Effects on domestication and feeding on the avian melanocortin system

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Title
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Sammanfattning
Domestication in chickens has made feed-restriction a necessity if broiler breeder hens should reach sexual maturity and be fertile. This is claimed to cause chronic hunger. To measure hunger the gene expression of the appetite regulators agouti-related peptide (AgRP), pro-opiomelanocortin (POMC), neuropeptide Y (NPY) and adenosine monophosphate-activated protein kinase (AMPK) of the melanocortin system was quantified with qPCR. This was done in feed-restricted Red Junglefowl and compared with the gene expression of two strains of feed-restricted broilers, Ross 308 and Rowan Ranger, to detect possible effects on domestication on appetite regulation. POMC-expression was upregulated 2-fold in the feed-restricted Red Junglefowl. POMC-expression was downregulated by half in the feed-restricted Ross 308. AgRP/NPY-expression was upregulated 4-fold in feed-restricted Rowan Rangers. A comparison between the control groups (ad libitum fed) of the breeds showed that the NPY-expression was lower in Ross 308 and Rowan Ranger compared with the ancestor. Results show no difference in body weight of ad libitum fed and feed-restricted Red Junglefowl. Conclusions were that the feed-restricted Red Junglefowl was not properly restricted in food supply since no difference in body weight between the treatment groups was detected. The upregulation of POMC in the feed-restricted Red Junglefowl could be stress-linked influenced by the feeding type (scattering of food in litter). No conclusions of the impact of domestication on chicken’s appetite could be drawn. Domestication has probably had its impact by altering other signaling pathways of the melanocortin system than in the arcuate nucleus.

Nyckelord
Feed-restriction, hunger, arcuate nucleus, gene expression, avian melanocortin system, domestication, broiler, Red Junglefowl, stress.
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1 Abstract
Domestication in chickens has made feed-restriction a necessity if broiler breeder hens should reach sexual maturity and be fertile. This is claimed to cause chronic hunger. To measure hunger the gene expression of the appetite regulators agouti-related peptide (AgRP), pro-opiomelanocortin (POMC), neuropeptide Y (NPY) and adenosine monophosphate-activated protein kinase (AMPK) of the melanocortin system was quantified with qPCR. This was done in feed-restricted Red Junglefowl and compared with the gene expression of two strains of feed-restricted broilers, Ross 308 and Rowan Ranger, to detect possible effects on domestication on appetite regulation. POMC-expression was upregulated 2-fold in the feed-restricted Red Junglefowl. POMC-expression was downregulated by half in the feed-restricted Ross 308. AgRP/NPY-expression was upregulated 4-fold in feed-restricted Rowan Rangers. A comparison between the control groups (ad libitum fed) of the breeds showed that the NPY-expression was lower in Ross 308 and Rowan Ranger compared with the ancestor. Results show no difference in body weight of ad libitum fed and feed-restricted Red Junglefowl. Conclusions were that the feed-restricted Red Junglefowl was not properly restricted in food supply since no difference in body weight between the treatment groups was detected. The upregulation of POMC in the feed-restricted Red Junglefowl could be stress-linked influenced by the feeding type (scattering of food in litter). No conclusions of the impact of domestication on chicken’s appetite could be drawn. Domestication has probably had its impact by altering other signaling pathways of the melanocortin system than in the arcuate nucleus.

2 Introduction
Domestication in chickens has led to a negative relationship between body weight and reproductive efficiency (Decuypere et al., 2010). In the meat industry, broilers are selected for high growth rate to make the production more effective. Meat-chickens are fed ad libitum, but the breeding generation needs to be restricted in order to ensure proper body development. Broilers are claimed to be chronically hungry due to increased motivation to eat compared with broilers fed ad libitum (D’Eath et al., 2009). With ad libitum feeding risks of developing abnormalities and mortality rate increases, which can happen before the chickens reach sexual maturity. The problem that broilers are claimed to be chronically hungry and that risks of developing abnormalities without feed-restriction is commonly referred to as “The Broiler Breeder Paradox” (Decuypere et al., 2010). According to commercial feeding regimes, studies have shown that the breeding population only gets to
consume a quarter of ad libitum intake (Savory et al, 1993). This results in a constant motivation to eat, which means that the breeding population is chronically hungry (Savory et al, 1993).

