS	0.03	14	NS
Body mass (g)		0.17	14	NS	0.12	14	NS
Condition (g/cm ³)		0.40	14	NS	0.31	14	NS

Table 5. Correlations between sexual ornaments, display rate and body condition, and the primary antibody response against SRBC in control animals. Correlations with ornament size, display rate and body condition both at the time of challenge and response are depicted. NS: not significant. *: $P < 0.05$. P-values are without Bonferoni correction.

		Time of challenge			Time of response		
		<i>r</i>	<i>N</i>	<i>P</i>	<i>r</i>	<i>N</i>	<i>P</i>
Stage of mask		-0.23	9	NS	-0.14	9	NS
Bill colour Above nostrils	Chroma	0.27	8	NS	0.14	9	NS
	Value	-0.04	8	NS	0.00	9	NS
	Hue	-0.35	8	NS	-0.44	9	NS
	Index	0.71	8	*	0.53	9	NS
Leg colour	Chroma	0.00	9	NS	-	9	-
	Value	-0.01	9	NS	0.12	9	NS
	Hue	0.25	9	NS	0.20	9	NS
	Index	-0.15	9	NS	-0.22	9	NS
Extreme display		-	9	-	-	9	-
Total display		0.37	9	NS	-	8	-
Body mass (g)		-0.54	9	NS	-0.41	9	NS
Condition (g/cm ³)		-0.78	9	*	0.71	9	*

Table 6. Correlations between sexual ornaments, display rate and body condition, and the secondary antibody response against SRBC in testosterone treated black-headed gulls. Correlations with ornament size, display rate and body condition both at the time of challenge and response are depicted. NS: not significant, *: $P < 0.05$. P-values are without Bonferoni correction.

		Time of challenge			Time of response		
		<i>r</i>	<i>N</i>	<i>P</i>	<i>r</i>	<i>N</i>	<i>P</i>
Stage of mask		0.06	12	NS	-0.01	12	NS
Bill colour above nostrils	Chroma	-	-	-	-0.03	12	NS
	Value	-	-	-	-0.38	12	NS
	Hue	-	-	-	-0.10	12	NS
	Index	-	-	-	0.20	12	NS
Bill colour below nostrils	Chroma	-	-	-	0.05	12	NS
	Value	-	-	-	0.08	12	NS
	Hue	-	-	-	0.03	12	NS
	Index	-	-	-	-0.02	12	NS
Leg colour	Chroma	-	-	-	0.03	12	NS
	Value	-	-	-	-0.39	12	NS
	Hue	-	-	-	-0.27	12	NS
	Index	-	-	-	-0.34	12	NS
Extreme display		-0.45	12	NS	-0.68	12	*
Total display		-0.33	12	NS	-0.34	12	NS
Body mass (g)		-0.40	12	NS	-0.41	12	NS
Condition (g/cm ³)		-0.02	12	NS	0.05	12	NS

Table 7. Correlations between sexual ornaments, display rate and body condition, and the secondary antibody response against SRBC in control animals. Correlations with ornament size, display rate and body condition both at the time of challenge and response are depicted. NS: not significant, (*): $P < 0.10$, *: $P < 0.05$, *: $P < 0.005$. P-values are without Bonferoni correction.**

		Time of challenge			Time of response		
		<i>r</i>	<i>N</i>	<i>P</i>	<i>r</i>	<i>N</i>	<i>P</i>
Stage of mask		0.94	7	***	0.93	7	***
Bill colour above nostrils	Chroma	-	-	-	0.53	7	NS
	Value	-	-	-	-0.16	7	NS
	Hue	-	-	-	-0.09	7	NS
	Index	-	-	-	0.52	7	NS
Bill colour below nostrils	Chroma	-	-	-	0.01	7	NS
	Value	-	-	-	-0.74	7	(*)
	Hue	-	-	-	0.21	7	NS
	Index	-	-	-	0.17	7	NS
Leg colour	Chroma	-	-	-	-	7	-
	Value	-	-	-	-0.08	7	NS
	Hue	-	-	-	-0.71	7	(*)
	Index	-	-	-	0.52	7	NS
Extreme display		-	7	-	-0.06	7	NS
Total display		-0.12	7	NS	-0.26	7	NS
Body mass (g)		0.53	7	NS	0.56	7	NS
Condition (g/cm ³)		-0.22	7	NS	-0.16	7	NS

White Blood Cells, Foot Infections and Sexual Ornamentation

Also the percentage white blood cells and the size of the foot infections are measures of immune function. Therefore I investigated whether sexual ornament size was an honest signal for immunocompetence as measured by these parameters. I correlated the percentage of white blood cells and the size of the foot infections with mask stage and bill and leg colour. I only did this for the data that we collected before testosterone treatment had started, on day 0, to be able to pool all animals in one analysis. This gave us the substantial sample size of 35 animals. On day 0 the gulls did not show any behavioural displays yet. Therefore it was impossible to correlate display rate with the immunological parameters. Also the relationships with body mass and condition were investigated. We only found a significant relation between bill chroma and foot infection size (table 8). However this correlation would be absolutely not significant after Bonferoni correction. This result leads to the conclusion that there are no clear and consistent associations between foot infection size, percentage of white blood cells, body condition and sexual ornament size.

Table 8. Day 0: Correlations between sexual ornament size and body condition, and the % white blood cells (WBC) and the size of the foot infections in black-headed gulls. NS: not significant, (*): $P < 0.10$, *: $P < 0.05$. P-values are without Bonferoni correction.

		Volume % WBC			Size of foot infections		
		<i>r</i>	<i>N</i>	<i>P</i>	<i>r</i>	<i>N</i>	<i>P</i>
Stage of mask		0.24	35	NS	-0.01	35	NS
Bill colour above nostrils	Chroma	0.10	35	NS	-.34	35	*
	Value	0.10	35	NS	-0.20	35	NS
Leg colour	Hue	0.07	35	NS	0.12	35	NS
	Index	-0.02	35	NS	-0.20	35	NS
Extreme display Total display	Chroma	-0.16	35	NS	-0.19	35	NS
	Value	-0.02	35	NS	-0.33	35	(*)
	Hue	0.04	35	NS	-0.08	35	NS
	Index	-0.11	35	NS	0.14	35	NS
Body mass (g)		-	35	-	-	35	-
Condition (g/cm ³)		-	35	-	-	35	-
Body mass (g)		-0.18	35	NS	0.22	35	NS
Condition (g/cm ³)		-0.01	35	NS	-0.05	35	NS

Most gulls were still in winter plumage on day 0. This might have caused the lack of clear relationships between the immunological parameters and the secondary sexual characters. On day 0 the gulls had much duller bill and leg colour than later in the season. There was also less variation in these traits among the gulls than later in the season. It might have been that the gulls only signalled about their immunological state during the breeding season, when they had fully developed sexual ornaments and were competing for mates.

To check this hypothesis I correlated foot infection size with mask stage, and bill and leg colour on day 87, long after the termination of the actual experiment. At that time the gulls had much brighter bill and leg colour and more developed masks (see figures 7, 9 and 11). Associations with body mass and condition were investigated as well. We do not have data on display rates on day 87. Testosterone treated animals and controls were separately analysed. No clear and consistent associations were found. The only significant correlation between body condition and foot infection size would disappear after Bonferoni corection (table 9).

Table 9. Day 87: Correlations between sexual ornaments and body condition, and the size of the foot infections in testosterone treated and control black-headed gulls. NS: not significant, *: $P < 0.05$. P -values are without Bonferroni correction.

		Testosterone			Control		
		r	N	P	r	N	P
Stage of mask		0.29	11	NS	0.13	8	NS
Bill colour below nostrils	Chroma Value	0.41	11	NS	0.37	7	NS
	Hue	0.58	11	NS	0.05	7	NS
	Index	-0.07	11	NS	-0.61	7	NS
		-0.08	11	NS	-0.51	7	NS
Leg colour	Chroma Value	-0.03	11	NS	-	-	-
	Hue	-0.23	11	NS	0.03	7	NS
	Index	0.50	11	NS	0.41	7	NS
		-0.30	11	NS	-0.32	7	NS
Extreme display		-	-	-	-	-	-
Total display		-	-	-	-	-	-
Body mass (g)		0.23	11	NS	0.50	8	NS
Body condition (g/cm^3)		-0.61	11	*	-0.16	8	NS

Sexual Ornamentation, Display and the Immunological Memory

Another question is whether ornament size during the forming of the immunological memory predicted the height of the secondary response afterwards. If sexual ornament size were indeed an honest signal of immunocompetence, I would expect gulls with a higher signal level during the memory-forming period to develop their immunological memory more adequate. In that case gulls with a high signal level during the four weeks after the first challenge with SRBC should have had a higher response to the second challenge with SRBC.

This hypothesis was tested by correlating the secondary response with the average display rate and the average mask stage during the four-week memory-forming period. In the control, but not in the testosterone treated animals there was a significant positive correlation between average mask stage during the memory phase and the secondary response (figure 19, testosterone: $r=0.0659$, $N=12$, $P=0.839$, control: $r=0.8746$, $N=7$, $P=0.010$). The same was true when the within individual differences in response height between the primary and the secondary response were correlated with mask stage (figure 20, testosterone: $r=0.2085$, $N=12$, $P=0.515$, control: $r=0.9675$, $N=7$, $P=0.000$). I assume that the within individual difference in height between the primary and the secondary response was caused by the presence of IgG antibodies produced by the immunological memory system.

