Quantification and localisation of StAR expression in Atlantic cod ovaries

Master thesis

Aquaculture

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June 2009

Acknowledgements

The work of this thesis was carried out at the Institute of Marine Research, Bergen.

First of all, I would like to thank my supervisors, Eva Andersson, Birgitta Norberg and Ian Mayer, for providing guideline and valuable feedback on my work throughout the study. A special thank to Eva, for teaching me about the field of quantification and localization of gene expression, and for having numerous of valuable discussions with me. I would also like to express my gratitude to Geir Lasse Taranger, who not only helped with statistics, but also contributed with feedback on my work.

Thank you Elin Sørhus, for participating in my sampling, teaching me how to perform the laboratory methods, and always being willing to help whenever I had a question. I also appreciate the help from Ørjan Karlsen with catching of the large broodstock cod, and the valuable advices from Ingrid Uglenes Fiksdal and Anne Torsvik, concerning the histological part of my laboratory work.

All the people that work in the laboratory of molecular biology, thank you all for maintaining a positive environment.

Cathrine Kristoffersen, thank you for your encouragement and all the laughs and conversations in our office.

And off course: thank you all my friends and family for always being there for me.

Lene Kleppe

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Abbreviations

AcAh-TEA	Acetic anhydride/TEA
ACTH	Adrenocorticotropic hormone
ARA	Arachidonic acid
BCIP	5-bromo-4-chloro-3-indolyl-phosphate-4-toluidine salt
bp	Base pairs
BPG	Brain-pituitary-gonad
BSA	Biovine serum albumin
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary/copy DNA
cRNA	RNA obtained from cDNA
Ct	Cycle times
ΔCt	Normalized cycle times
ΔΔCt	The calibrated Ct value
CV	Coefficient of variation
DEPC	Diethyl pyrocarbonate
DIG	Digoxigenin
DNA	Deoxyribonucleic acid
dNTPs	Nucleotide triphosphates
EDTA	Ethylenediamine tetra acetic acid
EtOH	Ethanol
E2	Estradiol-17β
FSH	Follicle stimulating hormone
GSI	Gonadosomatic index
IMR	Institute of Marine Research
ISH	In Situ Hybridization
kDa	Kilo Dalton
LB-medium	Luria-Bertani-medium
LH	Luteinizing hormone
LL	Continuous artificial light
mRNA	Messenger RNA
MIH	Maturatin inducing hormone
NBT	Nitroblue tetrazolium chloride
NIFES	National Institute for Nutrition- and Seafood Research
NTC	No template control
OD260	Optical Density readings at 260 nm
ORF	Open reading frame
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
qPCR	Quantitative real-time PCR
RNA	Ribunucleic acid

RPM	Revolutions per minute
StAR protein	Steroidogenic acute regulatory protein
TE	Tris-HCl/EDTA
TEA	Triethanolamin/DEPC-H2O

Abstract.

Steroidogenesis is essential for the production of sex steroids, and therefore also the sexual maturation process. The rate-limiting step in steroidogenesis is the movement of cholesterol from the outer to the inner mitochondrial membrane, a process that is controlled by the steroidogenic acute regulatory (StAR) protein. To date, limited information exists about StAR expression and regulation.

Today's cod farming industry is facing different challenges, among them are problems associated with final gonad maturation and spawning, especially in females. Numerous factors may affect this, and suboptimal nutrition is suggested as one of them. One ingredient in cod broodstock feed that an optimal amount is not yet established for is Arachidonic acid (ARA). In addition to affect different aspects of spawning results, ARA is suggested to be involved in the regulation of steroid production.

In this thesis work StAR transcripts (mRNA) in cod ovaries were quantified over a whole reproductive cycle to investigate if the transcript levels changed over time, and to check if they showed a connection with the amount of ARA in the diet; this was performed by applying quantitative real-time PCR (qPCR). Furthermore, StAR mRNA was localized in ovaries by applying *In Situ* hybridization.

The samples used for quantification of StAR originated from cod from a previous experiment at the Institute of Marine Research (IMR), Austevoll, where they were fed different amounts of dietary ARA; 0.5, 1, 2 and 4% ARA of total fatty acids. StAR expression levels showed the same general pattern in all groups, with a peak during the spawning season (February and March 2006), but also elevated and variable values after their first spawning season (June 2005). There were no significant differences in StAR expression between groups except in July ($p = 0.03485$) and January 2005 ($p = 0.00349$), but which group was different

could only be revealed for January; here fish fed 0.5% ARA had significantly higher StAR expression than fish fed 4% ARA. Fish used for localization of StAR were reared at IMR, Austevoll, and randomly sampled during the thesis study. No StAR expression was detected in follicles of early primary growth (early previtellogenic). However, in both late primary growth (late previtellogenic)-, and late vitellogenic follicles StAR was detected in the ooplasm. Furthermore, StAR was detected in granulosa- and theca cells of late vitellogenic follicles.

These findings suggest that StAR expression levels change over a reproductive cycle in cod, with a peak in the spawning months February and March. Furthermore, ARA may affect StAR expression levels in early spawning. Additionally, StAR expression changes with degree of follicle development.

1. Introduction.

1.1 Today's cod farming industry and some of its challenges.

The Atlantic cod (*Gadus morhua L.*) is one of the major new species in marine aquaculture. During the last few years the cod farming industry has grown markedly, notably in Norway. In 2007 about 13 million hatchery-produced cod were stocked for marine farming in Norway, representing an increase of around 5 million individuals from 2006 (Kyst og havbruk, 2008). However, today's cod farming is not without challenges, among them are continuing problems that occur during final gonad maturation and spawning, especially in females. Problems associated with spawning irregularities and high mortality in female cod represent a major production limitation and welfare problem in cod farming. Different causal factors have been suggested for this reproductive dysfunction, including unfavourable/high water temperatures, stress during handling, uneven female/male ratios, suboptimal feeding regimes and impaired/disrupted spawning behaviour (Birgitta Norberg, Institute of Marine research (IMR), Austevoll, Norway, pers. comm.).

The development of a successful aquaculture industry of new species requires the optimisation of the different parts in the production cycle, including broodstock management and juvenile production. Nutrition has been recognized as one of the key components of both broodstock management and juvenile production. Optimal nutrition is required to ensure good fish health, production efficiency and gamete quality, and has also impact on fish welfare. In spite of this, the information concerning effects of the broodstock's feed composition on reproductive physiology and performance is still scarce.

1.2 Steroid production and StAR.

Steroid hormones are a group of physiologically vital body components, produced through a process called steroidogenesis in specific body tissues. Several types of steroid hormones exist, and they all have different functions, including regulation of carbohydrate metabolism and stress responses, regulation of salt balance, maintenance of blood pressure, and being vital components for reproductive function, fertility and secondary sex characteristics (Stocco,

2001a). In teleost fish, the important end-products of the steroid synthesis in connection with sexual maturation are estradiol-17β (E2), maturation inducing hormone (MIH) and 11-ketotestosterone, that mediate oocyte growth, oocyte- and sperm maturation, and spermatogenesis, respectively (figure 1.1). Furthermore, steroids have functions in the brain connected to for example memory (Flood et al., 1992).

Steroidogenesis, and in turn gonadal development, is regulated by the tropic hormones of the brain-pituitary-gonad (BPG) axis, notably the gonadotropic hormones luteinizing hormone (LH) and follicle stimulating hormone (FSH) produced by the pituitary (Stocco, 2001a). This regulation can be divided into two phases – one acute and one chronic; where the acute regulation takes place in order of minutes and leads to the production of steroids as a reply to acute requirements, while the chronic regulation includes synthesis of mRNAs and enzymes needed for production of steroids, in order to improve the cells' synthetic capacity (Stocco, 2001a). Rapid synthesis of steroids takes place in response to trophic hormone signals from the pituitary gland, and such hormones mediate their actions through the cyclic AMP (cAMP) second messenger pathway (Stocco, 2000). This is the main pathway in the regulation of steroidogenesis, but other non-cAMP pathways might also be involved (Stocco et al., 2005), including through arachidonic acid (see 1.3 "*Arachidonic acid*").

Regardless of the fact that different steroid hormones have different physiological actions in the body, they all have to be synthesized from cholesterol. This biosynthesis of steroids (steroidogenesis) starts with the conversion of cholesterol to pregnenolone (figure 1.1), the precursor of all steroids (Stocco, 2000). This reaction is catalyzed by an enzyme called the cytochrome P450 side chain cleavage enzyme (P450scc) (figure 1.1), which has been shown to be located in the matrix side of the inner mitochondrial membrane (Farkash et al., 1986).

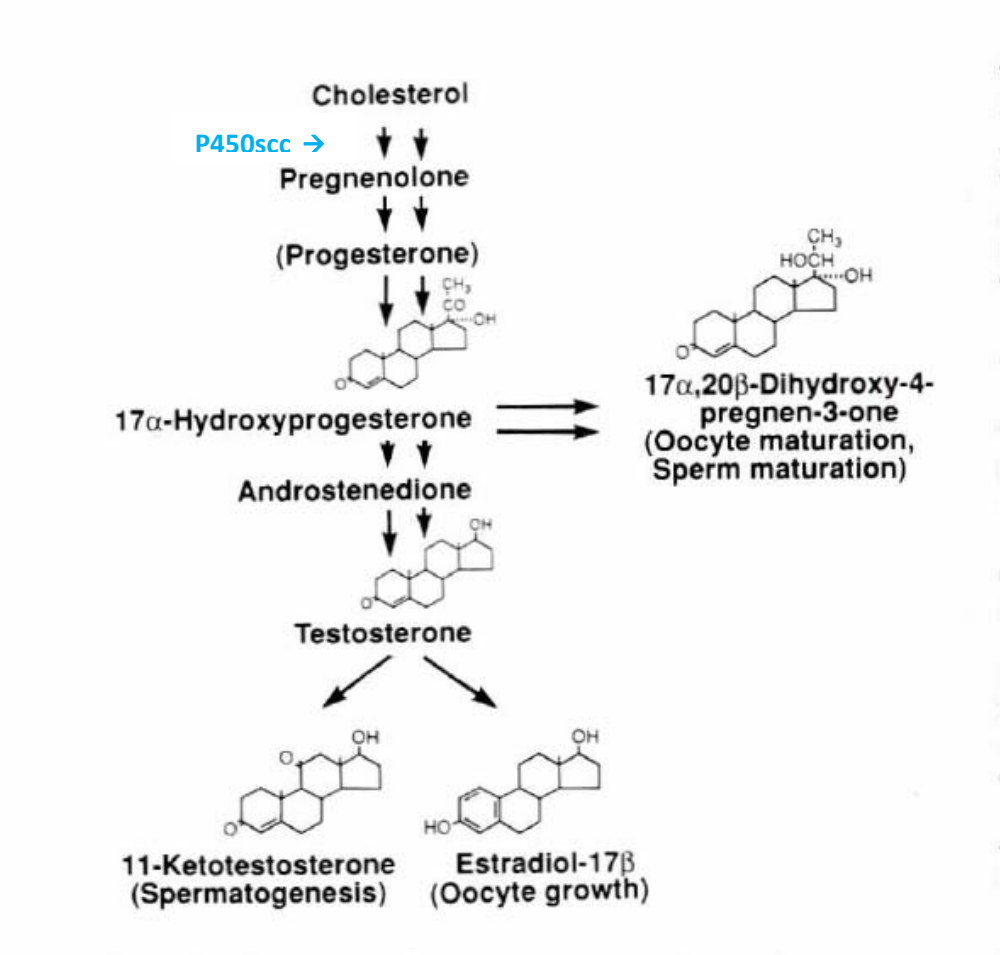


Figure 1.1 Simplified pathway in teleost fish showing the final steroids responsible for the regulation of spermatogenesis, oogenesis and final gamete maturation (modified from Nagahama, 1994).

It is suggested that the rate limiting step of steroidogenesis is the movement of cholesterol across the mitochondrial membrane (Crivello and Jefcoate, 1980, Privalle et al., 1983), where cholesterol is the substrate for P450scc. In this connection the steroidogenic acute regulatory (StAR) protein has been shown to play an important role in mammals; it mediates this movement of cholesterol across the mitochondrial membrane (Stocco, 2001a, b).

StAR is a 30 kDa protein, and was first described in 1983 (Krueger and Orme-Johnson, 1983), but it was not until 1994 that the protein was purified, and the cDNA encoding the 37-kDa precursor protein was cloned and sequenced (Clark et al., 1994). StAR expression is regulated through the cAMP pathway by trophic hormones (Clark et al., 1995, Sugawara et al., 1995), although all the molecular details in relation to this regulation are not yet acknowledged. The mechanism of action of StAR is also a subject that is not fully

understood, but most probably, in order to perform the transfer of cholesterol to the inner mitochondrial membrane, it works in connection with other compounds like lipids and other proteins that exist outside the outer mitochondrial membrane (Stocco, 2001a). However, we do know that StAR plays a vital role in all steroid production, including the sex steroids. Thus it can be concluded that StAR also is essential for the sexual maturation process; an indicator for this is elevated StAR expression during late stages of spermatogenesis (Maugars and Schmitz, 2008) and oogenesis (Kusakabe et al., 2002, Nakamura et al., 2005, Rocha et al., 2009).

StAR transcript expression has been found in steroidogenic tissues, including gonads, adrenals (Clark et al., 1995, Pollack et al., 1997) and brain (King et al., 2002). Furthermore, it has been suggested that this expression is restricted to the steroidogenic cells of such tissues (Clark et al., 1995); in both mammalian and nonmammalian female gonads (ovaries), steroidogenic cells are the granulosa- and theca cells (also called follicle cells). These cells surround the oocyte, and together these three components form a follicle (figure 1.2).

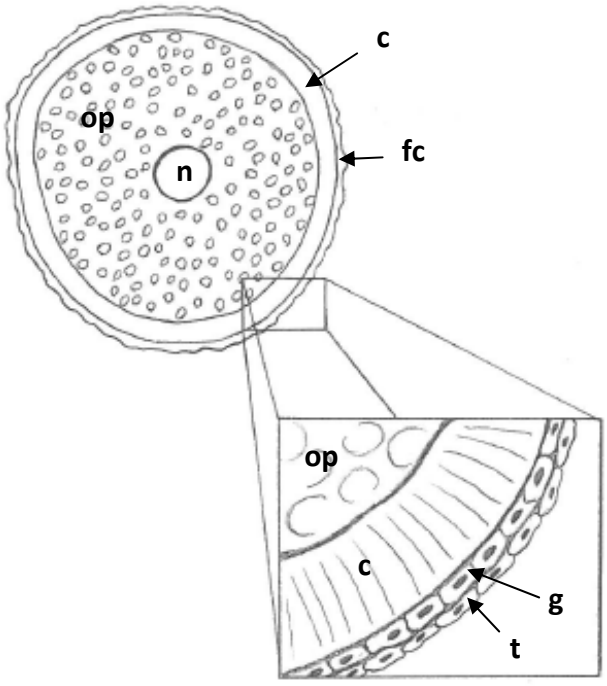


Figure 1.2 Illustration of an ovarian follicle in cod, showing the location of the steroidogenic cells (follicle cells, or granulosa- and theca cells) of the follicle. c = chorion, fc = follicle cells, g = granulosa cells, n = oocyte nucleus, op = ooplasm, t = theca cells.

On the other hand, weaker signals have also been found in other non-steroidogenic tissues like intestine, pyloric caeca, spleen and kidney (Kusakabe et al., 2002). However, the potential function of StAR outside adrenals and gonads is currently not known (Miller, 2007). Tissues that express StAR are found in a variety of mammalian species including human (Sugawara et al., 1995, Gradi et al., 1995) mouse, (Clark et al., 1994), rat (Mizutani et al., 1997, Minegishi, et al., 2002) and buffalo (Malhotra et al., 2007), as well as the chicken (Bauer et al., 2000). Furthermore, StAR transcript expression has also been found in a number of teleost fish, including the cod (Goetz et al., 2004), zebrafish (Bauer et al., 2000), rainbow trout (Kusakabe, 2002) and European sea bass (Rocha et al., 2009). However, to date knowledge about StAR expression and regulation is still limited, especially in vertebrates other than mammals.

1.3 Arachidonic acid.

Arachidonic acid (ARA) is an omega-6-fatty acid (20:4n-6) that has been shown to positively affect fecundity and egg quality in marine fish species like Atlantic cod (Salze et al., 2005) and Atlantic halibut (*Hippoglossus hippoglossus L.*) (Mazorra et al., 2003). ARA is a precursor for prostaglandins, that again affects development of steroids (Wales, 1986, Wales, 1988, Van Der Kraak et al., 1990, Mercure et al., 1996), can induce ovulation (Døving & Reimers, 1992) and can function as pheromones (Moore et al., 2002, Laberge & Hara, 2003). In addition, it has been suggested that ARA can affect larval growth, survival (Castell et al., 1994) and pigmentation (McEvoy et al., 1998, Estevez et al., 1999, Copeman et al., 2002).

Studies on different vertebrates, including mammals (Wang and Leung, 1988, Abayasekara et al., 1990, Romanelli et al., 1995), birds (Johnson and Tilly, 1990) and fish (Van Der Kraak and Chang, 1990, Wade and Van Der Kraak, 1993, Mercure and Van Der Kraak, 1995, Mercure and Van Der Kraak, 1996) have demonstrated that ARA affects steroid production. As mentioned above, steroid production is regulated by trophic hormones like LH, FSH and ACTH; it also appears that LH (Cooke et al., 1991, Moraga et al., 1997) and cAMP (Wang et al., 2002) induce release of ARA. Moreover, it has been indicated that release of ARA is vital for StAR protein expression, something that gave reason to suggest that ARA regulates steroidogenesis through its regulation of StAR protein expression (Wang

et al., 1999). In fact, this regulation takes place on the level of gene transcription (Wang et al., 2000).

Today's feed for cod broodstock normally contains ARA at 1% of total fatty acid content. However, it has been shown that eggs from wild cod contain more ARA than eggs from farmed cod (Salze et al., 2005), and so far an optimal amount of ARA in feed for farmed cod broodstock cod is not known. For this reason an experiment was carried out at the Institute of Marine Research, Austevoll, where cod were fed diets with different ARA content, from the end of their first spawning season to the end of their second spawning season. Sample material from this experiment has been used in this thesis work.

1.4 Hypotheses and aim of the study.

This thesis work is based on the following hypotheses:

1) It is well known that the amount of sex steroids increases over the spawning season, in parallel with follicle growth and maturation in female fish. When considering that StAR plays an important role in the regulation of steroidogenesis, and thus also in sex steroid production, we hypothesize that its transcript levels vary over the reproductive cycle of cod.

2) ARA has been shown to affect steroid production in different species; therefore it may also, like StAR, be connected to the sexual maturation process. Additionally, it has been shown a relation between the amount of ARA in the diet of marine fish species and their spawning success; we therefore hypothesize that there is a connection between the amount of ARA in the diet and the levels of StAR transcripts in cod ovarian follicles.

3) StAR is essential in the steroid production, and it has previously been suggested that it is restricted to the steroidogenically active cells within steroidogenic tissues. We therefore hypothesize that StAR expression exists in major steroid producing cells in cod follicles, which are the theca and granulosa cells.

4) The cod is a batch spawner with several consecutive steroid egg maturation cycles over one spawning season. As StAR is a vital component in steroidogenesis, we expect that its activity will vary between follicles over the spawning season, and therefore we hypothesize that StAR transcripts are expressed differently in follicles of different sizes/degrees of development.

The aim of this thesis study was to quantify the transcript levels (mRNA) of StAR in cod broodstock follicles during a full reproductive cycle, then relate this to the amount of ARA in the fish diet, and also to localize StAR within ovarian follicles of different developmental stages. This was performed using modern methods for the quantification and localization of gene expression; quantitative real-time PCR (qPCR) and *In Situ* Hybridization (ISH).

qPCR is a method used, among other purposes, for quantifying nucleic acids (Wilhelm and Pingoud, 2003). During the course of the polymerase chain reaction in q-PCR the amount of product formed can be quantified; this is done by the help of fluorescent dyes or probes introduced into the reaction. The fluorescence (which is monitored) of such dyes or probes is proportional to the amount of product formed, and further the number of amplification cycles required to achieve a determined amount of fluorescence (and thus DNA molecules) is registered (Kubista et al., 2006). In the histological technique ISH, a labeled nucleic acid probe (DNA or RNA) is hybridized to a sequence of mRNA that is complementary to the probe, and this takes place in tissue sections. Localization of gene expression is then possible (Wilcox, 1993) through visualization of the hybridized probe. Development of such methods is a key for the study of gene expression in fish, which will be important for increasing our understanding of essential physiological processes in fish, including gonadal development and spawning in captive cod stocks.

2. Materials and methods.

2.1 Animals and biological material.

Three main groups of fish were used in this thesis work, all of which were female Atlantic cod (*Gadus morhua L.*) One group (that was further divided into four different diet groups, see *2.2 "Experimental design"*) was used for quantification of StAR by applying complementary DNA (cDNA) previously synthesized from isolated RNA, originating from gonadal tissues from these fishes; they were cod broodstock from Parisvatnet, with an initial age and mean weight of 2 years and 2.3 kg, respectively. The other two groups were used for localization of StAR by applying gonadal tissues sampled from these fishes during this thesis work; one group was maturing cod from IMR, station Austevoll, with an age of 1 year and 8 months and with a mean weight of 1.2 kg, and the other group was spawning cod from the same location, with an age of 5 years and with a mean weight of 5.4 kg.

2.2 Experimental design.

This thesis did not include its own experimental design; most of the material used (cDNA) had previously been collected in an experiment that was part of a cooperation project led by Skretting ARC and where the National Institute for Nutrition- and Seafood Research (NIFES), the Institute of Marine Research (IMR) and three Norwegian cod hatcheries (Cod culture Norway, Havlandet marin yngel and Sagafjord sea farm) participated as collaborating partners; "Optimised nutrition in Atlantic cod broodstock, with the underlying project "Effect of arachidonic acid on spawning performance". In this project 3200 cod were distributed in 8 sea cages (5*5*5 m) at IMR, Austevoll. This took place in May 2005, after the first spawning. These fishes were then fed 4 experimental diets (1 diet per duplicate) containing different amounts of arachidonic acid (ARA); 0.5, 1, 2 and 4% ARA of total fatty acids. Monthly samplings were collected (with the exception of April 2006) during the whole project period, ending in May 2006. 384 female gonad (ovary) samples from these fishes were used as material in this thesis work (originating from the fish group for quantification of StAR, see

2.1 "*Animals and Biological material*"), with one ovary sample representing one fish from one of the diet groups.

In the fish groups used for localization of StAR (see 2.1 "*Animals and Biological material*"), no experimental design was necessary; cod are batch spawners and thus have oocytes of different developmental stages present in the ovary during most of the spawning season, and one or two random samplings are therefore sufficient to collect ovaries with oocytes of a variety of sizes. Consequently, two samplings were performed.

2.3 Sampling of the material.

Most of the material (all the cDNA samples) had previously been collected (see 2.2*" Experimental design"*), and this had been stored at IMR, Bergen. The numbers of cDNA samples from each sampling date and diet group that is included in this thesis for quantification of StAR are listed in table 2.1.

Table 2.1 Overview of the number of samples (n) from each sampling date and diet group (groups A-D) included in this thesis work for quantification of StAR..

Sampling date	n for group A	n for group B	n for group C	n for group D	Total n
13.06.2005	3	4	6	4	17
11.07.2005	8	7	5	5	25
17.08.2005	2	6	4	4	16
19.09.2005	5	3	4	2	14
17.10.2005	9	7	9	6	31
21.11.2005	10	12	13	8	43
19.12.2005	5	4	6	5	20
23.01.2006	9	10	9	11	39
21.02.2006	20	24	31	26	101
21.03.2006	13	17	14	11	55
15.05.2006	7	6	6	4	23

When it comes to the sample material for localization of StAR, this was sampled on two different occasions; 5 random female gonad samples from maturing cod (see 2.1 "*Animals*

and Biological material") were collected 01.12.08 at IMR, Austevoll, and 3 random samples from mature/spawning cod (see 2.1 "*Animals and Biological material*") were collected 23.02.09 at the same location.

The selected individuals were stunned by a blow to the head, in agreement with Norwegian regulations for fish sacrifice, and then the samples were collected; a small tissue sample was taken from the ovary for each fish, and put into 4% buffered paraformaldehyde fixation in the ratio 1:25 tissue:fixation. These were stored chilled (4ºC) over night for further processing in the laboratory.

2.4 Processing of the material.

2.4.1 Quantification of StAR.

The processing of the material for quantification of StAR (the cDNA samples, see 2.1 "*Animals and Biological material*") consisted of making the samples ready to be analyzed by qPCR (see 1.4 "*Hypotheses and aim of the study" and 2.5.1.1 Real-time quantitative polymerase chain reaction (q-PCR)"*). As previously mentioned cDNA had already been synthesized from the gonad tissues collected in 2005-2006; however, since this is an important part in the quantification process, the protocols for isolating RNA and synthesizing cDNA are included in this thesis (see appendix 2). Gloves were used all the time to keep the material RNase- and DNase-free.

2.4.1.1 Constructing the StAR qPCR assay for cod.

For an introduction to the principles of qPCR analysis and the connected terms Ct, ΔCt and ΔΔCt, see 2.5.1.1."*Real-time quantitative polymerase chain reaction (qPCR)*" and 2.5.1.2 "*qPCR calculations in Microsoft excel*".

The cloning and sequencing of StAR (cStAR1 and cStAR2, GenBank accession no. AY291434 and AY291435, respectively) in cod has previously been performed (Goetz et al.,

2004). The designing of primers and probe had been done at IMR earlier: for alignment of genomic DNA and complementary DNA, and thus also figuring out where on the sequence to put primers and probe , Vector NTI 10 (Invitrogen) had been applied, and by using Primer express 3 (Applied biosystems), the most suitable primers and probe had been chosen. Finally the chosen primers and probe had been tested on both genomic DNA and complementary DNA by using gel electrophoresis.

A qPCR assay also needs a housekeeping/reference gene, to which the gene of interest (StAR) can be compared to. In this thesis work 18S was chosen; this is frequently used in qPCR assays at IMR, and from experience it is acknowledged as a suitable housekeeping/reference gene when quantifying gene expressions in fish follicles; consequently, primers and probe for 18S were also already designed. The primers used in the StAR qPCR assay are listed in table 2.2, and the probes are listed in table 2.3.

Table 2.2 Overview of the primers used in the StAR qPCR assay of this thesis work.

Primer name	5' to 3' sequence	Manufacturer/supplier
StAR forward primer	CATCCACCATGAACTCAACAGAA	Invitrogen
StAR reverse primer	TCGATCCTGGAGCTGAGGAA	Invitrogen
18S forward primer	CCCTGTAATTGGAATGAGTGTACTTT	Invitrogen
18S reverse primer	ACGCTATTGGAGCTGGAATTACC	Invitrogen

Table 2.3 Overview of probes used in the StAR qPCR assay of this thesis work.

Probe name	5' to 3' sequence	Manufacturer/supplier
StAR probe	6-FAM-CCCGGCTCTGGCA-MGB	Applied biosystems
18S probe	6-FAM-CACCAGACTTGCCCTCC-MGB	Applied biosystems

2.4.1.2 Optimisation of the StAR qPCR assay.

Before starting qPCR analysis of a target gene, an optimisation of the system is necessary; the purpose is to check the amplification efficiency of the genes, and to choose a suitable cDNA dilution to use in further quantification; too diluted template often results in uneven amplification, and too dense template can lead to PCR-poisoning, again causing uneven amplification. Using 2 µl template, the following dilutions were tested in a dilution series:

1x (undiluted), 2x, 4x, 8x, 16x, 32x (table 2.4).

Table 2.4 Dilution series of 18S and StAR, showing the cDNA dilution, the log of the cDNA dilution, mean Ct and ΔCt.

cDNA dilution	log (cDNA dilution)	Mean Ct 18S	Mean Ct StAR	dCt
1,0000 (1)	0,00	14,33	30,02	15,69
0,5000 (1:2)	-0,30	15,34	31,01	15,67
0,2500 (1:4)	-0,60	16,30	31,93	15,63
0,1250 (1:8)	-0,90	17,40	32,77	15,37
0,0625 (1:16)	-1,20	18,38	33,68	15,30
0,0313 (1:32)	-1,50	19,52	35,06	15,53

Ideally, the target amplification efficiency and the reference amplification efficiency are more or less equivalent; this gives a valid ΔΔCt calculation (see 2.5.1.2 *"qPCR calculations in Microsoft excel").* By looking at how the normalized cycle times (ΔCt) varies with template dilution, it is possible to check if the PCR efficiency is the same for the two amplification reactions (AppliedBiosystems/

http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_042380.pdf). However, first it is useful to check if the amplifications are exponential: by plotting mean Ct against the logarithm of ovary input cDNA (the cDNA dilution), the slope reveals to what extent the reaction is exponential. A Slope of 3.32 represents a totally exponential amplification, which is ideal. This was checked for the dilution series, and the slopes were 3.4341 and 3.2327 for 18S and StAR, respectively (figure 2.1). These values are quite close to 3.32, and consequently the degree of exponentiality is acceptable.

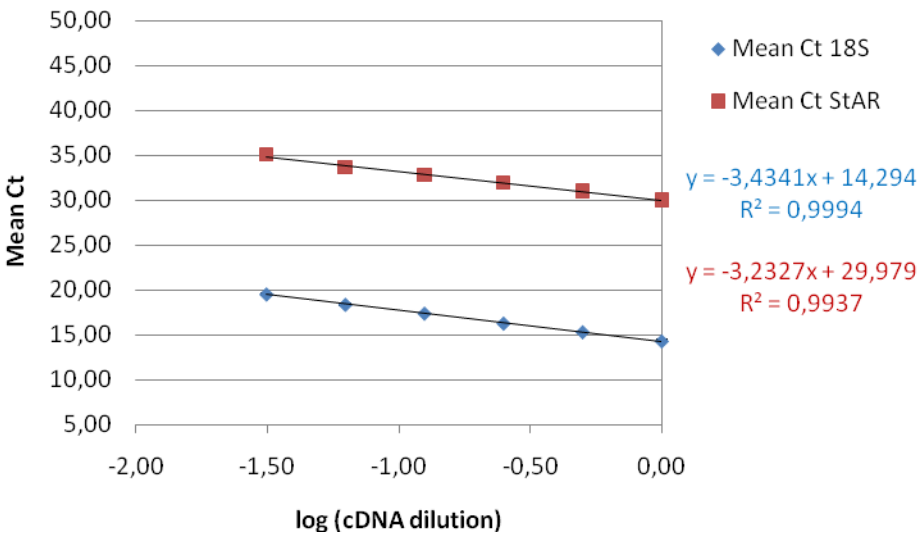


Figure 2.1 Plot of Mean Ct's of 18S and StAR vs. log (cDNA dilution), from the dilution series, shown with standard deviation of the mean. The slopes are 3.4341 and 3.2327 for 18S and StAR, respectively. The y-axis is removed from the right and added to the left of the figure so that the slope value is in reality the opposite than the one in the formula.

Furthermore, a plot of ΔCt vs. log (cDNA dilution) from the dilution series was made (figure 2.2). Ideally, the absolute value of the slope should not exceed ±0.1 (Applied Biosystems/

http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_042380.pdf); a dilution area that has a slope value within ±0.1 represents an area with stable amplification.

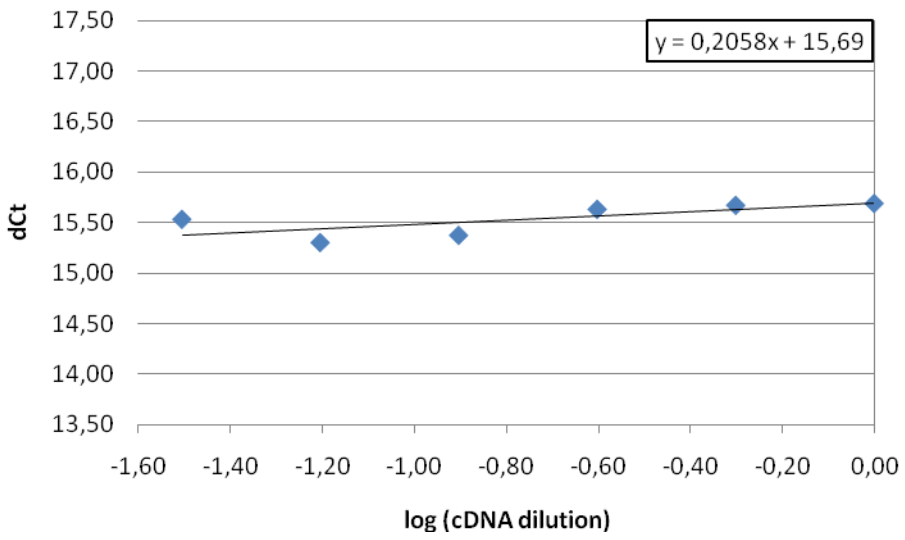


Figure 2.2 Plot of ΔCt vs. log (cDNA dilution) from the dilution series of 18S and StAR, where all dilutions are included. The absolute value of the slope is -0.2058. The y-axis is removed from the right and added to the left of the figure so that the slope value is in reality the opposite than the one in the formula.

When all points in the dilution series were included, the slope value was -0.2058 (figure 2.2). In order to find a stable amplification area, the points representing the most diluted cDNA were excluded from the graph, so that the slope value changed into an acceptable value. When including dilutions 1x (undiluted/1.0000), 2x (0.500) and 4x (0.2500), the amplification was stable (slope was -0.0967, figure 2.3), and the dilution 4x was chosen to be used for the quantification analysis in this thesis work.

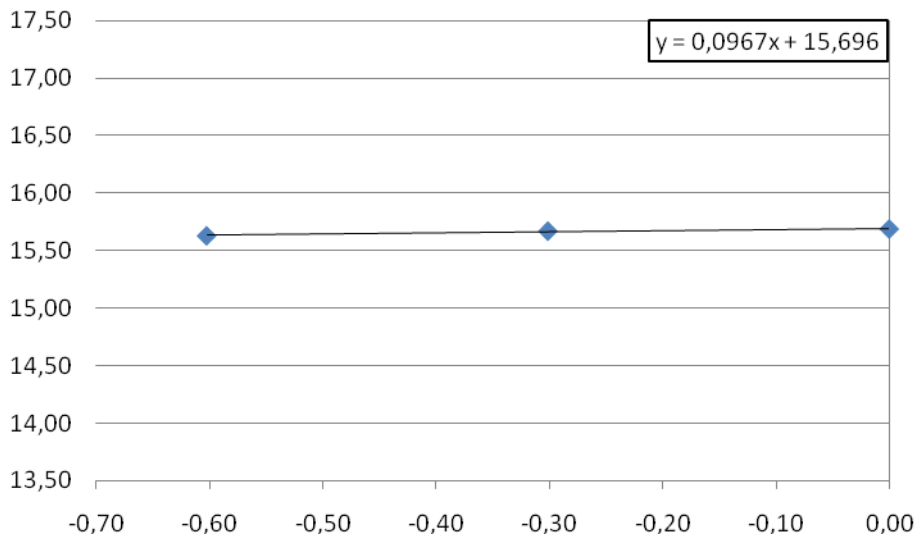


Figure 2.3 Plot of ΔCt vs. log (cDNA dilution) from the dilution series of 18S and StAR, where dilutions 1x (undiluted/1.0000), 2x (0.500) and 4x (0.2500) are included. The absolute value of the slope is -0.0967. The y-axis is removed from the right and added to the left of the figure so that the slope value is in reality the opposite than the one in the formula.

Another challenge for optimisation of the assay was that StAR was weakly expressed; therefore more template than 2 µl would be more suitable. At the same time the reference gene (18S) is highly expressed, so that too much template needed to be avoided; a final template volume of 4 µl (6.666 ng RNA) was chosen to be applied.

2.4.1.3 Pre-qPCR.

For each gene a reaction solution (mastemix) containing probe, primers, PCR mastermix and H_2O (see mastermix in appendix 1 for full recipe) was made (kept chilled). After vortexing and centrifuging the mastermixes for a few seconds, they were distributed on an optical reaction plate (kept chilled), using a multipipette; for each reaction plate containing 96 wells, 23 duplicate samples could be run. Half of the reaction plate was used for the StAR gene and the other half of the plate was used for 18S; one duplicate (two wells) per gene per sample, and one duplicate per gene for a no template control (NTC). 21 µl mastermix was added per well on the reaction plate, making it ready to have template (cDNA) added. Further, 23 cDNA 1:10 samples were thawed, vortexed and centrifuged for a few seconds, before 4 µl from each

sample was added in duplicates for each gene on the plate. For the NTC wells, 4 µl H_2O was added instead of template. An optical adhesive cover was then placed on top of the reaction plate to make sure that the solutions in the different wells would not mix or disappear during the following centrifugation (1000 RPM) for 20-30 seconds. Finally the reaction plate could be placed in the qPCR instrument (7900HT Fast Real-Time PCR system); this runs for 40 minutes to complete the program for one reaction plate.

2.4.2 Localization of StAR.

The processing of the material for localization of StAR (see 2.1 *"Animals and Biological material"*) consisted of making the samples ready to be analyzed by ISH (see 1.4 "*Hypotheses and aim of the study" and 2.5.3 In Situ Hybridization*). As mentioned, these samples were stored chilled over night after the sampling. Gloves were used all the time to keep the material RNase- and DNase-free.

2.4.2.1 Dehydration.

The day after sampling, the samples were placed in a clean histokinette (Leica TP 1020); this instrument gradually dehydrates the samples from 50% ethanol to 100% ethanol, and finishes by soaking the tissue in liquid paraffin (histowax tissue processing/embedding medium). It takes 22 hours to complete the process.

2.4.2.2 Embedding.

On the second day after sampling the samples were embedded in paraffin (Histowax tissue processing/embedding medium); this was performed by placing them in heated/liquid paraffin, and then chilled (-20 ºC) for 15 minutes so that the paraffin became solid.

2.4.2.3 Sectioning.

For the paraffin sectioning, an instrument of the type Leica RM2255 was used; the tissues were sectioned into 3 µm layers and put into heated (46-48 ºC) DEPC water to stretch out. After a few seconds the sections were placed on superfrost plus microscope slides and finally dried in a heating chamber (Melag incubat) holding 60 ºC, for 30 minutes so that they would stick properly to the slides.

2.4.2.4 Midi-prep.

Before starting cRNA probe synthesis (see 2.4.2.5 "cRNA probe synthesis"), the gene of interest has to be subcloned into a suitable plasmid/vector. Basically this involves releasing and purifying of the insert (in this case StAR) from a parent vector, ligation of the insert into a new vector, and then transforming this new vector (now containing the insert) into capable bacteria. This had previously been done for StAR at IMR where a plasmid containing the ORF of 858 bp of Atlantic cod StAR (cStAR1, GenBank accession no. AY291434) has been constructed. Plasmids containing StAR were available at IMR, Bergen; but in order to increase the number of these plasmids a midi-prep was performed.