Hunger can be quantified by understanding the physiology of appetite. The hypothalamus controls hunger by regulating the energy homeostasis (Schwartz et al., 2000). After fasting, food intake must increase. The arcuate nucleus is the center of the hypothalamus that regulates hunger (Schwartz et al., 2000). Adiposity signals regulates either anabolic or catabolic peptides. Variants of neurons in the arcuate nucleus activates or gets inhibited depending on the type of signal. The neurons releases hormones that controls the energy homeostasis (Schwartz et al., 2000). Neuropeptide Y (NPY) and Agouti-related peptide (AgRP) are specifically co-localized in neurons from the arcuate nucleus which increase appetite when stimulated by releasing NPY and AgRP hormones (Schwartz et al., 2000). Melanocortins and its precursor pro-opiomelanocortin (POMC) suppresses food intake (Schwartz et al., 2000). The specific melanocortin controlling food intake is α-Melanocyte-stimulating hormone (α-MSH). Another peptide inhibiting food intake is cocaine- and amphetamine-regulated transcript (CART) (Schwartz et al., 2000). To regulate cellular signaling, adenosine monophosphate-activated protein kinase (AMPK) activates when AMP levels are high to increase catabolism (Lei and Lixian, 2012).

The avian melanocortin system appears to regulate hunger differently than the mammalian system does (Dunn and Boswell, 2015). The latest research suggests that birds have a more short-term influence on regulation of the energy balance compared with mammals. In mammals AgRP and POMC are long-term regulators (Dunn and Boswell, 2015). Studies have presented various results of gene expression of the regulators of the avian melanocortin system over the years, but the trend seems to be that AgRP expression is upregulated during feed-restriction (Dunn and Boswell, 2015). In that manner, Dunn et al (2013) suggested that AgRP is more important for maintaining energy balance in birds compared with anorexic regulators as POMC.

To improve the welfare of broilers, the industry is trying to find solutions to reduce frustration and suffering. Feed-restriction can be done by reducing the quantity or quality of the food and there are arguments about which of the alternatives leads to a better welfare (D´Eath et al., 2009). The discussion is mostly about how different types of feeding can reduce frustration in birds. Frustration can be shown by developing of stereotypic behavior such as object pecking (Dixon 2008). By studying behavior and the melanocortin system based on different feeding types
the understanding of hunger can be improved. To reduce the fast growth rate for broilers, a new breed was launched a couple a years ago (Clarke 2014). The Rowan Ranger is a slow-growing broiler intended for the organic food industry (Clarke 2014). Despite the slower growth rate of the Rowan Ranger, feed-restriction for the breeder hens are still recommended according to the commercial feeding regimes for regular broilers (Clarke 2014).

In this study the melanocortin system of the ancestor of domesticated chickens, the Red Junglefowl, was studied by quantifying gene expression of its main regulators. The gene expression of the anorexic regulators (POMC and CART), the orexigenic regulators (NPY and AgRP) and the catalyst AMPK from the arcuate nucleus with effect on appetite was studied. By analyzing the expression of regulators from the arcuate nucleus, changes in hunger regulation can be detected. The results was compared with gene expression of POMC, AGRP, NPY and AMPK the arcuate nucleus from two domesticated strains of broilers, Ross 308 broilers and the slow-growing Rowan Rangers. Ross 308 is widely used for breeding (Sandilands et al., 2005) and therefore used in studies concerning feed restriction. By comparing the gene expression of appetite regulators in the arcuate nucleus of the ancestor of chickens with the gene expression of two strains of domesticated broilers, perhaps a difference in appetite could be detected. Detection of differentiating appetite between the ancestor and the broilers could then be possibly be explained by domestication. All of the breeds were divided into two groups where one was fed ad libitum and the other was feed-restricted according to commercial feeding regimes for broiler breeder populations. It was hypothesized that the feed-restricted chickens would have an increased AgRP/NPY-expression as Dunn et al. (2013) suggested.

3 Material & methods

3.1 Origin of samples

Ten, five week old Red Junglefowl chickens (Gallus gallus), where of five were fed ad libitum and five were feed-restricted, were used in this study. The feed-restricted animals had been fed according to commercial feeding programs for broiler breeder chickens. The ad libitum fed chickens had food available in a feeder. The feed restricted chickens was given food spread in the litter (on the floor) once a day. The feed-restriction started at day 25 where the birds was given 140 g of food to share. For every day, until day 35, the total food amount was increased with 5 g. The weights of the animals was noted seven times during the 35 days of life.
The chickens were euthanized at day 35 and the arcuate nucleus was extracted through dissection and frozen in liquid nitrogen.

The arcuate nucleus is located in the hypothalamus of the brain. In figure 1 the location of the arcuate nucleus in the chicken brain is shown.

Figure 1. An illustration of the chicken brain. The location of the arcuate nucleus is marked with a black box. Figure reproduced from Puelle et al 2007.