We should still be careful to conclude that average mask stage is an honest signal for the state of the immunological memory. Average mask stage during the memory phase was highly correlated with mask stage at the second challenge ($r=0.9955$, $N=19$, $P=0.000$). As we already know mask stage at second challenge was associated with the secondary antibody response afterwards.

No significant correlation between display rate during the memory-forming period and secondary response was found (testosterone, total display: $r=-0.2229$, $N=12$, $P=0.486$, extreme display: $r=-0.3038$, $N=12$, $P=0.337$, control: total display: $r=-0.1312$, $N=7$, $P=0.779$, the controls showed no extreme displays).

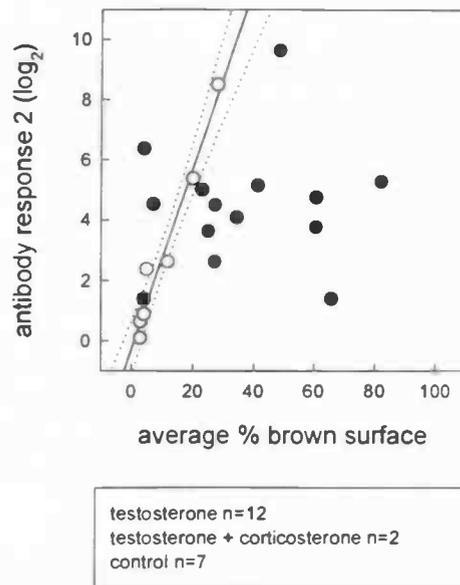


Figure 19. Correlation between the average mask stage during the period of memory forming and the secondary antibody response. Linear regression line with 95 % confidence intervals was plotted through the control animals.

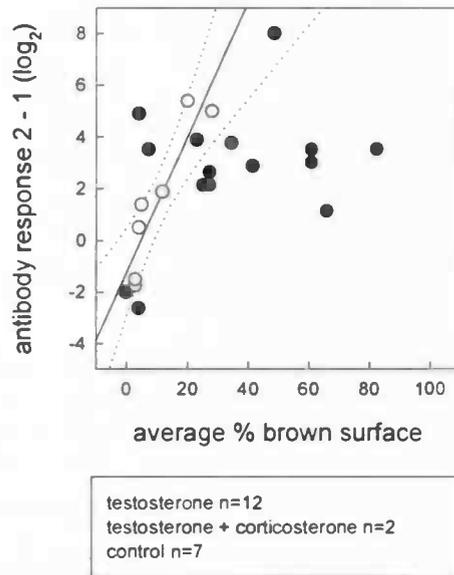


Figure 20. Correlation between the average mask stage during the period of memory forming and the within individual differences between the primary and the secondary antibody response. Linear regression line with 95 % confidence intervals was plotted through the control animals.

Costs of Antibody Response and Sexual Ornamentation

The adaptive resource allocation version of the immunocompetence handicap hypothesis suggests that there is a trade-off in the allocation of resources over sexual ornaments and immune system (Wedekind and Folstad 1994). In that case it is possible that *differences* in the level of sexual ornaments and display are related to the height of the antibody response.

To test this idea, within individual differences in secondary sexual traits at challenge and at response were correlated with the height of the response. This was done for both the primary response (response 1) and the secondary response (response 2, table 10: response 1, table 11: response 2). These analyses yielded one significant correlation in the control birds, between the within individual difference in mask stage between challenge and response, and the second response. As opposed to what would be expected on the basis of the adaptive resource allocation hypothesis this was a positive association. This association could be evidence for ornaments being honest signals.

Table 10. Primary response: Within individual differences in sexual ornaments, display and body condition, between the time of challenge and response correlated with the antibody response level. Testosterone treated and control black-headed gulls. NS: not significant. P-values are without Bonferoni correction.

		Testosterone			Control		
		<i>r</i>	<i>N</i>	<i>P</i>	<i>r</i>	<i>N</i>	<i>P</i>
Stage of mask		-0.38	14	NS	0.18	9	NS
Bill colour below nostrils	Chroma Value	-0.9	10	NS	-0.11	8	NS
	Hue	0.14	10	NS	0.10	8	NS
	Index	-0.13	10	NS	-0.22	8	NS
		-0.05	10	NS	0.03	8	NS
Leg colour	Chroma Value	-	14	-	-0.00	9	NS
	Hue	0.10	14	NS	0.35	9	NS
	Index	-0.10	14	NS	-0.03	9	NS
		0.02	14	NS	-0.07	9	NS
Extreme display		0.32	14	NS	-	9	-
Total display		0.16	14	NS	-0.37	9	NS
Body mass (g)		-0.13	14	NS	0.43	9	NS
Body condition (g/cm ³)		-0.10	14	NS	0.46	9	NS

Table 11. Secondary response: Within individual differences in sexual ornaments, display and body condition, between the time of challenge and response correlated with the antibody response level. Testosterone treated and control black-headed gulls. NS: not significant, *: P<0.05. P-values are without Bonferoni correction.

		Testosterone			Control		
		<i>r</i>	<i>N</i>	<i>P</i>	<i>r</i>	<i>N</i>	<i>P</i>
Stage of mask		-0.04	12	NS	0.82	7	*
Extreme display		-0.41	12	NS	-0.06	7	NS
Total display		-0.15	12	NS	-0.37	7	NS
Body mass (g)		0.16	12	NS	0.27	7	NS
Body condition (g/cm ³)		0.08	12	NS	0.33	7	NS

Conclusion

We found no convincing evidence for sexual ornaments and display being honest signals of immunocompetence in the Black-headed Gull.

5.5. Male Immunology, Sexual Ornaments and Female Mate Choice

Important questions are whether sexual ornaments indeed play an important role in mate choice and whether preferred mates are more immunocompetent, as predicted by the immunocompetence handicap hypothesis. Unfortunately we could only use pair bonds formed in four aviaries in our analysis (Zwiggelaar 1996). The rest of the aviaries were left from the analysis because in those were either only animals of the one sex or one of the males died, so that the females could not choose. Because of the low sample size our results in respect to female mate choice are not very conclusive. To not further diminish the sample size testosterone treated and control animals were pooled.

Male Sexual Ornaments and Female Mate Choice

Mean frequencies of male oblique and forward displays during the whole experiment were summed together. The sum of the mean frequencies of *extreme* oblique and *extreme* forward display were also calculated separately. Mated males tended to show consistently more extreme forward and oblique displays, which have a higher intensity and are probably more difficult to perform than the normal oblique and forward displays (figure 21, paired *t*-test: $T=2.95$, $df=3$, $P=0.060$). There was no difference in total display rate between mated and unmated individuals (figure 21, paired *t*-test: $T=0.38$, $df=3$, $P=0.727$).

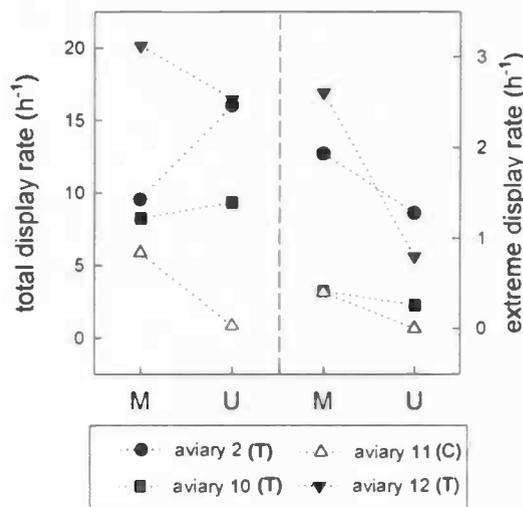


Figure 21. Female mate choice and male display rates. Mean frequencies of oblique and forward display over the whole experiment were summed together. Total display rate is all displays, including the extreme displays. Extreme display rates are also depicted separately. Males in the same aviary are connected by dotted lines. M: mated male, U: unmated male, (T): testosterone treatment, (C): control treatment.

There was no effect of the stage of the brown mask and the bill and leg colour of the male on female mate choice. To test this, average mask stage and bill and leg colour index during the whole experiment were calculated and compared between mated and unmated males (figure 22, Wilcoxon-signed-rank, A. mask: $P=0.86$, B. bill above: $P=0.79$, C. bill below: $P=0.36$, D. leg: $P=0.20$).

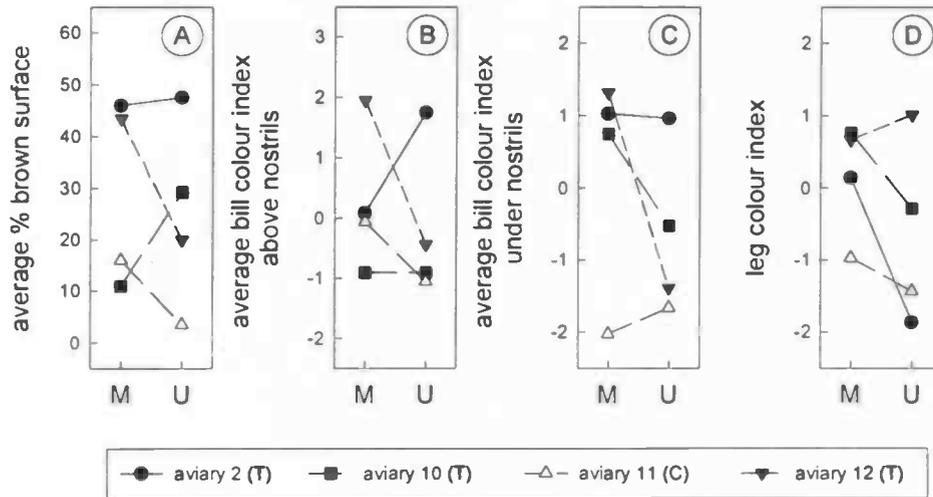


Figure 22. Female mate choice and male sexual ornaments. Average ornament size over the whole experimental period is depicted. Males in the same aviary are connected by lines. M: mated, U: unmated, (T): testosterone treatment, (C): control treatment.