Approximately 50 ml of LB-medium with ampicillin was distributed into three Erlenmeyer flasks. Furthermore, 10 µl of glycerol stock (including the bacteria *Escherichia coli*, containing "StAR plasmids") was added to each of the flasks. As mentioned, these "StAR plasmids" had earlier been made and put into bacteria at IMR, and the glycerol stock is the medium in which they had been stored. Finally the colonies were incubated in 37 ºC over night. The next day the plasmids were purified using a QIAGEN plasmid midi protocol (QIAGEN plasmid purification handbook 09/2000) and kit (HiSpeed plasmid midi kit). The plasmids were then stored in -80 ºC.

2.4.2.5 cRNA probe synthesis.

Two kinds of probes were necessary to synthesize; one control (sense) probe that would not bind to the mRNA of interest (StAR) in the ovary tissue, and one anti-sense probe that would bind. Two different probes were therefore synthesized at the same time using the same protocol (see appendix 3), with the exception of what kind of RNA polymerase was added: using T7 produced the sense probe and T3 produced the anti-sense probe.

For linearization of vector and insert, OD260 was measured (240.76 ng/µl), 1µg DNA was used for each cutting reaction, and the cutting reaction was calculated: 1000 ng/240.76 ng/µl = 4.2 µl of plasmid. Furthermore, sterile deionized water, RE 10 x buffer, 1:10 Acetylated BSA and DNA (1 µg) was added. After mixing restriction enzyme R. E was added, the solution was spun down and then kept at 37 ºC for 1-4 hours. Then TE and phenol/chloroform was added, followed by vortexing and spinning. The supernatant was decanted into a new tube, chloroform was added and the solution was vortexed and spun. The supernatant was again decanted into a new tube and precipitation of the linearized DNA was performed by adding 3M NaAc and 100% EtOH, then the solution was put in -20 ºC for at least 1 hour, cool centrifuged to 4 ºC, and spun. The supernatant was discarded, 170 µl 70% EtOH was added and the solution was spun, and this was then repeated with 100% EtOH. The supernatant was again discarded, the pellet was air dried and then resuspended in nuclease-free water.

To do Digoxigenin (DIG) RNA labeling (cRNA probe synthesis), OD260 was measured: T7 = 16.28 ng/µl and T3 = 14.61ng/µl. Template DNA (1 µg) in nuclease-free water, NTP labeling mixture 10x, transcription buffer 10x, RNase inhibitor and RNA polymerase (T3 or T7) was added to an eppendorf tube on ice, followed by mixing, centrifugation and incubation for 2 hours at 37 ºC. Furthermore, DNaseI was added, followed by incubation for 15 minutes at 37 ºC. The reaction was stopped by adding 0.2 M EDTA. To precipitate the cRNA, 4 M LiCl and prechilled (-20 ºC) 100% EtOH was added, followed by mixing and leaving the solution for at least 30 minutes at -70 ºC. The solution was centrifuged, the supernatant was discarded, prechilled (-20 ºC) 70% EtOH was added, the solution was centrifuged and the supernatant was discarded again. The pellet was air dried and then resuspended in DEPC-water and RNase inhibitor, and OD260 was measured: T7 = 366.51 ng/µl and T3 = 356.76 ng/µl.

In order to control the DIG-incorporation in cRNA probes, graded dilutions were made of probe and control DIG-RNA (1:10, 1:100, 1:1000). 1µl of each dilution was pipetted on to a Hybond-N+ membrane, and this was then air dried and put in a crosslinker. The membrane was washed in buffer B1 and then in buffer B2, followed by incubation in buffer B2 and anti-DIG (= 1:5000 dilution) for 30 minutes. After washing in buffer B1 and then in buffer B3, the membrane was incubated in buffer B3, NBT and BCIP in dark for at least 5 minutes. To stop the reaction, TE was added. The probe quantity and quality was checked by using a bio-analyzer (Agilent 2100 Bioanalyser, Agilent technologies).

The degree of incorporated DIG could be seen on the membrane for each of the probes; this was then compared with the control (which showed a perfect dilution series). If no color had been seen on the membrane, no DIG would have been incorporated in the probe; however, the dilution series looked good in this case.

2.5 Analyses.

2.5.1 Quantification of StAR.

2.5.1.1 Real-time quantitative polymerase chain reaction (q-PCR).

The basics behind the real-time polymerase chain reaction is described in detail in Kubista et al., 2006. The mechanism of the program of the q-PCR instrument is temperature cycling; first the DNA template in the reaction plate is subjected to high temperature, which separates the strands of the DNA (denaturation). Furthermore, a lowering of the temperature allows primers to anneal to the template (annealing), and a final temperature of around 72ºC gives an optimum temperature for the polymerase, which incorporates nucleotide triphosphates (dNTPs) and thus extends the primers (Kubista et al., 2006). The software of the instrument, SDS RQ manager, produced an amplification plot for each sample (figure 2.4); the increase in fluorescence for each cycle of PCR could then be read from these plots. In addition, a cycle time (Ct) value was calculated for each sample; this represents the number of cycles required to reach the set threshold fluorescence value. The threshold value is based on the variability of the baseline data; in this case it was manually set to 0.06.

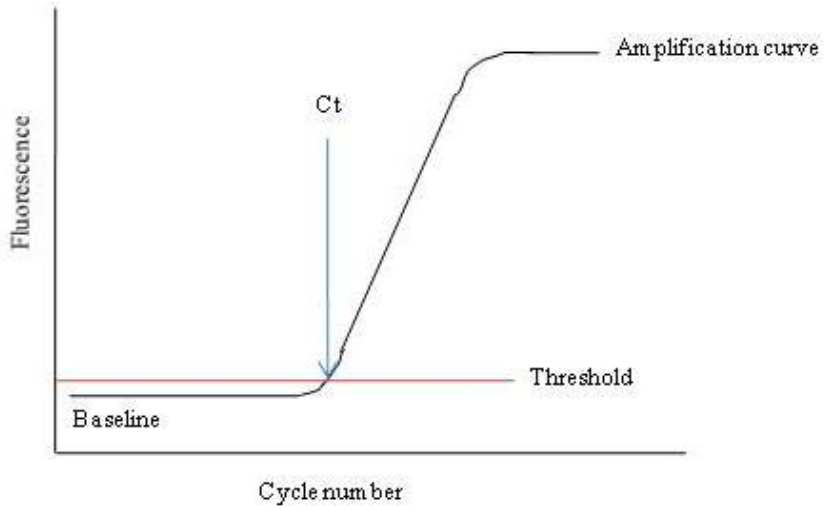


Figure 2.4 Illustration of a general qPCR amplification plot of fluorescence vs. cycle number for one sample, showing the baseline, threshold and cycle times (Ct).

In order to transform the relative Ct values to quantitative ones, the data were further processed in Microsoft excel, using the arithmetic comparative method (ΔΔCt method), according to the manufacturer's guidelines (Applied Biosystems).

2.5.1.2 qPCR calculations in Microsoft excel.

For each of the 2 replicates of a sample, the following was calculated:

The average of the cycle time (Ct)

Standard deviation (Stdev) of the Ct's

Coefficient of variation (CV), given by: CV = Stdev/average transcript quantity

Outlier values were removed, following two different criteria:

1) For both of the genes, $Stdev^2_{mean\ Ct's}$ needed to be below 0.05.

2) For the reference gene (18S), mean Ct needed to be within ± 2 x $Stdev_{mean\ Ct}$.

To calculate the normalized Ct (ΔCt) for StAR, the Ct's of each sample of the gene of interest (StAR) were normalized to those of the reference gene (18S) for the same sample:

$$\Delta Ct_{StAR} = \text{average } Ct_{StAR} - \text{average } Ct_{18S}$$

Stdev of ΔCt was calculated:

$$Stdev_{\Delta Ct} = \sqrt{(Stdev_{18S})^2 + (Stdev_{StAR})^2}$$

The fish group with lowest gonadosomatic index (GSI) values, and thus representing the group with least mature ovaries was the group sampled in August 2005; therefore this group was chosen to be the calibrator group, to which the other samples were compared to in the next calculations.

The calibrated value (ΔΔCt) was calculated for each sample:

$$\Delta\Delta Ct = \Delta C_{Sample} - \Delta C_{calibrator}$$

The fold-change for each sample relative to the calibrator was calculated:

$$\text{Fold-change} = 2^{(-\Delta\Delta Ct)}$$

The fold-change value expresses the relative amount of target mRNA, normalized to the reference gene and relative to the calibrator group.

2.5.2 Gonadosomatic index (GSI) and Estradiol-17β (E2).

Although the GSI- and E2-values from the fish used in this thesis work were not analysed here, but previously when the main project was running (see 2.2 "*Experimantal design*"), they were included in the results of this thesis; both of these parameters are connected to follicle growth, and it was therefore interesting to see if any correlations with StAR could be found.

The gonadosomatic index is the percentage ratio between gonad- and somatic weight, given by: GSI = (gonad weight/body weight)*100. As a result of the sexual maturation process with ovary- and testis growth, this ratio changes markedly during the spawning

season; consequently, it was interesting to see if the StAR expression followed the same pattern. As mentioned, E2 mediates follicle growth in connection to sexual maturation, and it was therefore interesting to see how the changing amount of this hormone correlated with StAR expression. The raw data for GSI and E2, as well as sampling date, which is also the sampling dates for the cDNA samples used for quantification of StAR, are listed in appendix 6.

2.5.3 In Situ Hybridization (ISH).

The ISH method used in this thesis work is non-radioactive, with digoxigenin (DIG)-labeled cRNA probes (see *2.4.2.5 "*c*RNA probe synthesis")*, for detailed protocol, see appendix 4. The sections were rehydrated in xylene, 100%-, 70%-, and 50% EtOH, and finally in 1xPBS. Furthermore, the sections were rinsed in TrisHCl and permeabilized by being treated for 5 minutes with proteinase K, followed by rinsing in TrisHCl again. Acetic anhydride treatment was performed by leaving the sections in TEA for 10 minutes, then in AcAh-TEA for another 10 minutes, followed by rinsing in PBS. The sections were prehybridized in hybridization buffer for 2 hours in a moisture chamber, and then hybridized in preheated (80 ºC for 5 minutes, then placed on ice) hybridization buffer with added cRNA probe (2000 ng/ml) in a moisture chamber for 16 hours at 65 ºC. Post-hybridization wash included 30 minutes in 5xSSC, 15 minutes in 30% formamide in 5xSSC at 65 ºC, 2x30 minutes in 0.2 x SCC at 65 ºC (followed by cooling to room temperature) and then 5 minutes in 0.2 x SCC. The slides were RNase treated by washing in RNase buffer at 37 ºC, incubation for 30 minutes in 2 µg/ml RNase A, washing in RNase buffer at 37 ºC again, and 2x30 minutes in 0.2 x SSC.

Immunodetection and visualization of DIG-alkaline phosphatase was performed by washing the sections in buffer B1, incubate them in buffer B2 for 1 hour and then incubate them in buffer B2 containing sheep anti-DIG 1:2000 for 16 hours at 8 ºC (antibody incubation). The sections were then rinsed in buffer B1 and buffer B3, and incubated in buffer B4 for 1-6 hours (in this thesis work, up to two days were sometimes necessary) in the dark, so that the alkaline phosphatase reaction could take place. The reaction was then stopped by adding TEN to the sections for 10 minutes. In order to get rid of brown color and enhance the blue color representing StAR transcripts, the slides were put in 100% EtOH for 30 seconds.

The slides were then coverslipped in 50% glycerol in TEN and nailpolish was used to seal the slides. After the nailpolish had dried, the sections were studied and analyzed in a microscope (Nikon eclipse 80i); as mentioned, blue staining of tissue represented StAR expression. In order to classify the different developmental stages of the follicles, an overview was made, using descriptions from Kjesbu and Kryvi, 1989 as a guideline (table 2.5): here the main developmental traits for each follicle stage during oogenesis are listed.

Table 2.5 An overview over main developmental traits taking place in the follicle during oogenesis in cod (Kjesbu and Kryvi, 1989 is used as a guideline).

Main follicular stage	**Follicle sub-stage and its main developmental traits**
Previtellogenic	*Early primary growth*: - Oogonia are transformed to oocytes. - Follicle cells (granulosa- and theca cells) start to envelope the oocyte. - Peripheral nuclei are created. *Late primary growth*: - A circumnuclear ring appears. - Chorion starts to form between the oocyte and the follicle cells. *Yolk vesicle (cortical alveoli) formation*: - The circumnuclear ring gradually breaks down. - Cortical alveoli (spherical and transparent look) appear in the outer part of the ooplasm.
Vitellogenic	*Early true vitellogenesis*: - Yolk granules form in the oomplasm. At first they appear peripherally, but later they are located throughout the ooplasm, and their size increase markedly. *Middle of true vitellogenesis*: - The yolk increases enormously. - The number of cortical alveoli increases. - Chorion develops and becomes striated and two layered. *Late true vitellogenesis*: - This stage is recognized by large cortical alveoli and yolk granules, in addition to an irregular nucleus. - During follicle maturation a migration of the nucleus to the animale pole of the oocyte, hydration of the oocyte, follicle growth and ovulation take place.

2.6 Quality checking: the quantification method.

The quantification of gene expression is a complex process, and involves several different steps before the results are obtained. However, in addition to the optimisation of the quantification system (see *2.4.1.2 "Optimisation of StAR qPCR assay"*), several parts of the process can be tested in order to ensure applicable results; looking at the 18S mean Ct is one possibility.

18S is the reference/housekeeping gene used in this thesis work, and therefore it should represent the stable one of the two genes that are quantified. A stable expression pattern over the experimental period is then expected, something that would ideally be shown by stable mean Ct-values. These were therefore analyzed to check if the expression changed significantly over the experimental period.

2.7 Statistical tests.

The statistics and analytics software Statistica 8.0 was used for all statistical tests in this thesis work. A one-way ANOVA was used for comparing the StAR gene expression between the diet groups per month, and if a significant difference was found ($p<0.05$), the post hoc test Unequal N NSB was applied to reveal which group was different; this was also applied for GSI analyses. Furthermore, to check if and where the StAR expression (in all diet groups together) changed over time, the non-parametric Kruskal-Wallis test was used. This test was also used to check if the 18S mean Ct changed significantly over the experimental period. In addition, a possible correlation between StAR expression and GSI, and StAR expression and Estradiol levels was tested with a multiple regression (bivariate correlation). In order to obtain better normality in the StAR data, the fold-change values (relative Q) were transformed to log (Q+1), and these were used in some of the statistical tests; the bivariate correlation (in addition to the relative Q values) and the one-way ANOVA. All the statistical test results for this thesis work are listed in appendix 5.

3. Results.

3.1 Quantification of StAR.

3.1.1 StAR expression over time.

The StAR expression in ovaries showed a similar seasonal development in all 4 diet groups with a peak in the spawning period in Ferbruary to March at 3 years of age, but also variable and elevated and level at the start of the experiment in June at the end of spawning season at 2 years of age (figure 3.1). In fish fed diet A (0.5% ARA), StAR expression changed significantly over the experimental period (Kruskal-wallis test; $p = 0.0000$) (figure 3.1; diet A). In February and March StAR expression was higher than in October and November. Additionally, the value of March was higher than that of July. The expression for the fish fed diet B (1% ARA) did also change significantly (Kruskal-wallis test; $p = 0.0000$) (figure 3.1; diet B); in February and March this was higher than in July, August and November. Also, in March there was a higher value than what was found in October and January. A significant change in StAR expression over the experimental period was also found in the fish fed diet C (2% ARA) (Kruskal-wallis test; $p = 0.0000$) (figure 3.1; diet C); in February and March this was higher than the one in October and November. Furthermore, March showed a higher value than June, July, August, September and January. Finally, a significant change in StAR expression was found in the fish fed diet D (4% ARA) (Kruskal-wallis test; $p = 0.0000$) (figure 3.1; diet D); in February and March this was higher than in July, October and January.

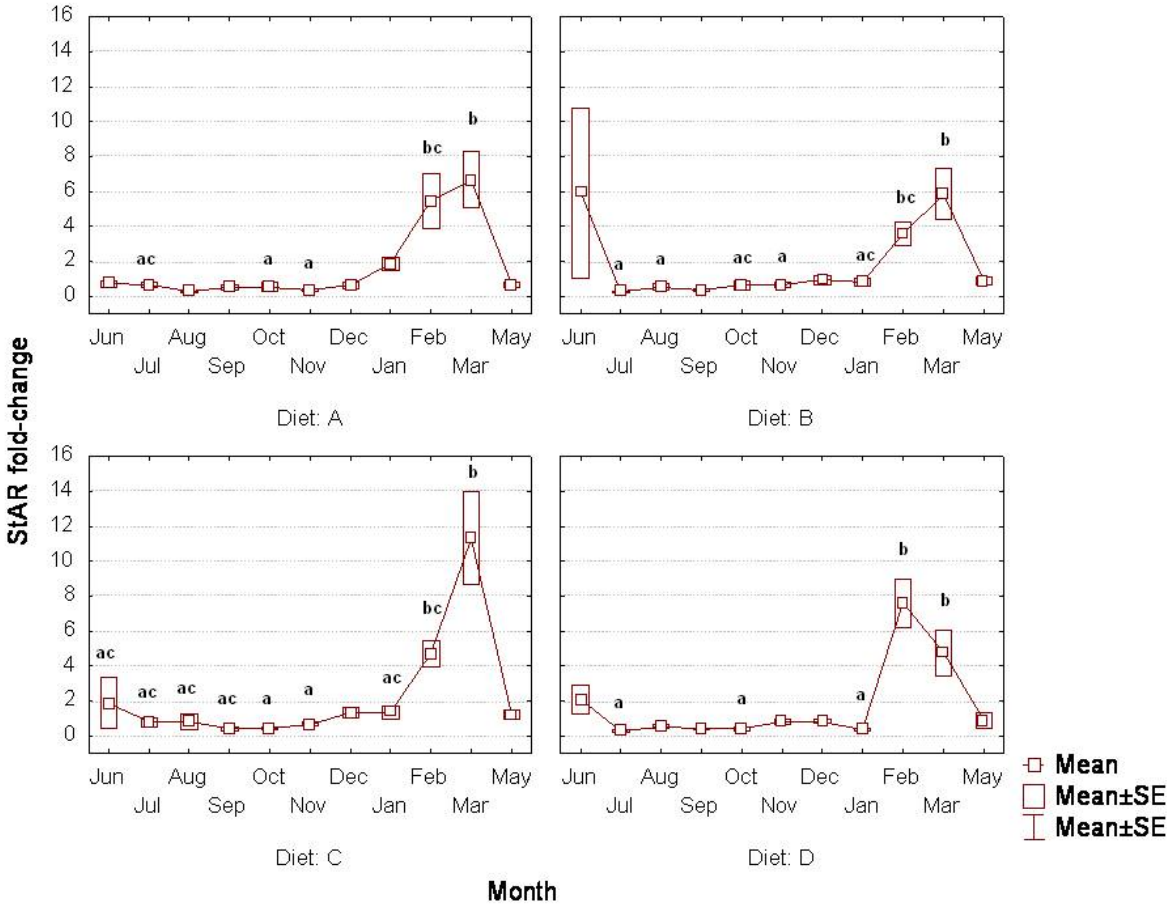


Figure 3.1 StAR expression in the four experimental diet groups, shown by mean ± se. Boxes marked with different letters differ significantly from each other, and boxes without marking do not differ significantly from any of the other boxes. The experimental period goes from June 2005 to May 2006.

In summary it tended to be a general trend; The StAR expression changed over a time from June 2005 to May 2006; in February and March the expression was higher than the other months, for all diet groups. Of the two peak months February and March, March represented the month with the most significant differences from the other months of the experimental period, with the exception of the fish fed diet D (4% ARA), where the expression in February differed equally much from the expression in the other months, as that of March.

3.1.2 Differences in StAR expression between the diet groups.

The StAR expression varied between diet groups at some of the sampling points over the experimental period (figure 3.2). In June the group fed diet B (1% ARA) tended to have relatively elevated StAR expression compared to the groups fed the other diets; however, no significant differences were found at this sampling time. In July a significant difference between groups was detected by one-way ANOVA ($p = 0.03485$), but the post hoc test did not reveal which group was different. In the following months all the way to January 2006, the StAR expression in all groups was relatively low, with no significant differences between any of the groups. However, by January, when the StAR expression started to increase, a difference between two of the diet groups was found; fish fed diet A (0.5% ARA) had significantly higher StAR expression than fish fed diet D (4% ARA) ($p = 0.00349$). In February the fish fed diet D (4% ARA) seemed to reach its maximum StAR expression, in contrast to the other groups of fish, which seemed to reach their maximum values in March. It appears that fish fed diet C (2% ARA) had the highest StAR expression in this peak month, followed by fish fed diet A (0.5% ARA) and finally fish fed diet B (1% ARA). However, although there seemed to be differences in StAR expression in February and March, no significant differences were found between any of the groups, and that was also the situation in May 2006, when the gene expression had decreased in all fish groups.

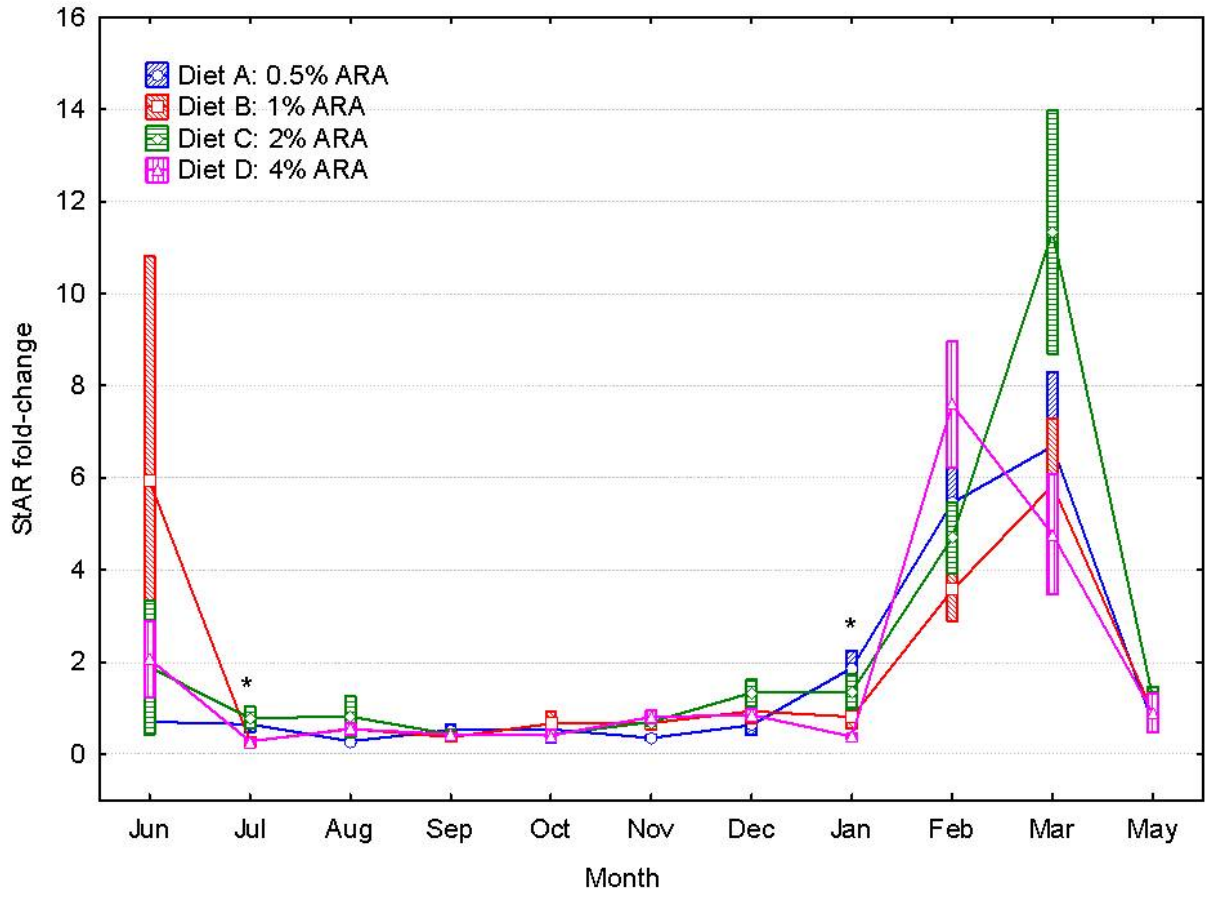


Figure 3.2 StAR expression in the four experimental diet groups, shown by mean ± se. Sampling points showing significant differences in StAR expression between groups are marked with "*" The experimental period goes from June 2005 to May 2006.

3.1.3 18S mean Ct.

The (mean) Ct varied significantly over the experimental period in all four diet groups (figure 3.3). In the group fed diet A (0.5% ARA), the mean Ct in December was different from the ones in October and February, and in January, March and May the values were also different from that of February. In the fish fed diet B (1% ARA), the mean Ct's in December and May were different from the ones in July, October, January and February. In addition, the value for January further differed from the ones in August and November. When it comes to diet group C (2% ARA), the mean Ct's of August, November, December, March and May were all different from the one in January. Furthermore, the values of March and May differed from

the ones in October, January and February. In the fish fed diet D (4% ARA), the mean Ct's of June, November, December, March and May differed from the one in January. In addition, the value in February was different from the ones in November and March.

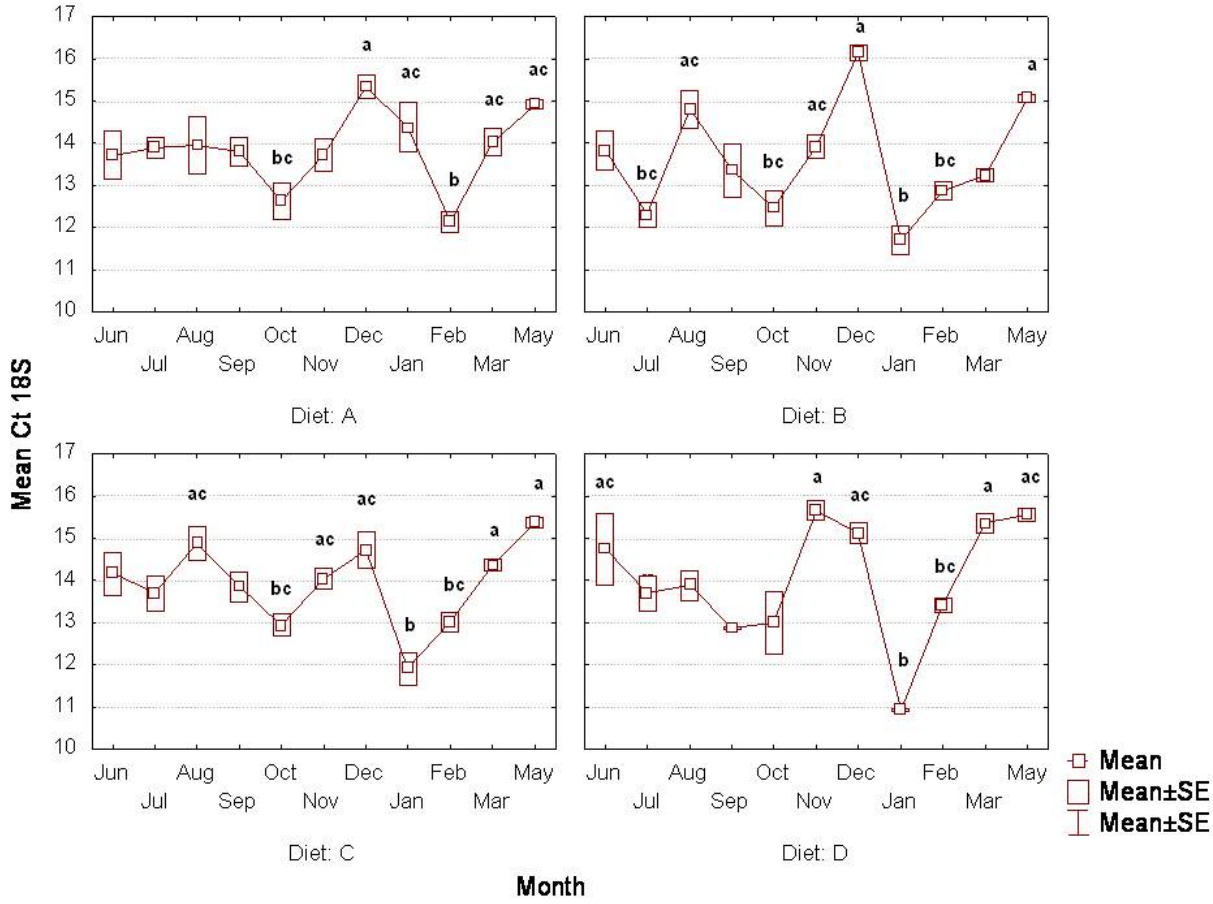


Figure 3.3 18S Ct values in the four experimental diet groups, shown as mean ± se. Boxes marked with different letters differ significantly from each other, and boxes without marking do not differ significantly from any of the other boxes. The experimental period goes from June 2005 to May 2006.

When comparing the mean Ct's for 18S with the ones for StAR, they tend to show a slightly similar pattern over the experimental period (figure 3.4); from June to December the expression is relatively stable, before it increases in January and February (and March for StAR). However, the StAR expression varies more (from ~29.5 to ~36.5 in fold change) than the 18S expression (from ~11 to ~16 in fold change).

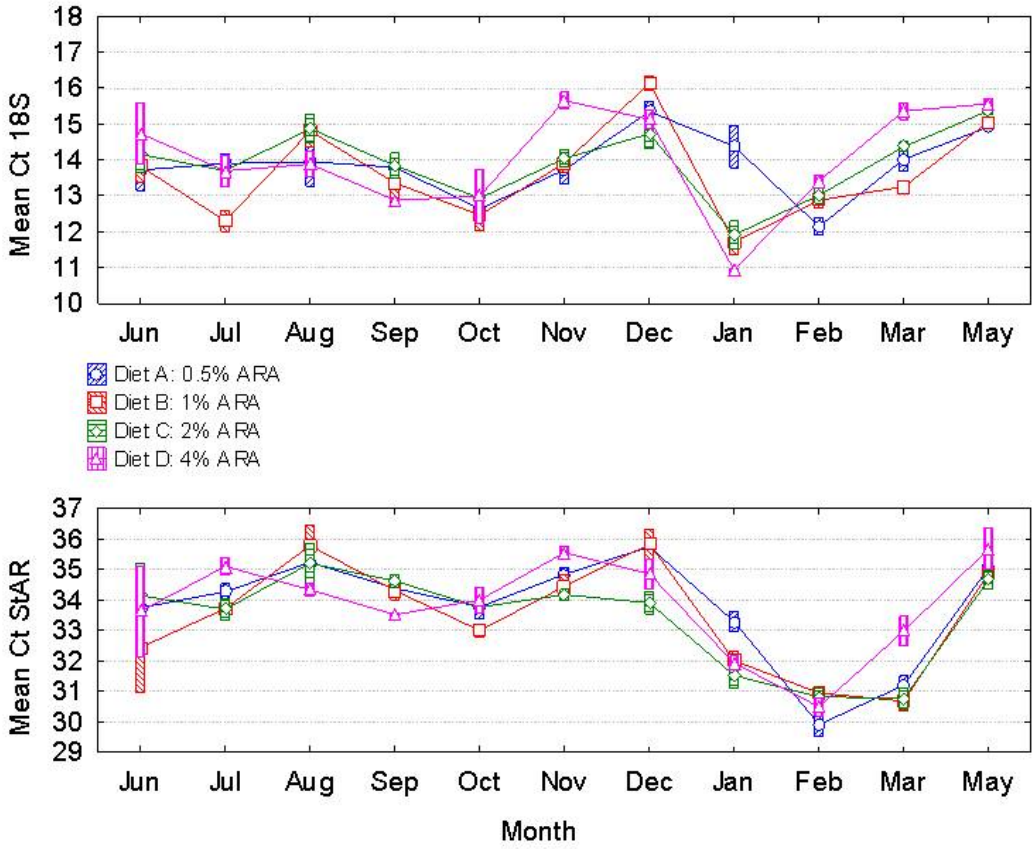


Figure 3.4 18S- and StAR Ct values in the four experimental diet groups, shown as mean ± se. The experimental period goes from June 2005 to May 2006.

3.2 Gonadosomatic index (GSI).

The GSI values were generally low (GSI < 5) in June to October, with the exception of the group fed diet D (4% ARA) in June. From November the values started to increase, and the highest values were reached in February and March. By May the GSI had decreased markedly (figure 3. 5).

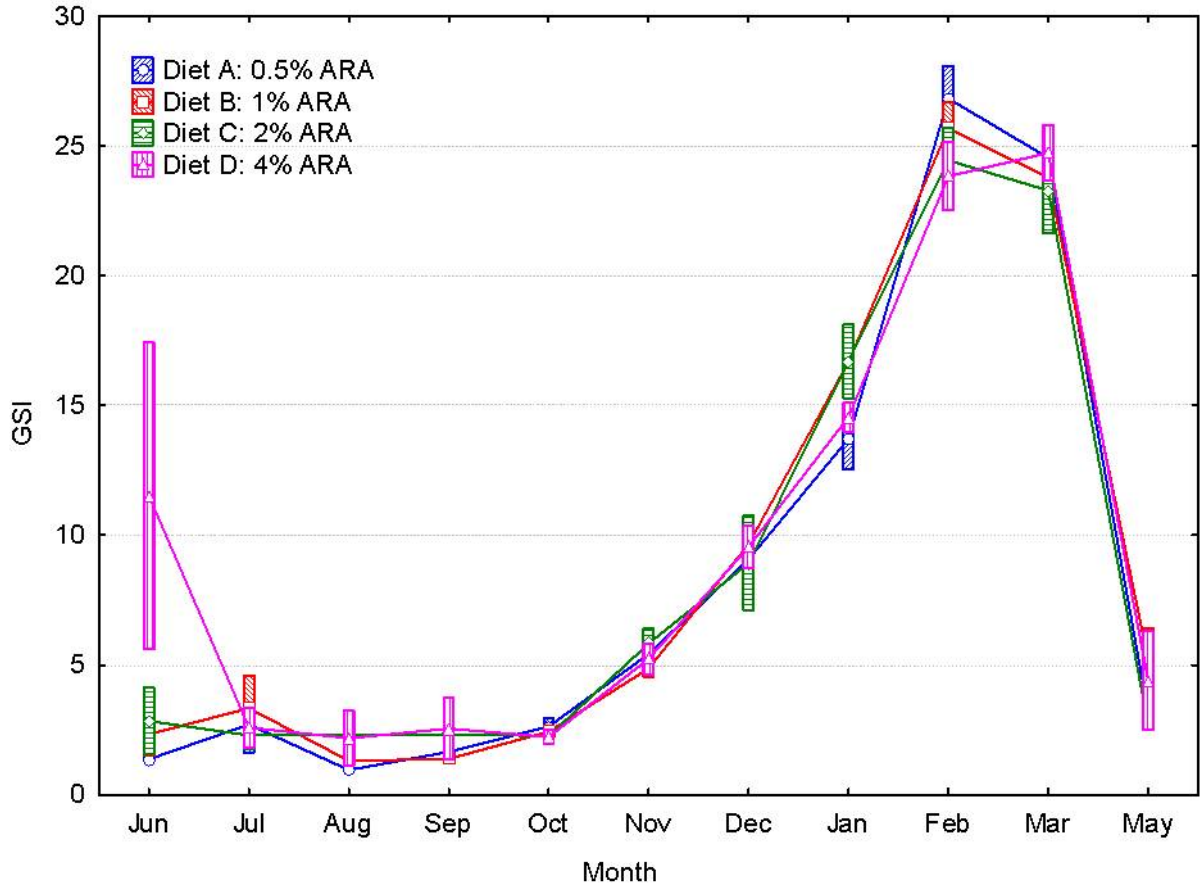


Figure 3.5 GSI in the four experimental diet groups, shown by mean ± se. The experimental period goes from June 2005 to May 2006.

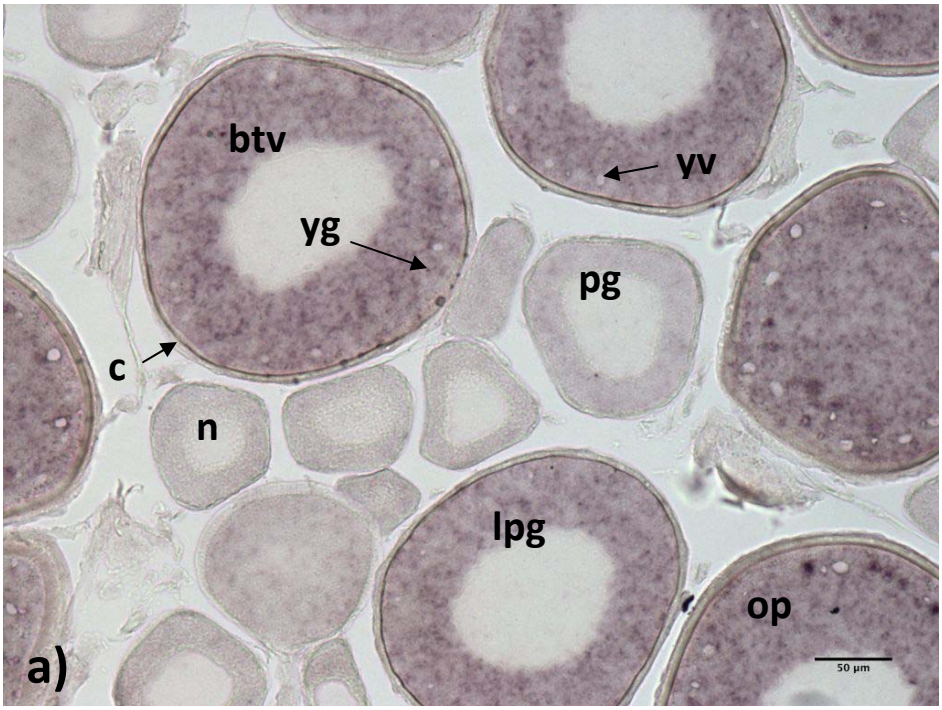
The group fed diet D (4% ARA) showed a slightly different pattern than the other three groups; first of all, this group had a marked variation in GSI values in June compared to the other groups; however, no significant differences were found. Second, this group seemed to have its peak values in March, compared to the other groups which seemed to have their peak values in February. The fish fed diet A (0.5% ARA) appeared to have the highest peak GSI in

February, followed by fish fed diet B (1% ARA) and finally fish fed diet C (2% ARA), but no significant differences were found (figure 3.5).