The gene expression of the appetite regulators POMC, AgRP, NPY and AMPK was of interest in this study to quantify hunger in feed-restricted chickens. By quantification of expression of the regulators, the appetite could be measured relatively to the ad libitum fed chickens. As reference gene Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used. The expression of the gene CART was also included in the study, but due to a small amount of tissue from the arcuate nucleus the efficiency of the primers could not be analysed. There was only enough material of the standard dilution series to analyse the efficiency of POMC, AgRP, NPY and AMPK.

The gene expression data from two broiler strains Ross 308 and Rowan Rangers was obtained from work in progress in my supervisor’s lab for comparison with the ancestor. A comparison was done to detect the possible effects on appetite regulators by domestication. The expression of the CART gene was not included in the collected data and therefore not included in the comparison between the breeds. The feed-restricted
chickens from both breeds had been fed according to commercial feeding regimes for broiler breeders. Both of the breeds were given the same amount of food as the Red Junglefowl.

RNA-extraction using RNAqueous®-Micro Total RNA Isolation Kit was done from the tissue according the accompanying manual (Thermofisher scientific). RNA-concentration was determined using a NanoDrop® ND-100 Spectrophotometer. The RNA-samples were stored at -80°C until cDNA-synthesis.

The primers for AMPK, NPY, AgRP and POMC used was ordered according to sequences found in a paper by Lei and Lixian (Lei and Lixian, 2012). The rest of the primers were designed using NCBI-blast primer designing tool. The functioning primer sequences are presented in Table 1 below. The efficiency of the primers (not CARTPT1 and CARTPT2) were tested using a standard curve before the qPCR-analysis.

**Table 1. Sequence of the primers used for amplification in the qPCR**

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Forward sequence (5’ to 3’)</th>
<th>Reverse sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMPKa2</td>
<td>GGGACCTGAAACCAGAGAACG</td>
<td>ACAGAGGAGGGCATAGAGGATG</td>
</tr>
<tr>
<td>NPY</td>
<td>GAGGCACACATCAACCTCATCAC</td>
<td>TGGTTTCTGTGCTTTCCCTCAAA</td>
</tr>
<tr>
<td>AgRP</td>
<td>GGAACCGCAGGGCATTGTC</td>
<td>GTAGCAGAAGGCCTGAAGAA</td>
</tr>
<tr>
<td>POMC</td>
<td>CGCTACGGCGCGCTTCA</td>
<td>TCTTGTAGGCGGCTTTGACGAT</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GTCAAGGCTGAGAACCGGAA</td>
<td>GCCCATTTGATGTTGCTGGG</td>
</tr>
<tr>
<td>CARTPT1</td>
<td>GTCACTTTAGCGGAGGCCACC</td>
<td>GGTAGTTTGCAACGCATCCA</td>
</tr>
<tr>
<td>CARTPT2</td>
<td>GATATCCAGCCGCCTCAAC</td>
<td>TACCTGCGGAGGACAGACAT</td>
</tr>
</tbody>
</table>

**3.2 cDNA-synthesis**

As a preparation step all genomic DNA was removed from the samples. This was done by mixing 0.5 µg of Total RNA (see appendix), 1 µl of 10X Reaction Buffer (with MgCl₂), 1 µl of DNase I (RNase-free) and water (nuclease-free) up to 10 µl in a RNase-free tube. The tube was then incubated at 37 °C for 30 minutes. 1 µl 50 mM EDTA was added and incubated at 65 °C for 10 minutes. The RNA-samples were placed on ice.
A master mix was done in a sterile, nuclease-free tube on ice by adding 1 µl Oligo(dT)primer, 4 µl 5X Reaction Buffer, 0.5 µl Ribolyck RNase Inhibitor (40 u/µl), 2 µl 10 mM dNTP Mix, 1 µl RevertAid M-MuLV Rev Transcriptase (200 u/µl) and the total volume of the RNA-sample (11µl). A master mix was prepared for (x+2) in case of pipetting errors. 8.5 µl of the master mix was added to each RNA-sample. The tubes were incubated at 42 °C for 60 minutes. The reaction was terminated by heating the samples at 70 °C for 5 minutes. The cDNA-samples were stored at -80°C between laboratorial use.

3.3 Primer efficiency

Using a standard curve the efficiency of the primers could be determined. By pooling 2 µl from every cDNA-sample, a dilution series with dilution factor 1:4 was done (1/4 – 1/8 – 1/32 – 1/128). For dilution, nuclease-free water was used. The qPCR was run and the efficiency for each dilution was measured by using LightCycler® 480 Software (release 1.5.0 SP4). Standard efficiency is between 1.8-2.1. The annealing temperature most efficient for the primers used in this study is 60 °C.