Male Immunocompetence and Female Mate Choice

A major prediction of the immunocompetence handicap theory is that females prefer immunocompetent males. In contrast with this prediction we did not find any preference of females for males that have a higher antibody response (figure 23, paired-*t*-test, response 1: $T = -0.86$, $df=3$, $P=0.45$, response 2: $T = -0.27$, $df=2$, $P=0.81$, response 3: $T = -2.10$, $df=2$, $P=0.17$).

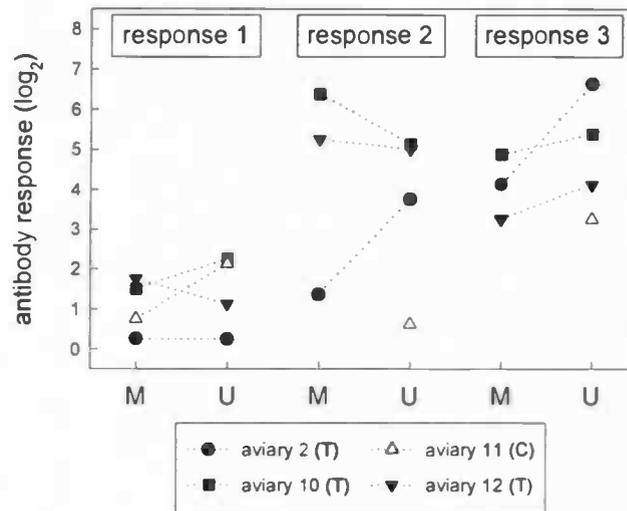


Figure 23. Male antibody response against SRBC and female mate choice. Males in one aviary are connected with lines. The second and the third response of the mated male in aviary 11 are missing. M: mated males, U: unmated males, (T): testosterone treatment, (C): control treatment.

There was also no effect of male foot infections, sedimentation rate or percentage white blood cells on female mate choice (figure 24, paired-*t*-test, A. infections: $T = -0.25$, $df=3$, $P=0.82$, B. sedimentation rate: $T = -0.05$, $df=3$, $P=0.9631$, C. white blood cells: $T = 0.33$, $df=3$, $P=0.76$).

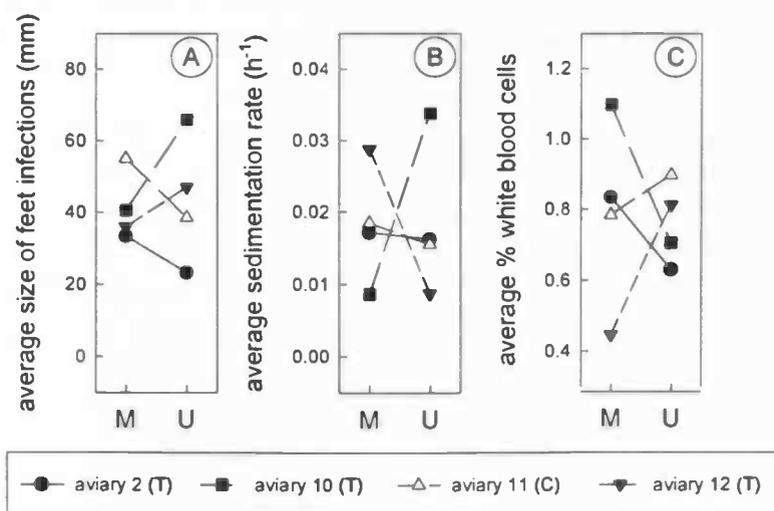
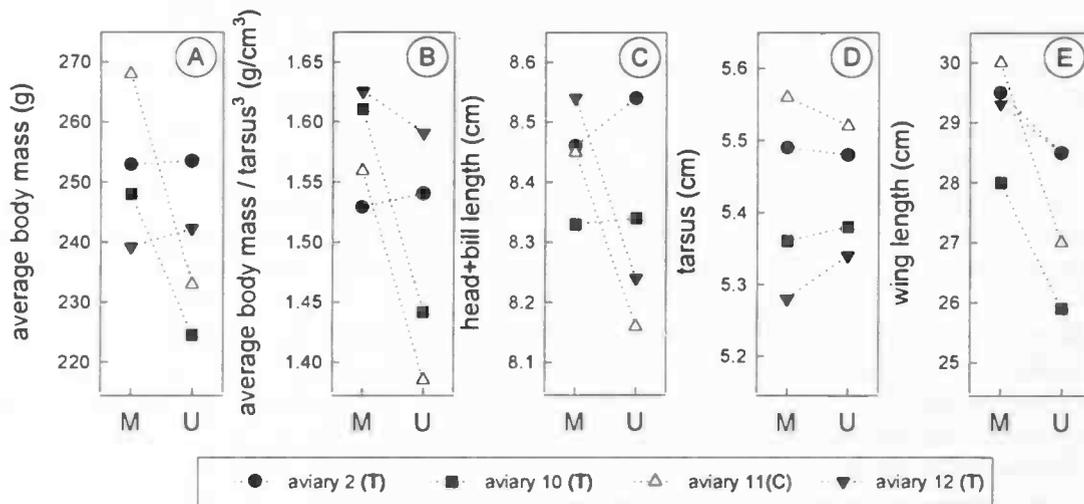


Figure 24. Male immunocompetence and female mate choice. A. foot infections, B. sedimentation rate, C. % white blood cells. Averages were calculated over all measurements during the whole experimental period. Males in one aviary are connected with lines. M: mated males, U: unmated males, (T): testosterone treatment, (C): control.

Male Body Size and Female Mate Choice

I also investigated the effect of body size parameters of the males on female mate choice. Male body size may be important for female mate choice, as it may determine physical strength. The females show a preference for the males with the longest wings (figure 25E, paired *t*-test: $T=3.37$, $df=3$, $P=0.043$). There was no effect of male body mass, condition, head+bill or tarsus length on female mate choice (figure 25 A-D, paired *t*-tests, body mass: $T=1.48$, $df=3$, $P=0.234$, condition: $T=1.94$, $df=3$, $P=0.147$, head+bill: $T=1.26$, $df=3$, $P=0.297$, tarsus: $T=-0.35$, $df=3$, $P=0.749$)

Figure 25. Male body size and female mate choice. A. body mass, B. condition, C. head+bill length, D. tarsus length, E. wing length. Average body mass and condition were calculated over the whole experimental period. Males in one aviary are connected with lines. M: mated males, U: unmated males, (T): testosterone treatment, (C): control treatment.



Conclusion

Overall we found no clear evidence of male immunological state, ornamentation, display rate or body size being involved in female mate choice in the Black-headed Gull. However our results are not conclusive, due to a very low statistical power, as a result of the small sample size.

6. DISCUSSION

6.1. Our Results and the Immunocompetence Handicap Hypothesis

The Dual Effect Testosterone:

Immunosuppressive and Sexual Ornamentation Promoting

The immunocompetence handicap hypothesis states that testosterone promotes the development of sexual ornaments and simultaneously suppresses immune function. This is the so-called dual effect of testosterone.

In agreement with the hypothesis testosterone indeed had a positive effect on sexual ornamentation and display rate. Display rates were strongly enhanced by testosterone treatment (Zwiggelaar 1996). Also the development of the brown mask was promoted by testosterone. And although we had difficulties with measuring bill colour changes, because of injured bills, there is evidence that the bill colour of testosterone treated gulls was darker and more saturated. This is confirmed by the observation of clear visual differences between the bill colour of the two experimental groups during the winter season after the experiment.

However, in contradiction with the original immunocompetence hypothesis of Folstad and Karter (1992) we did not find evidence of an *obligatory* immunosuppressive effect of testosterone in black-headed gulls. There was no negative effect of testosterone on the primary antibody response against SRBC. Also the secondary antibody response was not affected either by testosterone. Neither any other aspect of immune function, like the volume percentage of white blood cells, the sedimentation rate and the occurrence of foot infections (Zwiggelaar 1996).

Our results do not necessarily contradict the 'adaptive resource allocation' version of the immunocompetence handicap hypothesis (Wedekind and Folstad 1994). In this version of the hypothesis testosterone only regulates the trade-off of limited resources between the immune system and sexual ornamentation. It could be that the testosterone treated gulls compensated in some way for the extra energy expenses, needed for the development of secondary sexual traits. A possibility is that testosterone treated gulls lowered their body mass, but this was not observed. Another possibility is that testosterone treated gulls save energy by reducing the energetic demands of physiological systems other than the immune system. Testosterone treated gulls could for example have saved energy by showing less behavioural activity such as walking, flying and bathing. However this was probably also not the case. My impression is that the testosterone treated gulls tended to be even more active than the control animals. A final possibility is that testosterone treated gulls compensate for their increased energy demands by simply having a higher food intake. The gulls could easily have realised this, because food was available *ad libitum*.