3.3 Localization of StAR.

3.3.1 Previtellogenic ovarian follicles.

StAR expression was found in previtellogenic ovarian follicles using ISH (figure 3.6), including those of late primary growth and the ones facing yolk vesicle formation, but also in follicles starting to enter true vitellogenesis (for guideline, see table 2.6). StAR transcripts were found in large areas of the oocyte cytoplasm (ooplasm) (figure 3.6 a), but wheter the follicle cells (granulosa- and theca cells) also showed this expression was difficult to determine (figure 3.6 c). Follicles of early primary growth did not clearly reveal the presence of StAR transcripts, but a weak staining could still be seen (figure 3.6 a); however, these follicles did not show noticeably more staining than the follicles with the control (sense) probe (figure 3.6 b)


btv
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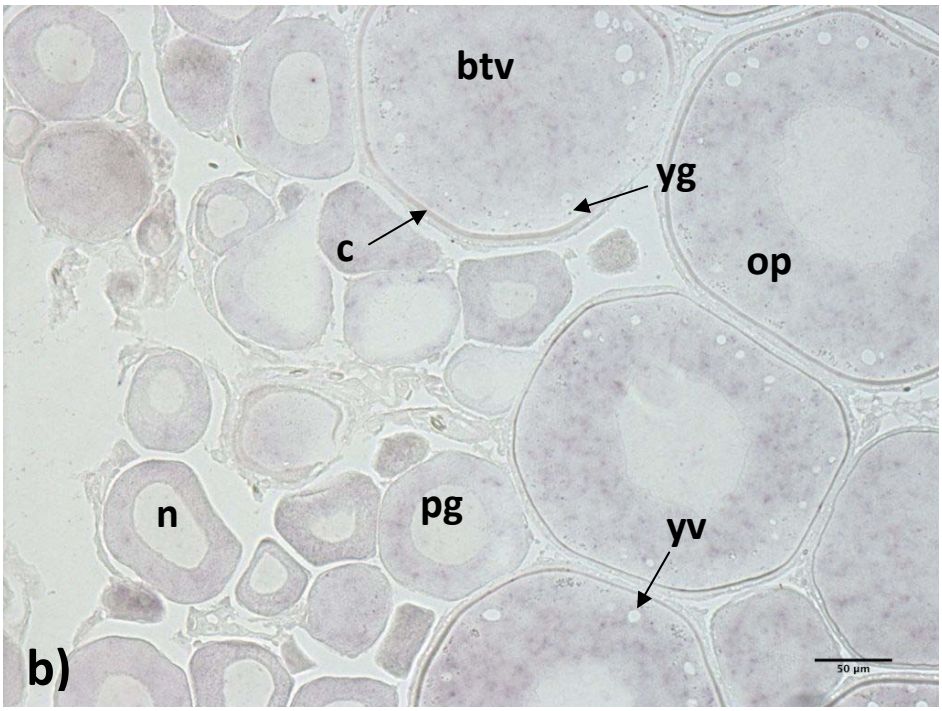
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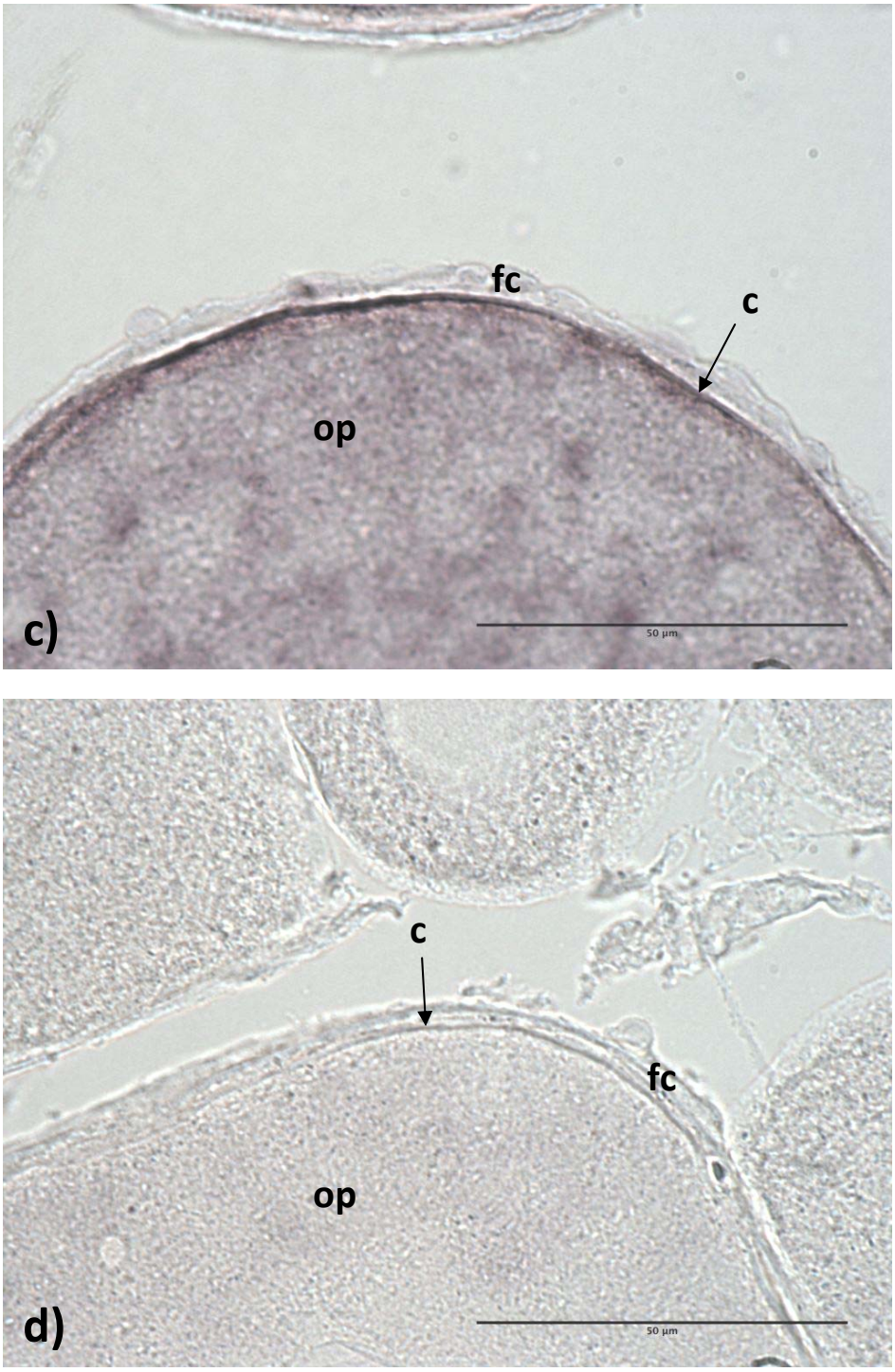
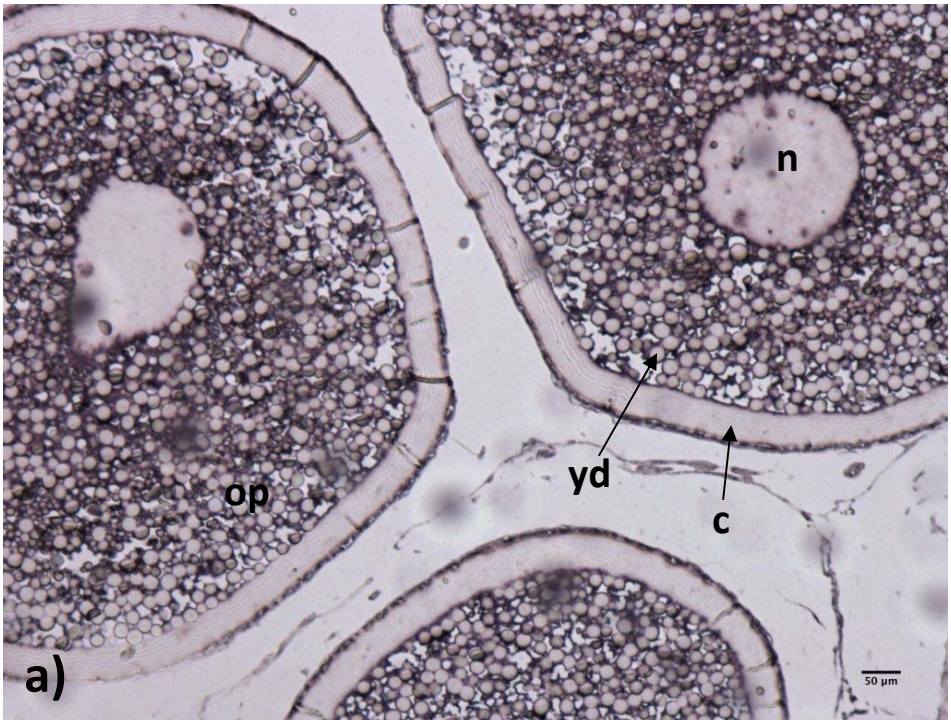
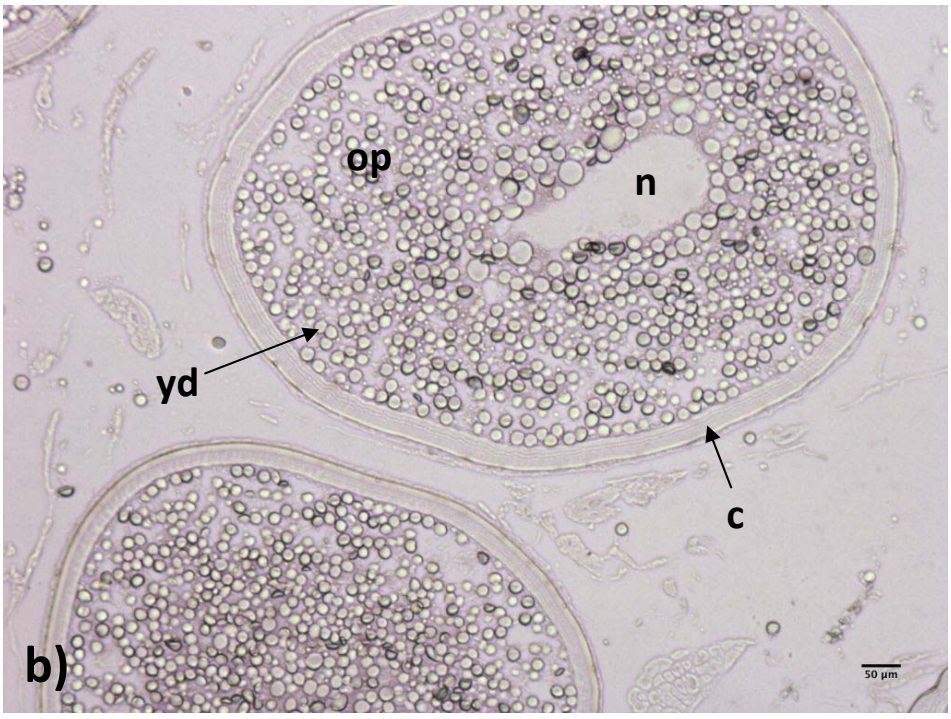


Figure 3.6 *In Situ* Hybridization on Atlantic cod ovarian follicles of varying developmental stages ranging from early primary growth to beginning of true vitellogenesis using an anti sense probe targeting StAR transcripts. btv = follicle of beginning of true vitellogenesis, c = chorion, fc = follicle cells, n = oocyte nucleus, lpg = follicle of late primary growth, op = ooplasm, pg = follicle of primary growth, yg = yolk granule, yv = yolk vesicle (cortical alveoli). All slides are 3 µm thick. Scale bar is 50 µm. a) 20x enlarged follicles with anti sense probe, StAR expression is detected in the ooplasm (op). b) 20x enlarged follicles with control (sense) probe; no StAR expression is detected c) 100x enlarged follicle with anti sense probe; StAR expression is detected in the ooplasm (op). d) 100x enlarged follicle with control (sense) probe; no StAR expression is detected.

3.3.2 Vitellogenic ovarian follicles.

StAR expression was also found in follicles of late true vitellogenesis (figure 3.7); here both the ooplasm (figure 3.7 a) and the follicular granulosa- and theca cells (figure 3.7 c) were stained using the anti sense probe against StAR transcripts.





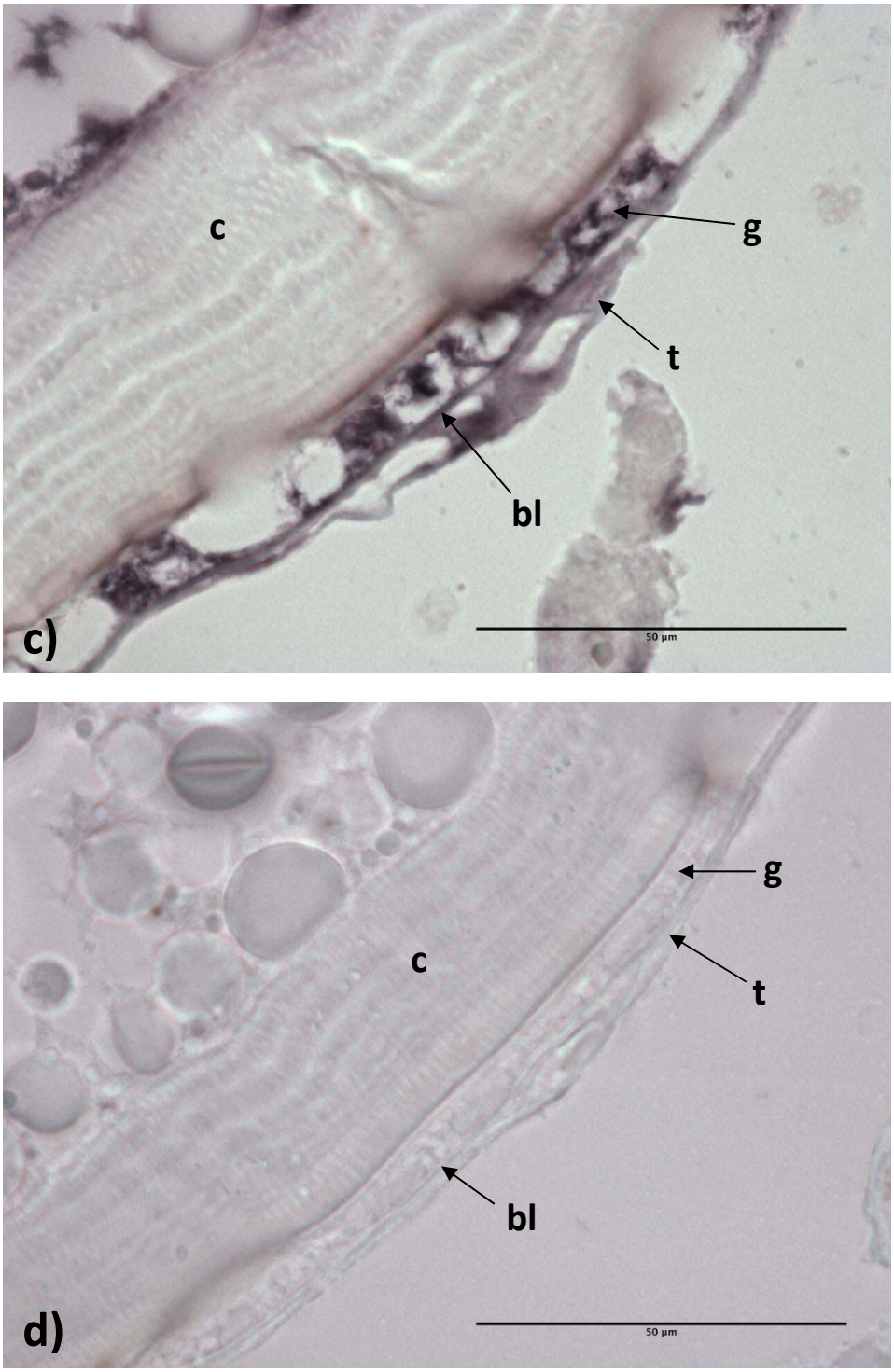


Figure 3.7 *In Situ* Hybridization on follicles of late true vitellogenesis using an anti sense probe targeting StAR transcripts. bl = basal lamina, c = chorion, g = granulosa cells, n = oocyte nucleus, op = ooplasm, t = theca cells, yd = yolk droplet. All sections are 3 µm thick. Scale bar is 50 µm. a) 10x enlarged follicles with anti sense probe; StAR expression is detected in the ooplasm (op). b) 10x enlarged follicles with control (sense) probe; no StAR expression is detected. c) 100x enlarged follicle with anti sense probe; StAR expression is detected in granulosa (g)- and theca (t) cells. d) 100x enlarged follicle with control (sense) probe; no StAR expression is detected.

3.4 Correlation analyses.

3.4.1 Correlation between StAR expression and GSI.

A correlation was found between StAR expression and GSI, both with comparing relative Q (fold-change) values ($p < 0.00000$) and log (Q+1) values ($p < 0.00000$) with GSI. A stronger correlation was found by using log (Q+1) values ($r = 0.607470549$, $R^2 = 0.369020468$) (figure 3.9) than by using relative Q values ($r = 0.405158254$, $R^2 = 0.164153211$) (figure 3.8).

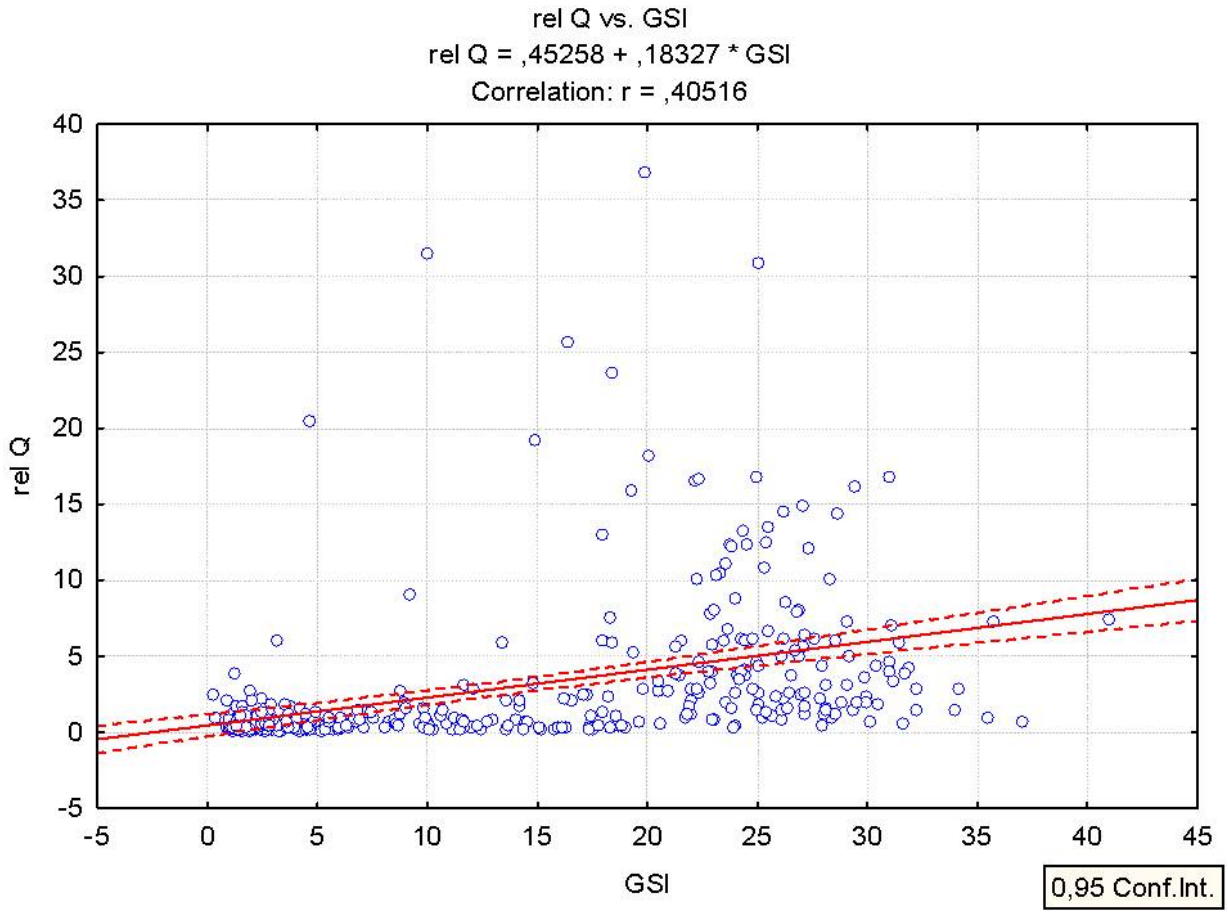


Figure 3.8 Correlation between StAR expression (rel Q) and GSI.

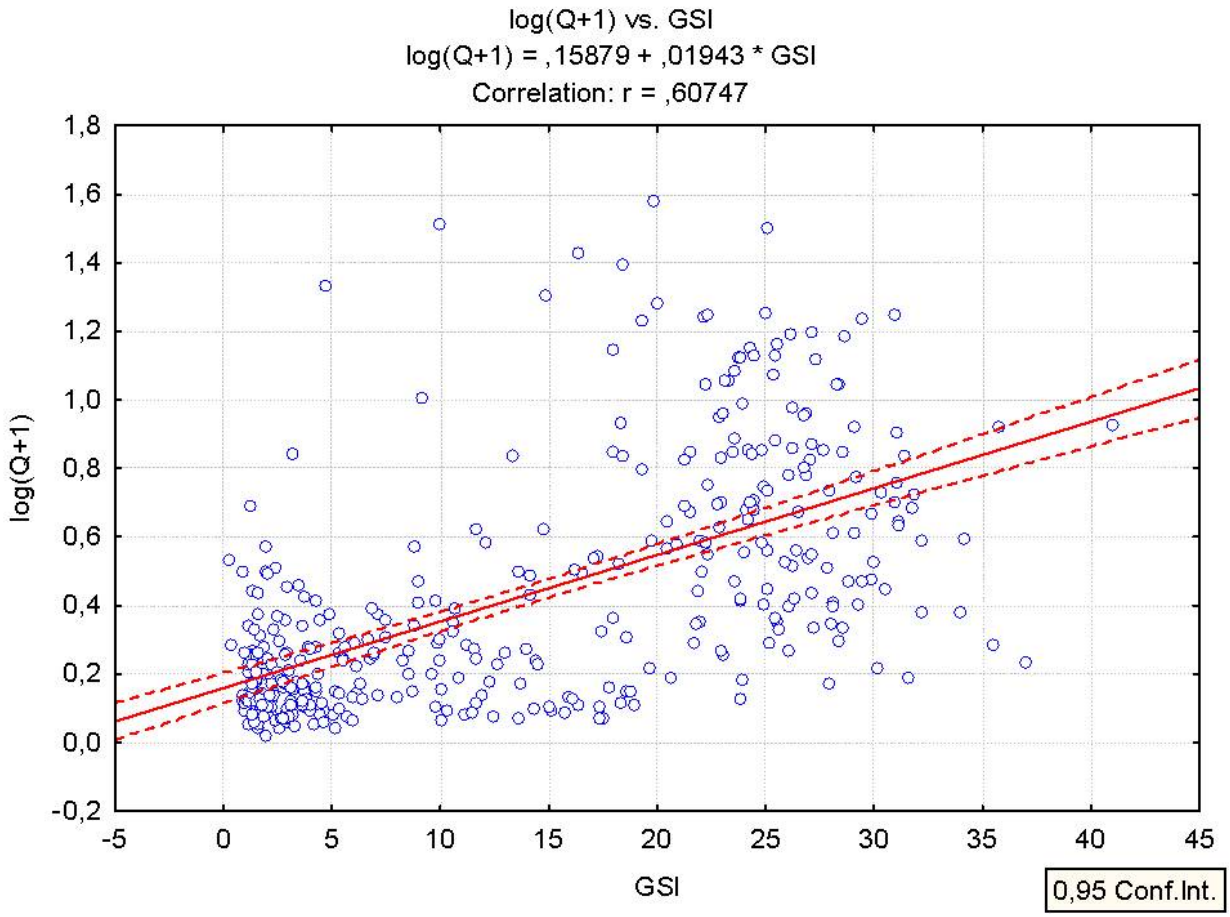


Figure 3.9 Correlation between StAR expression (log (Q+1)) and GSI.

3.4.2 Correlation between StAR expression and E2.

A correlation was found between StAR expression and estradiol (E2), both with comparing relative Q values ($p < 0.00000$) and log (Q+1) values ($p < 0.00000$) with E2. A stronger correlation was found by using log (Q+1) values ($r = 0.38209316$, $R^2 = 0.145995183$) (figure 3.11) than by using relative Q values ($r = 0.25750242$, $R^2 = 0.0663074962$) (figure 3.10).

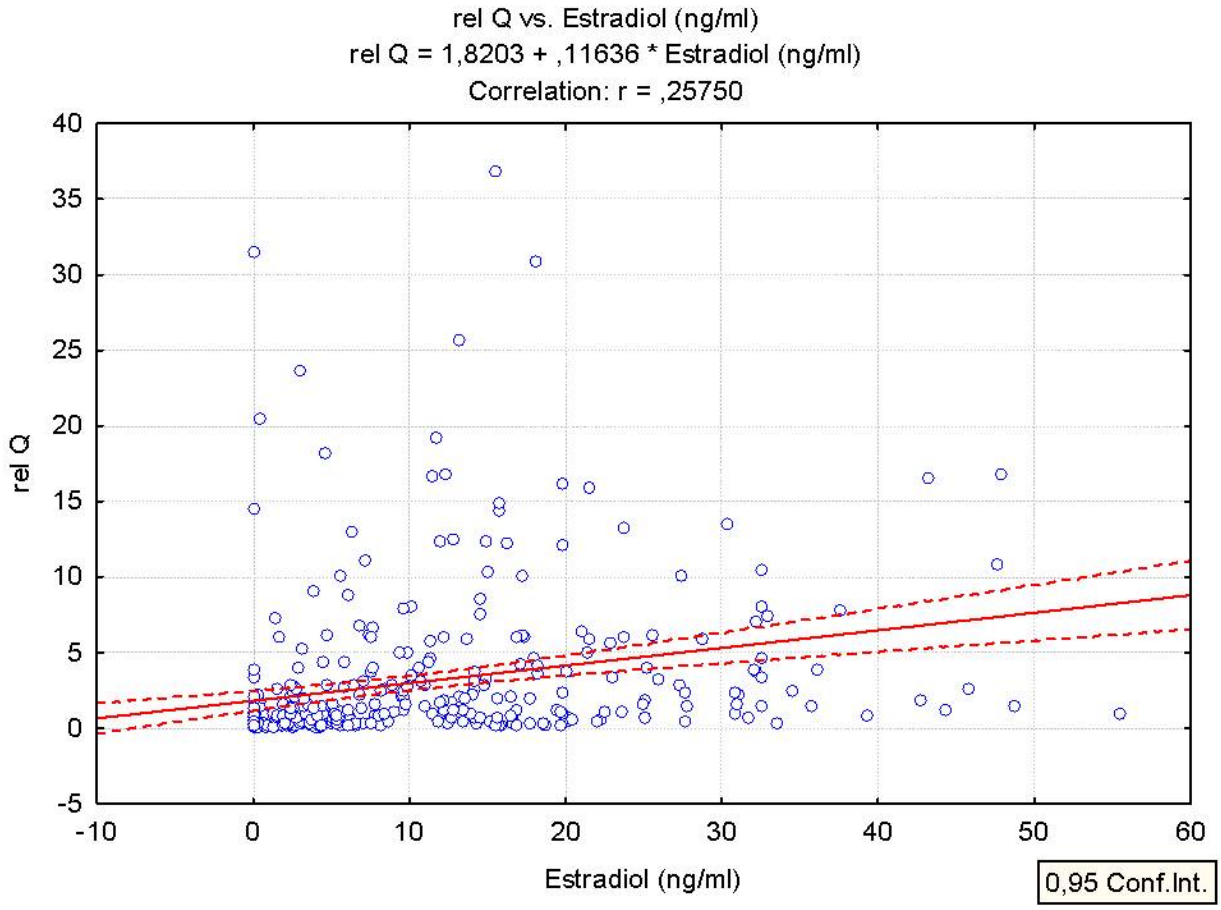


Figure 3.10 Correlation between StAR expression (rel Q) and E2.

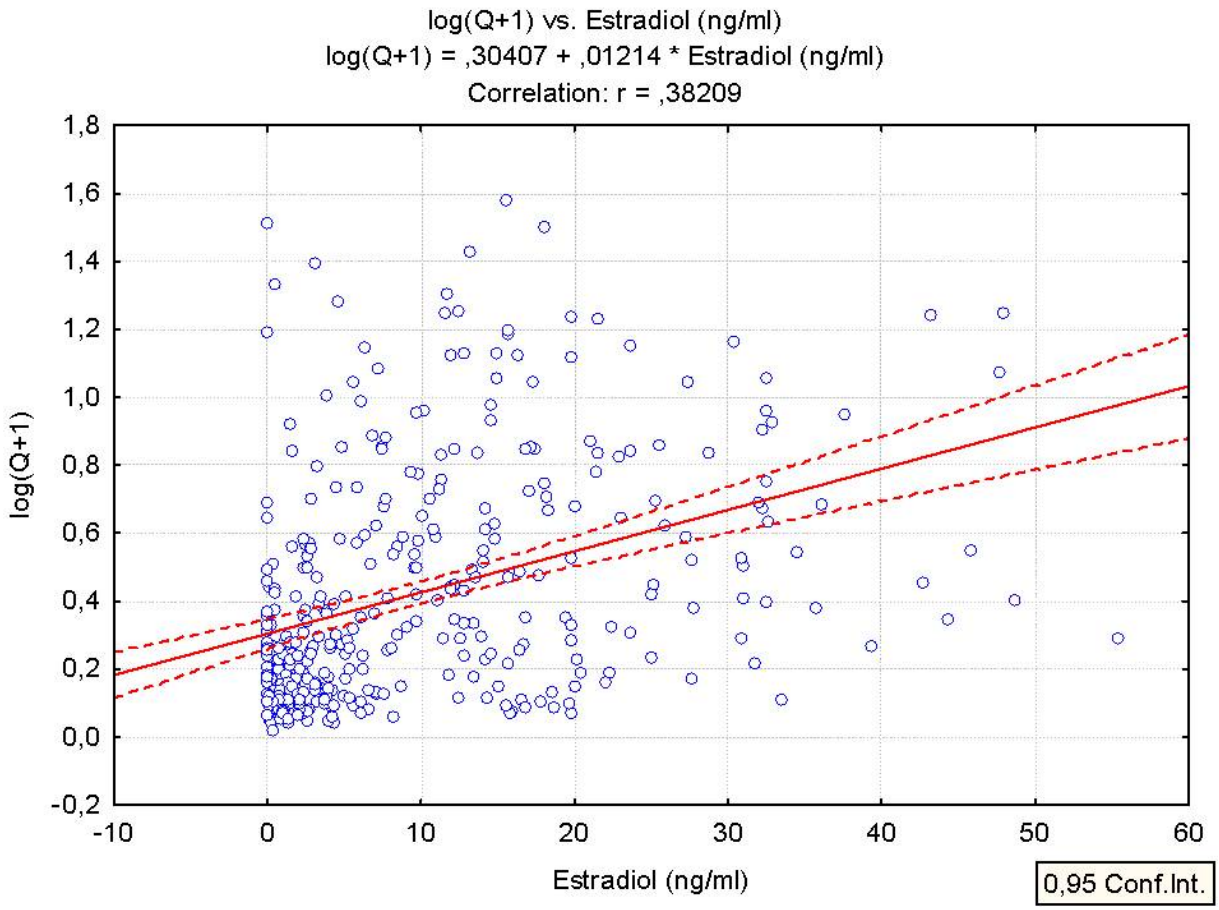


Figure 3.11 Correlation between StAR expression (log (Q+1)) and E2.

4. Discussion.

4.1 Quantification of StAR.

4.1.1 StAR expression over time.

StAR expression changed significantly over time in ovaries of Atlantic cod, and this was true for all experimental diet groups. It tended to be a common pattern; in February and March the StAR expression peaked, and was significantly higher than the expressions in a number of the other sampling times. This is expected, since these months represent the peak spawning season for Atlantic cod; an active steroid production, and therefore also StAR activity, is required in order to produce essential hormones for oogenesis, including testosterone, estradiol and maturating inducing hormone (MIS). This finding that StAR expression in cod ovaries increased and peaked during the spawning season, when a number of the follicles present in the ovary are in late stages of the sexual maturation process, is supported by previous findings of elevated StAR expression around final maturation and ovulation in ovaries of other fish species, including rainbow trout (Kusakabe et al., 2002, Nakamura et al., 2005) and European sea bass (Rocha et al., 2009),

The StAR expression pattern over time observed in this thesis work coincides to a great extent with the pattern of GSI for the same individuals; here the values were relatively low (<5%) from June until October (with the exception of fish fed 4% ARA in June), and then from November the GSI increased toward its maximum in February and March, the same peak months as for the StAR expression. A GSI pattern reminding of the one for StAR activity makes sense in connection to sexual maturation; An increase in StAR transcripts leads to increased steroid production and therefore also increased levels of hormones like estradiol, which again mediates follicular growth and therefore also increased ovary weight and GSI.

Compared to the levels of StAR expression at the other sampling points (excluding the spawning peak months February and March), the expression in June tended to be elevated in three of the four experimental diet groups. A noticeable character of the data from this sampling time, and especially the data representing the fish fed 1% ARA, was a more marked variation in the transcript levels than at later sampling points. This corresponded also to larger

variation in GSI values. Further analysis revealed that individuals that showed elevated StAR levels at this time also had elevated GSI. This is most likely due to some individuals being delayed in the completion of their spawning season at two years of age. In contrast to the next spawning season at 3 years of age, when all the females had low GSI values by May indicating that the spawning season was completed, many of the females had still elevated GSI in June at the end of the spawning in their second year of age. This was particularly pronounced in the group fed 4% ARA, but also in the groups fed 1 and 2% ARA. The reason for this delayed termination of the spawning in some individuals at 2 years of age could be due to the use of artificial continuous light (LL) in some neighboring cages, as LL treatment can delay sexual maturation in cod in sea cages (Taranger et al., 2006).

4.1.2 StAR expression between the diet groups.

In June, the fish fed 1% ARA tended to have elevated StAR expression compared to the other groups; here the mean fold change value was around 6, compared to around 2 or less in the other groups. However, this difference was not found to be significant, and as discussed above this was most likely due to different stages of completion of the previous spawning season among the individual females.

In July, one-way ANOVA showed significant difference in StAR expression between the diet groups; however, the post hoc test did not reveal which groups that were different. Further inspection of the data showed that a few individuals had elevated StAR transcript levels and some had also elevated GSI at this time, possibly since they had completed their spawning late in the season. Therefore, it is not likely that this apparent difference in StAR was due to the diet but just a random effect by the variable time of completion of the spawning season among individual females.

In the following months all the way until January, there were no significant differences in StAR expression between the four diet groups. This may reflect that these months are outside the spawning season and the steroid production is relatively low compared to the actual spawning season. This was also reflected in relatively low StAR transcript levels in all groups until January, and these relatively low StAR transcript levels may have masked any

potential effects of ARA on StAR expression. It may be that any connection between ARA and StAR expression only becomes evident in periods with high StAR activity.

In January, fish fed 0.5% ARA had significantly higher StAR expression than fish fed 4% ARA. This could be an indication of an influence of the amount of dietary ARA on the StAR expression in early spawning in cod; Fish fed 4% ARA seemed to have a slower initiation of increased StAR activity in connection to spawning than what was the situation for fish fed 0.5% ARA. Although there were no significant differences between groups in the following months, fish fed 4% ARA tended to show a slightly different pattern in StAR expression than the other groups from January an onwards; this group seemed to reach its highest expression levels already in February, in contrast to what appears to be the tendency in the other groups, which is highest levels in March. This suggests that increasing dietary ARA may have a negative influence on StAR expression in cod ovaries early in the spawning season. Although little information exists on the effects of different amounts of dietary ARA on StAR gene transcripts, several in vitro studies on other vertebrates have investigated effects of ARA on steroid production, including those of Wang and Leung, 1988, Abayasekara et al., 1990, Johnson and Tilly, 1990, Romanelli et al., 1995, in addition to studies on teleost fish, where ARA has been found to be stimulating (Van Der Kraak and Chang, 1990, Wade and Van Der Kraak, 1993) and both stimulating and inhibitory, depending on the ARA dosage (Mercure and Van der Kraak, 1995, Mercure and Van Der Kraak, 1996). Alternatively, the lower StAR expression in the 4% ARA group in January in this study may reflect effects of ARA on the timing of the spawning season, and not a direct effect of ARA on StAR expression. On the other hand, there were only minor differences in GSI between the 0.5% and 4% diet groups in January, weakening this notion.

Furthermore, it seems like fish fed 2% ARA reached the highest StAR expression compared with the other groups, with a maximum of around 11 compared to a maximum of 6-7 for the fish fed 0.5, 1 and 4% ARA; however, these differences were not significant. It is worth noticing that over the spawning season there was an increase in variation of the data despite an increase in the number of individuals sampled in this part of the study. This spawning-related variability in the data within each fish group, even though they have been sampled at the same date and time, could be explained by the fact that cod spawn every 60-70 hours (depending on the temperature); this means that at the time of sampling, individuals within the same diet group may exist in different spawning stages, and therefore may also

differ in their StAR gene expression levels. Additionally, mRNA levels may be more dynamic than levels of other parameters like steroids, which are end products; consequently, when quantifying transcript levels in different individuals at the same time, a certain variation is expected.

4.2 Localization of StAR.

In this thesis work StAR expression was detected in cod ovarian follicles of various developmental stages. In both previtellogenic (excluding follicles of early primary growth) and vitellogenic follicles, StAR expression was detected in large areas of the oocyte cytoplasm (ooplasm). Furthermore, StAR expression was found in follicle cells (granulosa- and theca cells) of late vitellogenic follicles. In previtellogenic follicles it was difficult to determine if StAR was expressed or not in these cells.

The observation that no expression was visible before the follicles had reached late primary growth indicates that StAR transcripts do not appear until the follicles reach a certain developmental stage. This is reasonable, when considering that StAR expression stays low and does not increase until the spawning season, when the follicles start to grow and develop. This is supported by a study on human ovaries, where StAR expression was not detected in immature follicles (Pollack et al., 1997). Furthermore, StAR expression was not detectable in trout ovarian follicles until the beginning of final maturation (Kusakabe et al., 2002).