3.4 qPCR

For each gene every sample was replicated three times for the run in the qPCR. For every run per gene, the standard series was included to calculate the efficiency of the primers. Three NTC:s were run per gene. The master mix was prepared for (x+3) reactions in case of pipetting errors. For each reaction 3 µl water (nuclease-free), 0.5 µl forward primer, 0.5 µl reverse primer and 5 µl SYBR green Master Mix was put into the master mix. 1 µl of template was used in each reaction. Nuclease-free water was used as template for the NTC:s. The cDNA was diluted 1:12. The annealing temperature was set to 60 °C. The run of the template was set to 40 cycles.

3.5 Statistical analysis

The gene expression was quantified using the Pfaffl-method (Pfaffl, 2004). The method uses the efficiency calculated by the Lightcycler machine and gives a relative quantification of the changes of mRNA-levels. The quantification of the CART-gene was done by using the Livak and Schmittgen method (Livak and Schmittgen, 2001). The Livak and Schmittgen method assumes that the efficiency of the primer is 100%. The Livak and Schmittgen method was used on the CART-gene because the efficiency could not be calculated due to restricted sample volumes. The quantification of the gene expression was normalized against the reference gene, GAPDH. The ratio between the target gene and the reference gene was used for comparison between the treatment groups.
The samples were run in triplets in the qPCR. The mean value of the CT (cycle threshold) of the triplets was used for calculating the expression levels.

The statistics was done by using Minitab® 17 software. A 2-paired t-test was done comparing the two treatment groups of the Red Junglefowl because the weight curves (see figure 2) aligned. For the gene expression of Red Junglefowl, Ross 308 and Rowan Ranger a comparison of the treatment groups (feed-restricted and ad libitum) was done with a one-way ANOVA analysis. To compare the different breeds, the ad libitum fed Red Junglefowl was used as a control group and set to 1. For comparison between the breeds and treatments a two-way ANOVA analysis was done. If significant differences between breeds were observed, a Tukey comparison test was included. For comparison between breeds in the ad libitum fed chickens a one-way ANOVA analysis combined with a Tukey-test was done.

4 Results

4.1 Body weights

![Figure 2. Weight curves of the mean weights of the breeds. The grey line indicates the start of treatment where the animals were separated with different feeding types. AL stands for ad libitum and these birds had access to food through a feeder at all times. FR stands for feed-restricted and these birds was fed once a day through scattering of the food. Ross is Ross 308 broilers, Rangers is Rowan Rangers and RJF is Red Junglefowl.](image-url)
The Red Junglefowls did not differ in body weights between the two treatment groups. Figure 2 below presents all of the animals weights at day 35. The 2-paired t-test showed no significant difference in body weight between treatment groups in Red Junglefowl (T-Value=0.7, P-Value=0.797).

![Graph showing body weights at day 35](image)

*Figure 3. The body weights at day 35. The mean of the body weights is presented with 95% confidence interval. AL stands for ad libitum and these birds had access to food through a feeder at all times. FR stands for feed-restricted and these birds were fed once a day through scattering of the food.Ross is Ross 308 broilers, Rangers is Rowan Rangers and RJF is Red Junglefowl. NS stands for non-significant.*

### 4.2 Gene expression of Red Junglefowl

The one-way ANOVA analysis of Red Junglefowl showed that feed-restricted Red Junglefowls have an increased expression of POMC (F=13.35, P=0.008).

![Graph showing gene expression](image)
Figure 4. The relative gene expression of Red Junglefowl normalized with GAPDH mRNA. The mean ratio of ad libitum is set to 1 with included standard deviation. The mean ratio of feed-restricted is presented with a 95 % confidence interval. (A) AMPK mRNA-expression, (B) POMC mRNA-expression with a significant difference (p<0.05), (C) AgRP mRNA-expression, (D) NPY mRNA-expression, (E) CART1 mRNA-expression, (F) CART2 mRNA-expression
4.3 Gene expression of Ross 308 broiler
The one-way ANOVA analysis of Ross 308 broiler showed that feed-restricted Ross 308 has a downregulated expression of POMC (F=8.70, P=0.026).

Figure 5. The relative gene expression of Ross 308 normalized with GAPDH mRNA. The mean ratio of ad libitum is set to 1 with included standard deviation. The mean ratio of feed-restricted is presented with a 95% confidence interval. (A) AMPK mRNA-expression, (B) POMC mRNA-expression with a significant difference (p<0.05), (C) AgRP mRNA-expression, (D) NPY mRNA-expression
4.4 Gene expression of Rowan Ranger broiler

The one-way ANOVA analysis of Rowan Ranger broiler showed that feed-restricted Rowan Rangers have an increased expression of AgRP (F=5.80, P=0.047) and NPY (F=7.92, P=0.026).