How to Rescue the Original Immunocompetence Handicap Hypothesis?

I could think of different arguments that rescue the original version of the immunocompetence handicap hypothesis in the case of the Black-headed Gull. It could be argued that we did not find an immunosuppressive effect of testosterone, because not testosterone, but some other hormone or 'biochemical' is involved in the connection between the immune system and sexual ornamentation in this species (Owens and Short 1995). However, this argument is opposed by the fact that testosterone plays an important role in the development of sexual ornaments in the Black-headed Gull.

Another argument is that we can not exclude that we would have found an immunosuppressive effect of testosterone if we had given a higher dose. However testosterone titers would then easily come outside the physiological range. Furthermore, the dose we gave was sufficient to have a clear effect on several secondary sexual characters. Analysis of the testosterone levels of the stored blood samples can give us a decisive answer about the height of the titers in the gulls.

Finally, we have a considerable chance of making a second order error when we state that testosterone has no effect on antibody response. On the basis of the variation in antibody response, that we found, and $\alpha = 0.05$, I calculated that we would need 47 individuals in each treatment group to have a power of 0.90 in detecting a one-unit difference in primary antibody response. To have a power of 0.50 in detecting a one-unit difference, we would still need 18 individuals in each group. For having a power of 0.90 in detecting a difference of two units in the secondary response we would need 35 individuals per group and for a power of 0.50 we would need 14 individuals per group (Zar 1984). We eventually had data on 12 testosterone treated and 7 control animals. Hence the statistical power of our analyses was fairly low.

However there have been done more experiments that found no effect of testosterone antibody response. Ros et al. (1997) did not find a negative effect of testosterone on antibody response in black-headed gulls, and also in a similar experiment carried out in adult black-headed gulls testosterone did not affect antibody response (Scheepstra pers. com.). Combining the data of all three experiments would drastically increase the statistical power.

Relationships among Immune Function, Sexual Ornamentation and Display

We found just weak evidence for secondary sexual characters being an honest signal for immunocompetence, which is another premise of the immunocompetence handicap hypothesis. Only in the control animals we found a significant positive correlation between the stage of the brown mask and secondary antibody response. It should be noted that this relationship was found by *post hoc* statistical testing. The relationship should be confirmed by a new experiment. In testosterone treated animals this association was not found.

To my opinion it is not surprising that we find such a relationship in control animals, and not in testosterone treated animals. The testosterone treated gulls were not able to 'choose' the height of their own testosterone levels. It is possible that they were just 'forced' by the administered testosterone doses to develop a certain display level, while suppressing their immune function to a certain extent, independent of their immunological state. As a result correlations between immune function and mask stage will have been obscured in testosterone treated gulls. However Ros *et al.* did find a correlation between display rate and antibody response in testosterone treated gulls, which contradicts this idea.

We could not reproduce the correlation Ros *et al.* (1997) found between display rate and primary antibody response. A possible explanation for this is that Ros *et al.* housed six gulls together in one aviary, whereas we housed the gulls in groups of three individuals, sometimes even less because of mortality. The small group size caused huge differences in mean display rates between the different aviaries, as display rates of the individuals within one aviary were highly dependent. This could have clouded possible relationships between display rate and antibody response.

Male Immunology, Sexual Ornaments and Female Mate Choice

An important aim of this study was to investigate the role of immunocompetence and secondary sexual ornaments in female mate choice. We did not find any indication for female preference for immunocompetent males. Females did not prefer males with a high antibody response against SRBC. Male sedimentation rate, percentage white blood cells or number of foot infections did not affect female mate choice either.

Overall we found no clear evidence of male sexual ornaments and display being involved in female mate choice either. Mated males tended to perform the *extreme* oblique and forward displays at higher frequencies than unmated males. However, the higher display rate of the mated males was not necessarily the reason that the females selected them. Higher frequencies of extreme display could also be caused by the fact itself that they were mated. Already mated black-headed gulls often perform many extreme displays to their partners. There was no difference between mated and unmated males in total display rate. We did not find any effect of bill colour, leg colour, or stage of the brown mask on female mate choice.

Probably sexual display is only one aspect on which females base their mate choice. Also male body size may be important, since this may determine physical strength. However we found just little evidence for this idea, as only wing length was greater in mated males. There was no relation of being mated with body mass and condition, head+bill length and tarsus length. I want to remark that, given that there was a difference in body size between mated and unmated males it actually remains unclear whether this was caused by female choice or by male competition.

Further factors that could have played a role in female mate are the colours of the males in the ultraviolet spectrum and the odour of the males. Birds possess vision in the UV spectrum (Maier 1994). It has been found that colour perception in the UV spectrum influences mate choice in zebra finches (Bennett *et al.* 1996). Many bird species have probably a good sense of smell (e.g. Jones 1997). From mammals it is known that smell could be important in mate choice (Wedekind 1995). The same may be true in birds.

Artefacts like bill damage, or broken feather tips, or even the colour rings we used to mark the birds (Burley 1982), may have influenced female mate choice. Thus we might have introduced several confounding factors.

Effects of Stress and Suboptimal Body Condition

We have several indications that the condition of our gulls was far from optimal, and that the birds suffered from considerable stress. Firstly, our animals had a much lower body mass than the birds of Ros *et al.* at the start of the experiment (day 0, implantation). Furthermore there was a very high mortality among our animals. Fourteen of the 35 animals that we implanted died before the end of the experiment. This was partly due to an unexpected strong synergetic effect of the testosterone + corticosterone treatment (8 of the 10 TB-animals died, Zwiggelaar 1996). However also among the animals that were only treated with testosterone or were controls, 5 of the 25 birds died before the end of the experiment. Of the 22 birds that survived until the end of the experiment, 11 had damaged bills, caused by the wire netting.

Several factors may have caused a lot of stress in the gulls. Firstly, the birds were caught many times in the course of the experiment (7 x, e.g. for implantation, SRBC challenges etc). After catching the birds were sometimes kept in boxes for a couple of hours, because the measuring of bill and leg colour, feet infections, etc. was very time consuming. Secondly, all birds have been re-housed at least two times. As mentioned already re-housing is probably a very stressful event for black-headed gulls. A third factor could be the small group size in which the birds were kept. Black-headed gulls are social birds living in large groups, and the birds did not seem to be at ease in the small groups in which we kept them. Finally, the fact that it was a very cold spring that year may have caused extra difficulty for the gulls.

The stress that the gulls suffered from could have influenced our results on immune function. It is known that a stress-caused elevation of corticosterone titers can seriously suppress immune function. This immunosuppression can sometimes even lead to death, caused by infectious diseases (Bradley 1987). If the immune system of all animals was already heavily compromised by stress, this could have masked an additional negative effect of testosterone. Analysis of the corticosterone concentration in the stored blood samples could reveal the height of the corticosterone titers in our animals during the experiment. Also our results on the effect of testosterone on body mass, and the relationship between body mass change and display rate may have been influenced by stress. It could explain several discrepancies between the body mass results in our experiment and in the experiment of Ros *et al.* (see results-section 5.3. *Our experiment versus the Experiment of Ros et al.*). Furthermore the poor condition of the gulls could have influenced the results on mate choice. Besides sexual displays and ornaments, the general condition, body weight, plumage condition (broken feathertips), injuries (damaged bills), may have influenced our results on mate choice.

In conclusion I believe that it is not unlikely that stress and suboptimal body condition seriously affected the results of our experiment. Therefore more weight should be added to the well-being of the animals in future experiments. However, the most important reason to add more weight to the well-being of the animals may be their well-being in itself.

6.2. The Immunocompetence Handicap Hypothesis: State of the Art

Functional Explanations for the Dual Effect of Testosterone

The original immunocompetence handicap hypothesis (Folstad and Karter 1992) assumes testosterone to be *obligatory* immunosuppressive, and because development of sexual ornaments would be dependent on testosterone, these ornaments would be honest signals of immunocompetence. However, cheaters, which lost for example the connection between testosterone and immune function (e.g. by losing receptors for testosterone), could evolve in such a system (Wedekind and Folstad 1994). Cheating would only be impossible if the immunosuppressive effect of testosterone is in the animals' own interest.

The possibility of cheating, in case the immunosuppressive effect of testosterone has no adaptive value for an individual, is a serious problem for the immunocompetence handicap theory. New hypotheses try to explain why the immunosuppressive effect of *testosterone* would be adaptive. An example of this is the recently postulated hypothesis that suggests that testosterone acts as a suppressor of immune function to prevent sperm cells of being attacked by the immune system (Hillgarth *et al.* 1997, Folstad and Skarstein 1997). Spermatocytes are considered as non-self by the immune system. Suppression of auto-immune responses to the antigenic spermatocytes by testosterone would facilitate the production of high quality sperm during the breeding season.

To my opinion the approach of formulating hypotheses, which only focus on the immunosuppressive effect when explaining the adaptive value of immunosuppression by testosterone, is not very fruitful. The sperm-quality hypothesis, for example, raises only new problems.