The detection of StAR expression in granulosa- and theca cells reflects that these are the steroidogenic cells of the ovary tissue. Together these two cell layers produce steroidogenic components needed for follicular growth and maturation (Nagahama, 1994), and when considering that they are important sites for steroid production, it makes sense to find StAR transcripts here. Additionally, it has previously been suggested that StAR expression in steroidogenic tissues is restricted to steroidogenic cells within these tissues (Clark et al., 1995), and other studies have found StAR expression in either theca cells or both theca- and granulosa cells in ovaries of different vertebrate species, among them are human (Pollack et al., 1997), cow (Soumano and Price, 1997) swine (Garmey et al., 2000), trout (Kusakabe et al., 2002) and sheep (Logan et al., 2002). However, in previtellogenic follicles it was difficult to determine if StAR was expressed in these cell types; this was due to small

sized follicles with less developed follicle cells. In these follicles it was challenging to separate chorion, granulosa- and theca cells from each other, and consequently no clear statements could be made about the exact localization of the expressed StAR transcripts. Logan et al., 2002 suggested that theca cells of sheep ovarian follicles do not become steroidogenically active until later in development, regardless of the earlier formation of these cells; this may also be true for cod ovarian follicles, if StAR is not expressed in these follicle cells.

StAR transcripts were also detected in the ooplasm in both late previtellogenic and vitellogenic ovarian follicles. This is interesting, considering that the theca- and granulosa cells are the sites for steroid production in ovaries, and not the ooplasm. This may be expressions for maternally transferred StAR transcripts that are essential components for later steroid production; the reason for this theory is that unfertilized eggs (ovulated oocytes) contain, in addition to a number of different components like yolk proteins, maternal mRNAs, hormones, proteins and vitamins (Bobe et al., 2009), several sex steroids (Feist et al., 1990). StAR may also have other functions than regulating steroidogenesis, as is suggested in Logan et al., 2002, where StAR expression was detected in oocytes that lacked accompanying expressions of steroidogenic enzymes. Furthermore, StAR expression has been found in non-steroidogenic tissues (Kusakabe et al., 2002), something that indicates additional roles for StAR than a regulator of steroid production.

4.3 Correlation analyses.

4.3.1 Correlation between StAR expression and GSI.

A positive correlation was observed between StAR expression and GSI, and by converting the data to a log function (log of rel Q + 1), it gave the best correlation (r = 0.60747). As mentioned, it makes sense that StAR activity and GSI follows a similar pattern in connection to spawning. Both parameters start to increase in the beginning of the spawning season, and they also have peak values in February and March; they are linked together through steroid production and ovary growth. Given this, it is expected that these two parameters correlate.

4.3.2 Correlation between StAR expression and E2

A positive correlation was observed between StAR expression and E2, and by converting the data to a log function (log of rel Q + 1), it gave the best correlation (r = 0.38209). As with the GSI, a correlation was expected to be found between E2 and StAR expression; these two parameters are connected through the steroid synthesis. E2 is one of the end products of the steroidogenesis, and StAR controls the rate limiting step of this process; elevated StAR activity would then be expected to be followed by elevated levels of steroids, and therefore also E2. This observed correlation is in agreement with the finding of another study, where elevated StAR expression coincided with an increase in plasma E2 levels (Rocha et al., 2009). On the other hand, Nakamura et al., 2009 found that E2 downregulates expression of StAR in rainbow trout previtellogenic ovaries, suggesting a possible negative feedback effect. However, limited information exists about the effects of sex steroids on the steroid production of the gonads.

4.3 The performance of this thesis work.

The performance of this thesis work was carried out without any problems, through careful guidelines from superviors and laboratory personnel. However, if I was to repeat the study, a few changes would be considered. First of all, a major advantage would be for me to participate in all procedures that needed to be performed in order to obtain the results; in this way I would have more control over what had been done, and possible sources of errors would be easier for me to detect. Second, a higher number of fish would be advantageous to include in the quantification of StAR. Despite more individual samples originally available, quite few samples were suitable to include in the qPCR analysis in some groups at certain sampling points (see table 2.1). A high n is necessary to create a good basic for statistical analyses.

4.4 The methods used in this thesis work.

Two methods were used in this thesis work to analyze the sampled material; Real-time quantitative PCR (qPCR) and *In Situ* Hybridization (ISH). As mentioned in the introduction, qPCR is a method used, among other purposes, for quantification of nucleic acids (Wilhelm and Pingoud, 2003). By introducing fluorescent dyes or probes to the polymerase chain reaction in qPCR, the amount of product formed can be quantified; this is possible by monitoring the fluorescence of the probes or dyes, which is proportional to the amount of product formed (Kubista et al., 2006). In this case, the amount of StAR transcripts (StAR mRNA) in cod ovaries has been quantified. This method is advantageous compared to standard PCR because it quantifies the nucleic acids over the whole amplification period, so that differences in amount of product between samples can be detected; by applying standard PCR, only an end-point measurement is achieved; differences between samples can therefore not be detected. However, in qPCR precise processing of the samples is required, and the method includes numerous processing steps before the qPCR instrument is able to analyze the samples. In order to assure acceptable result values, several aspects can be tested. With the exception of optimalization of the StAR qPCR assay and being strict in which Ct-values that were accepted for further analyses (see *2.5.1.2 "qPCR calculations in Microsoft excel"*), the mean Ct-values of the reference gene (18S) were analyzed and then compared to the values of the gene of interest (StAR) in this thesis work.

The purpose of having a reference gene in qPCR is to have a relatively stable gene expression to which the gene expression of interest can be compared to, in order to measure how much this expression changes relative to the reference. For this to give reliable results the expression of the reference gene should be quite stable over the experimental period. In this thesis work 18S changed significantly over time, in all groups of fish. This is unfortunate, and many different factors could have affected this. One factor could be physiological; no known gene expressions are 100% stable in all tissues at all times, live animals are dynamic and follow both biological and seasonal rhythms. When comparing 18S expression with that of StAR, there was a tendency of parallel expression patterns in the two genes; from June to December the expressions were relatively stable, followed by elevated values in January and February (and March for StAR). This might be a response to physiological processes that take

place in the fish during this time of year – the spawning season. However, the StAR expression changed more than the 18S expression; this is predictable since StAR is not a housekeeping gene, and is therefore expected to be able to change is activity markedly in response to various stimuli. Another factor that could possibly affect the stability of 18S expression in this case is the processing of the material for quantification, notably the RNA isolation and cDNA synthesis. As mentioned these steps had previously been performed at IMR, Austevoll; during this work the samples had not been handled randomly, but in numerous order. Consequently, samples from one month might have been handled slightly different from samples from other months etc. Additionally, a spectrophotometer was used instead of a nanodrop to determine the amount of isolated RNA from each sample; a nanodrop is more accurate than a spectrophotometer, something that may indicate a source of error, that again could lead to an uneven reference gene expression.

In ISH a labeled nucleic acid probe (DNA or RNA) is hybridized to a sequence of mRNA that is complementary to the probe; this allows for localization of gene expressions in tissue sections (Wilcox, 1993) by visualization of the hybridized probe. In this thesis work, StAR transcripts (StAR mRNAs) were localized in cod oocytes by applying non-radioactive digoxigenin (DIG) labeled cRNA probes. Both isotopic and nonisotopic labels can be applied; however, despite the fact that isotopic labels might have better sensitivity, a nonisotopic system is not only more practical to apply (Kadkol et al., 1999), but it also gives better cytology. In this thesis work the tissues for localization of StAR were fixated in paraformaldehyde and embedded in paraffin; although it would be interesting to see some additional results on cryo sections, paraffin sections were chosen to be prioritized because they give better cytology. As mentioned, the fixation ratio was 1:25 tissue:fixation; however, this is somewhat challenging to achieve with high accuracy for an untrained eye. Consequently, this might have contributed to a possible source of error, considering that correct fixation is vital for accomplishing good results. Furthermore, the probe type (single stranded RNA) and the ISH protocol used in this thesis work was chosen after experience at the molecular laboratory at IMR; this type of probe and protocol is often used when working with gonadal tissues, and has repeatedly shown to work very well.

4.5 Statistics.

All the statistical tests used in this thesis work were performed in Statistica 8.0. To check for significant differences in StAR expression and GSI between groups of fish fed different amounts of dietary ARA, a one-way ANOVA was used, and if a significant difference ($p<0.05$) was found, the post-hoc test Unequal N NSB was applied to check which group was different. This parametric test is statistically stronger than a non-parametric test, so when possible, this is a preferred test to apply. However, it assumes normal distribution, and the data for quantification of StAR did not always show this. However, we decided that the homogeneity- and normal distribution tests performed (see appendix 5) gave acceptable results, so that the one-way ANOVA, could be applied. Furthermore, since the StAR data did not contain equal n, both between sampling dates and between groups within the same sampling date, the Unequal N NSB was chosen because it does not assume equal n. In order to check if the StAR expression in general changed over time, the choice of statistical test was a non-parametric Kruskal-Wallis; this test was chosen because when putting all fish groups together for each sampling date, the variation increased, and the data set was no longer suitable for a parametric test. This test was also used to check for differences over time in the Ct values, because it is a suitable test when checking for changes over time. As mentioned, some of the data values in this thesis work were transformed to log values (see *2.7 "statistical methods*"); this was done to obtain better normality, so that the data would be more suitable for statistical analyses.

4.6 Conclusions.

This thesis work was based on four hypotheses, where the first one hypothesized that StAR transcript levels vary over the reproductive cycle in cod. This was verified since all diet groups had significant changes in StAR expression over the experimental period, with a peak in the spawning season.

Second, it was hypothesized that there is a connection between the amount of dietary ARA and the levels of StAR transcripts in cod ovaries. The results did not strongly indicate such a connection, since there were only minor effects on ARA on StAR expression

throughout the study. However, this hypothesis can not be rejected, since fish fed 0.5% ARA had significantly higher StAR expression than fish fed 4% ARA in January; this indicates that dietary ARA does have some effects on StAR transcript levels, in particular early in the spawning season.

Furthermore, it was hypothesized that StAR expression is confined to the steroid producing granulosa- and theca cells of cod ovaries. Although it was difficult to localize the signal in previtellogenic follicles, StAR expression was clearly detected both in granulosa- and theca cells in late vitellogenic follicles. This hypothesis could therefore be verified for late vitellogenic follicles in cod ovaries.

The last hypothesis of this work was that StAR transcripts are expressed differently in cod ovary follicles of different sizes/degrees of development. Although it is difficult to quantify the degree of expression of gene transcripts in a localization technique like ISH, some conclusions could still be made in this connection; StAR transcripts did not become visible in the ooplasm until the follicles had reached late primary growth stage, and for the granulosa- and theca cells, StAR expression was not clearly detected until the follicles were late vitellogenic. The hypothesis could therefore be verified.

The aim of this thesis was to quantify the transcript levels of StAR in cod broodstock ovaries over a full reproductive cycle, then relate this to the amount of dietary ARA, and to localize StAR within follicles of different developmental stages; all of this was achieved during the time frame for this master thesis. Additionally, through this thesis work, a qPCR assay was created for StAR in cod, something that has not been performed before.

4.7 Future perspectives.

Through this thesis study I have gained knowledge in the field of the StAR gene in female cod; both how its transcript levels change over the reproductive cycle, where it is expressed in ovaries and how it may respond to the amount of arachidonic acid in the feed. These are important aspects within cod farming, when trying to understand the mechanisms behind the sexual maturation process and how we can supply optimal nutrition for the broodstock. Further research on StAR and especially on its relation to dietary arachidonic acid would be

interesting, to increase our knowledge about the effects of this fatty acid on steroid production and therefore also the sexual maturation process, and then to search for a possible connection to the spawning problems that represent a major concern in today's cod farming.

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Appendix 1

Ingredients used in the laboratory.

Chemicals.

The chemicals used in this thesis work are listed in table 1.

Table 1 Overview of the chemicals used (including buffers ready for use from kits).

Chemical	Manufacturer/supplier
Acetic Anhydride	Sigma-Aldrich
Acetylated BSA (bovine serum albumin) 1:10	In kit from Invitrogen
Agarose, LE	SeaKem®
Ampicillin, D(-)α-Aminobenzylpenicillin	Sigma-Aldrich
Anti-digoxigenin-AP, Fab fragments	Roche
5-bromo-4-chloro-3-indolyl-phosphate-4-toluidine salt (BCIP, 4-toluidine salt)	Roche
Bromphenol blue	Riedel-de Haën
Buffer P1	In kit from Qiagen
Buffer P2	In kit from Qiagen
Buffer P3	In kit from Qiagen
Buffer QBT	In kit from Qiagen
Buffer QC	In kit from Qiagen
Buffer QF	In kit from Qiagen
Chloroform	Sigma-Aldrich
Denhardt's solution 50x concentrate	Sigma-Aldrich
Dextran sulfate sodium salt	Sigma-Aldrich
DNA-ladder, 1Kb	Invitrogen
Diethyl pyrocarbonate (~97% NMR)	Sigma-Aldrich
Ethanol, absolute	Kemetyl
Ethidium bromide	Boehringer Mannheim
Formamide	Fluka
Formamide, deionized	Sigma-Aldrich
Glycerol, 87%	Merck
Goat serum	Dako
Levamisole/tetramisole	Sigma-Aldrich
LiCl	Sigma-Aldrich
MgCl2	Merck
NaCl	Merck

NaAc, pH 5.2	Sigma-Aldrich
NaOH, 1N	Merck
Nitroblue tetrazolium chloride (NBT)	Roche
NTP labeling mixture	Roche
PAP-pen	Dako
Paraffine, histowax 56-58°C	Leica
Paraformaldehyde	Merck
Phenol chloroform isoamyl	Sigma-Aldrich
Phosphate buffered saline (PBS) tablet	Calbiochem
Protector RNase inhibitor	Roche
RE 10x buffer	In kit from Invitrogen
Saline-sodium citrate (SSC) buffer, 20x	Sigma-Aldrich
TacMan Fast universal PCR mastermix (2x)	Roche
TBE-buffer 10 x pH 8.3 tris-borat-EDTAbuffer	Merck
Transcription buffer 10x	Roche
Triethanolamine	Fluka
Tris buffer, pH 8.0	Calbiochem
Tris-HCl, 1M, pH 8.0	Invitrogen
Tryptone soyabroth	Oxoid
Water, Rnase-, Dnase-, and protease-free	5 Prime
Xylene	Merck

***Solutions*:**

The following solutions were used in this thesis work:

Acetic anhydride solution:

Acetic anhydride 0.25 %

TEA buffer

The ingredients were mixed just before use.

50% glycerol in TEN:

Glycerol

TEN buffer

50% of glycerol was added to the wanted volume of TEN.

Buffer B1:

Tris-HCl 0.1 M

NaCl 0.15 M

The ingredients were mixed, and the pH was adjusted to pH 7.5.

Buffer B2:

B1

Heat inactivated goat serum 1%

The goat serum was inactivated by keeping it at 56ºC for 3 minutes. Then the ingredients were mixed.

Buffer B3:

Tris-HCl 0.1 M

NaCl 0.1 M

$MgCl_2$ 0.05 M

The ingredients were mixed, and the pH was adjusted to pH 9.5.

Buffer B4:

Buffer B3

Nitroblue tetrazolium chloride (NBT) 3.4 µl (stock 100 mg/ml)/ml B3

BCIP, 4-toluidine salt 3.5 µl (stock 50 mg/ml)/ml B3

Levamisole 1 mM (0.24 mg/ml)

The ingredients were mixed in dark.

DEPC-H_2O, 1L:

Diethyl pyrocarbonate (~97% NMR) 1ml

Destilled H_2O 1L

The Diethyl pyrocarbonate was added to the water, and heated for 1-2 hours in a 37 ºC water bath. Then the solution was autoclaved.

EDTA (Ethylenediamine tetra acetic acid**):**

EDTA-pellets	186.2 g
NaOH	~20 g
Destilled H_2O	1 L

The EDTA pellets and NaOH were solved in the destilled water, and pH was adjusted to pH 8.0.

Hybridization buffer:

Formamide	50 %
SSC	5x
Denhardt's solution	5x
tRNA	250 µg/ml
Fish sperm DNA	500 µg
Dextran sulfate	10 %

Recipe for 20 slides:

1) Dextran sulfate	1.2 g
2) Water, Rnase-, Dnase-, and protease-free	480 µl
3) SSC (20x)	3 ml
4) Denhardt's solution (50x)	1.2 ml
Fish sperm DNA	1.2 ml
tRNA	120 µl
Formamide	6 ml

Ingredients 1-3 were mixed, vortexed and left for a while until the dextran sulfate was dissolved. Then the rest of the ingredients were added.

Loading dye, 10x:

EDTA	20 mM (23 mg)
Glycerol	50 %
Bromphenol blue	0.05 % (4 mg)

Destilled H_2O was added until a final volume of 4 ml was reached.

Luria-Bertanina (LB)-medium:

Tryptone	10 g
NaCl	10 g
Destilled H2O	1L
Ampicillin	100 µg/ml

The pH was adjusted with 5M NaOH to pH 7.0, and the solution was autoclaved. After the solution had reached a temperature below 60 ºC, ampicillin was added to the final concentration of 100 µg/ml.

Mastermix (for both StAR and 18S):

RNase-, DNase-, and protease-free H_2O	3.5 µl
TaqMan Fast universal PCR mastermix (2x)	12.5 µl
Forward primer	2.25 µl
Reverse primer	2.25 µl
Probe	0.5 µl

The ingredients were mixed by vortexing and centrifuging for a few seconds.

The primers and the probe in the recipe are specific for each gene. For every qPCR reaction plate the volume of each ingredient in this recipe needs to be multiplied with at least the number of wells in the plate. In this case they were multiplied with 53, which gives a safe amount of mastermix for a plate containing 48 wells.

4 % Paraformaldehyde fixation solution:

Paraformaldehyde	40 g
NaOH (1N)	3-10 droplets
2x PBS	500 ml
Destilled H_2O	500 ml

The paraformaldehyde was added to the water and heated (with magnet stirrer) until the temperature of the solution reached 70-80 ºC. Then the heat was turned off, and 3-10 droplets of NaOH were slowly added until the solution was totally clear. Finally the PBS was added.

Phosphate buffered saline (PBS):

PBS-tablet	1 tablet
Destilled H_2O	1L

The tablet was dissolved in the water.

Probe 1:10 (for qPCR) (for both StAR and 18S):

Stock solution was diluted with RNase-, DNase-, and protease-free H_2O in the ratio 1:10. Then the primer solution was vortexed and centrifuged for a few seconds.

RNase buffer:

Tris-HCl, pH 7.5	0.01 M
NaCl	0.5 M
EDTA	0.005 M

The ingredients were mixed.

18S primer 1:10 (for qPCR):

Stock solution	100 µl
RNase-, DNase-, and protease-free H_2O	890 µl
Tris buffer, 1M, pH 8.0	10 µl

The ingredients were mixed by vortexing and centrifuging for a few seconds.

StAR primer 1:10 (for qPCR):

Stock solution was diluted with RNase-, DNase-, and protease-free H_2O in the ratio 1:10. Then the primer solution was vortexed and centrifuged for a few seconds.

TE-buffer:

Tris-HCl 10 mM

EDTA 1 mM

The ingredients were mixed, and by using 6 M HCl the pH was adjusted to pH 7.5. The buffer was then sterile filtrated.

TEA, 0.1 M, pH 8.0, 300 ml:

Trietanolamin 5 ml

DEPC-H_2O 295 ml

The ingredients were mixed just before use.

TEN buffer:

TRIS-HCl 10 mM

EDTA 1 mM

NaCl 0.9 %

The ingredients were mixed, and the pH was adjusted to pH 8.0.

Tris-HCl, 0.05 M, pH 7.5:

Tris-HCl stock solution 1 M

DEPC-H2O

Stock solution was diluted with the DEPC-H_2O.

Reagent kits:

The reagent kits used in this thesis work are listed in table 2.

Table 2 Overview of the reagent kits used.

Kit name	Manufacturer/supplier
HiSpeed plasmid midi kit	Qiagen
Not I	Invitrogen
Spe I	Invitrogen

Enzymes:

The enzymes used in this thesis work are listed in table 3.

Table 3 Overview of the enzymes used.

Enzyme name	Manufacturer/supplier
DNase 1, RNase-free	Roche
Not I restriction enzyme	In kit from Invitrogen
Protector Rnase inhibitor	Roche
Proteinase K	Roche
Spe I restriction enzyme	In kit from Invitrogen
T3 RNA polymerase	Roche
T7 RNA polymerase	Roche
Taq DNA polymerase	Promega

Plasmids:

The plasmids used in this thesis work are listed in table 4.

Table 4 Overview of the plasmids used.

Plasmid name	Manufacturer/supplier
pCR®4-TOPO®	Invitrogen

Nucleic acids.

The nucleic acids used in this thesis work are listed in table 5.

Table 5 *Overview of the nucleic acids used.*

Nucleic acid name	Manufacturer/supplier
Control DIG-RNA	Roche
Fish sperm DNA	Roche
tRNA	Roche

TaqMan primers:

The TaqMan primers used in this thesis work (for qPCR) are listed in table 6.

Table 6 Overview of the TaqMan primers used.

Primer name	5' to 3' sequence	Manufacturer/supplier
StAR forward	CATCCACCATGAACTCAACAGAA	Invitrogen
StAR reverse	TCGATCCTGGAGCTGAGGAA	Invitrogen
18S forward	CCCTGTAATTGGAATGAGTGTACTTT	Invitrogen
18S reverse	ACGCTATTGGAGCTGGAATTACC	Invitrogen

TaqMan probes:

The probes used in this thesis work (for qPCR) are listed in table 7.

Table 7 Overview of the TaqMan probes used.

Probe name	Method	5' to 3' sequence	Manufacturer/supplier
StAR probe	qPCR	6-FAM-CCCGGCTCTGGCA-MGB	Applied biosystems
18S probe	qPCR	6-FAM-CACCAGACTTGCCCTCC-MGB	Applied biosystems

Appendix 2

Protocol for RNA isolation Fastprep and Tri-reagent.

Work RNase-free and wear gloves.

Cool centrifuge and ice/cool blocks are needed.

- Mark FastPrep tubes, add 1 ml of Tri-reagent, keep on ice (cool blocks).

- Add tissue (50-80 mg) to Tri-reagent, leave on ice/cool block for at least 5 minutes.

- FastPrep machine: put tubes in and fasten the lids with the three screws.

- Run on speed 4 for 20 seconds (the machine makes swootching noice; if it makes more noise, tighten the lid-screws).

- Take out the tubes and leave at room temperature for 5 minutes.

- Add 200 µl chloroform, wortex for 1 minute.

- Centrifuge for 15 minutes at 4ºC and max speed (prepare new tubes to take supernatant).

- Take supernatant to new tube (do not take all – leave if cloudy).

- Add 500 µl isopropanol, invert tubes 5x, leave at room temperature for 10 minutes.

- Spin for 10 minutes at 4ºC and max speed.

- Decant over sink – one swift move.

- Wash pellet with 1 ml 80% EtOH.

- Spin for 5 minutes at 4ºC at 7.500 g (9000 rpm).

- Remove supernatant.

- Flash spin (small centrifuge), remove last drop of EtOH – carefully.

- Leave to dry for 5-10 minutes (do not over-dry, then the pellet will become difficult to dissolve again).

- Reconstitute pellet in nuclease-free water (50-100 µl, dependent on pellet size).

- precipitate by adding 1/10 of volume of 3M NaAc, pH 5.2 and 2 - 2.5 x volume of 20ºC, 100% EtOH, leave at -80ºC for at least 20 minutes.

- Centrifuge for 30 minutes, decant supernatant, wash sup with 130 µl 80% EtOH, spin for 5 minutes with max speed, remove sup, flash spin – remove last drop of EtOH.

- Air dry for 5-10 minutes (do not over-dry), reconstitute in nuclease-free water.

Protocol for cDNA synthesis.

Reverse transcription Core kit. EUROGENTEC via MedProbe cat. No RT-RTCK-05.

Work RNase-free and wear gloves.

- In a pcr-tube (on ice), add:

    X µl 500 ng total RNA

    X µl RNase-free water

to the final volume of 12.15 µl.

- In a pcr-tube (at room temperature), add:

    3 µl 10x reaction buffer, then spin

    6 µl $MgCl_2$ solution, then spin

    6 µl dNTP solution, then spin

    1.5 µl random nonameres, then spin

    0.6 µl RNase inhibitor, then spin

    0.75 µl Euroscript RT

Keep the enzyme on ice and pipette directly in the bottom of the tube.

- Add the template:

    X µl 500 ng total RNA

    X µl RNase-free water

to the total volume of 12. 15 µl.

- ABI PCR mashine, protocol Eva1 (cDNA-rt), follow on screen instructions.

Cycling parameters are:

    10 minutes at 25ºC

    30 minutes at 48ºC

    5 minutes at 95ºC

- Store cDNA at -20ºC.

Appendix 3

Protocol for cRNA probe synthesis.

***Linearization of vector and insert*:**

- Measure OD260, and use 1µg DNA for each cutting reaction.

- Calculate the cutting reaction – final volume should be 40 µl.

- Add:

    26.8 µl sterile, deionized water

    4 µl RE 10 x buffer

    4 µl 1:10 Acetylated BSA

    4.2 µl DNA (1 µg)

Mix by pipetting, add 1 µl restriction enzyme R. E, spin down and keep at 37 ºC for 1-4 hours.

- Add 360 µl TE, pH 8.0 and 400 µl phenol/chloroform, vortex for 1 minute and spin down for 5 minutes at room temperature.

- Take supernatant to new tube carefully and add 400 µl chloroform.

- Vortex for 1 minute and spin for 5 minutes at room temperature.

- Take supernatant to new tube carefully and precipitate by adding:

    40 µl 3M NaAc, pH 5.2

    1000 µl 100% EtOH

- Put in -20 ºC freezer for at least 1 hour, then cool centrifuge to 4 ºC, and spin for 30 minutes.

- Discard supernatant, add 170 µl 70% EtOH and spin for 10 minutes.

- Discard supernatant, add 180 µl 100% EtOH and spin for 10 minutes.

- Discard supernatant, air dry the pellet for 5 minutes and resuspend in 15 µl nuclease-free water.

***Digoxigenin (DIG) RNA labeling (cRNA probe synthesis)*:**

- Measure OD260.

- In a sterile, RNase-free eppendorf tube, on ice, add:

    13 µl template DNA (1 µg) + nuclease-free water

2 µl NTP labeling mixture 10x

2 µl transcription buffer 10x

1µl RNase inhibitor

2 µl RNA polymerase (T3 or T7)

Mix gently, centrifuge briefly and incubate for 2 hours at 37 ºC.

- Add 2 µl DNaseI and incubatie for 15 minutes at 37 ºC.

- Add 2 µl 0.2 m EDTA (pH 8.0) to stop the reaction.

- Precipitate by adding 2.5 µl 4 M LiCl and 75 µl prechilled (-20 ºC) 100% EtOH, mix and leave for at least 30 minutes at -70 ºC.

- Centrifuge for 15 minutes at 14 000 rpm, and discard supernatant.

- Add 50 µl prechilled (-20 ºC) 70% EtOH, centrifuge for 10 minutes at 14 000 rpm, and discard supernatant.

- Air dry the pellet for 10 minutes and resuspend in 49 µl DEPC-water and 1 µl RNase inhibitor.

- Measure OD260, aliquote the rest of the probe and store in -70 ºC.

Control of the DIG-incorporation in cRNA probes:

- Make graded dilutions of probe and control DIG-RNA (1:10, 1:100, 1:1000).

- Pipett 1µl of each on to a Hybond-N+ membrane, and

  - Air dry for 5-10 minutes

  - Put in crosslinker

- Wash the membrane with buffer B1 for 1 minute, and then with buffer B2 for 30 minutes (with gentle shaking).

- Incubate the membrane with 5 ml buffer B2 and 1µl anti-DIG (= 1:5000 dilution) for 30 minutes (with gentle shaking).

- Wash the membrane with buffer B1, 2 x 15 minutes, and then with buffer B3 for 2 minutes (with gentle shaking).

- Incubate the membrane with 10 ml buffer B3, 45 µl NBT and 35 µl BCIP in the dark for >5 minutes.

- Stop the reaction with TE, pH 8.0.

- To check the probe quantity and quality, use a bio-analyzer.

Appendix 4

Protocol for In Situ hybridization.

Digoxigenin labeled cRNA probes on paraffin sections.

Day 1:

Rehydration.

Rehydrate sections by leaving them in the following solutions:

10 minutes in xylene x 2

2 minutes in 100% EtOH x 2

2 minutes in 70% EtOH

2 minutes in 50% EtOH

2 minutes in 1xPBS

Perform all of these baths with the slides kept in a glass kyvette. Then transfer the sections to moisture chambers, containing wipes soaked in 5xSSC. Use wipes to dry the slides around the sections, so that PAP-pen can be applied.

Permeabilization.

Treat the sections with proteinase K:

5 minutes in TrisHCl (0.05 M, pH 7.5)

5 minutes in 5 µg proteinase K/ml trisHCl at room temperature

Rinse in tris-HCl

Acetic anhydride treatment.

10 minutes in TEA (0.1 M, pH 8.0)

10 minutes in AcAh-TEA at room temperature

Rinse for 3 x 5 minutes in PBS

The TEA and the AcAh-TEA are made just before use.

Prehybridization.

Add hybridization buffer (250 µl/slide) and incubate the sections in the moisture chambers for 2 hours (<6 hours) at room temperature.

Hybridization.

Preheat hybridization buffer (500 µl) with added probe to 80 ºC for 5 minutes, and place directly on ice.

Incubate the sections in the moisture chamber with cRNA probe (2000 ng/ml hybridization buffer) for 16 hours at 65 ºC.

Day 2:

Post-hybridization wash.

Heat 30% formamide in 5 x SSC and 0.2xSSC to 65 ºC, then pour off the hybridization buffer from the sections. Wash the sections:

30 minutes in 5 x SSC at room temperature

15 minutes in 30% formamide in 5 x SCC at 65 ºC

2 x 35 minutes (up to 1 hour) in 0.2 x SCC at 65 ºC

Cooling to room temperature

5 minutes in 0.2 x SCC at room temperature

RNase treatment.

Wash for 5 minutes at 37 ºC in RNase buffer

Incubatie for 30 minutes with 2 µg/ml RNase A

5 minutes at 37 ºC in RNase buffer

2 x 30 minutes in 0.2 x SSC at room temperature

Antibody incubation.

5 minutes in buffer B1 at room temperature

1 hour in buffer B2 at room temperature

16 hours at 8 ºC in sheep anti-DIG 1:2000 in buffer B2 (1-6 hours at room temperature)

Day 3:

Rinse the sections 3 x 5 min in buffer B1 at room temperature.

Alkaline phosphatase reaction.

5 minutes in buffer B3 at room temperature

Incubate in buffer B4 (NBT/BCIP, made fresh and kept in dark), 1-6 hours in the dark at room temperature.

Short antibody incubation needs longer reaction time (>6 hours), while long antibody incubation needs shorter reaction time (1-6 hours). If the transcripts are abundant, a relatively shorter antibody incubation and reaction time is needed; in this case the slides needed to be kept in incubation for more than 6 hours.

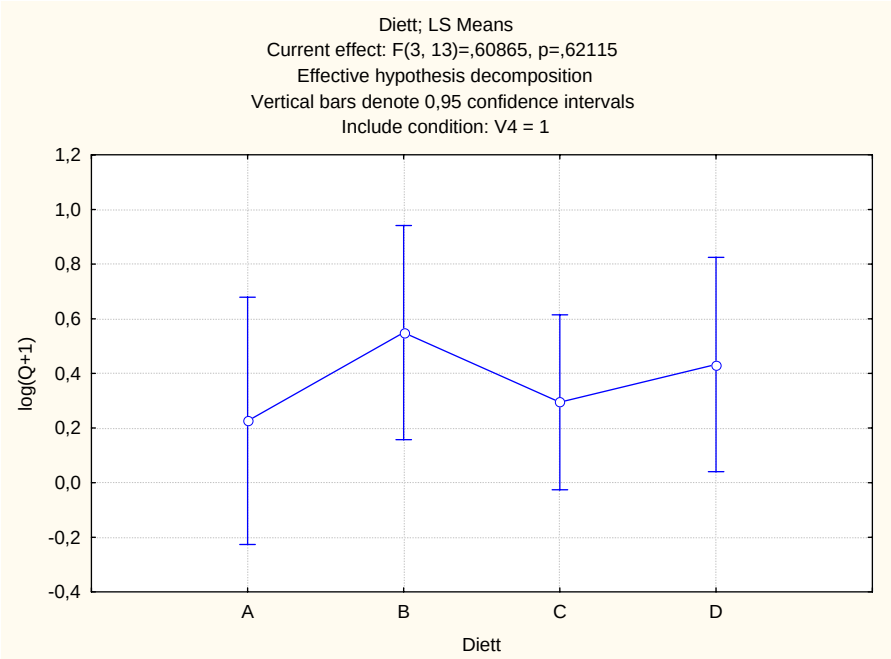
Stop the reaction in TEN for 10 minutes.

In order to get rid of brown color and enhance the blue color representing StAR transcripts, put the slides in 100% EtOH for 30 seconds. Then coverslip in 50% glycerol in TEN and use nailpolish to seal the slides.

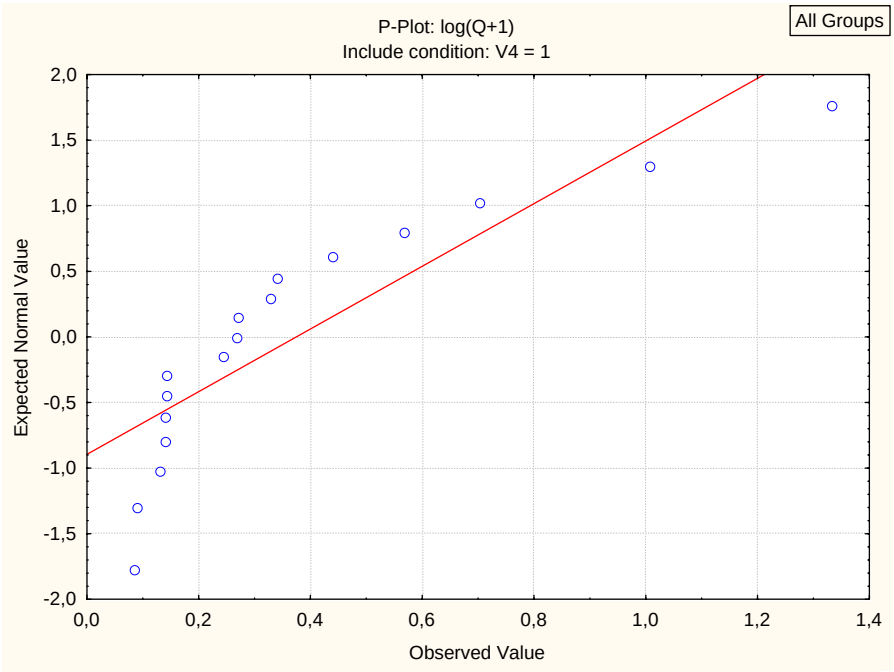
Appendix 5

Statistical tests.