![Graphs showing gene expression of AMPK, POMC, AgRP, and NPY](image)

Figure 6. The relative gene expression of Rowan Ranger normalized with GAPDH mRNA. The mean ratio of ad libitum is set to 1 with included standard deviation. The mean ratio of feed-restricted is presented with a 95 % confidence interval. (A) AMPK mRNA-expression, (B) POMC mRNA-expression, (C) AgRP mRNA-expression with a significant difference (p<0.05), (D) NPY mRNA-expression with a significant difference (p<0.05)
4.5 Comparison between breeds and treatment

The comparison between the breeds and the treatments the two-way ANOVA analysis showed differences between breeds in AMPK (F=8.4, P=0.002), NPY (F=10.35, P=0.001) and POMC (F=10.29, P=0.001). A Tukey-test showed that the Red Junglefowl was the deviating group. This means that the gene expression of AMPK, NPY and POMC is higher in Red Junglefowl compared with Ross 308 and Rowan Rangers.

Between treatments there were differences in NPY and AgRP. This means that the gene expression of NPY (F=4.32, P=0.049) and AgRP (F=7.62, P=0.011) is upregulated in the feed-restricted chickens.
Figure 7. The relative gene expression of all breeds normalized with GAPDH mRNA. The mean ratio of Red Junglefowl ad libitum is set to 1 with included standard deviation. The mean ratio of feed-restricted and Ross and Rangers ad libitum is presented with a 95 \% confidence interval. (A) AMPK mRNA-expression, (B) POMC mRNA-expression, (C) AgRP mRNA-expression, (D) NPY mRNA-expression. \textquoteleft{*}\textquoteleft stands for a significant difference (P<0.05) and NS stand for non-significant.
4.6 Comparison between breeds in ad libitum fed groups

Since there was no difference in body weight between the ad libitum fed and feed-restricted Red Junglefowl, the commercial feeding regimes for feed-restricted broilers is probably too abundant for proper feed-restriction for Red Junglefowls. A comparison between the breeds in the groups where it was certain the same treatment was given, the ad libitum fed chickens, is therefore a better approach for investigating the effect of domestication on hunger. The one-way ANOVA analysis combined with Tukey comparison showed that gene expression of NPY of Ross 308 and Rowan Rangers was downregulated compared to the Red Junglefowl (F=10.47, P=0.003).

![Figure 8](image_url)

**Figure 8.** The relative gene expression of all ad libitum fed breeds normalized with GAPDH mRNA. The target and reference gene expression ratio of Red Junglefowl has been set to 1 for comparison with included standard deviation. The mean ratio of Ross and Rangers is presented with a 95 % confidence interval. (A) AMPK mRNA-expression, (B) POMC mRNA-expression, (C) AgRP mRNA-expression, (D) NPY mRNA-expression. ‘*’ stands for a significant difference (P<0.05).
5 Discussion

This study showed that feed-restricted broiler chickens had an increased AgRP/NPY gene expression while the ancestor, the Red Junglefowl did not. A plausible explanation to this disagreement is that the body weights of the Red Junglefowl could not ensure that the feed-restricted group was properly restricted there was because no difference between the body weights of the treatment groups. Therefore a comparison between the gene expression results of the feed-restricted Red Junglefowl and the feed-restricted broiler strains could not detect changes in appetite due to domestication. It was not known if the feed-restriction treatment had triggered hunger in the Red Junglefowl. A comparison between the breeds of the ad libitum fed groups showed a higher gene expression of NPY in Red Junglefowl.

For the broilers, Ross 308 and Rowan Ranger, the feed-restriction results in smaller body weights compared to ad libitum fed chickens. Interestingly, the ad libitum fed Red Junglefowls are not larger than the feed-restricted group. What needs to be emphasized in this study is that the feed-restricted Red Junglefowls, that were fed once a day, were fed according to commercial feeding regimes for broiler breeder chickens. Broiler breeder chickens are heavily feed-restricted to ensure that they reach sexual maturity (Decuypere et al., 2010). They are strongly selected to have a fast and high growth rate which increases risks of abnormalities and death before sexual maturity is reached (Decuypere et al., 2010). The egg-production is also more effective during feed restriction (Zuidhof et al., 2007). Red Junglefowl differs a lot in body weight compared with Ross 308 and Rowan Ranger. As shown in figure 2, the broiler strains weighed more than double as much as the Red Junglefowl. The Red Junglefowl is the ancestor of broilers and reaches sexual maturity by being fed ad libitum (Schütz et al., 2002). Therefore, commercial regimes for feed restriction are not needed for the Red Junglefowl. If feed-restriction did not occur, a more interestingly approach to discuss regarding the results of the Red Junglefowl is therefore the two types of feeding; to place food in a feeder (ad libitum) or spreading it on the floor once a day (feed-restriction).