In the first place the model only gives an explanation for the evolution of a mechanism, in which sex hormones link immune function and sexual ornamentation, in *males*. However there are many species, as the Black-headed Gull, in which also females show sexual ornaments, which are probably important for male mate choice. These signals should be honest in females either. Not to speak about species with sex role reversal in which females show more exaggerated ornaments than males, like the Eurasian Dotterel (*Charadrius morinellus*). Moreover cheating could still be possible in this sperm-quality model. Males could arise, that lost the connection between testosterone and sexual ornaments by using another hormone to regulate ornament size.

I want to mention that the results of our study do not support the sperm-quality hypothesis. If the function of an immunosuppressive effect of testosterone is to prevent spermatocytes from attacks of the immune system, testosterone should be obligatory immunosuppressive. We did not find testosterone to be obligatory immunosuppressive. I am aware that our study, in which we treated both males and females, was not really well-suited for testing the sperm-quality hypothesis, because it does not necessarily predict an immunosuppressive effect of testosterone in females.

How should we proceed when focussing on the immunosuppressive effect of testosterone only is not a good approach in finding an adaptive reason for the link between immune function and sexual ornamentation? I believe the only solution is to assume that there is a *direct* trade-off between immune function and sexual ornamentation. Only then cheating would be impossible (Wedekind and Folstad 1994). Only such a direct trade-off could explain why testosterone (or some other biochemical) directly links the immune system and sexual ornaments. A possible trade-off could involve all kind of resources, like energy, metabolites, nutrients, time. Even conflicting physiological pathways, needed for either the immune system or the development of ornaments, may be traded off.

Genetic versus Non-Genetic Immunocompetence

There has been discussion over what 'part' of the immune system should be measured to assess 'immunocompetence'. Skarstein (1996) states that 'good genes' or heritable parasite resistance should be distinguished from immunocompetence. Skarstein (1996):

"...This distinction between immune responsiveness and the effects of 'good genes' is crucial, as the 'immunocompetence handicap' hypothesis implies that females are ultimately looking for the 'good genes' described by Hamilton and Zuk (1982).....behavioral ecologists need to define exactly what is formally meant with the term 'good genes' within the context of the 'immunocompetence handicap' idea. Then by excluding this aspect of the immune response from our measure of 'immunocompetence', we will be able to understand if variation between individuals in degree of immunosuppression is influenced by whether they possess 'good genes' or not, which is the mechanism by which the 'immunocompetence handicap' is supposed to work...."

As examples of how 'good genes' may be understood Skarstein mentions antigen recognition propensity and the ability to tolerate infection, i.e. minimise damage from already established infections. Also others make the distinction, for instance between 'genetic' and 'acquired' immunocompetence, which would not be affected by genes. I doubt whether it is useful to try to distinguish these so-called 'good genes', 'genetic immunocompetence' or 'heritable parasite resistance' from some other 'non-genetic' or 'acquired immunocompetence'. Also the acquired immune function is influenced by genetic background and shows heritable variation (Kreukniet 1996). In my view it is impossible to disentangle a genetic (= 'good genes') and a non-genetic part of the immune system. Trying to do this is as useless as trying to separate nurture and nature.

To my opinion the main premises that are important for the 'Hamilton and Zuk' hypotheses are:

1. There is heritable variation among individuals in the effect of parasites on fitness. Whether this variation is caused by variation in the ability to minimise damage from an already established parasite, or in the ability to 'throw out' harbouring parasites, or by behaviour that lowers infection risk does not make any difference.
2. Ornament size is related to this heritable variation in fitness effects of parasites.
3. The individuals of one or both sexes show a preference for mates with good parasite resistance over mates that are less resistant and, are able to discriminate between these by ornament size.

The 'immunocompetence hypothesis' adds a mechanism for the connection between ornamentation and immune function: testosterone or some other 'biochemical'.

How to Measure Immunocompetence?

An important question when testing the immunocompetence-handicap hypothesis is how to assay immunocompetence (Siva-Jothy 1995). Siva-Jothy raises the problem that most tests of immunocompetence were primarily designed to measure only one specific aspect of immune function. None was designed to provide an index of the general ability of an individual to withstand a pathogenic challenge. Lochmiller (1995) states that measuring a very broad array of immunological parameters could yield an adequate picture of immune function. He mentions a study of himself in food restricted Bobwhite chicks as an example. In this study many different immunological parameters were measured, like for example spleen and bursa weight, lymphocyte yields from dissociated lymphoid organs, *in vitro* lymphoproliferative response of cultured splenocytes, white blood cell counts, antibody response against SRBC. An interesting detail is that they found an effect of their food restriction in several parameters, but not in the antibody response against SRBC!

Can We Test the Hamilton and Zuk Hypothesis Experimentally?

Provided that we are able to adequately measure immune function of a host by methods as of Lochmiller *et al.*, we are still not able to translate this estimate of immune function in the fitness effects that a parasite will have on his host. We need to know the parasite-caused fitness effects to be able to test the Hamilton and Zuk hypothesis. It is possible that a host with very poor immune function still has hardly any fitness loss caused by parasitic infection, because it is very well able to minimise the damage caused by the parasites. In this way this host could even have a lower parasite-caused fitness loss than a host that has very good immune function, but that is very poor in tolerating an already established parasitic infection.

To be able to measure the real fitness effects of parasitic infection we could infect our study animals with parasites. However this would not solve the problem, because it could be that differences in parasite-caused fitness effects between hosts are related to variation in parasite-avoidance behaviour.

These considerations lead to the conclusion that it is very difficult, to experimentally test the Hamilton and Zuk hypothesis. The best approach could be to first describe the interactions between hosts and parasites in one system. Also study the associations between parasites, host ornamentation and immune function should be described. It should be investigated whether the host shows parasite-avoidance behaviour. When all these interacting factors have been well described, a start could be made with manipulating all these factors one by one to tear the different causal relationships apart.

Is there a Difference between Ecto- and Endoparasites?

It may be important to distinguish between ecto- and endoparasites when investigating relationships among ornament size, testosterone levels, and these parasites. There is some evidence that the associations between ornament size and testosterone levels, and the intensity of infestation differ between these two types of parasites (e.g. Weatherhead 1993). A possible explanation for this is that the mechanism by which associations arise between, for instance, testosterone levels and intensity of parasite infestation, may differ between ecto- and endoparasites.

There are several potential mechanisms via which testosterone in could have an effect on numbers of ectoparasites. Firstly testosterone may change the behaviour of the host, which could lead to a change in infection risk, due to for example a change in the number of interactions with conspecifics. Furthermore the blood circulation could be affected by testosterone, which could change the food situation of bloodsucking parasites due to increased peripheral blood flow. Finally, testosterone could have a direct effect on the parasite itself. The parasite possibly uses an elevation of testosterone titer as a cue for the start of the reproductive season of its host. This to synchronise its own reproduction with the reproductive phase of its host.

None of the above mechanisms are mediated by the immune system of the host. It is difficult to see how the immune system could mediate an effect of testosterone on ectoparasite numbers. In contrast there are many mechanisms known by which the immune system influences numbers of endoparasites (Weir and Steward 1997). In conclusion associations between testosterone levels and ectoparasites may arise via a different mechanism as associations between testosterone and endoparasites. This could have implications for the interpretation of these different associations.

6.3. Further Research

An interesting follow-up of our study would be to carry out a similar experiment, but then to restrict the food intake of the gulls (at this moment such an experiment has been conducted yet). This could answer question whether testosterone is regulating a direct trade-off of resources between immune function and secondary sexual characters, as in a food limited situation the gulls will not be able to compensate for the development of sexual ornamentation by have a higher food intake.

Furthermore it would be useful to explore new methods that assess immune function. The antibody response to SRBC of black-headed gulls is not very high, compared to for example the antibody response in chickens (see Munns and Lamont 1991 for antibody response chickens). A higher response against some other antigen may give a higher resolution. The volume percentages of white blood cells measured from the capillaries are difficult to reproduce (appendix B). Hematological counts made on blood smears may be more precise. Determining the weight of the bursa and spleen could give additional information (Lochmiller 1995).

In new experiments that investigate correlational associations among immunological parameters and display behaviour, the gulls should be kept in as large groups together as possible. This to exclude variation in display rates caused by the subgroup in which animals are housed. Most preferable would be to house all gulls together in one big aviary.

Finally, it would be very interesting to gather more data on testosterone levels during different periods of the year in both males and females. This to gain more insight in both the seasonal changes in testosterone titers and sex differences in these titers. In our experiment we treated males and females with the same dose of testosterone. However although both sexes produce testosterone, females may have considerably lower peak levels than males. There is some evidence for this (Groothuis and Meeuwissen 1992). This would mean that we administer relatively higher doses to females than to males. More basic data on natural occurring testosterone titers could make the both the set-up and the interpretation of the results of these experiments more accurate.

6.4. Final Conclusion

Our results do not support the original immunocompetence handicap hypothesis of Folstad and Karter (1992). Testosterone had no immunosuppressive effect in the gulls, as predicted by their hypothesis. We also found just scanty evidence of sexual ornamentation and display being honest signals of immunocompetence, which is another prediction of the hypothesis. The results on the role of sexual ornamentation, display and immune function in mate choice were not conclusive due to low sample size.

Our results are not necessarily incompatible with the adaptive-resource-allocation version of the immunocompetence handicap hypothesis (Wedekind and Folstad 1994). A similar experiment in which the gulls are food limited should be conducted to examine whether testosterone plays a role in the regulation of resource allocation between the immune system and sexual ornamentation and display.