One-way ANOVA, Levene's test, P-plot and histogram of StAR log (Q+1) vs. diet group, sampling 1 (June):



	Levene's Test for Homogeneity of Variances (Lene data 030409) Effect: Diett Degrees of freedom for all F's: 3, 13 Include condition: V4 = 1			
	MS Effect	MS Error	F	p
log(Q+1)	0,065749	0,045019	1,460455	0,271004


All Groups
P-Plot: log(Q+1)
Include condition: V4 = 1
Expected Normal Value
Observed Value
2,0
1,5
1,0
0,5
0,0
-0,5
-1,0
-1,5
-2,0
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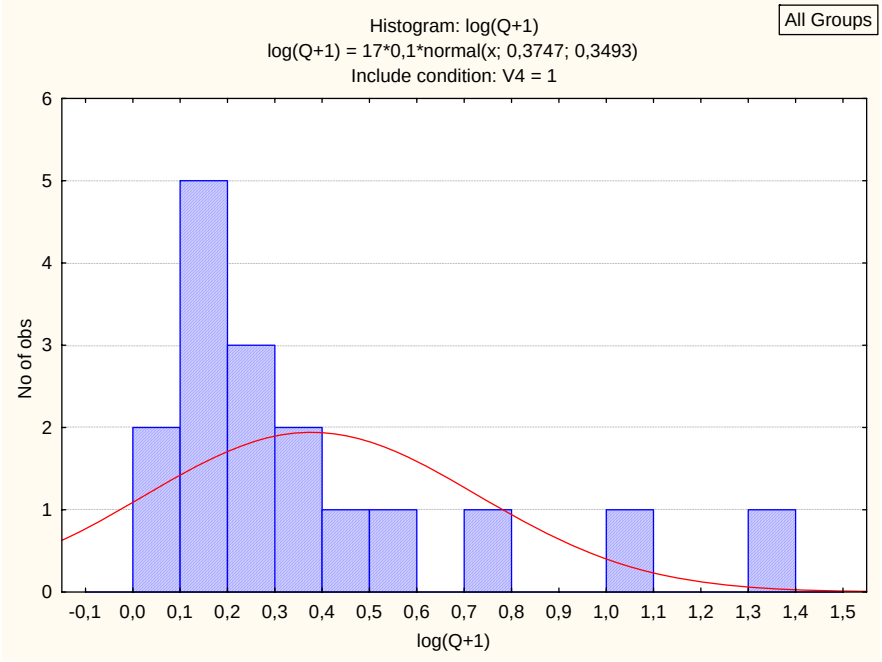
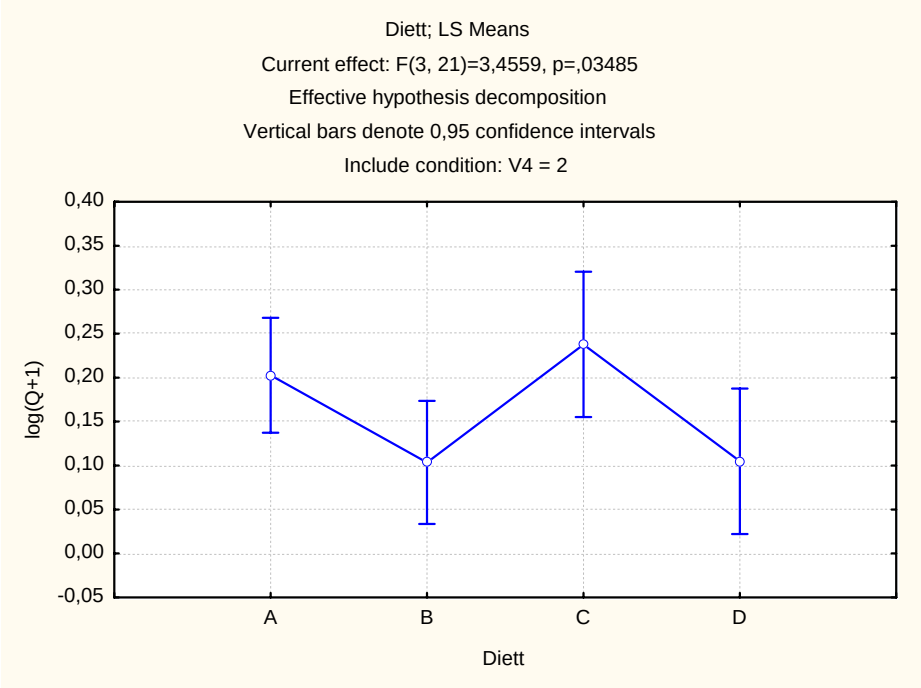
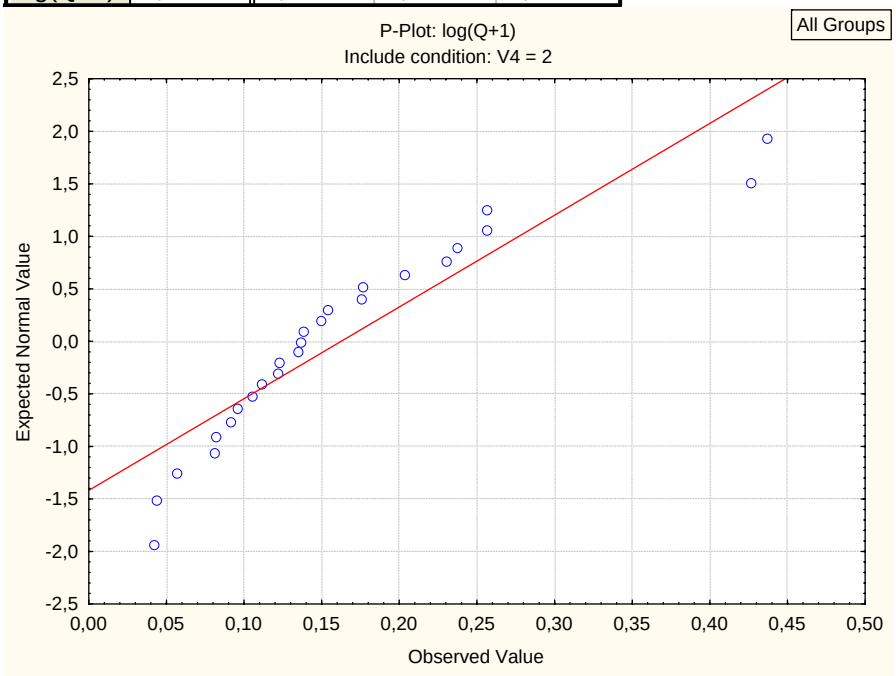
0,4
0,6
0,8
1,0
1,2
1,4

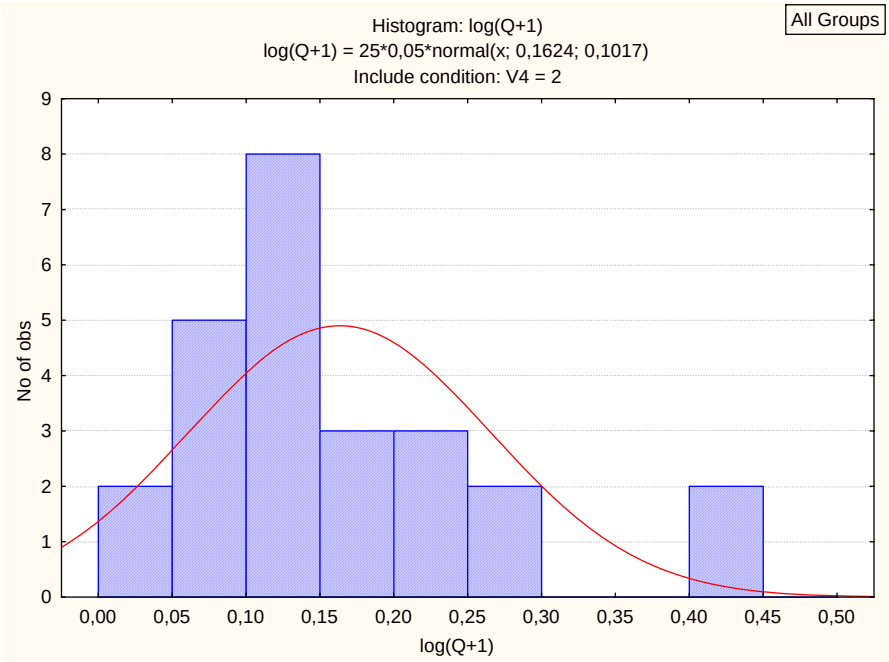

All Groups
Histogram: log(Q+1)
log(Q+1) = 17*0,1*normal(x; 0,3747; 0,3493)
Include condition: V4 = 1
No of obs
log(Q+1)
6
5
4
3
2
1
0
-0,1
0,0
0,1
0,2
0,3
0,4
0,5
0,6
0,7
0,8
0,9
1,0
1,1
1,2
1,3
1,4
1,5

***One-way ANOVA, Levene's test, P-plot, histogram and unequal N HSD of StAR log (Q+1) vs. diet group, sampling 2 (July)*:**



	Levene's Test for Homogeneity of Variances (Lene data 030409) Effect: Diett Degrees of freedom for all F's: 3, 21 Include condition: V4 = 2			
	MS Effect	MS Error	F	p
log(Q+1)	0,005136	0,003246	1,582163	0,223483

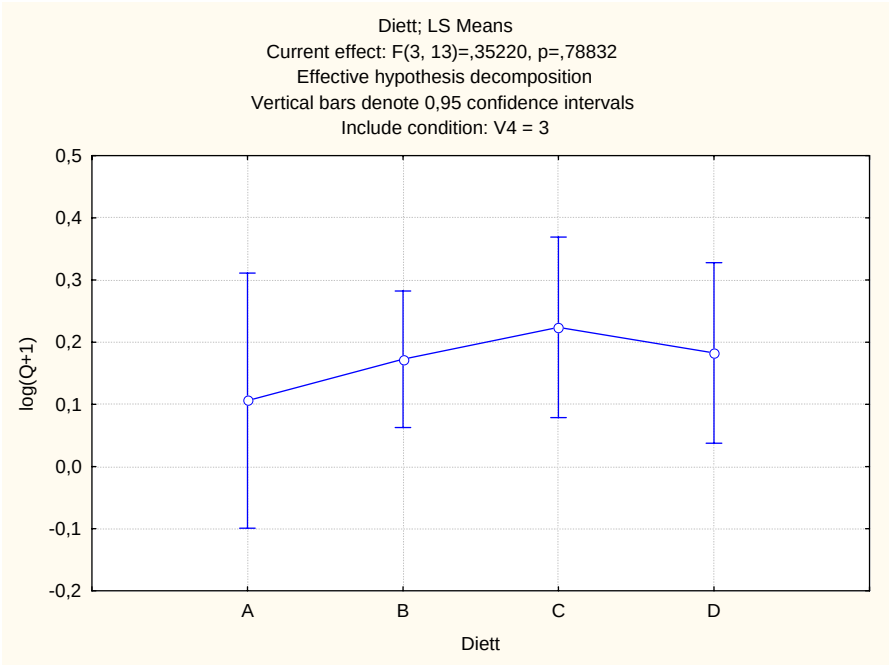




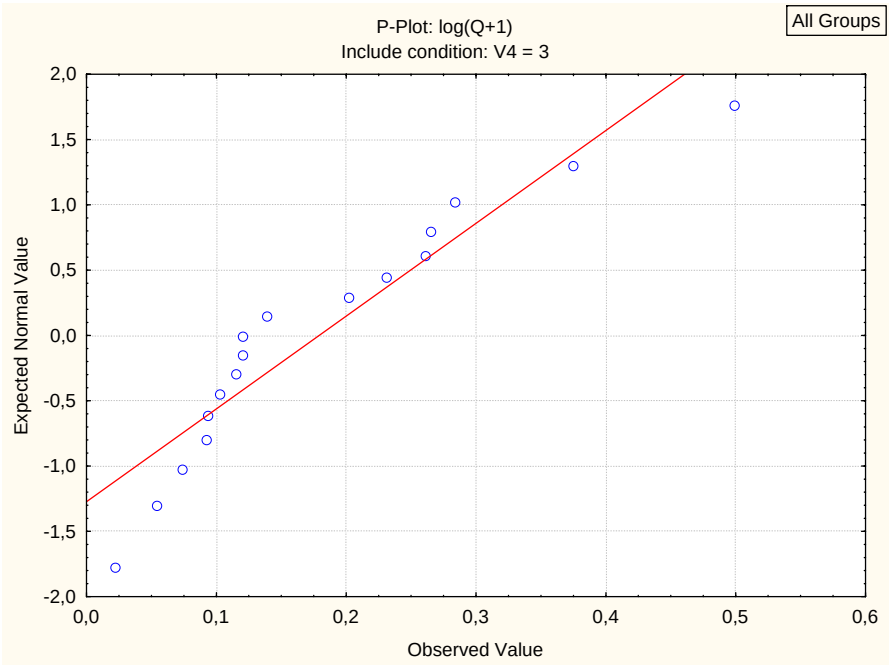
Unequal N HSD; variable log(Q+1) (Lene data 030409)
Approximate Probabilities for Post Hoc Tests
Error: Between MS = ,00792, df = 21,000
Include condition: V4 = 2

Cell No.	Diett	{1} ,20268	{2} ,10361	{3} ,23781	{4} ,10494
1	A		0,191384	0,923240	0,330795
2	B	0,191384		0,111250	0,999996
3	C	0,923240	0,111250		0,116275
4	D	0,330795	0,999996	0,116275	

***One-way ANOVA, Levene's test, P-plot and histogram of StAR log (Q+1) vs. diet group, sampling 3 (August)*:**



	Levene's Test for Homogeneity of Variances (Lene data 030409) Effect: Diett Degrees of freedom for all F's: 3, 13 Include condition: V4 = 3			
	MS Effect	MS Error	F	p
log(Q+1)	0,008844	0,003814	2,318959	0,123234


All Groups
P-Plot: log(Q+1)
Include condition: V4 = 3
Expected Normal Value
Observed Value
2,0
1,5
1,0
0,5
0,0
-0,5
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-1,5
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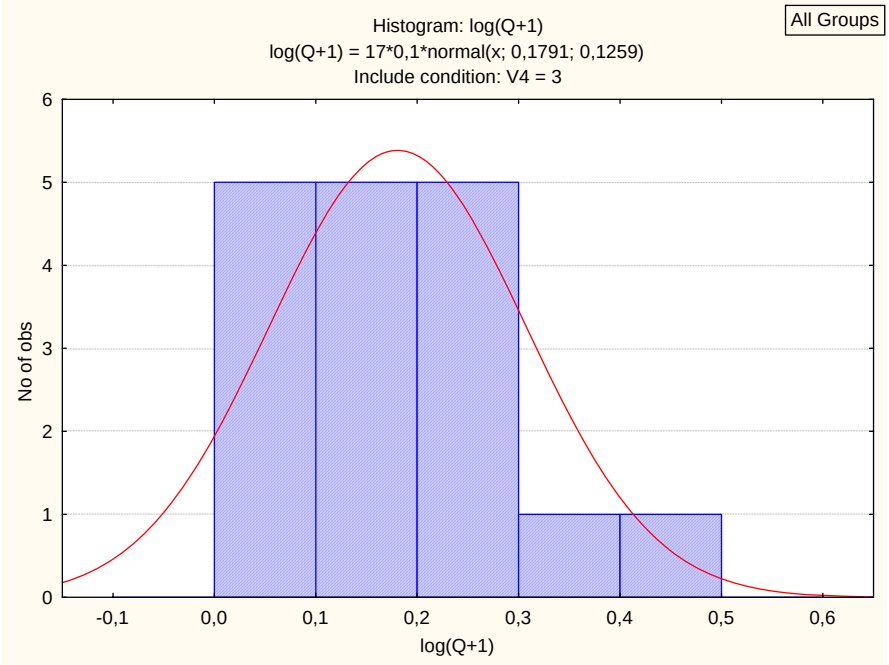
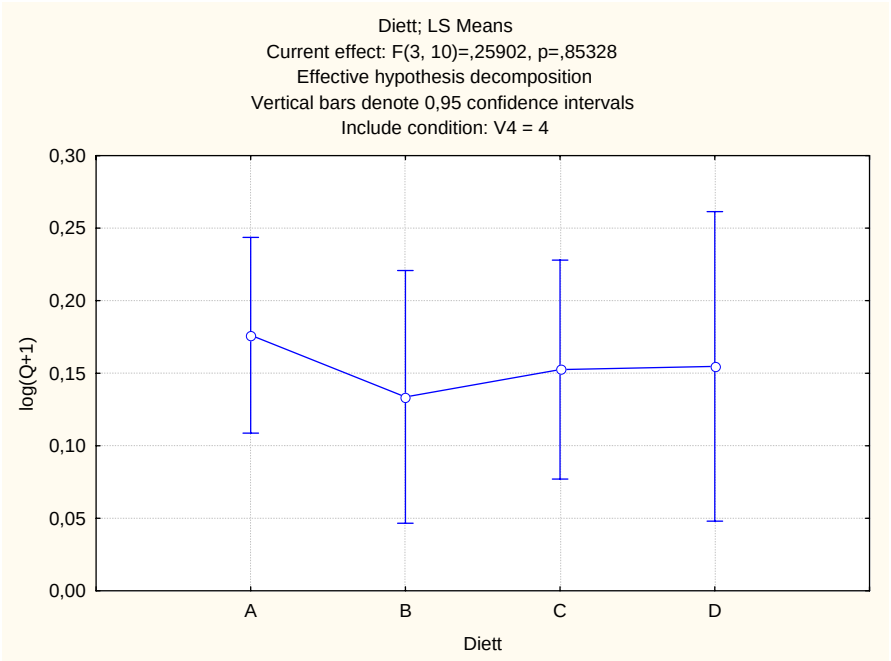
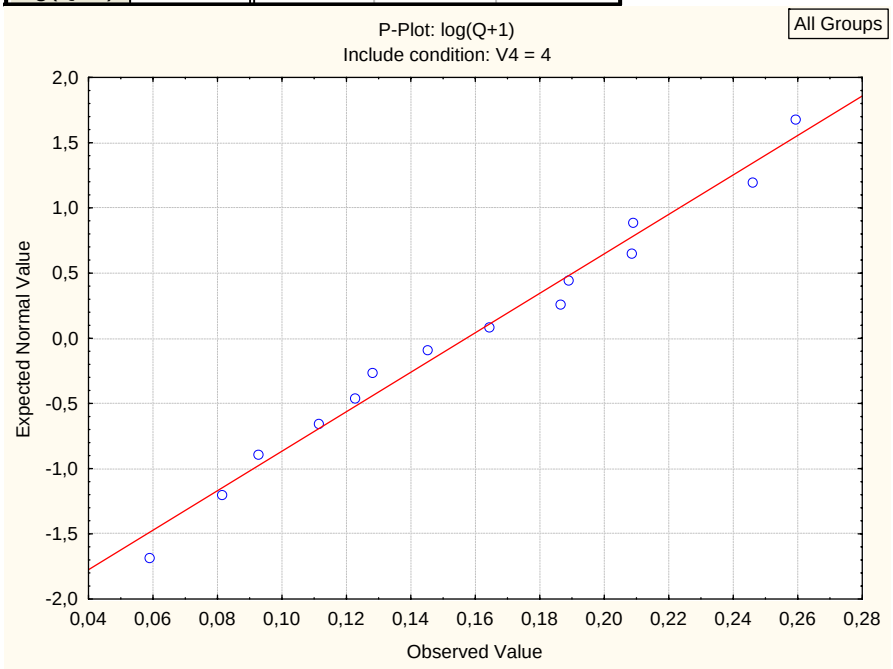
0,1
0,2
0,3
0,4
0,5
0,6

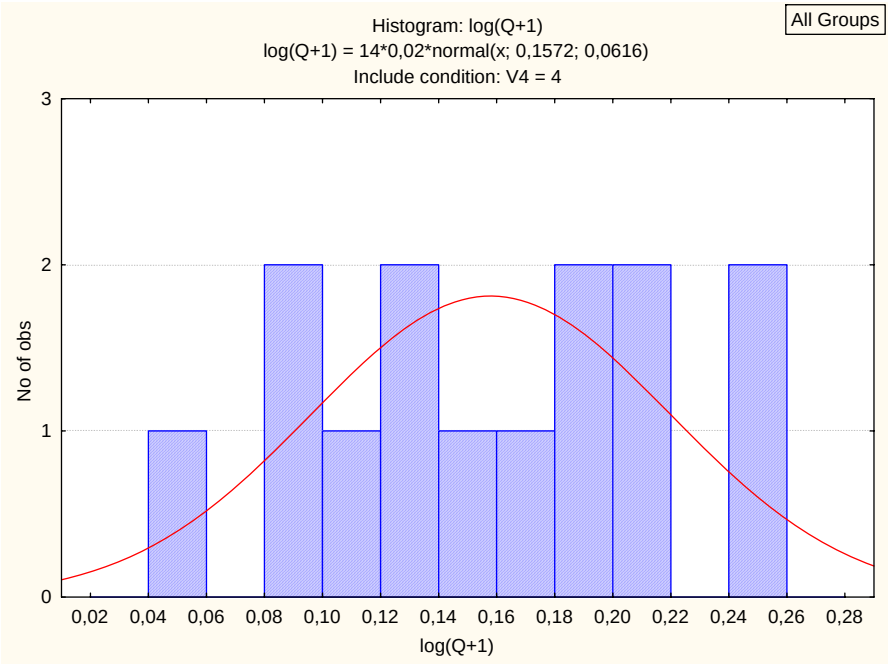

All Groups
Histogram: log(Q+1)
log(Q+1) = 17*0,1*normal(x; 0,1791; 0,1259)
Include condition: V4 = 3
No of obs
log(Q+1)
6
5
4
3
2
1
0
-0,1
0,0
0,1
0,2
0,3
0,4
0,5
0,6

***One-way ANOVA, Levene's test, P-plot and histogram of StAR log (Q+1) vs. diet group, sampling 4 (September)*:**

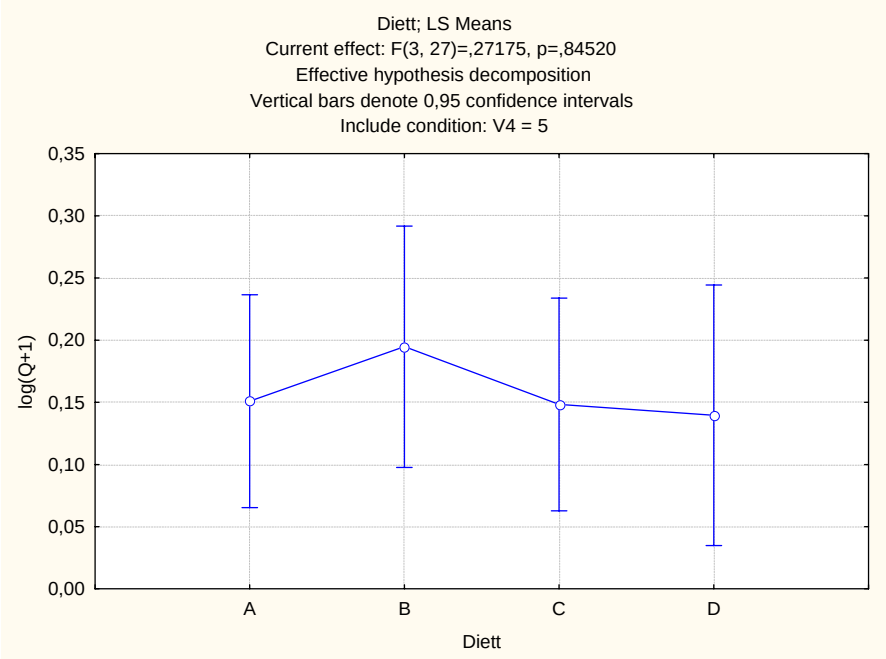


	Levene's Test for Homogeneity of Variances (Lene data 030409) Effect: Diett Degrees of freedom for all F's: 3, 10 Include condition: V4 = 4			
	MS Effect	MS Error	F	p
log(Q+1)	0,001794	0,000744	2,410631	0,127659

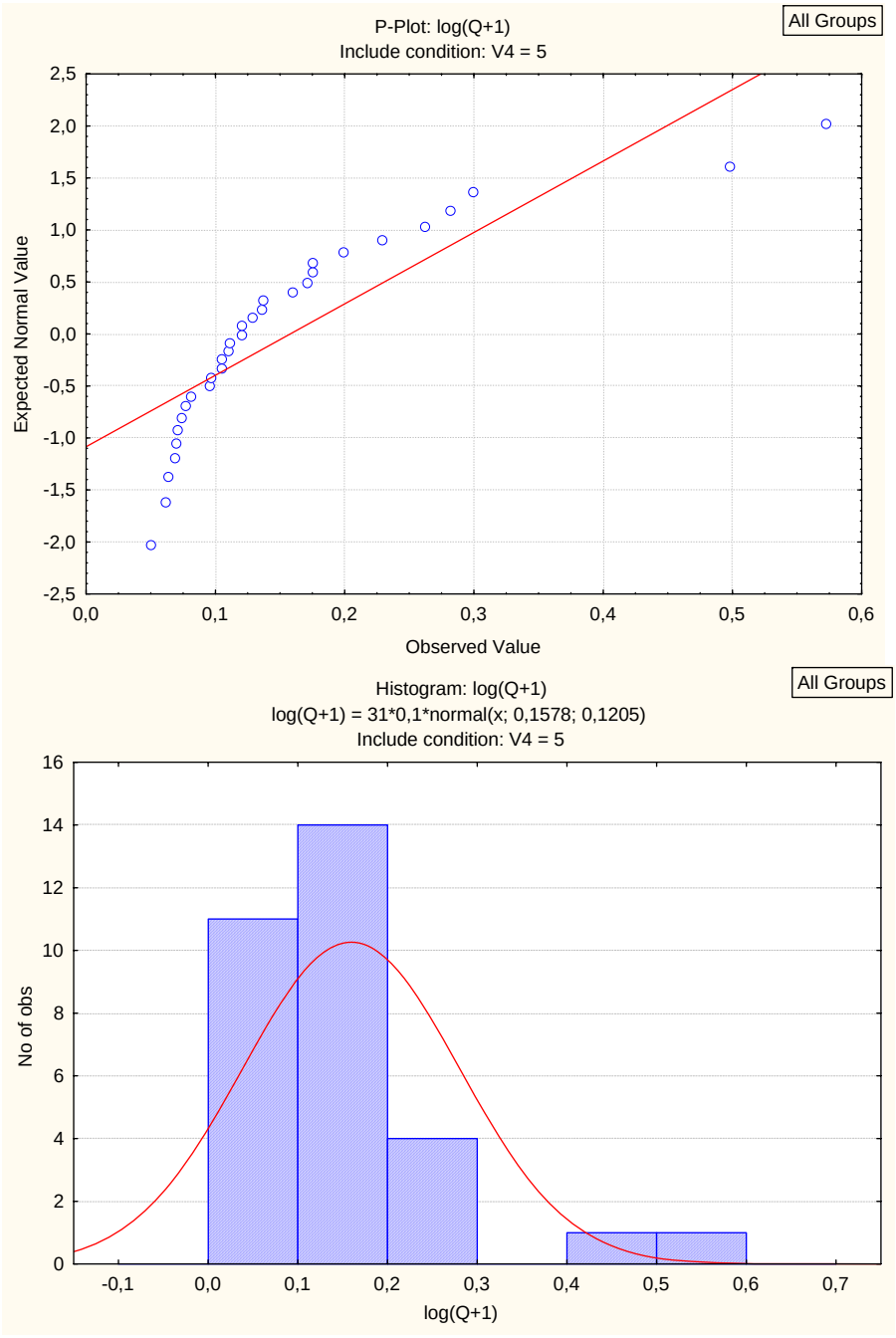




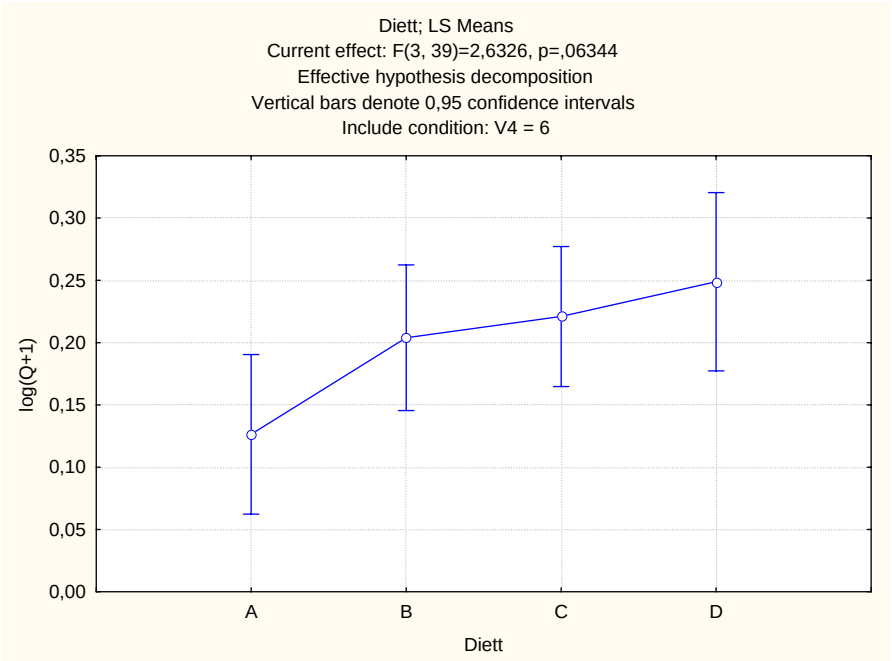
***One-way ANOVA, Levene's test, P-plot and histogram of StAR log (Q+1) vs. diet group, sampling 5 (October)*:**



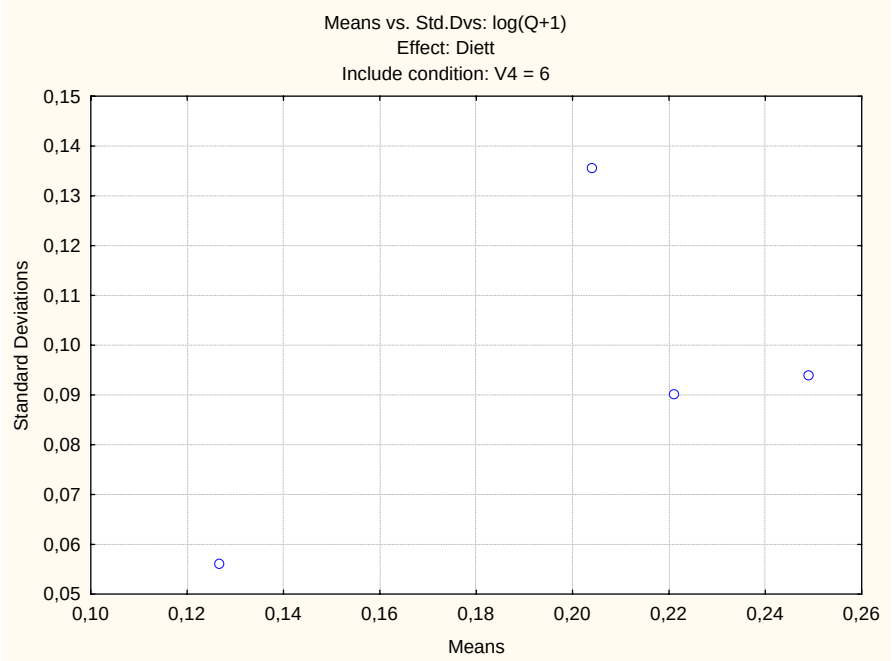
	Levene's Test for Homogeneity of Variances (Lene data 030409) Effect: Diett Degrees of freedom for all F's: 3, 27 Include condition: V4 = 5			
	MS Effect	MS Error	F	p
log(Q+1)	0,006853	0,007027	0,975275	0,418893

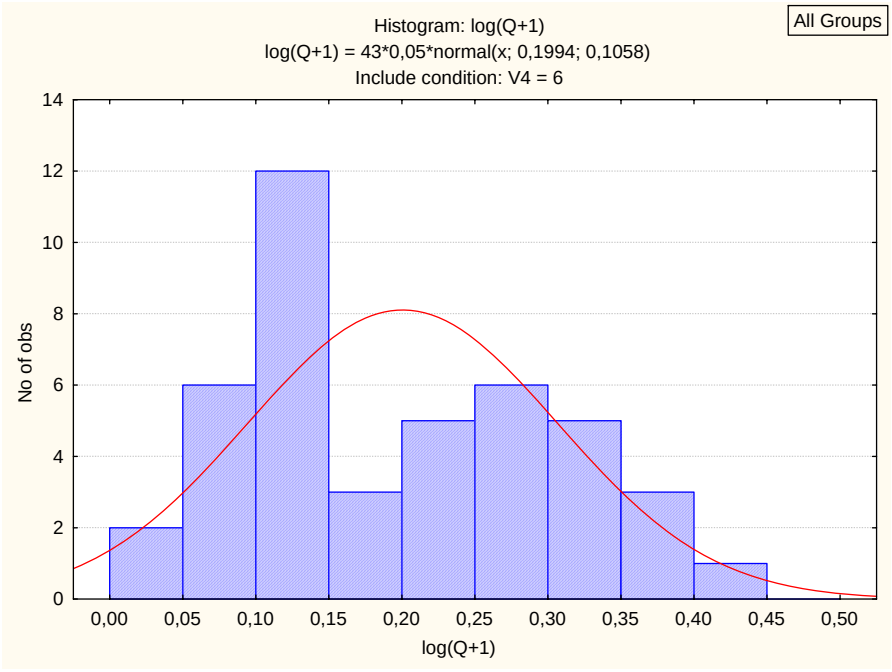

All Groups
P-Plot: log(Q+1)
Include condition: V4 = 5
Expected Normal Value
Observed Value
2,5
2,0
1,5
1,0
0,5
0,0
-0,5
-1,0
-1,5
-2,0
-2,5
0,0
0,1
0,2
0,3
0,4
0,5
0,6
All Groups
Histogram: log(Q+1)
log(Q+1) = 31*0,1*normal(x; 0,1578; 0,1205)
Include condition: V4 = 5
No of obs
log(Q+1)
16
14
12
10
8
6
4
2
0
-0,1
0,0
0,1
0,2
0,3
0,4
0,5
0,6
0,7

***One-way ANOVA, Levene's test, P-plot and histogram of StAR log (Q+1) vs. diet group, sampling 6 (November)*:**

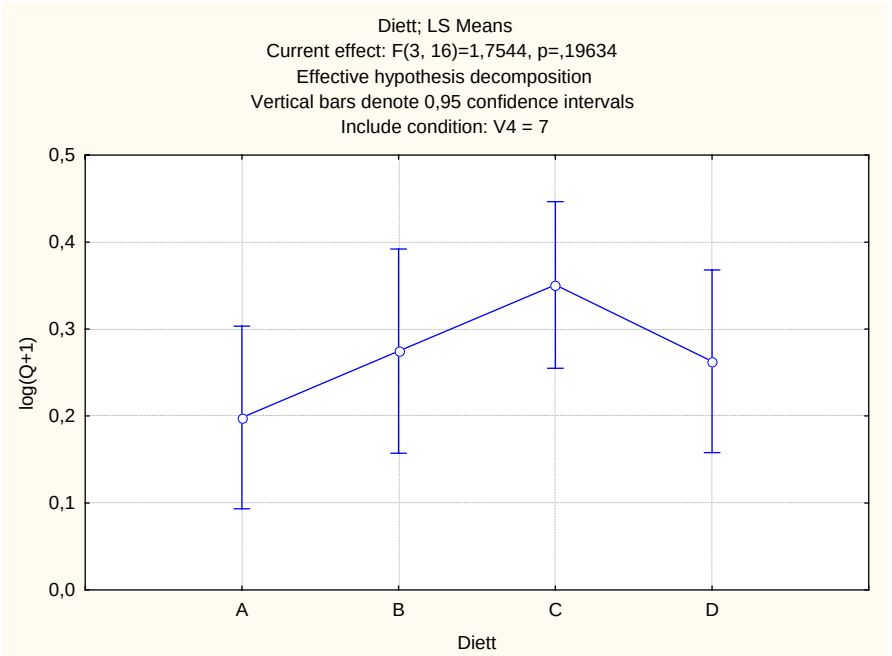


	Levene's Test for Homogeneity of Variances (Lene data 030409) Effect: Diett Degrees of freedom for all F's: 3, 39 Include condition: V4 = 6			
	MS Effect	MS Error	F	p
log(Q+1)	0,009923	0,001998	4,965595	0,005155

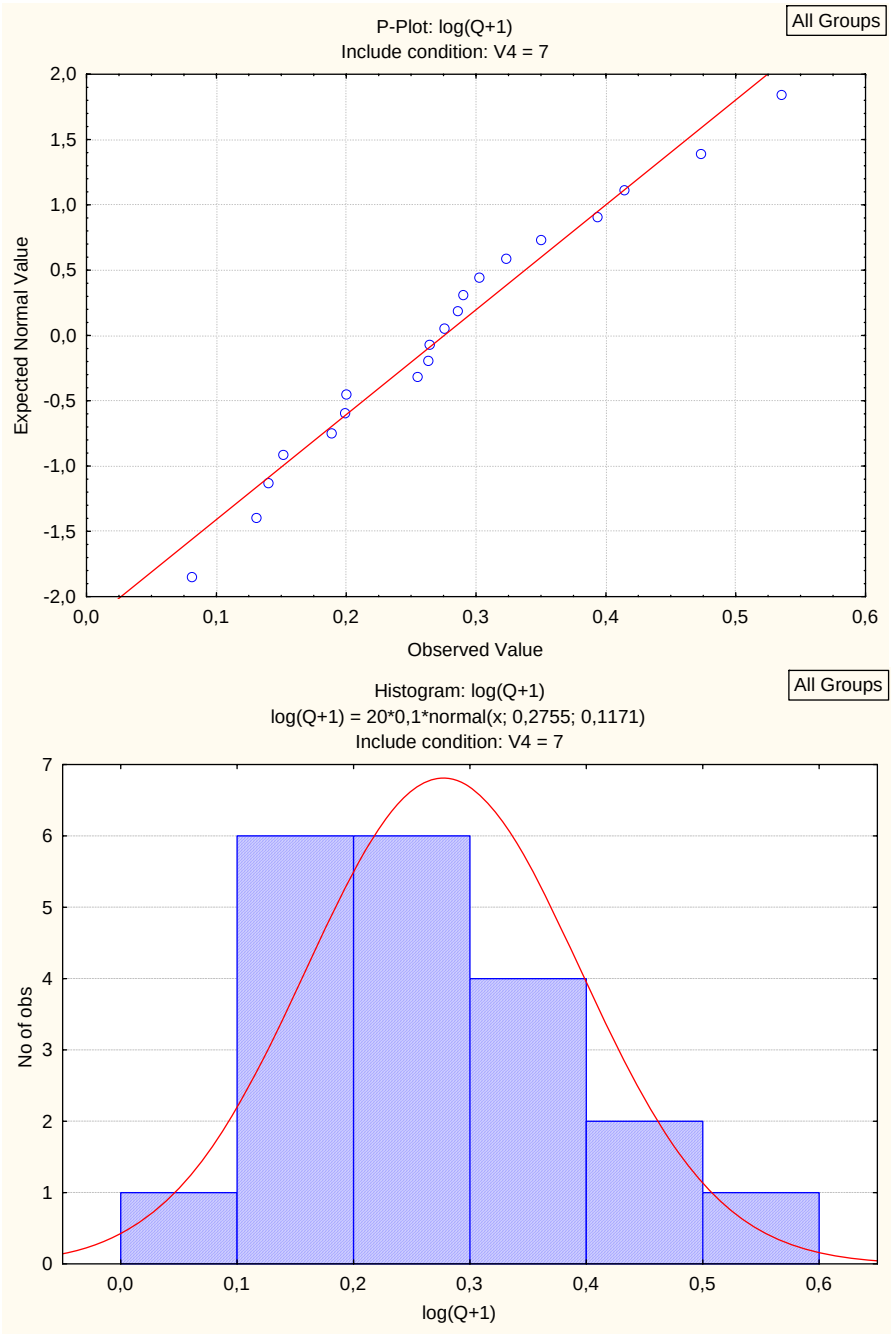




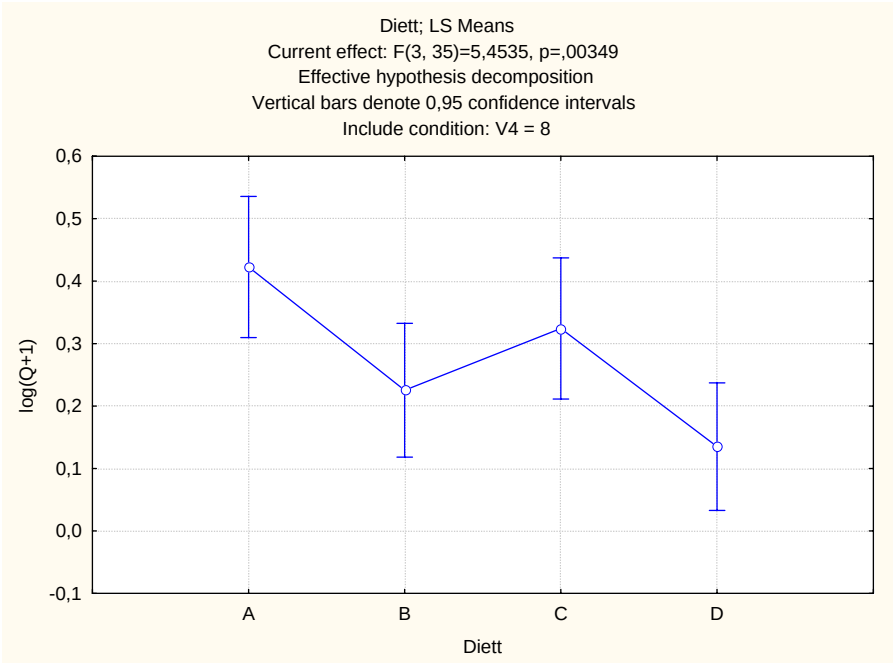
***One-way ANOVA, Levene's test, P-plot and histogram of StAR log (Q+1) vs. diet group, sampling 7 (December)*:**