Recently a study was done regarding which type of feeders broiler chickens preferred (Neves et al., 2015). The results suggested that broilers preferred feeders with a more open feeding area. When the area for food access was larger, the animals ate more. Interestingly, the same amount of animals positioned around the feeder with large access-area were positioned around the feeder with the restricted feeding area. The difference was the food intake. Perhaps the social behavior needs to be
taken into consideration in this study. In the study by Neves et al (2015), the birds positioned around the feeder with large access-area to food presumably ate more because more birds could reach the food. Results have indicated that broilers do exhibit social facilitation in feeding (Collins and Sumpter, 2007). The probability that more birds eats rises when the number of birds located/eating by the feeder increases (Collins and Sumpter, 2007). Scattering of the food can reduce, or even eliminate, stereotypic behavior as object pecking (Dixon 2008). Object pecking may be an indication of hunger and frustration. A study by Jong et al (2005) showed that object pecking was reduced by scattering the food in the litter, but no conclusions of the impact it had on the welfare of the animals could be drawn based on their study.

According to the results of the Red Junglefowls, only the expression of the gene pro-opiomelanocortin (POMC) of the genes included in this study is upregulated when Red Junglefowls are feed-restricted. POMC is an antagonist for appetite increasing agouti-related peptide (AgRP) (Song et al., 2013). In mammals it is well known that upregulation of POMC has an anorexic effect for the energy homeostasis (Cone 2005). The arcuate nucleus with neurons co-expressing neuropeptide Y (NPY) and AgRP or POMC is well studied in the mammalian central melanocortin system (Cone 2005). In birds, the understanding of the regulation of the energy homeostasis needs to be improved (Boswell and Dunn, 2015). The results may indicate that the feed-restricted Red Junglefowl has a decreased appetite compared to the ad libitum fed group. According to the results from this study, scattering of the food then reduces hunger. But, the the fact that the treatment groups of Red Junglefowl do not differ in body weight, suggest an equal hunger state in the two different treatment groups (ad libitum fed and feed-restricted). Another explanation could be that the body weights did not differ because the feed-restricted Red Junglefowls had not been properly restricted as mentioned earlier. To know if the Red Junglefowls were feed-restricted, measuring of the food consumption would have been needed.

Similar to the results in this study, Dunn et al published a paper (2013) presenting results where feed-restricted broilers showed a non-significant increase of POMC expression in the arcuate nucleus. If POMC is an anorexic regulator of energy homeostasis in chickens, the expression of POMC in feed-restricted animals should decrease. Dunn et al (2013) suggests that AgRP and NPY instead provides the main regulation of energy homeostasis in chickens because of the various results given by different studies. That AgRP/NPY is the main regulator of appetite in birds is aligned with the results of the gene expression of Rowan Ranger.
Some studies have shown a decreased or unchanged expression of POMC. Phillips-Singh et al (2003) showed results where no change in POMC regulation was obtained in feed-restricted Japanese quail. Unaffected POMC regulation was also found by Song et al (2012) in broiler chickens. As Dunn et al did (2013), Song et al (2012) also suggest that AgRP/NPY has the biggest role in mediation of the energy homeostasis in chickens. Higgins et al (2010) could show a non-significant decrease of POMC expression in broilers after feed-restriction. Hen et al (2006) compared layer chicks with broiler chicks and found a significant decrease POMC in both groups after 7 days of feed-restriction. Noteworthy with the study by Hen et al is that the sample sizes were quite small. Similar to earlier studies (Higgins et al., 2010) (Hen et al., 2006), the POMC in the feed-restricted Ross 308 broilers was decreased. The variation of results found in POMC expression in different studies could be an indication that the upregulation of POMC expression in Red Junglefowl fed by scattering of food, is not an indication of differing in appetite. Suggestions, according to Dunn et al (2013), would instead be that the appetite between the two treatment groups do not differ due to no change in AgRP/NPY expression levels. Dunn et al’s (2013) statement corresponds with the results of the gene expression of the Rowan Rangers broilers in this study where AgRP and NPY was upregulated 4-fold in the feed-restricted chickens while POMC-expression showed no difference.

CART inhibits food intake in mammals (Kristensen et al., 1998) and later it was also shown by injection of the peptide that it inhibited food intake in chickens (Tachibana et al., 2003). CART expression is included in food deprivation studies, but as with POMC, no conclusion of its impact on regulation of food intake can be drawn (Dunn et al., 2013) (Song et al., 2012). No study was found where RNA-extraction from only the arcuate nucleus of the hypothalamus had been done. For example, Dunn et al (2013) and Song et al (2012) dissected out other parts of or the whole hypothalamus for RNA-extraction in their studies. This study could then confirm that CART is expressed in the arcuate nucleus.