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APPENDIX A

THE SRBC PILOT EXPERIMENT AND RAINDROP DATA

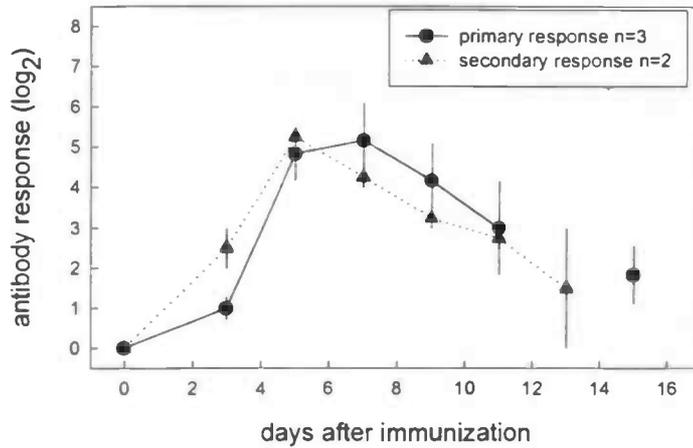


Figure A1. Representation of the peaks of the primary and secondary antibody response, which were determined in a pilot study.

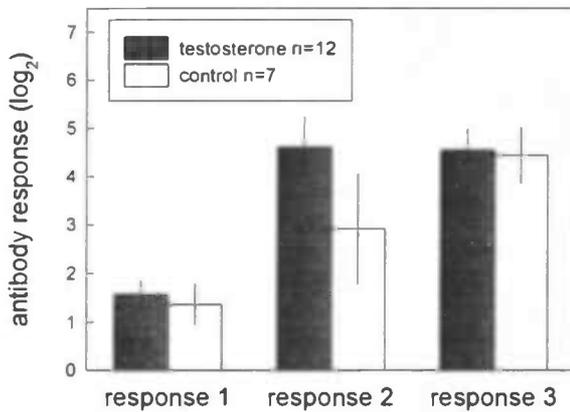


Figure A2. Antibody response against SRBC in testosterone treated and control animals as measured by the 'raindrop' method. (repeated measures MANOVA with 3 levels: response 1, 2 and 3, and 2 factors: sex and treatment, testosterone: $P = 0.168$, sex: $P = 0.884$, time: $P = 0.000$, no significant interaction effects). One control is missing, because we were not able to take a blood sample from this individual six days after the second challenge.

Table A1. Correlations between the 'raindrop' estimates' and 'rough' estimates of antibody titer and response.

		R_s	n	P
titer	initial	0.6703	36	0.000
	1	0.8559	28	0.000
	2	0.9332	22	0.000
	3	0.9240	22	0.000
response	1	0.7885	28	0.000
	2	0.8971	22	0.000
	3	0.8476	22	0.000

APPENDIX B

THE RELIABILITY OF SOME HEMATOLOGICAL PARAMETERS

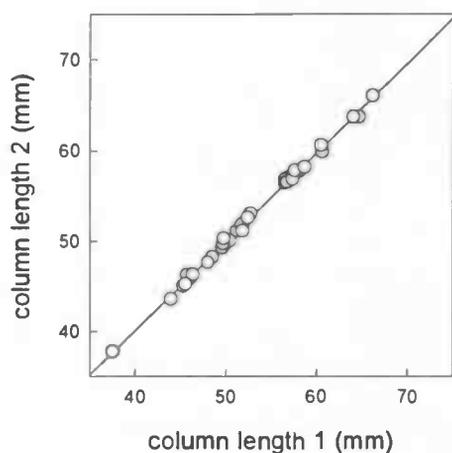


Figure B1. Correlation between two estimates of the length of the liquid column in the same capillary made by two different observers. Capillaries with broken liquid columns are not in the plot. Measures were done on the 8th of May. $R_s=0.996$, $N=36$, $P=0.000$. This figure indicates that the measuring of the column length by different observers did not introduce much variation.

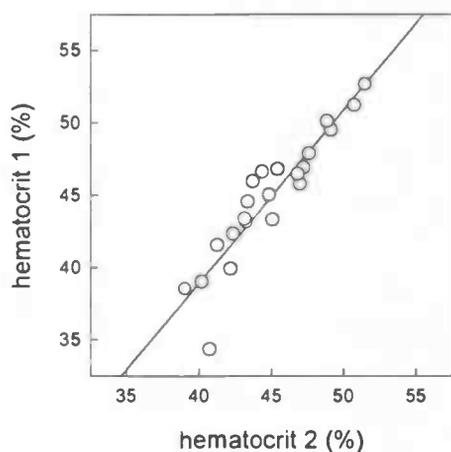


Figure B2. Correlation between the duplos of the measured hematocrit. Measures were done on the 8th of May. $R_s=0.9503$, $N=22$, $P=0.000$. From this follows that the repeatability of the hematocrit estimate was fairly high.

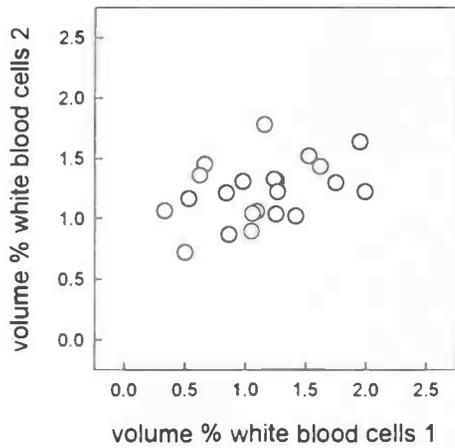


Figure B3. Correlation between the duplos of the measured % white blood cells. Measures were done on the 8th of May. $R_s=0.3529$, $N=22$, $P=0.107$. From this it follows that the repeatability of the white blood cells estimate was very poor.

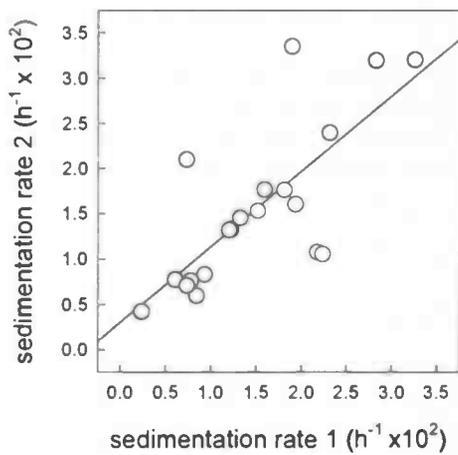


Figure B4. Correlation between the duplos of the measured sedimentation rate. Measures were done on the 8th of May. $R_s=0.6857$, $N=20$, $P=0.001$. From this figure it follows that the repeatability of sedimentation rate estimate was quite high, however there were a few strange outliers. These were possibly caused by capillaries that were not in exact vertical position during sedimentation or by variation in liquid column length between the capillaries.

Table B1 shows that the sedimentation rate was strongly related to the hematocrit. Presented are the within individual correlations between hematocrit and sedimentation rate. This association between hematocrit and sedimentation rate was not caused by the fact that the capillaries were so long in vertical position that all red blood cells were totally sedimented (see figure B5).

Date of measurement	R_s	N	P
26 th April	-0.6992	20	0.000
2 nd May	-0.6872	22	0.000
8 th May	-0.7628	22	0.000

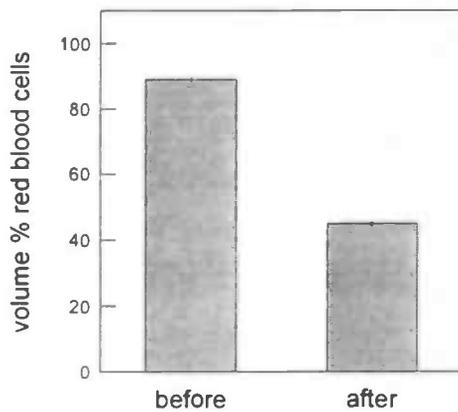


Figure B5. The volume % red blood cells before and after the capillaries were centrifuged. The estimate before centrifugation is the parameter measured to estimate the sedimentation rate. The estimate after centrifugation is the hematocrit. The difference between the % red blood cells before and after centrifugation is highly significant (Wilcoxon-signed-rank: $Z=-4.107$, $N=22$, $P=0.000$).

APPENDIX C

FOOT INFECTIONS: NUMBER VERSUS SIZE

Table C1. Correlations between the total size and number of foot infections. The number of foot infections was highly correlated with the total size of all foot infections. The total size was calculated by summing the diameters of all infections.