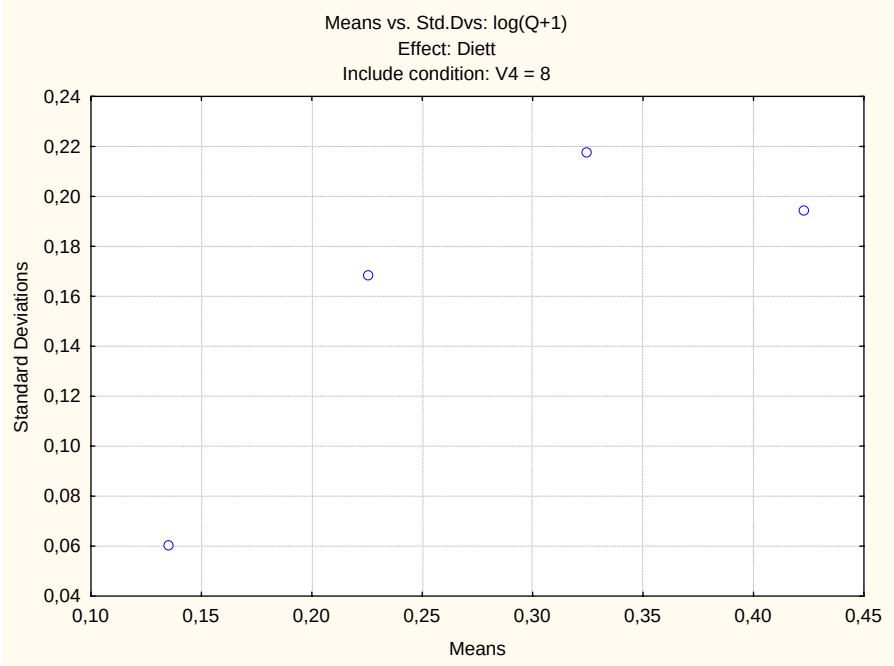
	Levene's Test for Homogeneity of Variances (Lene data 030409) Effect: Diett Degrees of freedom for all F's: 3, 16 Include condition: V4 = 7			
	MS Effect	MS Error	F	p
log(Q+1)	0,002981	0,004323	0,689548	0,571562

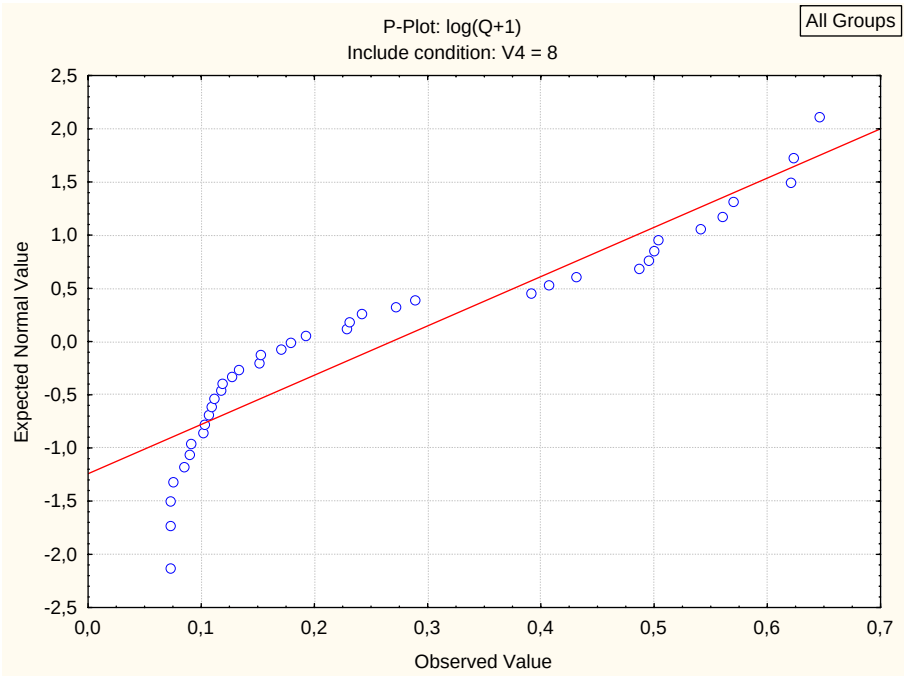

All Groups
P-Plot: log(Q+1)
Include condition: V4 = 7
Expected Normal Value
Observed Value
2,0
1,5
1,0
0,5
0,0
-0,5
-1,0
-1,5
-2,0
0,0
0,1
0,2
0,3
0,4
0,5
0,6
All Groups
Histogram: log(Q+1)
log(Q+1) = 20*0,1*normal(x; 0,2755; 0,1171)
Include condition: V4 = 7
No of obs
log(Q+1)
7
6
5
4
3
2
1
0
0,0
0,1
0,2
0,3
0,4
0,5
0,6

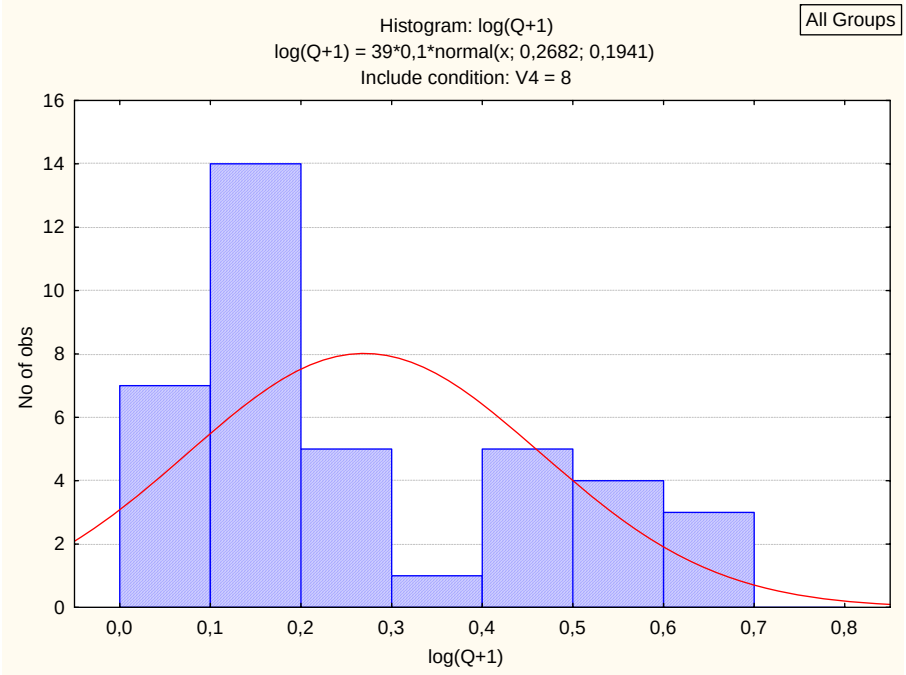
***One-way ANOVA, Levene's test, means vs. standard deviation, P-plot, histogram and unequal N HSD of StAR log (Q+1) vs. diet group, sampling 8 (January)*:**



	Levene's Test for Homogeneity of Variances (Lene data 030409) Effect: Diett Degrees of freedom for all F's: 3, 35 Include condition: V4 = 8			
	MS Effect	MS Error	F	p
log(Q+1)	0,038440	0,005142	7,475598	0,000541



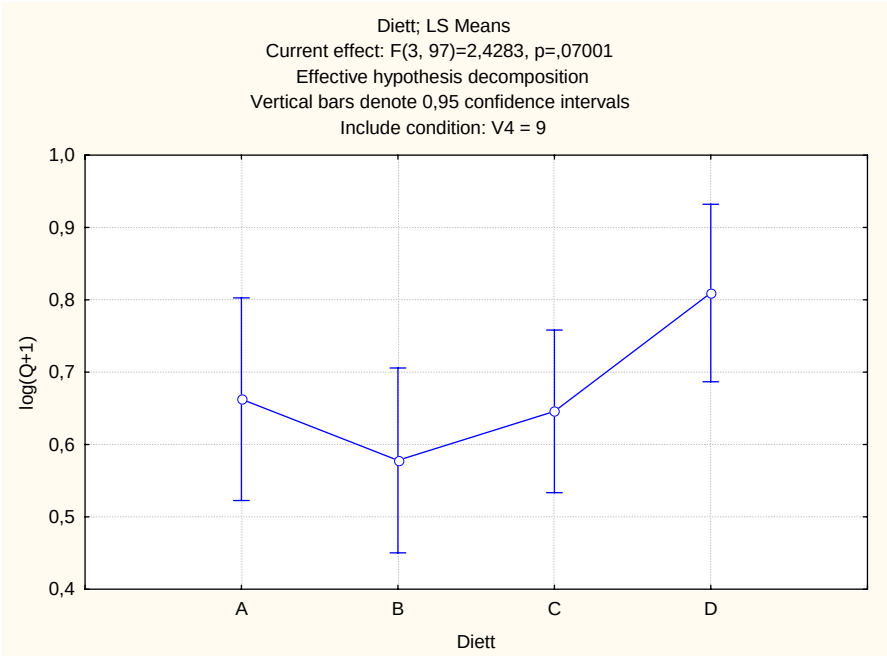




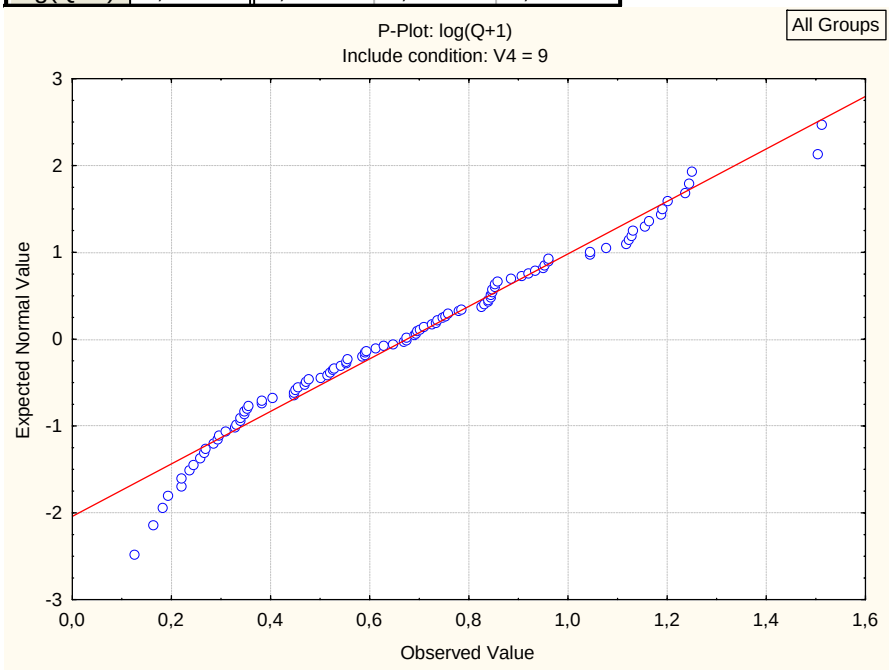
Unequal N HSD; variable log(Q+1) (Lene data 030409)
Approximate Probabilities for Post Hoc Tests
Error: Between MS = ,02788, df = 35,000
Include condition: V4 = 8

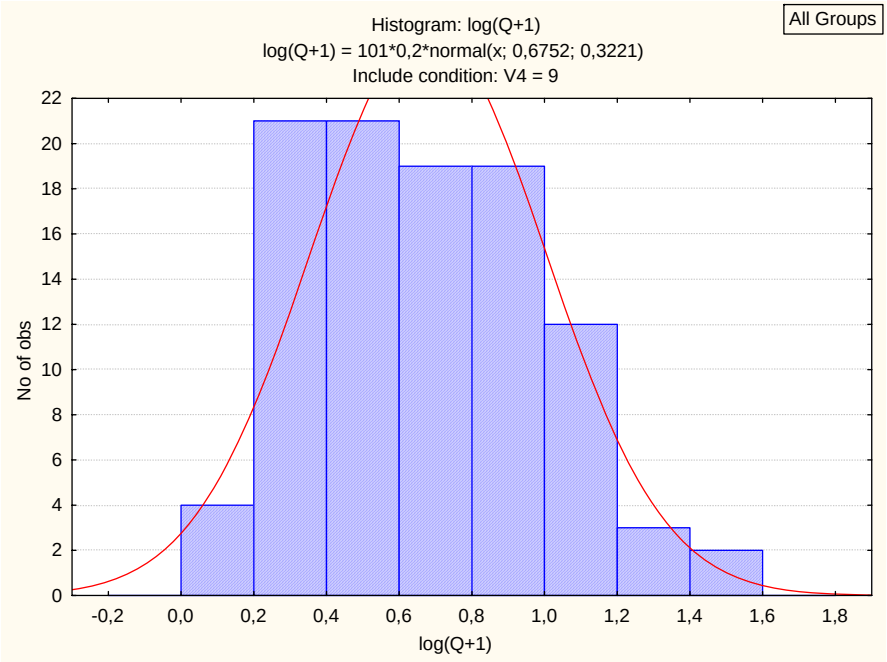
Cell No.	Diett	{1} ,42260	{2} ,22534	{3} ,32417	{4} ,13508
1	A		0,076476	0,599688	0,004568
2	B	0,076476		0,596615	0,625529
3	C	0,599688	0,596615		0,095306
4	D	0,004568	0,625529	0,095306	

***One-way ANOVA, Levene's test, P-plot and histogram of StAR log (Q+1) vs. diet group, sampling 9 (February)*:**

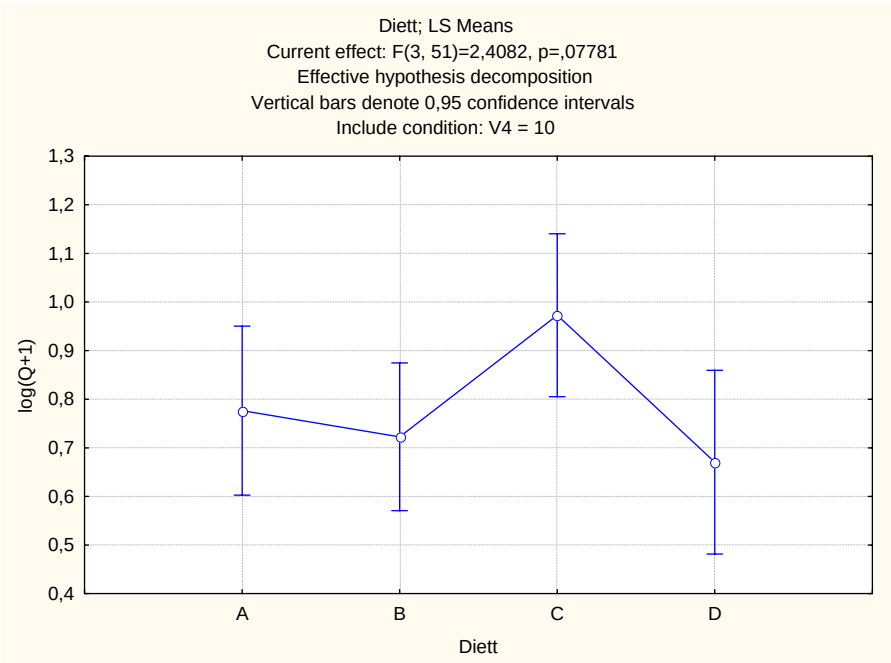


	Levene's Test for Homogeneity of Variances (Lene data 030409) Effect: Diett Degrees of freedom for all F's: 3, 97 Include condition: V4 = 9			
	MS Effect	MS Error	F	p
log(Q+1)	0,037053	0,029740	1,245885	0,297403

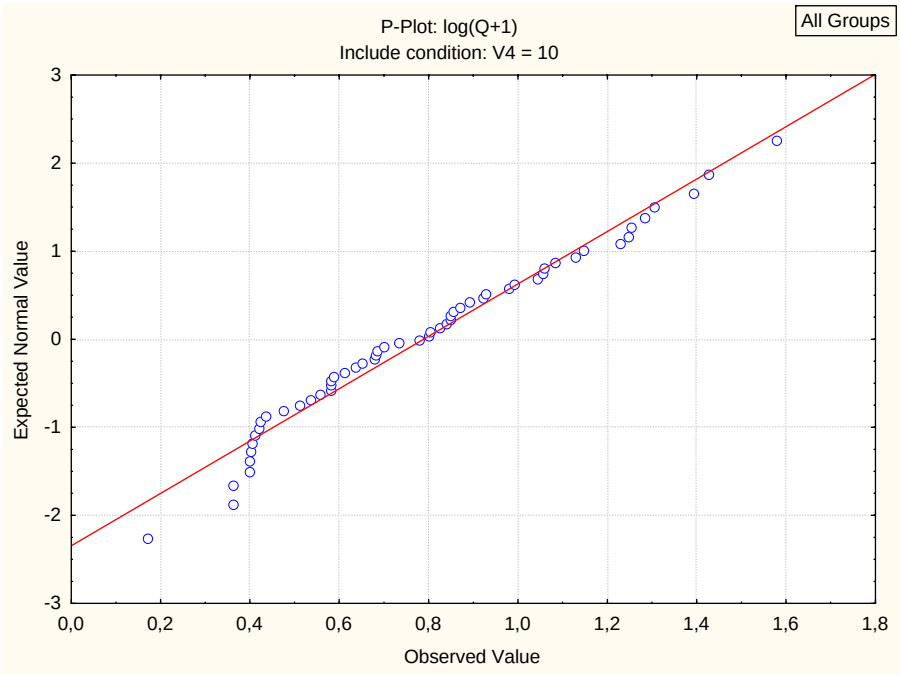


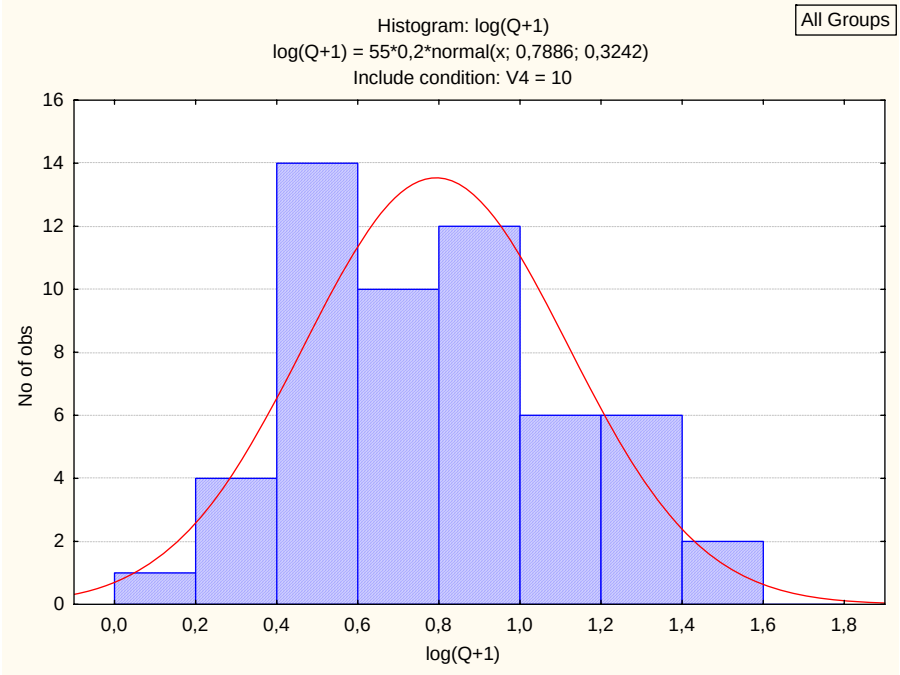


***One-way ANOVA, Levene's test, P-plot and histogram of StAR log (Q+1) vs. diet group, sampling 10 (March)*:**

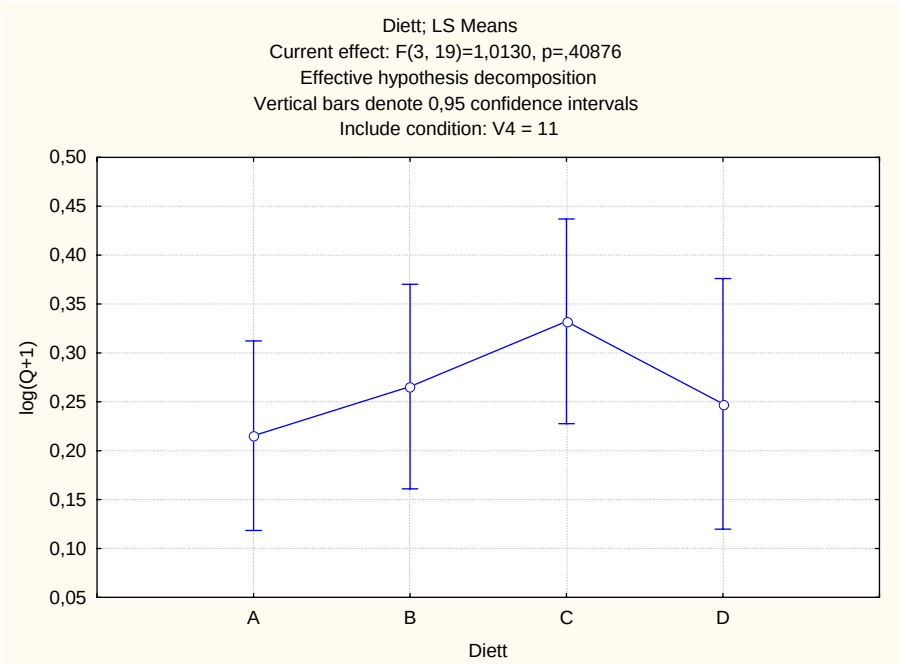


	Levene's Test for Homogeneity of Variances (Lene data 030409) Effect: Diett Degrees of freedom for all F's: 3, 51 Include condition: V4 = 10			
	MS Effect	MS Error	F	p
log(Q+1)	0,003255	0,027962	0,116411	0,950083

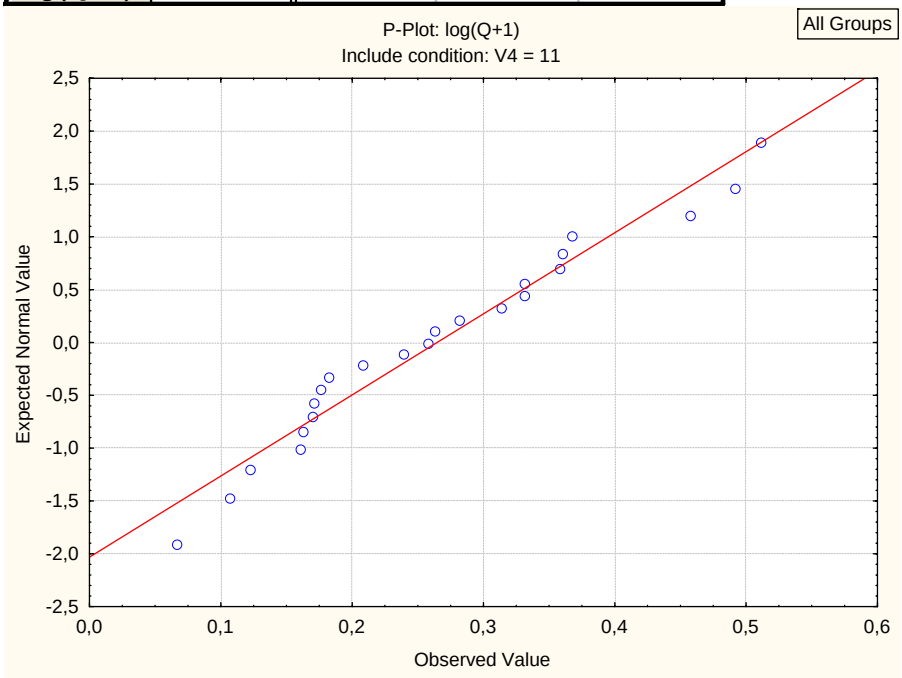

All Groups
P-Plot: log(Q+1)
Include condition: V4 = 10
Expected Normal Value
Observed Value
3
2
1
0
-1
-2
-3
0,0
0,2
0,4
0,6
0,8
1,0
1,2
1,4
1,6
1,8

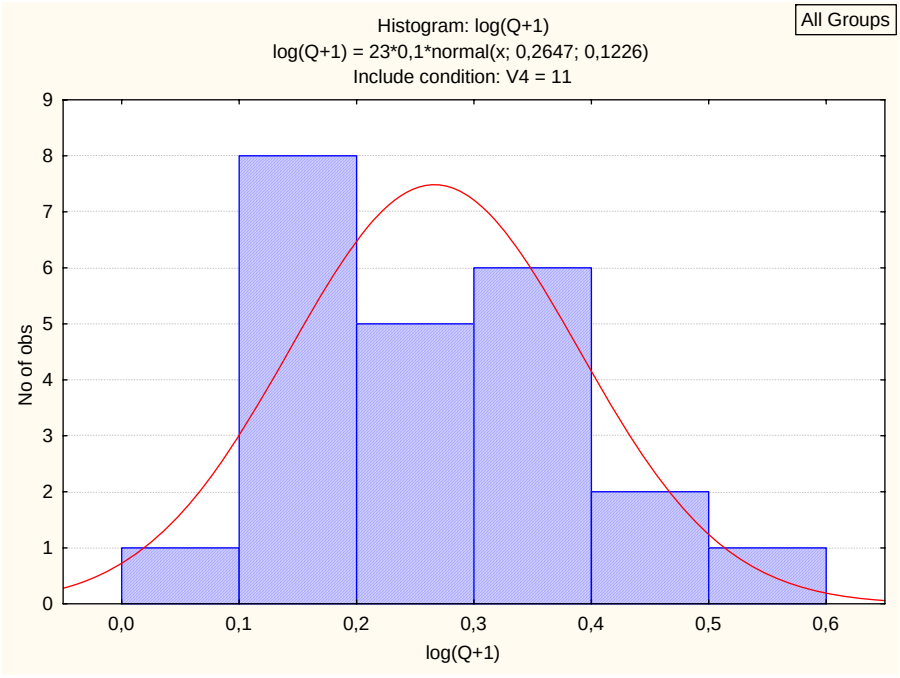

All Groups
Histogram: log(Q+1)
log(Q+1) = 55*0,2*normal(x; 0,7886; 0,3242)
Include condition: V4 = 10
No of obs
log(Q+1)
16
14
12
10
8
6
4
2
0
0,0
0,2
0,4
0,6
0,8
1,0
1,2
1,4
1,6
1,8

***One-way ANOVA, Levene's test, P-plot and histogram of StAR log (Q+1) vs. diet group, sampling 11 (May)*:**



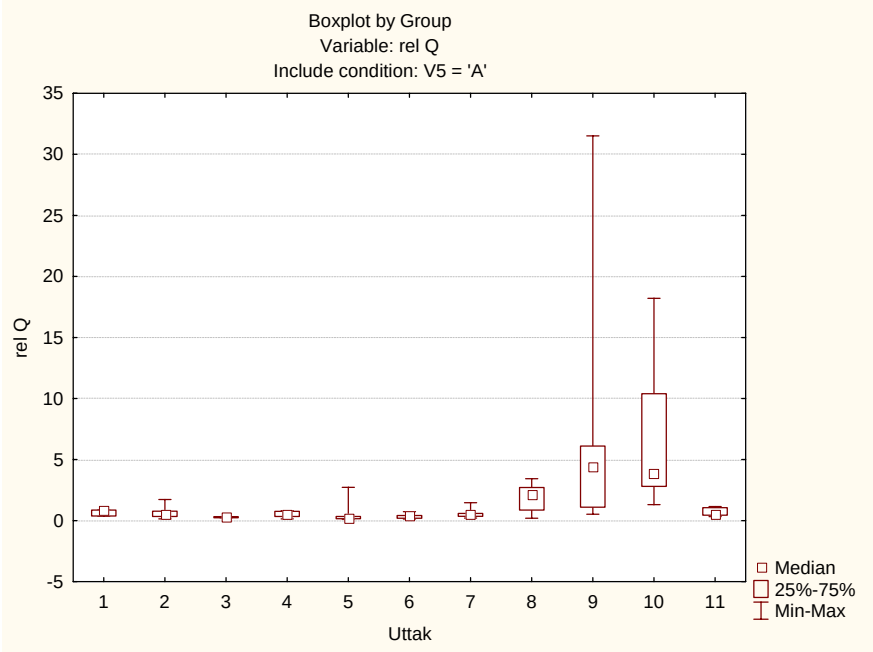
	Levene's Test for Homogeneity of Variances (Lene dat Effect: Diett Degrees of freedom for all F's: 3, 19 Include condition: V4 = 11			
	MS Effect	MS Error	F	p
log(Q+1)	0,003321	0,004735	0,701244	0,562887




All Groups
Histogram: log(Q+1)
log(Q+1) = 23*0,1*normal(x; 0,2647; 0,1226)
Include condition: V4 = 11
No of obs
0
1
2
3
4
5
6
7
8
9
0,0
0,1
0,2
0,3
0,4
0,5
0,6
log(Q+1)

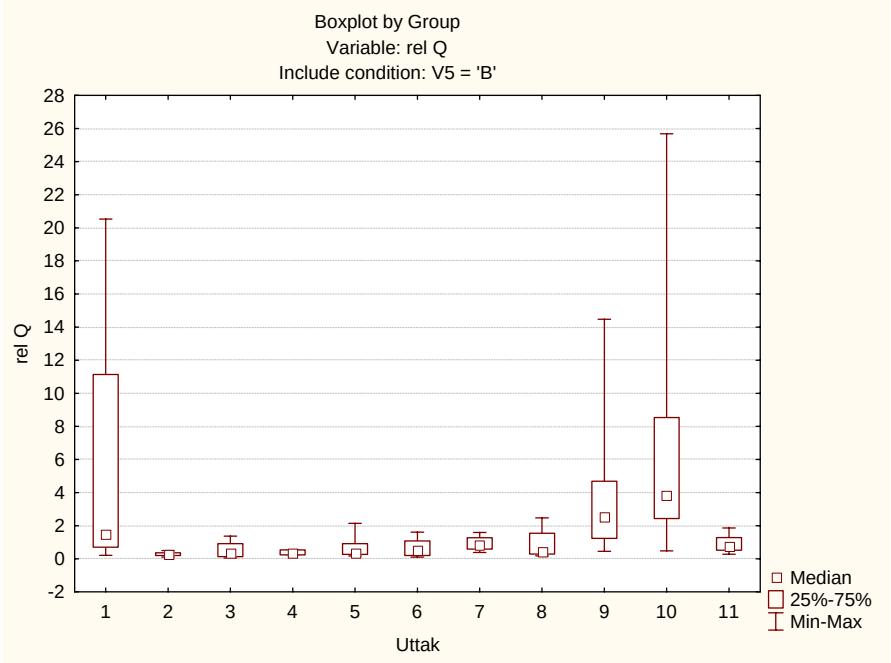
Kruskal-Wallis test of StAR relative Q vs. sampling no/date, diet group A:

Depend.: rel Q	Multiple Comparisons p values (2-tailed); rel Q (Lene data 030409) Independent (grouping) variable: Uttak Kruskal-Wallis test: H (10, N= 91) =58,10521 p =,0000 Include condition: V5 = 'A'										
	1 R:40,000	2 R:32,875	3 R:14,000	4 R:30,000	5 R:19,222	6 R:20,500	7 R:32,600	8 R:54,444	9 R:68,100	10 R:74,923	11 R:36,857
1		1,000000	1,000000	1,000000	1,000000	1,000000	1,000000	1,000000	1,000000	1,000000	1,000000
2	1,000000		1,000000	1,000000	1,000000	1,000000	1,000000	1,000000	0,078814	0,021786	1,000000
3	1,000000	1,000000		1,000000	1,000000	1,000000	1,000000	1,000000	0,316162	0,131560	1,000000
4	1,000000	1,000000	1,000000		1,000000	1,000000	1,000000	1,000000	0,215345	0,067619	1,000000
5	1,000000	1,000000	1,000000	1,000000		1,000000	1,000000	0,256993	0,000221	0,000064	1,000000
6	1,000000	1,000000	1,000000	1,000000	1,000000		1,000000	0,283706	0,000180	0,000053	1,000000
7	1,000000	1,000000	1,000000	1,000000	1,000000	1,000000		1,000000	0,395310	0,128015	1,000000
8	1,000000	1,000000	1,000000	1,000000	0,256993	0,283706	1,000000		1,000000	1,000000	1,000000
9	1,000000	0,078814	0,316162	0,215345	0,000221	0,000180	0,395310	1,000000		1,000000	0,388942
10	1,000000	0,021786	0,131560	0,067619	0,000064	0,000053	0,128015	1,000000	1,000000		0,116126
11	1,000000	1,000000	1,000000	1,000000	1,000000	1,000000	1,000000	1,000000	0,388942	0,116126	



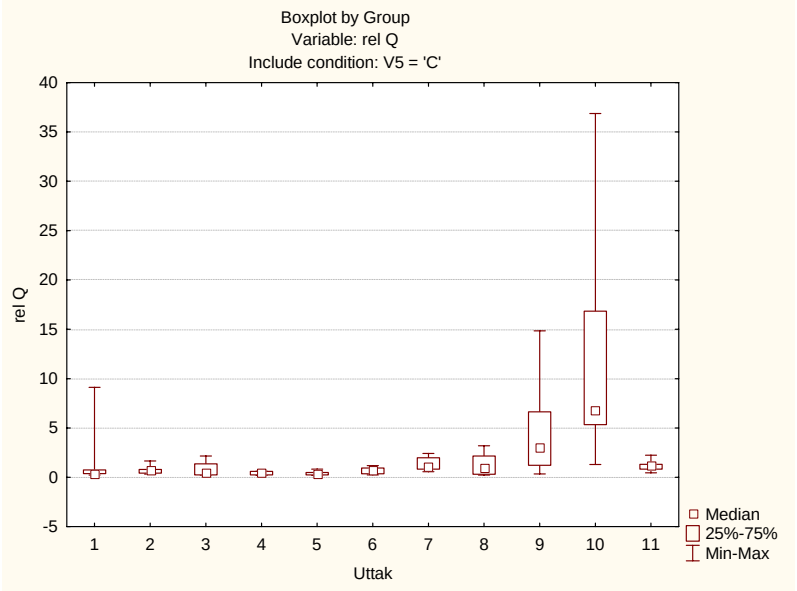
Kruskal-Wallis test of StAR relative Q vs. sampling no/date, diet group B:

	Multiple Comparisons p values (2-tailed); rel Q (Lene data 030409) Independent (grouping) variable: Uttak Kruskal-Wallis test: H (10, N= 101) =58,27628 p =,0000 Include condition: V5 = 'B'										
Depend.: rel Q	1 R:57,500	2 R:17,286	3 R:27,429	4 R:24,667	5 R:31,000	6 R:32,500	7 R:45,500	8 R:36,100	9 R:72,583	10 R:80,941	11 R:44,333
1		1,000000	1,000000	1,000000	1,000000	1,000000	1,000000	1,000000	1,000000	1,000000	1,000000
2	1,000000		1,000000	1,000000	1,000000	1,000000	1,000000	1,000000	0,000613	0,000072	1,000000
3	1,000000	1,000000		1,000000	1,000000	1,000000	1,000000	1,000000	0,018354	0,002621	1,000000
4	1,000000	1,000000	1,000000		1,000000	1,000000	1,000000	1,000000	0,416498	0,118926	1,000000
5	1,000000	1,000000	1,000000	1,000000		1,000000	1,000000	1,000000	0,052448	0,008108	1,000000
6	1,000000	1,000000	1,000000	1,000000	1,000000		1,000000	1,000000	0,006002	0,000638	1,000000
7	1,000000	1,000000	1,000000	1,000000	1,000000	1,000000		1,000000	1,000000	1,000000	1,000000
8	1,000000	1,000000	1,000000	1,000000	1,000000	1,000000	1,000000		0,051645	0,006762	1,000000
9	1,000000	0,000613	0,018354	0,416498	0,052448	0,006002	1,000000	0,051645		1,000000	1,000000
10	1,000000	0,000072	0,002621	0,118926	0,008108	0,000638	1,000000	0,006762	1,000000		0,468076
11	1,000000	1,000000	1,000000	1,000000	1,000000	1,000000	1,000000	1,000000	1,000000	0,468076	



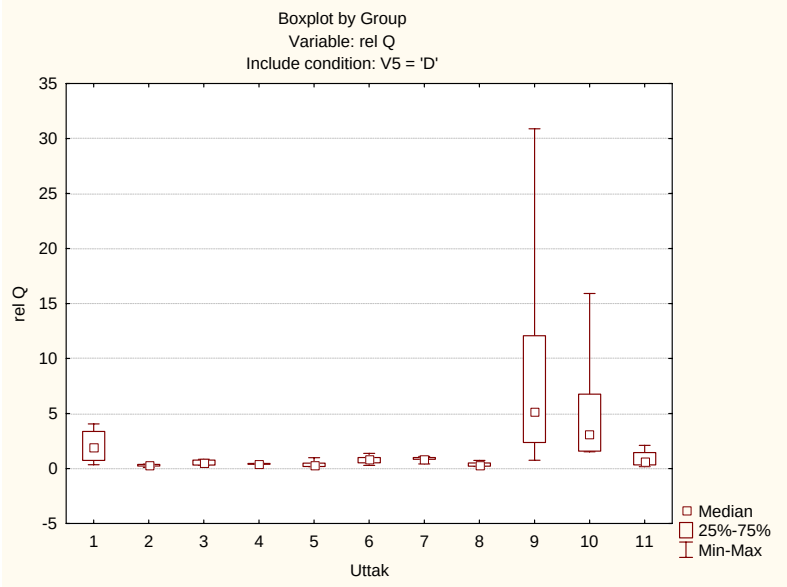
Kruskal-Wallis test of StAR relative Q vs. sampling no/date, diet group C:

Depend.: rel Q	Multiple Comparisons p values (2-tailed); rel Q (Lene data 030409) Independent (grouping) variable: Uttak Kruskal-Wallis test: H (10, N= 107) =62,48488 p =,0000 Include condition: V5 = 'C'										
	1 R:33,833	2 R:35,400	3 R:28,750	4 R:19,500	5 R:19,556	6 R:34,154	7 R:52,833	8 R:43,111	9 R:73,645	10 R:91,643	11 R:52,333
1		1,000000	1,000000	1,000000	1,000000	1,000000	1,000000	1,000000	0,221207	0,007406	1,000000
2	1,000000		1,000000	1,000000	1,000000	1,000000	1,000000	1,000000	0,580222	0,027704	1,000000
3	1,000000	1,000000		1,000000	1,000000	1,000000	1,000000	1,000000	0,355701	0,019281	1,000000
4	1,000000	1,000000	1,000000		1,000000	1,000000	1,000000	1,000000	0,056259	0,002267	1,000000
5	1,000000	1,000000	1,000000	1,000000		1,000000	1,000000	1,000000	0,000229	0,000003	1,000000
6	1,000000	1,000000	1,000000	1,000000	1,000000		1,000000	1,000000	0,006460	0,000083	1,000000
7	1,000000	1,000000	1,000000	1,000000	1,000000	1,000000		1,000000	1,000000	0,570735	1,000000
8	1,000000	1,000000	1,000000	1,000000	1,000000	1,000000	1,000000		0,514791	0,013848	1,000000
9	0,221207	0,580222	0,355701	0,056259	0,000229	0,006460	1,000000	0,514791		1,000000	1,000000
10	0,007406	0,027704	0,019281	0,002267	0,000003	0,000083	0,570735	0,013848	1,000000		0,518697
11	1,000000	1,000000	1,000000	1,000000	1,000000	1,000000	1,000000	1,000000	1,000000	0,518697	