The increasing POMC expression in the Red Junglefowl that was fed once a day was perhaps due to stress factors of the scattered food. A stress factor could be the competition of food that arises between the individuals or that the Red Junglefowls are not used to eat food from the floor. Elevated POMC expression is coupled with an anorexic response due to stress (Liu et al., 2007). In mammals it has been shown that stress causes an activation of POMC neurons and results in a decreased food intake (Liu et al., 2007). The same results was showed in birds where stress resulted in a voluntary food-restriction (Boswell and Dunn, 2015).
But, the study of voluntarily decreased food intake in birds could not indicate rise of the corticosterone levels (Boswell and Dunn, 2015). In mammals it was found that the decreased food intake and rising POMC-expression was coupled with increasing levels of cortisol (Liu et al., 2007). The upregulation of POMC in the feed-restricted Red Junglefowls may be stress induced, but the corticosterone levels and the food intake was not measured. Therefore no conclusions can be drawn if the upregulation is due to stress. In this study feed-restriction in the Red Junglefowls cannot be ensured. Also, the chickens of the treatment groups had the same body weight, which could indicates that the feed-restricted Red Junglefowls did not voluntarily reduce their food intake due to stress. If they reduced their food intake, the ad libitum fed chickens must have reduced their food intake as well since there was no difference in body weights between the treatment groups. If upregulation of POMC is stress induced, then the lowering of food intake in the ad libitum fed chickens would not be due to stress. A better control of the food intake was needed in this study.

As mentioned earlier, Collins and Sumpter (2007), found that broilers ate more when the number of actively eating chickens around them increased. If the individuals of the treatment groups of the Red Junglefowl ate the same amount of food, this means that the ad libitum fed chickens did not increase their food intake due to social behavior. This conclusion can only be drawn if the feed-restricted chickens were not feed-restricted and if they consumed the same amount of food as the ad libitum fed chickens. Supposed that the Red Junglefowls ate according to the conclusions, the social eating found in broilers could be a behavior evolved by domestication.

Comparison between the ad libitum fed breeds showed that the NPY-expression was lower in Ross 308 and Rangers compared with Red Junglefowl in the ad libitum fed groups. If Dunn et al’s statement (2013) that energy regulation for increasing appetite is mainly driven by the AgRP/NPY pathway is accurate, the downregulation of NPY-expression in Ross 308 and Rowan Rangers indicates that Red Junglefowl are hungrier than the domesticated chicken. But, since AgRP-expression did not correspond with the differing response of the NPY-expression, no conclusions of how appetite is affected by domestication could be done. AgRP/NPY are co-expressed neurons in the arcuate nucleus. Further studies are needed to see how the appetite of chickens has been affected by domestication. To improve the understanding of appetite the peripheral regulation of appetite can be studied. The peripheral regulation is controlled by signaling from tissues. Leptin is a hormone secreted from
adipose tissue (Schwartz et al., 2000). When the amount of adipose cells increase, the levels of the hormone secretion increases. This leads to a stimulation of the arcuate nucleus which results in an inhibition of food intake (POMC levels increase) (Schwartz et al., 2000). There has not fully been established if the LEP-gene exist in birds. LEP-like genes have been found and sequenced, but the tissue distribution has only been studied in some avian species (Boswell and Dunn, 2015). The expression of the LEP-like gene was low or absent in adipose tissue, but could be localized in the liver and gonads. The leptin receptor was most abundant in the brain and pituitary gland. This may give the information that leptin does not regulate the energy homeostasis as in mammals and instead targets the pituitary-gonads function (Boswell and Dunn, 2015). Even if the results indicates that the avian leptin has no regulatory impact of the energy intake, studies have shown that injection of mammalian leptin inhibits food intake (Boswell and Dunn, 2015). It is also suggested that domestication has led to a more ineffective role of leptin since most studies have been done in chickens, but that can only be true if leptin has a role in regulation of the energy homeostasis (Boswell and Dunn, 2015). If domestication had impact of the role of leptin it would be interesting to investigate its role in the ancestor of the domesticated chicken, the Red Junglefowl. The current understanding of the regulation of the appetite in birds is that it is not elevated leptin levels that reduces appetite. Instead it is the peptide cholecystokinin (CCK) that signals high energy levels and inhibits short-term food intake (Boswell and Dunn, 2015). A study by Dunn et al. (2013) presented a suggestion that it is presumably the CCK-receptor that has been suppressed by domestication. The study compared birds with high-growth allele to low-growth allele. The results showed an elevated CCK signaling in the birds with the high-growth allele combined with elevated AgRP-levels, which was not the case in the birds with the low-growth allele. This concluded that the CCK-receptor probably was suppressed due to domestication.