Date of measurement	R_s	N	P
12 th March	0.8782	35	0.000
28 th March	0.8302	28	0.000
2 nd May	0.8979	22	0.000
8 th May	0.8988	22	0.000
7 th June	0.8290	21	0.000

APPENDIX D

INJURED BILLS

The colour of the bill was measured at two different spots, above the nostrils and below the nostrils. Many gulls injured their bills in the wire-netting during the experiment. Mainly the part of the bill above the nostrils was injured. The injuries led to changes in the colour of the bill, due to hematomas and scar tissue. Therefore I expect that the bill colour above and below the nostrils did not correlate when a bill had been injured. I expect that if a bill was sound that the colour above and below the nostrils was similar. In that case the colour of the two different spots should have been positively correlated. It seems indeed to be true that bill colour above and below the nostrils correlated in birds with sound bills, whereas in birds with damaged bills there was no correlation (figure D1).

bill damage and colour

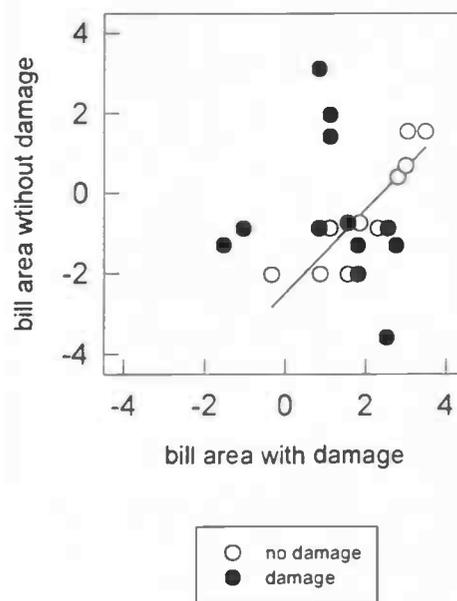


Figure D1. Correlation between the colour index of the spot above the nostrils and the spot below the nostrils. The spot above the nostrils was damaged in some gulls, the spot below the nostrils was never damaged. Black dots represent individuals with a injured bill, white dots represent individuals with a sound bill. There was a significant positive correlation between the colour of the two different spots of the bill in gulls that had no damaged bills ($R_s=0.92$, $N=10$, $P=0.000$). There is no correlation between the colour of the two bill spots in gulls that had damaged bills ($R_s=-0.4136$, $N=12$, $P=0.181$). The graph is based on the bill colour estimates made of the 8th of May (response 3). Animals of the three treatments were pooled. Bill area with damage: above nostrils, bill area without damage: below nostrils.

Figure D1 suggest that bill colour above the nostrils, but not below the nostrils, was affected when a gull injured its bill. This could have clouded a possible effect of the testosterone treatment on the bill colour above the nostrils. If this is true I expect an effect of testosterone on bill colour above the nostrils in birds with sound bills, but not in birds with damaged bills. I expect an effect of testosterone on the colour below the nostrils, if there is any, in both birds with damaged and in birds with sound bills. Figure D2 suggests indeed such a pattern.

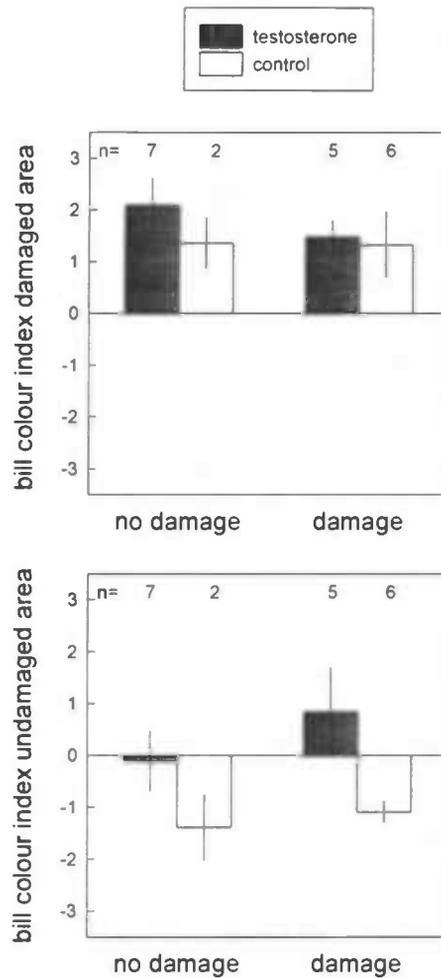


Figure D2. Bill colour of birds with damaged bills compared to the bill colour of birds without damaged bills. The bill colour of testosterone treated and control animals is separately depicted. In the upper graph the colour as measured above the nostrils is presented, whereas in the lower graph the colour measured below the nostrils is presented. The area above the nostrils had been damaged in some gulls. The area below the nostrils had not been damaged in any of the birds.

APPENDIX E

PROTOCOL OF THE SRBC IMMUNISATION METHOD

SHEEP RED BLOOD CELL (SRBC)
IMMUNIZATION METHOD

Victor Apanius, Charlotte Deerenberg, Nico Bos, Judy Bun
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University Groningen, The Netherlands

Inferences

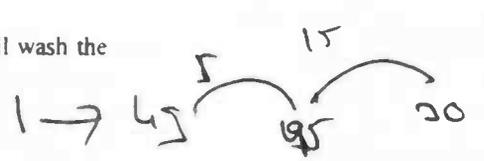
- * one aspect of immune function
- * generally correlates with resistance
- * antibodies are important for clearing bacterial infections

Making SRBC batches

Aim: Separate cells from plasma

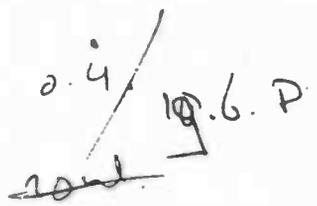
SRBC = sheep red blood cells in Alsever's solution
(ask the immunology department)
PBS = phosphate buffered saline

1. Make sure you keep things sterile.
2. Prepare the amount you want to use.
3. Transfer the blood into a plastic centrifuge tube (ca. 10 ml).
4. Balance the tube with blood exactly against a tube with the same weight (use water and a scale).
5. Centrifuge the blood: 10 minutes, 2000 rpm, lowest brake.
6. Remove with a pipette the liquid (plasma) from the condensed cells at the bottom of the tube.
7. Add PBS to the cells (up to ca. 10 ml). The following procedure will wash the cells and remove dead and/or lysed cells.
8. Return to 4. and repeat the procedure (4. to 6.) 3 times.



Caution: The batch of SRBC prepared this way will keep for 30 days in a fridge or on ice (4°C). NEVER below freezing-point: this will destroy the cells!

9. The batch of SRBC prepared this way is not yet suited to inject in the animal. It needs a 98% dilution to 5×10^8 cells per ml. Dilute as follows (eg.): take 0.1 (1) ml of the washed SRBC (be careful to exclude the white top layer). Add 4.9 (49) ml PBS. Now you have 5 (50) ml solution with 2 % washed SRBC. Count the cells: put a drop of the solution on either side of the haemocytometer, use a mean of 5 countings (Number of cells should be 50 per triple lined square). If necessary adjust the dilution. Now the cells are ready for injection.



Caution: The 98% diluted SRBC-samples keep for 14 days (at 4°C) when made from a fresh batch and only 5 days when made from an old batch.

counter counter
10 ml 3000 in 250 120 µl
aperture 4
threshold d count red
4.5
plate

- Adjust amount of SRBC to inject to the size of the animal (eg. 100 μ l=0.1 ml for 13 g bird. 500 μ l=0.5 ml for 250 g bird).

Caution: Always draw a blood sample before immunization to check for natural or general anti-SRBC antibodies.

Preparation of serum samples

- Draw at minimum 100 μ l blood from your animal and put the blood into marked (animal id., sample no., date) Eppendorf aliquots.
- Centrifuge the blood samples for 10 min, 13.000 rpm, brake 7 (slow).
- Transfer the serum into a new, marked aliquot. Use a pipette.

Caution: Take care to transfer serum only. no cells. and preferably no white layer (protein).

- Store the aliquots at -20°C till further analysis.

Analysis of serum samples

- Take the serum samples from the freezer and put them in a polystyrene foam grid.
- Place the grid with the samples in a 56°C waterbath for 30 min. This removes the protein, that would otherwise lyse the cells.
- Prepare the plates (see under 6.) as follows: take a plastic plate with 8*12 wells. The 12 rows are used to make a dilution series of 1:212. The 8 columns will contain the serum samples: mark the columns (animal id., sample no.). Fill all 96 wells with 20 μ l PBS (for mini samples or 50 μ l for big birds' samples), use a 12-fold pipette for convenience.
- Dilute the serum samples as follows: Add 20 (50) μ l serum to the 20 (50) μ l PBS in the first well of every 12 rows. Now the serum sample is diluted by a half. First, mix 3-5 times by sucking up with the pipette, next remove 20 (50) μ l from this well and add this to the next well (of the row) in the dilution series. Again, remove 50 μ l from this well, mix and add to the next well. Repeat for the remaining wells.
- Add 20 (50) SRBC (2%) to all 96 wells. Transfer the grid with serum samples to a incubator at 37°C for 1 hour. *also 20 μ l*
- Check for haemagglutination: in wells with a ~~high~~ concentration of SRBC-antibodies the antibody-bloodcell complexes will precipitate at the bottom. In wells with a ~~low~~ concentration of SRBC-antibodies the antibodies will keep the SRBC in suspension (hemagglutination, Hay and Hudson 1989 chap 4.6, 7.2). When no antibodies are present, the SRBC will clot at the bottom (hard to distinguish from high concentration of antibodies!).

grid \leftarrow
 plate
 PBS
 6x8x12x2
 12 rows
 8 columns
 12-fold
 pipette
 Serum

Strainer
 A-H
 1-12

1700

8. Keep a record of the (lowest) well number in the dilution series in which you observe hemagglutination (the SRBC are kept in suspension). The number gives the titer of the antibody concentration (10^x cells per ml). This titer is a discrete variable, but approaches continuity at high sample rates.