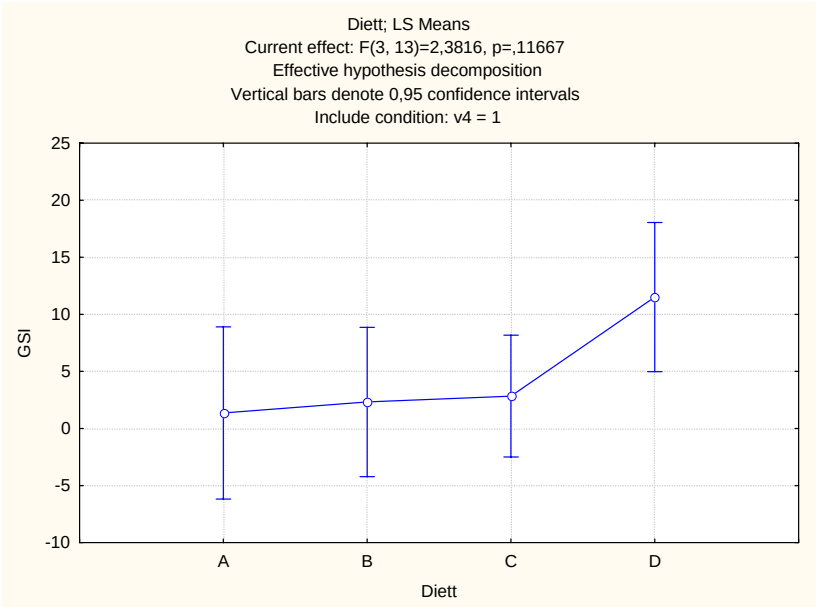


Kruskal-Wallis test of StAR relative Q vs. sampling no/date, diet group D:

Depend.: rel Q	Multiple Comparisons p values (2-tailed); rel Q (Lene data 030409) Independent (grouping) variable: Uttak Kruskal-Wallis test: H (10, N= 86) =63,48895 p =,0000 Include condition: V5 = 'D'										
	1 R:48,500	2 R:12,800	3 R:26,000	4 R:23,500	5 R:17,667	6 R:34,250	7 R:38,200	8 R:16,727	9 R:67,731	10 R:62,909	11 R:31,000
1		1,000000	1,000000	1,000000	1,000000	1,000000	1,000000	1,000000	1,000000	1,000000	1,000000
2	1,000000		1,000000	1,000000	1,000000	1,000000	1,000000	1,000000	0,000365	0,010929	1,000000
3	1,000000	1,000000		1,000000	1,000000	1,000000	1,000000	1,000000	0,102316	0,624481	1,000000
4	1,000000	1,000000	1,000000		1,000000	1,000000	1,000000	1,000000	0,867926	1,000000	1,000000
5	1,000000	1,000000	1,000000	1,000000		1,000000	1,000000	1,000000	0,000526	0,019630	1,000000
6	1,000000	1,000000	1,000000	1,000000	1,000000		1,000000	1,000000	0,050148	0,742983	1,000000
7	1,000000	1,000000	1,000000	1,000000	1,000000	1,000000		1,000000	0,849293	1,000000	1,000000
8	1,000000	1,000000	1,000000	1,000000	1,000000	1,000000	1,000000		0,000001	0,000793	1,000000
9	1,000000	0,000365	0,102316	0,867926	0,000526	0,050148	0,849293	0,000001		1,000000	0,339102
10	1,000000	0,010929	0,624481	1,000000	0,019630	0,742983	1,000000	0,000793	1,000000		1,000000
11	1,000000	1,000000	1,000000	1,000000	1,000000	1,000000	1,000000	1,000000	0,339102	1,000000	

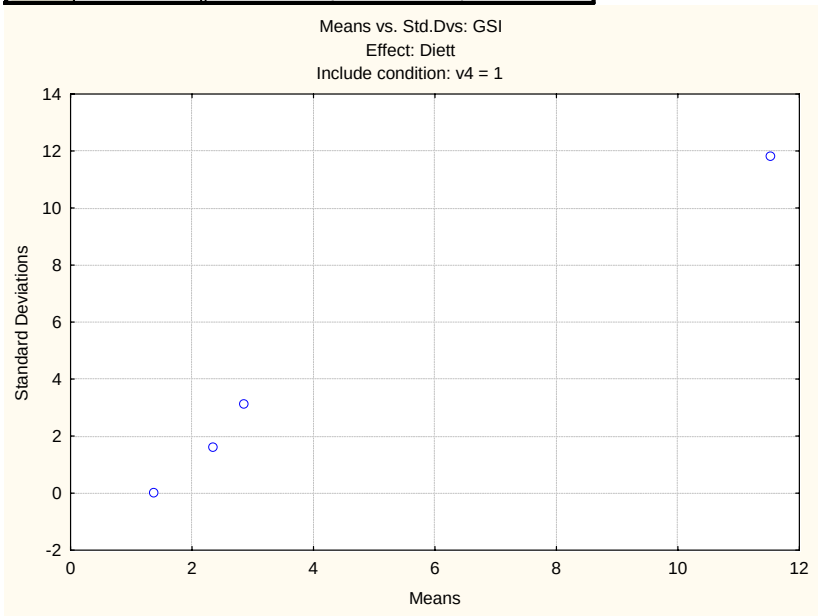


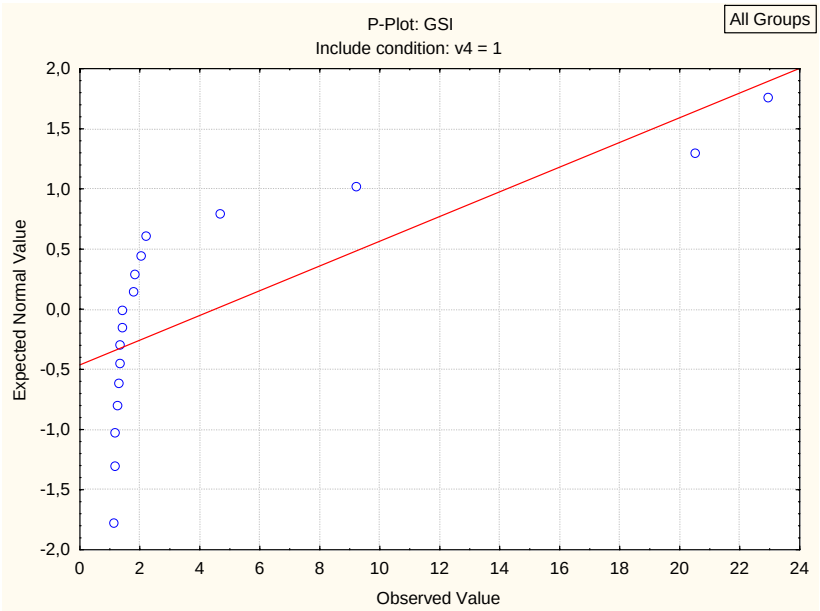
***One-way ANOVA, Levene's test, means vs. standard deviation, P-plot and histogram of GSI vs. diet group, sampling 1 (June)*:**

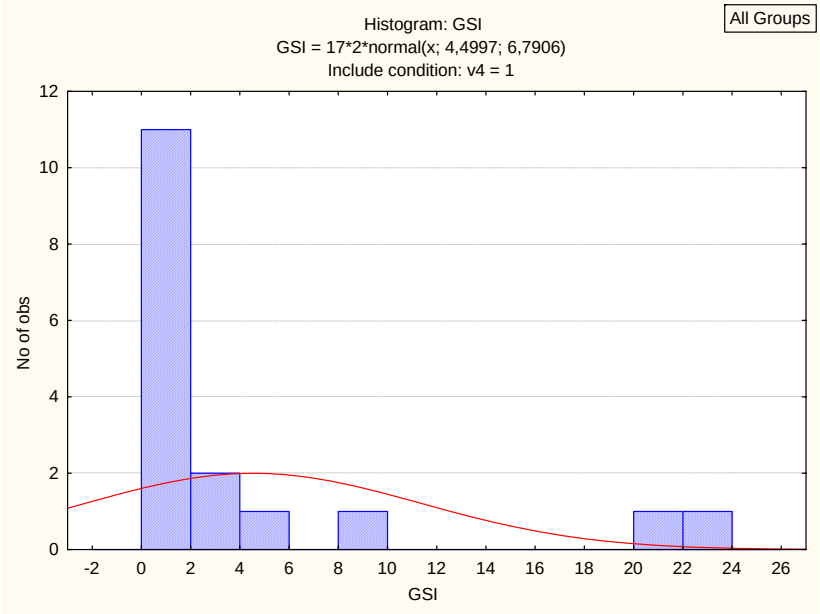


Levene's Test for Homogeneity of Variances (Lene data 030
Effect: Diett
Degrees of freedom for all F's: 3, 13
Include condition: v4 = 1

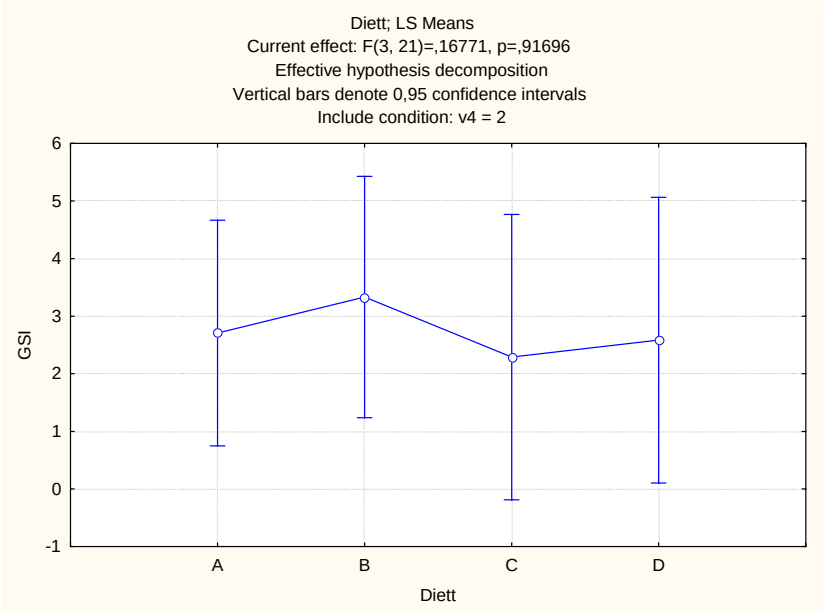
	MS Effect	MS Error	F	p
GSI	82,87721	2,126120	38,98048	0,000001



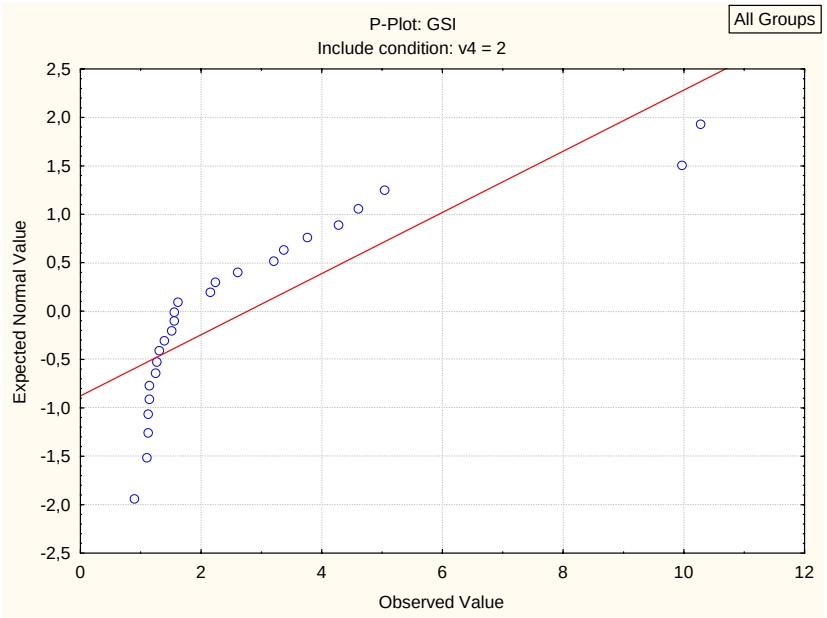

All Groups
P-Plot: GSI
Include condition: v4 = 1
Expected Normal Value
Observed Value
2,0
1,5
1,0
0,5
0,0
-0,5
-1,0
-1,5
-2,0
0
2
4
6
8
10
12
14
16
18
20
22
24

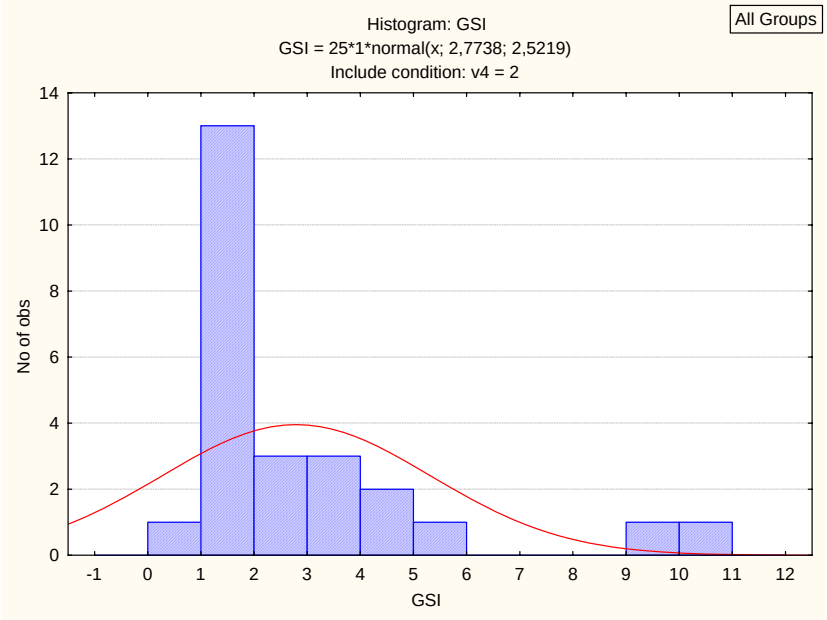

All Groups
Histogram: GSI
GSI = 17*2*normal(x; 4,4997; 6,7906)
Include condition: v4 = 1
No of obs
GSI
12
10
8
6
4
2
0
-2
0
2
4
6
8
10
12
14
16
18
20
22
24
26

***One-way ANOVA, Levene's test, P-plot and histogram of GSI vs. diet group, sampling 2 (July)*:**

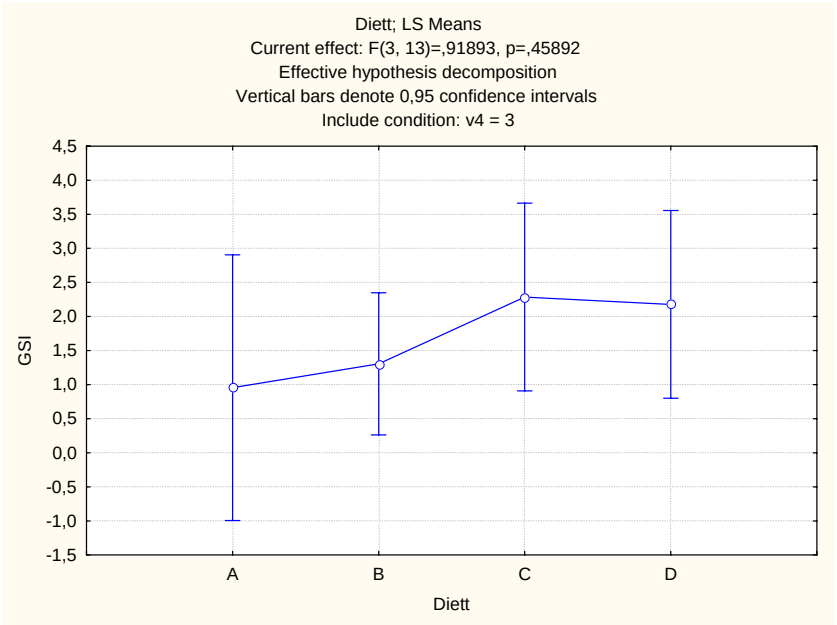


	Levene's Test for Homogeneity of Variances (Lene data 030 Effect: Diett Degrees of freedom for all F's: 3, 21 Include condition: v4 = 2			
	MS Effect	MS Error	F	p
GSI	2,494592	2,841808	0,877819	0,468408

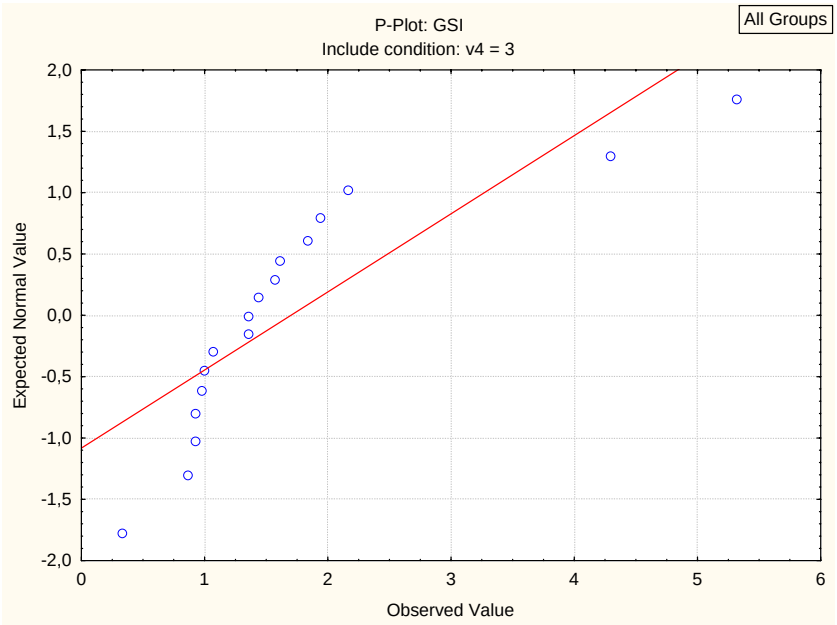

All Groups
P-Plot: GSI
Include condition: v4 = 2
Expected Normal Value
Observed Value
2,5
2,0
1,5
1,0
0,5
0,0
-0,5
-1,0
-1,5
-2,0
-2,5
0
2
4
6
8
10
12

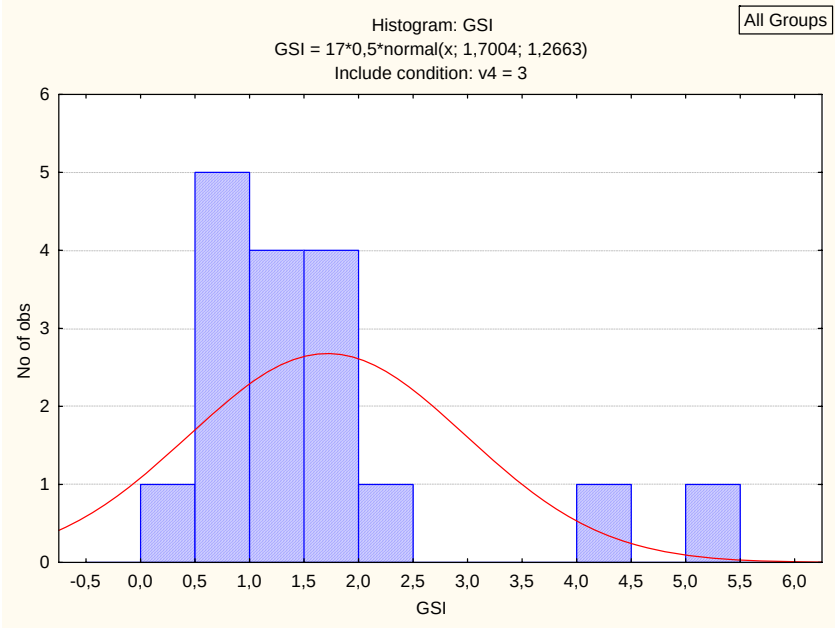

All Groups
Histogram: GSI
GSI = 25*1*normal(x; 2,7738; 2,5219)
Include condition: v4 = 2
No of obs
GSI
14
12
10
8
6
4
2
0
-1
0
1
2
3
4
5
6
7
8
9
10
11
12

***One-way ANOVA, Levene's test, P-plot and histogram of GSI vs. diet group, sampling 3 (August)*:**

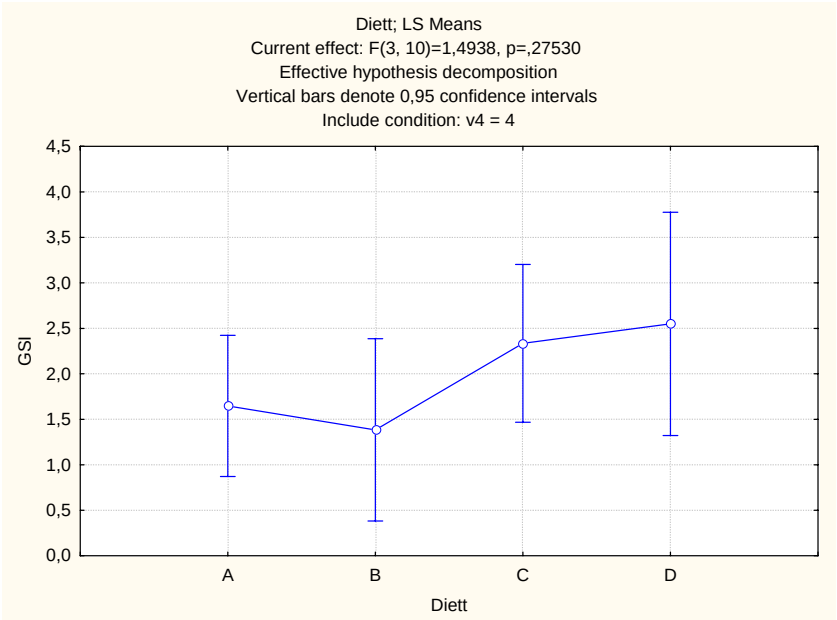


	Levene's Test for Homogeneity of Variances (Lene data 03C Effect: Diett Degrees of freedom for all F's: 3, 13 Include condition: v4 = 3			
	MS Effect	MS Error	F	p
GSI	1,619379	0,479562	3,376789	0,051332


All Groups
P-Plot: GSI
Include condition: v4 = 3
Expected Normal Value
Observed Value
2,0
1,5
1,0
0,5
0,0
-0,5
-1,0
-1,5
-2,0
0
1
2
3
4
5
6

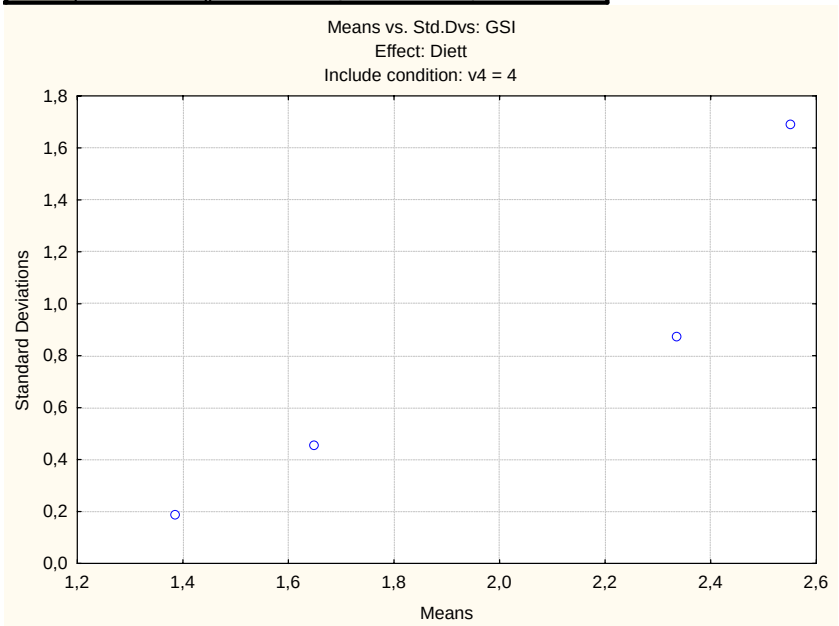

All Groups
Histogram: GSI
GSI = 17*0,5*normal(x; 1,7004; 1,2663)
Include condition: v4 = 3
No of obs
GSI
6
5
4
3
2
1
0
-0,5
0,0
0,5
1,0
1,5
2,0
2,5
3,0
3,5
4,0
4,5
5,0
5,5
6,0

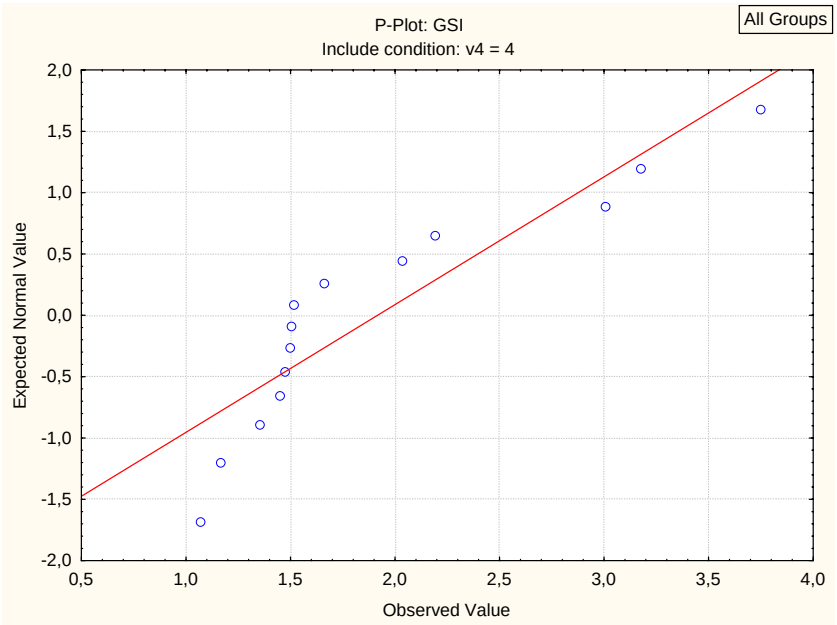
***One-way ANOVA, Levene's test, means vs. standard deviation, P-plot and histogram of GSI vs. diet group, sampling 4 (September)*:**

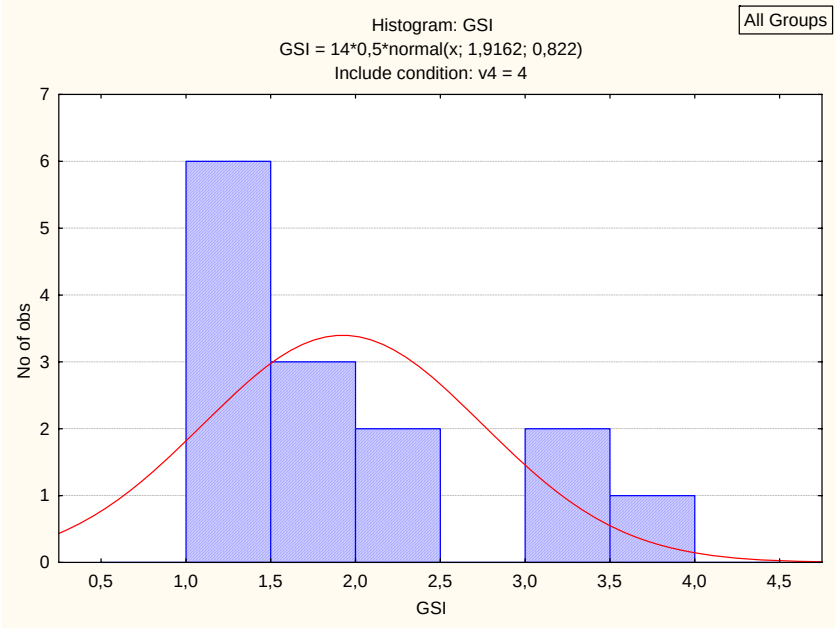


Levene's Test for Homogeneity of Variances (Lene data 030
Effect: Diett
Degrees of freedom for all F's: 3, 10
Include condition: v4 = 4

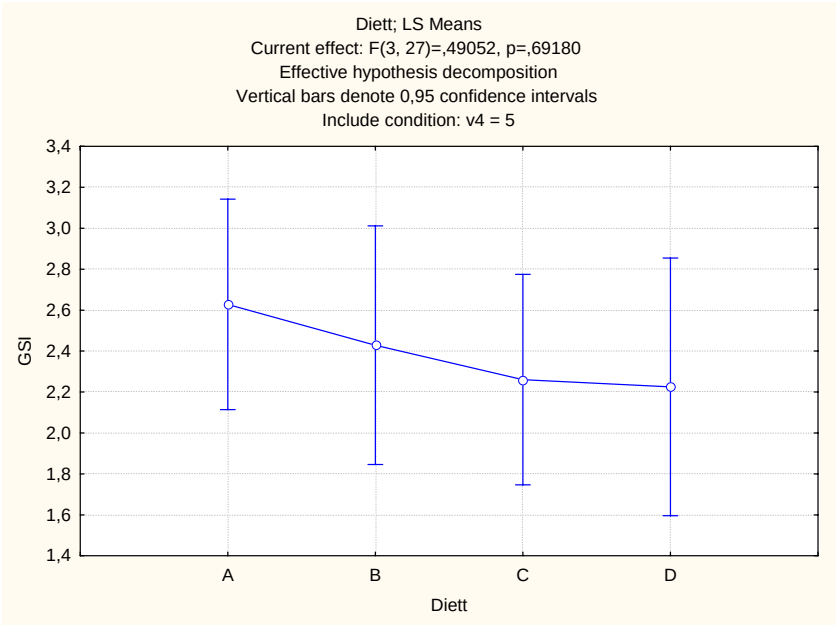
	MS Effect	MS Error	F	p
GSI	0,549414	0,018475	29,73828	0,000027



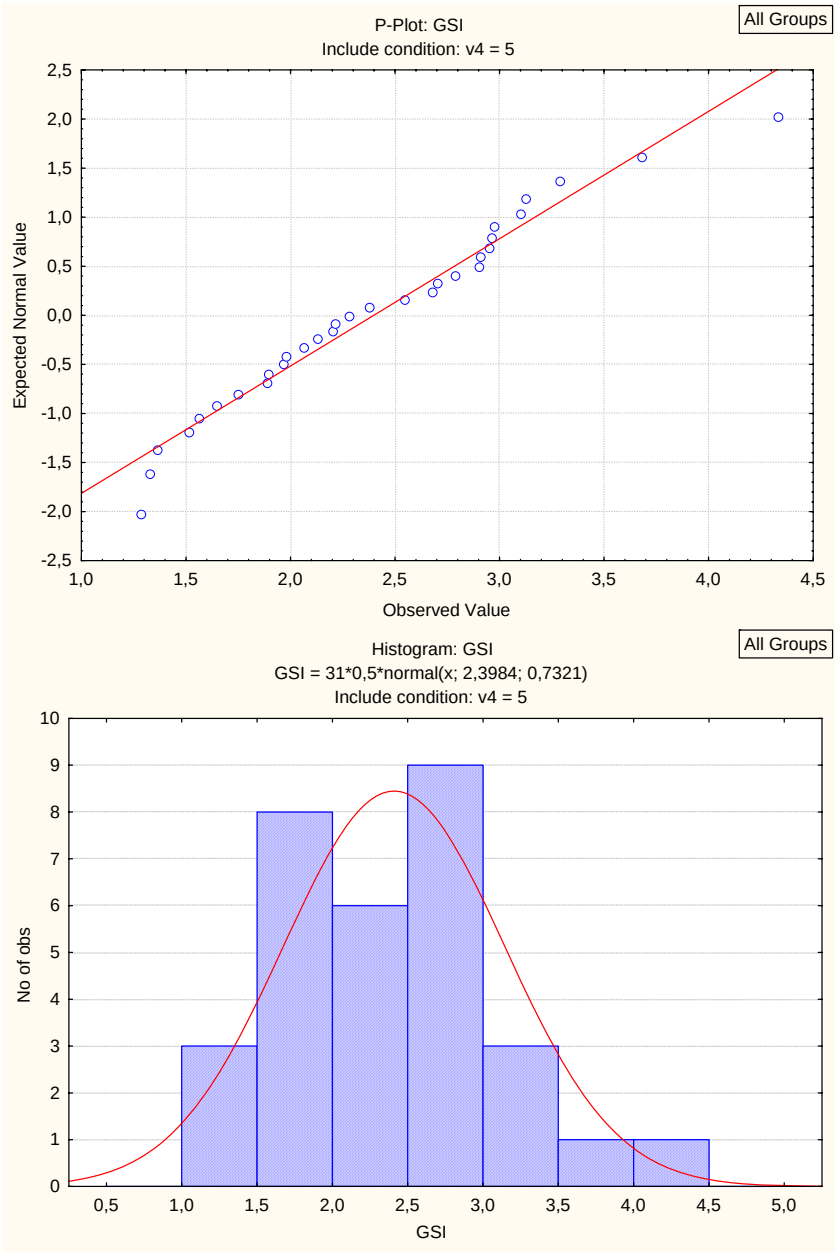

All Groups
P-Plot: GSI
Include condition: v4 = 4
Expected Normal Value
Observed Value
2,0
1,5
1,0
0,5
0,0
-0,5
-1,0
-1,5
-2,0
0,5
1,0
1,5
2,0
2,5
3,0
3,5
4,0


All Groups
Histogram: GSI
GSI = 14*0,5*normal(x; 1,9162; 0,822)
Include condition: v4 = 4
No of obs
GSI
7
6
5
4
3
2
1
0
0,5
1,0
1,5
2,0
2,5
3,0
3,5
4,0
4,5

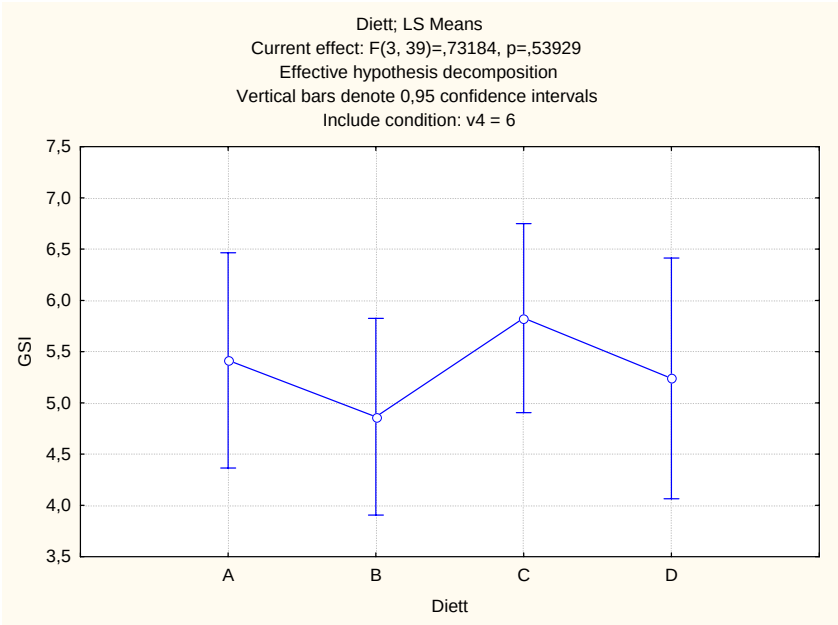
***One-way ANOVA, Levene's test, P-plot and histogram of GSI vs. diet group, sampling 5 (October)*:**



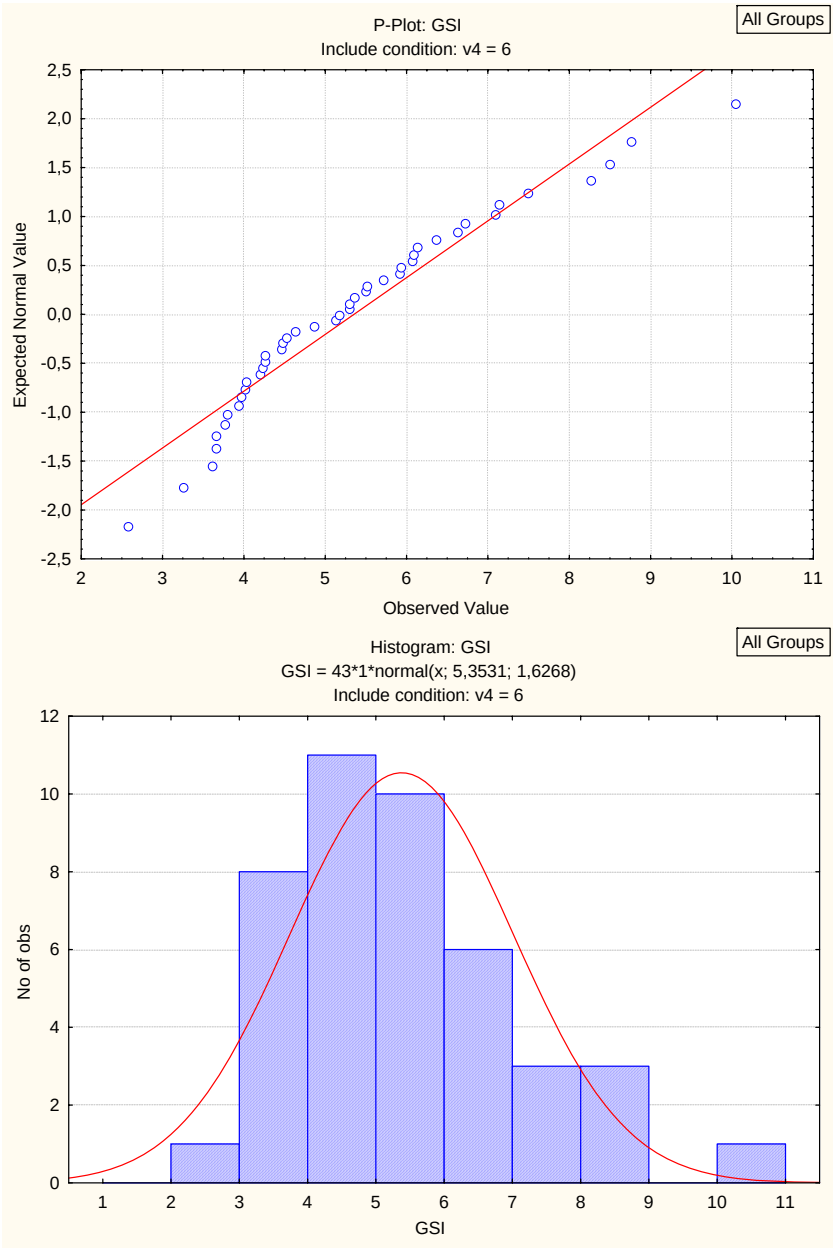
	Levene's Test for Homogeneity of Variances (Lene data 03C Effect: Diett Degrees of freedom for all F's: 3, 27 Include condition: v4 = 5			
	MS Effect	MS Error	F	p
GSI	0,044163	0,154635	0,285591	0,835356