5.1 Conclusions
This study provides a better understanding of the effects on the melanocortin system in birds. The increasing POMC expression of the Red Junglefowl that were fed once a day, could indicate that regulation of the melanocortin system is stress-linked. The results also indicates that the ancestor of the domesticated chicken had a higher appetite due to an increased level of NPY expression, but no conclusions can be made since AgRP was not elevated as well. Despite the higher appetite, the weight of the ad libitum fed Red Junglefowls did not differ with the weight of the Red Junglefowls that was fed once a day. A behavioral study combined with measuring of the food intake would therefore be helpful in the future
to further investigate how domestication has affected hunger in chickens. 
Lastly, a conformation of that AgRP/NPY neurons are the main regulator 
of appetite, as Dunn et al. (2013) stated, can be done according to the 
results of the Rowan Ranger broilers and by the comparison between all 
breeds and treatments

5.2 Societal and ethical aspects
Bettering of the understanding of hunger in chickens will help improve 
welfare in the chicken industry. Considering both the behaviourally and 
physiologically impacts of feed-restriction in chickens, the hunger of the 
chickens will be better understood. By studying the impact of 
domestication on appetite in chickens, the industry could get better 
information of how domesticated chickens regulates appetite compared 
with its ancestor. If it could be localized where the domestication has had 
its impact in the genome, birds with better habituation to hunger could be 
produced by selective breeding. This will help reduce suffering in 
chickens and could also results in reduced stress levels and stereotypic 
behaviour.

Killing and dissections of the animals were done by or under supervising 
of educated personnel. The dissection of the arcuate nucleus is critical 
due to its small size. By performing the dissections under supervising or 
by educated personnel, the risk of damaging the tissue was reduced.

6 Acknowledgement
I would like thank my supervisor Jordi Altimiras who provided the 
opportunity for me to perform this study. I also want to thank Petros 
Batakis for all the help with the laboratory work.

7 References
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8 Appendix

The RNA-concentration of the arcuate nucleus was determined using a NanoDrop® ND-100 Spectrophotometer.

Table 1. RNA-content and volumes used for qPCR reactions

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sample ID</th>
<th>RNA-concentration (ng/µl)</th>
<th>Volume for cDNA-synthesis (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feed restricted</td>
<td>60367</td>
<td>82</td>
<td>6,1</td>
</tr>
<tr>
<td>Feed restricted</td>
<td>60368</td>
<td>181</td>
<td>2,8</td>
</tr>
<tr>
<td>Feed restricted</td>
<td>60370</td>
<td>144</td>
<td>3,5</td>
</tr>
<tr>
<td>Feed restricted</td>
<td>60373</td>
<td>92</td>
<td>5,4</td>
</tr>
<tr>
<td>Ad libitum</td>
<td>60364</td>
<td>122</td>
<td>4,1</td>
</tr>
<tr>
<td>Ad libitum</td>
<td>60365</td>
<td>142</td>
<td>3,5</td>
</tr>
<tr>
<td>Ad libitum</td>
<td>60369</td>
<td>121</td>
<td>4,1</td>
</tr>
<tr>
<td>Ad libitum</td>
<td>60371</td>
<td>153</td>
<td>3,3</td>
</tr>
<tr>
<td>Ad libitum</td>
<td>60372</td>
<td>118</td>
<td>4,2</td>
</tr>
</tbody>
</table>

Primer efficiency for each gene was determined by calculating a standard curve using a dilution series of pooled cDNA from every sample. After the first run of every gene two samples were considered as outliers. The two samples ID were 60373 and 60372. Another qPCR-run was done on the two samples. The efficiency of the primers in the re-run differed a bit. The efficiency for sample ID 60373 and 60372 were only used for calculations of the relative expression of those two samples. The annealing temperature set for all primers was 60°C.

Table 2. Efficiency of the primers

<table>
<thead>
<tr>
<th>Primers</th>
<th>Efficiency</th>
<th>Efficiency for Sample ID 60373 and 60372</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>2,17</td>
<td>1,91</td>
</tr>
<tr>
<td>AgRP</td>
<td>1,88</td>
<td>1,87</td>
</tr>
<tr>
<td>NPY</td>
<td>2,01</td>
<td>2,05</td>
</tr>
<tr>
<td>POMC</td>
<td>2,09</td>
<td>2,01</td>
</tr>
<tr>
<td>AMPKα</td>
<td>1,99</td>
<td>2,15</td>
</tr>
</tbody>
</table>

Due to restricted sample volumes, the efficiency of the CARTPT1- and CARTPT2-primers could not be calculated.