Hay L. Hudson FC (1989) Practical immunology. Blackwell Scientific Publications, Oxford.

Lo. v. Ton GroothuisHAEMAGGLUTININATIE TEST

1. Alle cupjes vullen met 50 μ l PBS
2. In cupjes van de eerste rij 50 μ l van het te testen serum
3. 1:2 verdunningsreeks maken (multichannel pipet)
4. Aan alle cupjes 50 μ l 2% SRBC suspensie toevoegen aan de 50 μ l serum verdunning
5. Schudden (15 sec.)
6. Incuberen (kamertemperatuur, 2 uur)
7. Aflezen op lichtbak
8. Titer is de log waarde van de verdunning die nog meer dan 50% agglutinatie veroorzaakt.

HAEMOLYSE TEST (van hem, Rood)

1. Alle cupjes vullen met 50 μ l PBS
2. In cupjes van de eerste rij 50 μ l van het te testen serum
3. 1:2 verdunningsreeks maken (multichannel)
4. Aan alle cupjes 50 μ l 2% SRBC suspensie toevoegen aan de 50 μ l serum verdunning
5. Kort schudden (5 sec.)
6. Incuberen (broedstuf 37 $^{\circ}$ C, 15 min.)
7. Aan alle cupjes 25 μ l konijne-complement toevoegen
8. Kort schudden (5 sec.)
9. Incuberen (broedstuf 37 $^{\circ}$ C, $\frac{1}{2}$ uur)
10. Schudden (10 sec.)
Indien bezinksel op de bodem van de cupjes nog niet goed gemengd is, dan iets langer schudden.
11. Incuberen (broedstuf 37 $^{\circ}$ C, $\frac{1}{2}$ uur)
12. Aflezen op de lichtbak
13. Titer is de log waarde van de verdunning die nog meer dan 50% lysis veroorzaakt

MERCAPTOETHANOL-RESISTENTE HAEMAGGLUTININATIE TEST

1. Met uitzondering van de eerste rij, alle cupjes vullen met 50 μ l PBS
2. In cupjes van de eerste rij 50 μ l van het te testen serum doen
3. In de cupjes met het serum (eerste rij) 50 μ l 0,2 M 2-mercaptoethanol oplossing toevoegen (zuurkast)
4. Incuberen (broedstuf 37 $^{\circ}$ C, $\frac{1}{2}$ uur)
5. 1:2 verdunningsreeks maken (multichannel pipet)
6. Aan alle cupjes 50 μ l 2% SRBC suspensie toevoegen aan de 50 μ l serum verdunning
7. Schudden (15 sec.)
8. Incuberen (kamertemperatuur, 2 uur)
9. Aflezen op de lichtbak
10. Titer is de log waarde van de verdunning die nog meer dan 50% agglutinatie veroorzaakt.

ME-agglutinatie titer \leq agglutinatie titer

$$14,3 \text{ mol/liter} \\ 0,2 \text{ mol/liter} / 20 \\ = 0,01 \text{ mol/50 ml}$$

$$x \cdot 14,3 = 0,01$$

$$\text{ME} x = \frac{0,01}{14,3}$$

$$= 0,000699301 \text{ liter} = 700 \mu\text{l op 50 ml PBS}$$

APPENDIX F

MATERIALS AND PREPARATION

Vangen in Grote Vliegkooi

Nodig:

- ca. 8 personen
- groot net + schepnetten
- lieslaarzen, waadbroek, laarzen
- sleutels vliegkooi
- vangkisten

Vorbereiding:

- grote net uitvouwen + uit knoop halen
- mensen regelen

Wegen en Meten

Nodig:

- weegschaal
- schuifmaat
- theedoeken/weegbuis
- vangkisten

Vorbereiding:

- weegschaal waterpas zetten

Bepaling van Zwarte Masker

Nodig:

- referentie figuur
- goed licht

Bepaling Poot en Snavelkleur

Nodig:

- goed licht (=geen tl!!)
- Munsell Color book

Bloedprikken

Nodig:

- 1 persoon om vast te houden
- goed licht
- naalden 0.4-0.5 mm + spuiten 1 ml
- cupjes met heparine voor heparinizeren naalden
- watten
- water voor opzij poetsen veren
- ethanol voor ontsmetten
- cupjes met 5-10 μ l heparine voor monsters
- watervaste stift voor nummeren cupjes
- ijs + cupjesrek (+ extra ijs)
- centrifuge voor afdraaien cupjes bloed

- pipet voor afpipetteren plasma
- vriezer
- stopwatch in geval van bloedmonster voor corticosteron bepaling
- theedoeken
- afval Emmers voor watten + naalden

Hoeveelheden:

- testosteron-bepaling: 0.5 ml bloed
- corticosteron: 0.3 ml bloed
- SRBC: 0.3 ml bloed
- bloodsmear (2x): 2 druppels
- PCV (2x): 2 x 60 μ l

Opmerking

- 1 ml bloed geeft ca. 0.5 ml plasma

Vorbereiding:

- naalden + spuiten heparinizeren
- cupjes nummeren + vullen met 5-10 μ l heparine

Tijdsduur:

- ca. 10 min. per dier voor prikken + cappilair vullen + smears maken + immuniseren

Immuniseren

Nodig:

- PBS
- SRBC gewassen
- zie bloed prikken

Blood Smears

Nodig:

- beschrijfbaar objectglasjes
- potlood/watervaste stift
- ruimte om glasjes te laten drogen na het spoelen
- methanol
- methanol spoelbakje
- opbergdoos objectglazen

Hematocriet

Nodig:

- hematocriet-capillair
- afsluitwas
- watervaste stift
- eventueel plakband + strook karton om capillair op te plakken
- schuifmaat
- capillair-centrifuge

Opmerkingen:

- afdraaien: 1 duplo per keer, 90 % van 1500 rpm, 4-5 buisjes tegelijk, niet te veel anders duurt het te lang voor ze gemeten worden na het centrifugeren.
- meten met schuifmaat: 1. hele kolom 2. kolom rood + wit 3. rode kolom
- afdraaien binnen ca. 12 uur na prikken

SRBC Wassen

Nodig:

- centrifuge
- steriele 10 ml centrifugebuizen
- PBS of fysiologisch zout
- flesje SRBC in Alsever's oplossing (80 ml)

Tijdsduur:

- na 20 min 1^e keer centrifugeren
- na 40 min 2^e keer
- na 40 min 3 ×
- na 40 min 4 ×

Opmerking

- 100 ml SRBC in Alsever's oplossing geeft ongeveer 25 ml SRBC 100 %
- verlies bij vullen van injectiespuiten: 4.0 ml SRBC geeft 6 x 0.5 ml spuit, 25 % verlies dus

Hemagglutinatie

Nodig:

- titerplaten met ronde bodem of conische bodem (tip Sasha Norris) 8 × 12
- multi-pipet (microbiologie, let op juiste hoeveelheid)
- micropipet (dierfysiologie)
- SRBC (gewassen)
- PBS (212 g Na₂HPO₄, 42 g KH₂PO₄, 789 g NaCl)
- watervaste stift
- stopwatch
- warmwaterbad (dierfysiologie)
- piepschuim grid voor warmwaterbad
- thermometer
- broedstoof (anatomiezaal E-vleugel)
- eventueel: 'schudder' (dierfysiologie)

Vorbereiding:

- broedstoof + warmwaterbad regelen
- monsters ontdooien
- titerplaten laden met PBS
- monsters incuberen op 56° graden
- monsters laden
- verdunningsreeks maken
- SRBC toevoegen
- incuberen in broedstoof

Corticosteron Pellets Maken

Nodig:

- corticosteron
- cholesterol
- theelepeltje
- brander
- garen
- capsules
- weegschaal

Wingtaggen

Nodig:

- tags
- lucifers
- soldeerbouten
- theedoeken
- dikke injectienaalden

APPENDIX G

OVERVIEW OF MEASURED PARAMETERS

Table G1. Overview of the parameters that were measured in the course of the experiment. Behavioural observations were made between the 28th of February until the 8th of May, (Zwiggelaar 1996 for overview). Between the 28th of March and the 26th of April each individual was caught once to take a blood sample for determination of the corticosterone levels. ✓: measured, 1: response 1 measured, 2: response 2 measured, 3: response 3 measured, Bin: stage of mask scored with a binocular, A: measured above nostrils, B: measured below nostrils, A+B: measured both above and below nostrils.

Date	Hematology				Ornaments			Body mass	Foot infections
	SRBC titer	WBC	Sedimentation	Hematocrit	Mask	Leg colour	Bill colour		
13/2					✓	✓	A	✓	
29/2					Bin				
12/3	✓	✓		✓	✓	✓	A	✓	✓
21/3					✓	✓	A	✓	
22/3	First immunisation								
28/3	1	✓		✓	✓	✓	✓	✓	✓
9/4					Bin				
19/4					Bin				
26/4	✓	✓	✓	✓	✓			✓	
2/5	2	✓	✓	✓	✓	✓	A+B	✓	✓
8/5	3	✓	✓	✓	✓	✓	A+B	✓	✓
7/6					✓	✓	B	✓	✓
11/6								✓	
30/7					Bin				
3/9					Bin				
14/10					Bin				
19/11					Bin				