All Groups
P-Plot: GSI
Include condition: v4 = 5
Expected Normal Value
Observed Value
2,5
2,0
1,5
1,0
0,5
0,0
-0,5
-1,0
-1,5
-2,0
-2,5
1,0
1,5
2,0
2,5
3,0
3,5
4,0
4,5
All Groups
Histogram: GSI
GSI = 31*0,5*normal(x; 2,3984; 0,7321)
Include condition: v4 = 5
No of obs
GSI
10
9
8
7
6
5
4
3
2
1
0
0,5
1,0
1,5
2,0
2,5
3,0
3,5
4,0
4,5
5,0

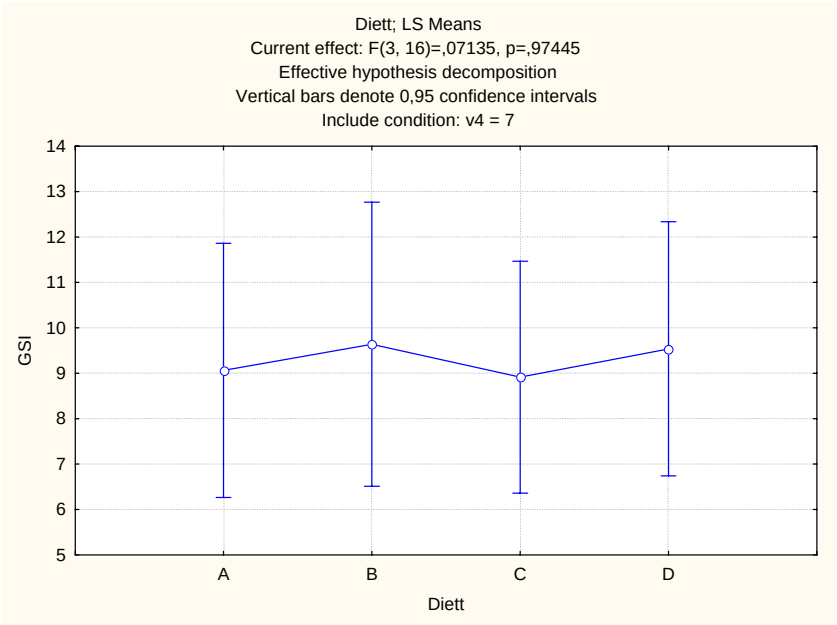
***One-way ANOVA, Levene's test, P-plot and histogram of GSI vs. diet group, sampling 6 (November)*:**



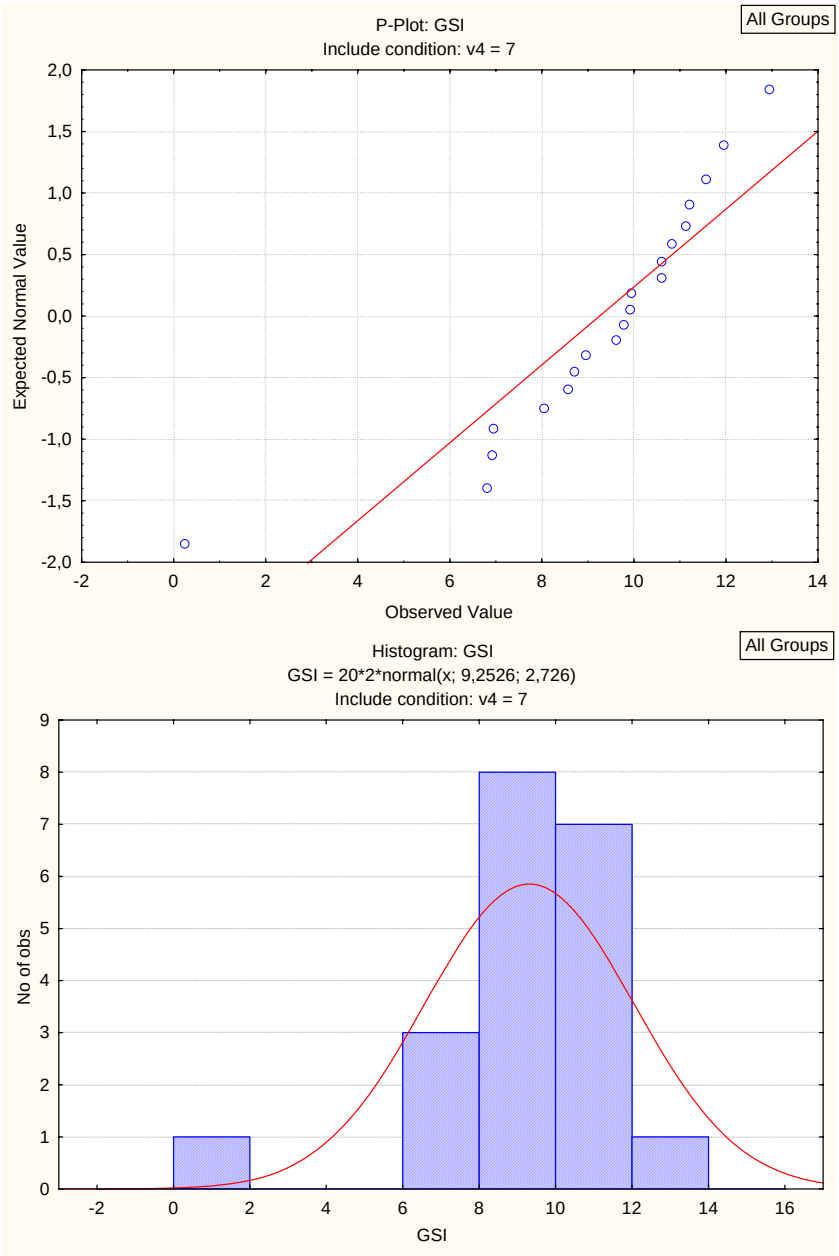
	Levene's Test for Homogeneity of Variances (Lene data 03C Effect: Diett Degrees of freedom for all F's: 3, 39 Include condition: v4 = 6			
	MS Effect	MS Error	F	p
GSI	1,252322	0,876165	1,429321	0,248861


All Groups
P-Plot: GSI
Include condition: v4 = 6
Expected Normal Value
Observed Value
2,5
2,0
1,5
1,0
0,5
0,0
-0,5
-1,0
-1,5
-2,0
-2,5
2
3
4
5
6
7
8
9
10
11
All Groups
Histogram: GSI
GSI = 43*1*normal(x; 5,3531; 1,6268)
Include condition: v4 = 6
No of obs
GSI
12
10
8
6
4
2
0
1
2
3
4
5
6
7
8
9
10
11

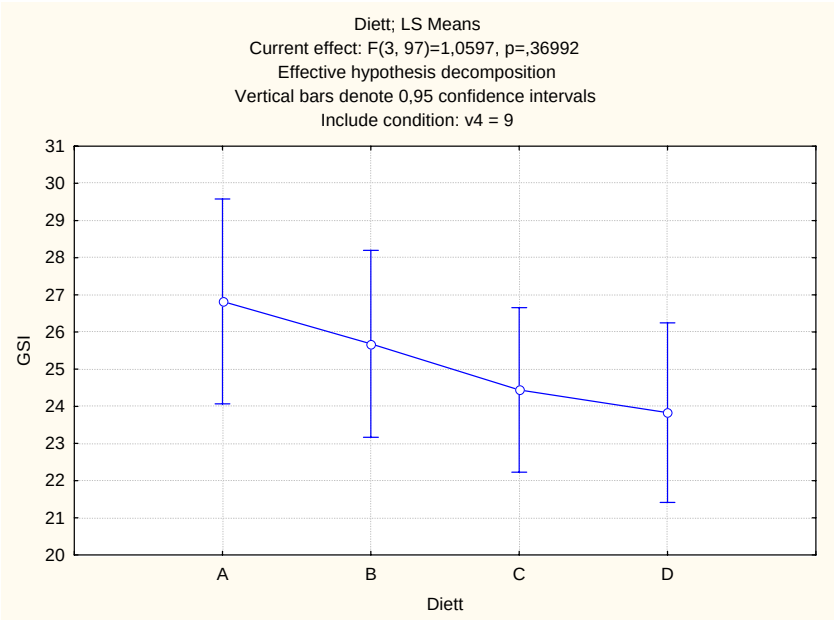
***One-way ANOVA, Levene's test, P-plot and histogram of GSI vs. diet group, sampling 7 (December)*:**



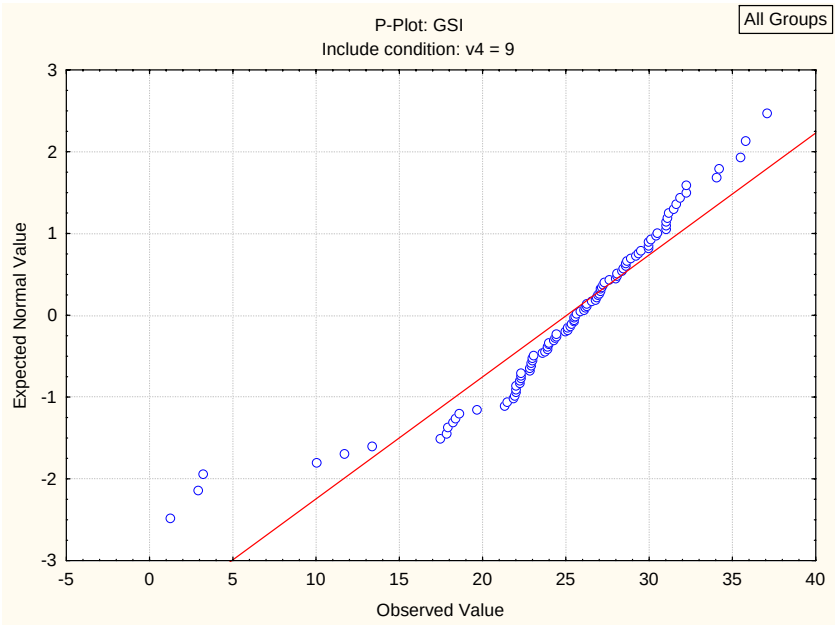
	Levene's Test for Homogeneity of Variances (Lene data 03(Effect: Diett Degrees of freedom for all F's: 3, 16 Include condition: v4 = 7			
	MS Effect	MS Error	F	p
GSI	3,046425	3,787694	0,804295	0,509648

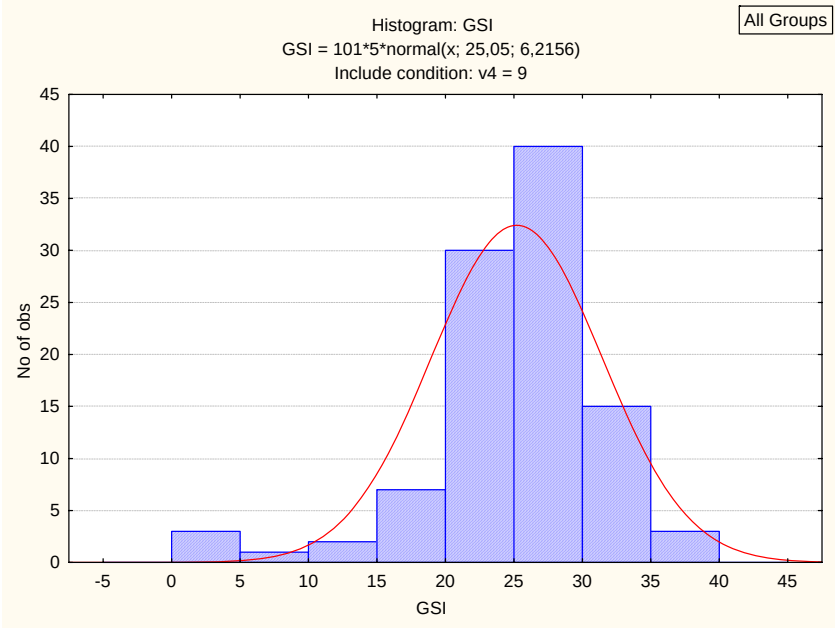

All Groups
P-Plot: GSI
Include condition: v4 = 7
Expected Normal Value
Observed Value
2,0
1,5
1,0
0,5
0,0
-0,5
-1,0
-1,5
-2,0
-2
0
2
4
6
8
10
12
14
All Groups
Histogram: GSI
GSI = 20*2*normal(x; 9,2526; 2,726)
Include condition: v4 = 7
No of obs
GSI
9
8
7
6
5
4
3
2
1
0
-2
0
2
4
6
8
10
12
14
16

***One-way ANOVA, Levene's test, P-plot and histogram of GSI vs. diet group, sampling 9 (February)*:**

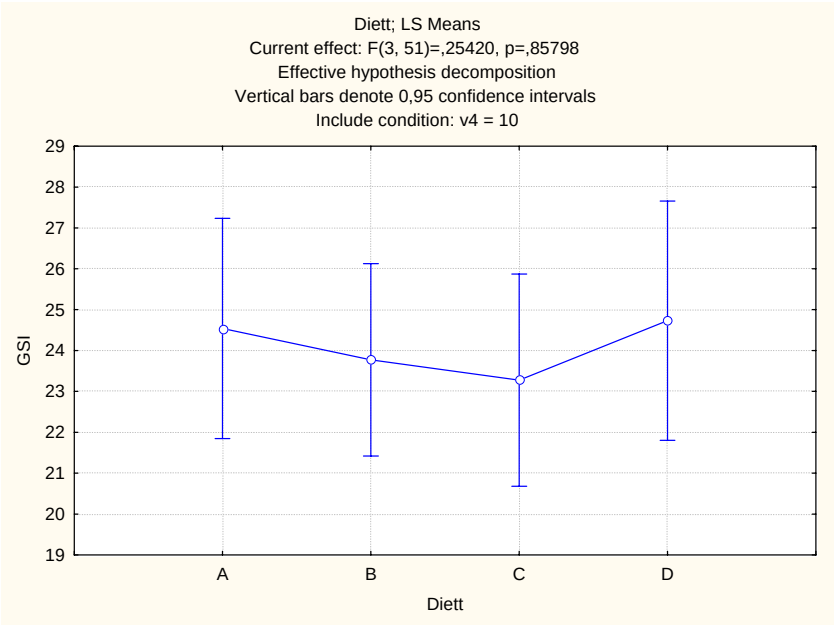


	Levene's Test for Homogeneity of Variances (Lene data 03(Effect: Diett Degrees of freedom for all F's: 3, 97 Include condition: v4 = 9			
	MS Effect	MS Error	F	p
GSI	3,971845	20,02554	0,198339	0,897292

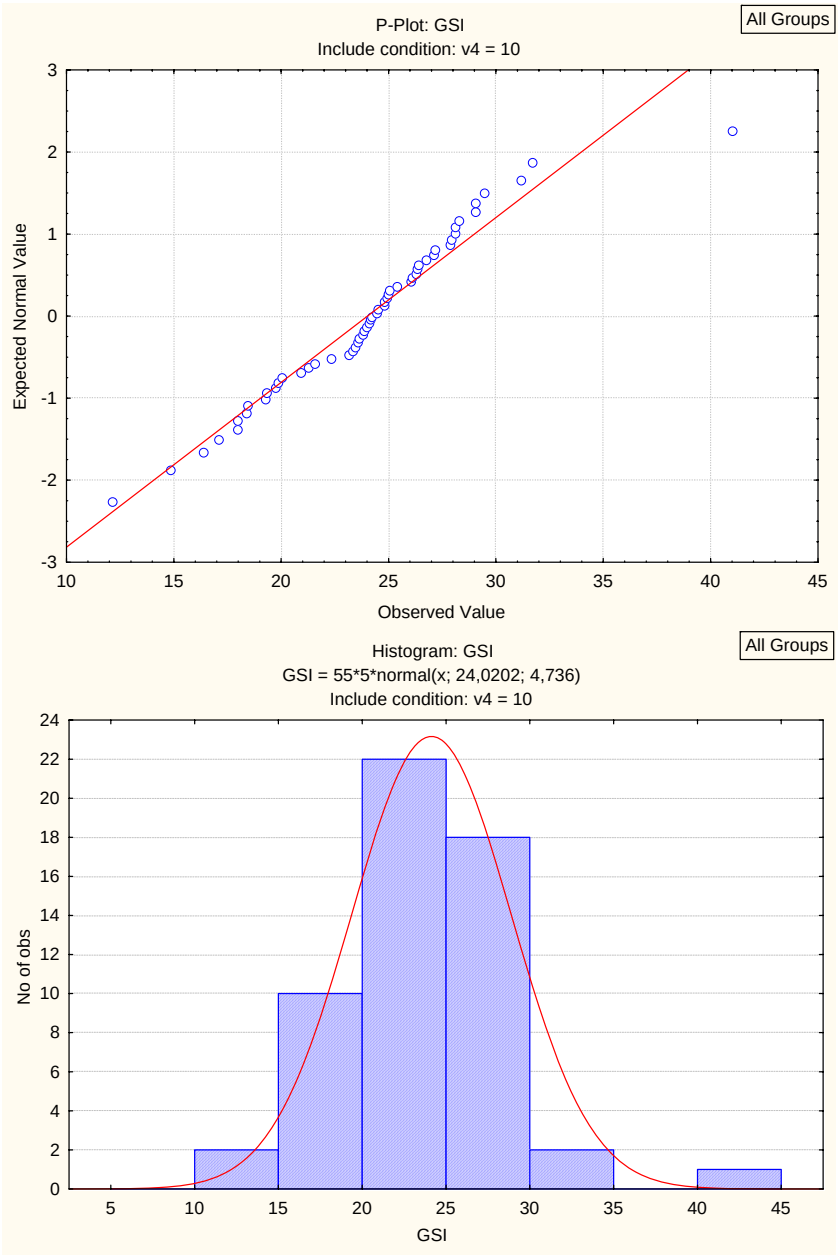

All Groups
P-Plot: GSI
Include condition: v4 = 9
Expected Normal Value
Observed Value
3
2
1
0
-1
-2
-3
-5
0
5
10
15
20
25
30
35
40


All Groups
Histogram: GSI
GSI = 101*5*normal(x; 25,05; 6,2156)
Include condition: v4 = 9
No of obs
GSI
45
40
35
30
25
20
15
10
5
0
-5
0
5
10
15
20
25
30
35
40
45

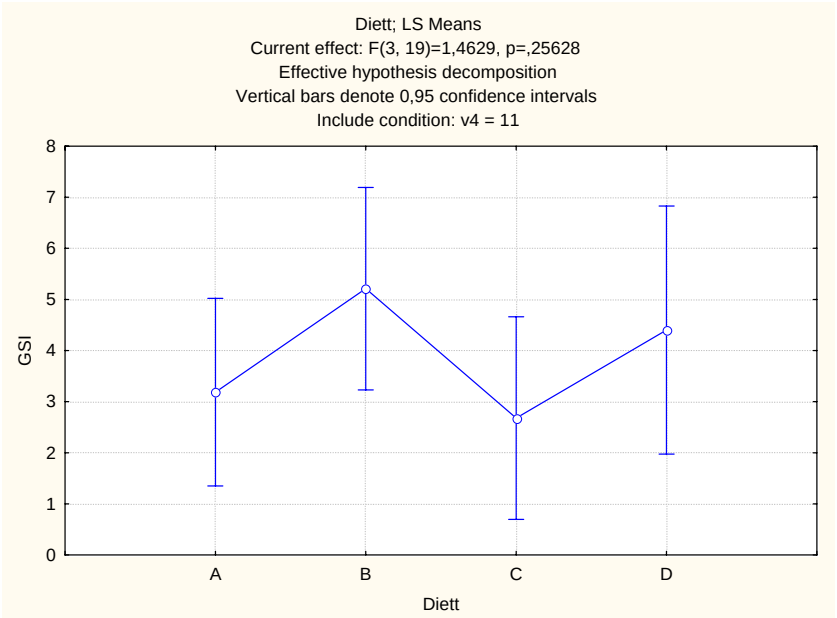
***One-way ANOVA, Levene's test, P-plot and histogram of GSI vs. diet group, sampling 10 (March)*:**



	Levene's Test for Homogeneity of Variances (Lene data 03(Effect: Diett Degrees of freedom for all F's: 3, 51 Include condition: v4 = 10			
	MS Effect	MS Error	F	p
GSI	3,354132	10,44854	0,321014	0,810125

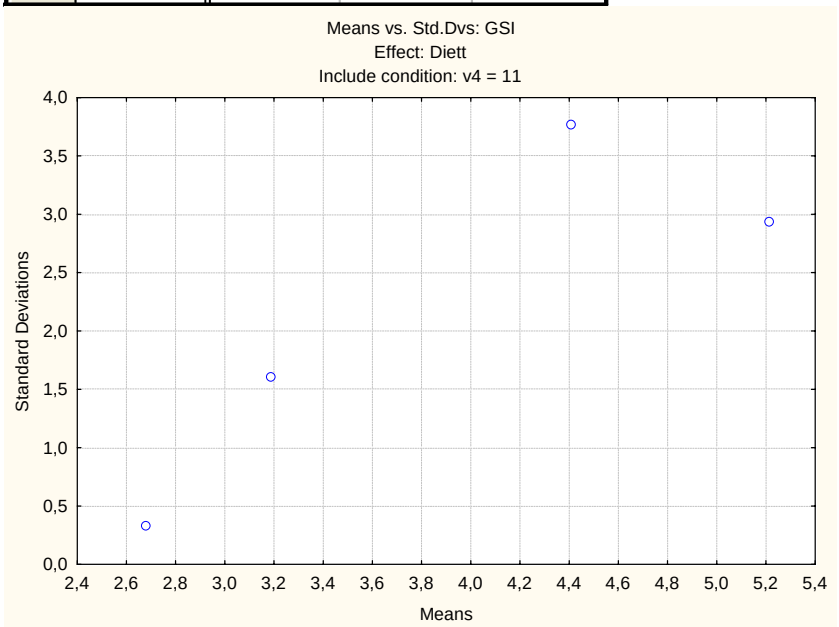

All Groups
P-Plot: GSI
Include condition: v4 = 10
Expected Normal Value
Observed Value
All Groups
Histogram: GSI
GSI = 55*5*normal(x; 24,0202; 4,736)
Include condition: v4 = 10
No of obs
GSI

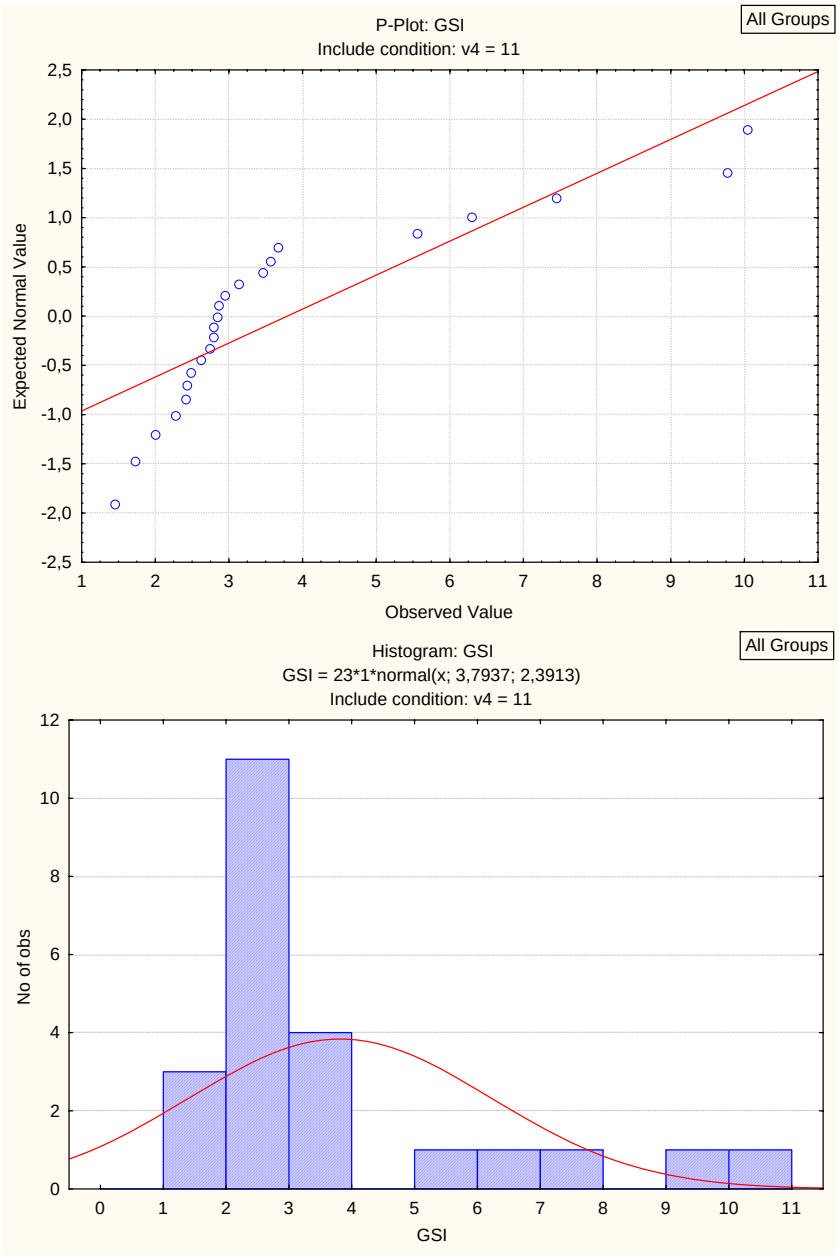
***One-way ANOVA, Levene's test, means vs. standard deviation, P-plot and histogram of GSI vs. diet group, sampling 11 (May)*:**



Levene's Test for Homogeneity of Variances (Lene data 03(
Effect: Diett
Degrees of freedom for all F's: 3, 19
Include condition: v4 = 11

	MS Effect	MS Error	F	p
GSI	7,080596	1,425801	4,966048	0,010357




All Groups
P-Plot: GSI
Include condition: v4 = 11
Expected Normal Value
Observed Value
2,5
2,0
1,5
1,0
0,5
0,0
-0,5
-1,0
-1,5
-2,0
-2,5
1
2
3
4
5
6
7
8
9
10
11
All Groups
Histogram: GSI
GSI = 23*1*normal(x; 3,7937; 2,3913)
Include condition: v4 = 11
No of obs
GSI
12
10
8
6
4
2
0
0
1
2
3
4
5
6
7
8
9
10
11

Correlation between GSI and StAR expression (relative Q):

Statistic	Summary Statistics; DV: rel Q (Lene data 030409)
	Value
Multiple R	0,405158254
Multiple R²	0,164153211
Adjusted R²	0,161970843
F(1,383)	75,2179471
p	1,21373786E-16
Std.Err. of Estimate	4,444475

N=385	Regression Summary for Dependent Variable: rel Q (Lene data 030409) R= ,40515825 R²= ,16415321 Adjusted R²= ,16197084 F(1,383)=75,218 p<,00000 Std.Error of estimate: 4,4445					
	Beta	Std.Err. of Beta	B	Std.Err. of B	t(383)	p-level
Intercept			0,452583	0,365667	1,237692	0,216589
GSI	0,405158	0,046716	0,183268	0,021131	8,672828	0,000000

Correlation between GSI and StAR expression (log (Q+1)):

Statistic	Summary Statistics; DV: log(Q+1) (Lene data 030409)
	Value
Multiple R	0,607470549
Multiple R²	0,369020468
Adjusted R²	0,367373002
F(1,383)	223,992748
p	3,36936611E-40
Std.Err. of Estimate	0,273084494

N=385	Regression Summary for Dependent Variable: log(Q+1) (Lene data 030409) R= ,60747055 R²= ,36902047 Adjusted R²= ,36737300 F(1,383)=223,99 p<0,0000 Std.Error of estimate: ,27308					
	Beta	Std.Err. of Beta	B	Std.Err. of B	t(383)	p-level
Intercept			0,158786	0,022468	7,06725	0,000000
GSI	0,607471	0,040589	0,019432	0,001298	14,96639	0,000000

Correlation between estradiol (E2) and StAR expression (relative Q):

Statistic	Summary Statistics; DV: rel Q (Lene data 030409) Value
Multiple R	0,25750242
Multiple R²	0,0663074962
Adjusted R²	0,0638042991
F(1,373)	26,4891235
p	0,000000429561879
Std.Err. of Estimate	4,74301957

N=375	Regression Summary for Dependent Variable: rel Q (Lene data 030409) R= ,25750242 R²= ,06630750 Adjusted R²= ,06380430 F(1,373)=26,489 p<,00000 Std.Error of estimate: 4,7430 Beta	Std.Err. of Beta	B	Std.Err. of B	t(373)	p-level
Intercept			1,820260	0,329526	5,523871	0,000000
Estradiol (ng/ml)	0,257502	0,050032	0,116363	0,022609	5,146759	0,000000

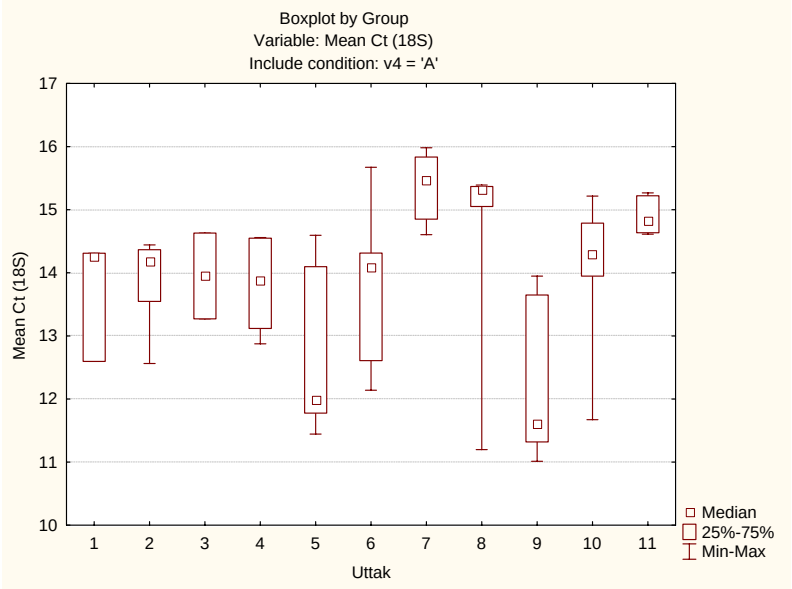
Correlation between estradiol (E2) and StAR expression (log (Q+1)):

Statistic	Summary Statistics; DV: log(Q+1) (Lene data 030409) Value
Multiple R	0,38209316
Multiple R²	0,145995183
Adjusted R²	0,143705626
F(1,373)	63,7656862
p	1,75567890E-14
Std.Err. of Estimate	0,318844014

N=375	Regression Summary for Dependent Variable: log(Q+1) (Lene data 030409) R= ,38209316 R²= ,14599518 Adjusted R²= ,14370563 F(1,373)=63,766 p<,00000 Std.Error of estimate: ,31884 Beta	Std.Err. of Beta	B	Std.Err. of B	t(373)	p-level
Intercept			0,304071	0,022152	13,72657	0,000000
Estradiol (ng/ml)	0,382093	0,047849	0,012137	0,001520	7,98534	0,000000

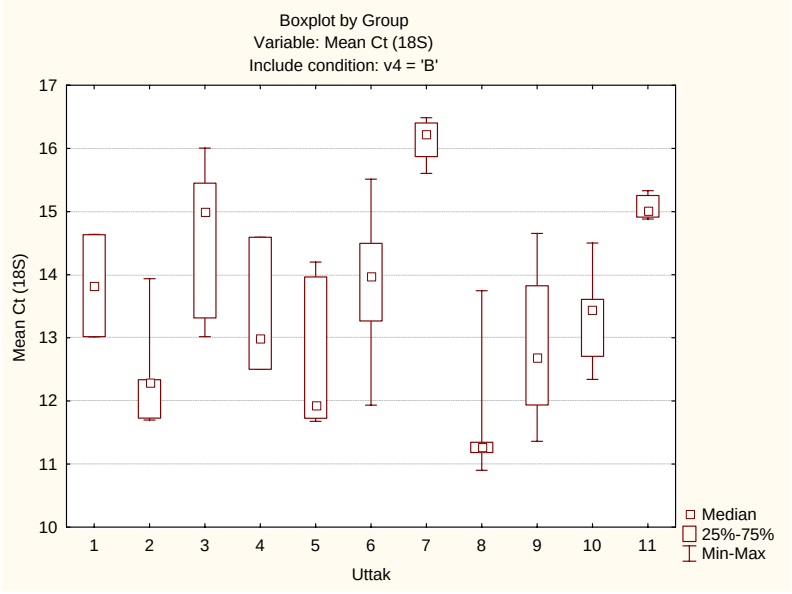
Kruskal-Wallis test of 18S mean Ct vs. sampling no/date, diet group A:

Depend.: Mean Ct (18S)	Multiple Comparisons p values (2-tailed); Mean Ct (18S) (StAR utregning threshold 20 april) Independent (grouping) variable: Uttak Kruskal-Wallis test: H (10, N= 91) =47,01716 p =,0000 Include condition: v4 = 'A'										
	1 R:45,000	2 R:47,625	3 R:51,500	4 R:46,600	5 R:30,667	6 R:47,200	7 R:81,600	8 R:65,000	9 R:18,850	10 R:53,692	11 R:74,000
1		1,000000	1,000000	1,000000	1,000000	1,000000	1,000000	1,000000	1,000000	1,000000	1,000000
2	1,000000		1,000000	1,000000	1,000000	1,000000	1,000000	1,000000	0,506507	1,000000	1,000000
3	1,000000	1,000000		1,000000	1,000000	1,000000	1,000000	1,000000	1,000000	1,000000	1,000000
4	1,000000	1,000000	1,000000		1,000000	1,000000	1,000000	1,000000	1,000000	1,000000	1,000000
5	1,000000	1,000000	1,000000	1,000000		1,000000	0,030024	0,320456	1,000000	1,000000	0,062271
6	1,000000	1,000000	1,000000	1,000000	1,000000		0,957916	1,000000	0,307082	1,000000	1,000000
7	1,000000	1,000000	1,000000	1,000000	0,030024	0,957916		1,000000	0,000111	1,000000	1,000000
8	1,000000	1,000000	1,000000	1,000000	0,320456	1,000000	1,000000		0,000739	1,000000	1,000000
9	1,000000	0,506507	1,000000	1,000000	1,000000	0,307082	0,000111	0,000739		0,011735	0,000109
10	1,000000	1,000000	1,000000	1,000000	1,000000	1,000000	1,000000	1,000000	0,011735		1,000000
11	1,000000	1,000000	1,000000	1,000000	0,062271	1,000000	1,000000	1,000000	0,000109	1,000000	



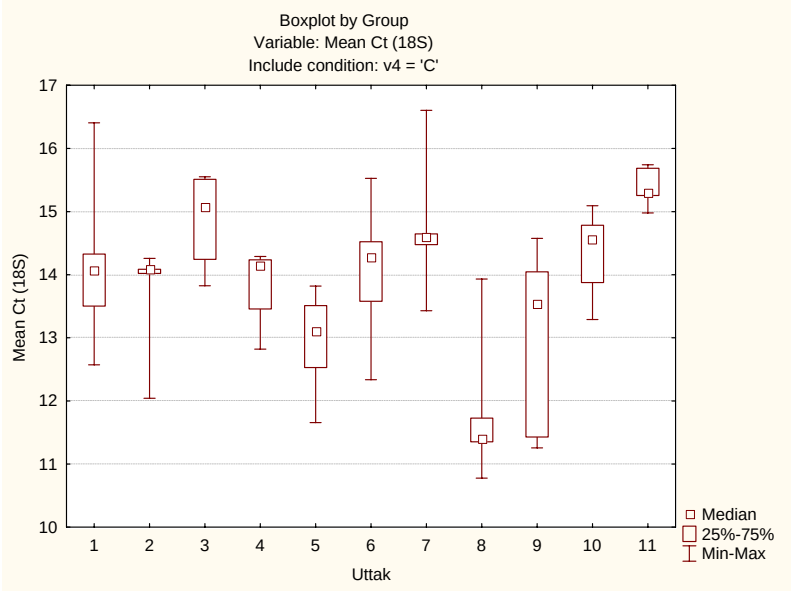
Kruskal-Wallis test of 18S mean Ct vs. sampling no/date, diet group B:

	Multiple Comparisons p values (2-tailed); Mean Ct (18S) (StAR utregning threshold 20 april) Independent (grouping) variable: Uttak Kruskal-Wallis test: H (10, N= 100) =52,86962 p =,0000 Include condition: v4 = 'B'										
Depend.: Mean Ct (18S)	1 R:62,750	2 R:28,286	3 R:77,000	4 R:52,000	5 R:32,714	6 R:65,417	7 R:98,250	8 R:16,300	9 R:42,458	10 R:50,529	11 R:89,167
1		1,000000	1,000000	1,000000	1,000000	1,000000	1,000000	0,374167	1,000000	1,000000	1,000000
2	1,000000		0,139883	1,000000	1,000000	0,391700	0,006561	1,000000	1,000000	1,000000	0,008909
3	1,000000	0,139883		1,000000	0,334067	1,000000	1,000000	0,002797	0,500144	1,000000	1,000000
4	1,000000	1,000000	1,000000		1,000000	1,000000	1,000000	1,000000	1,000000	1,000000	1,000000
5	1,000000	1,000000	0,334067	1,000000		0,978001	0,017231	1,000000	1,000000	1,000000	0,025824
6	1,000000	0,391700	1,000000	1,000000	0,978001		1,000000	0,004227	1,000000	1,000000	1,000000
7	1,000000	0,006561	1,000000	1,000000	0,017231	1,000000		0,000099	0,020330	0,169243	1,000000
8	0,374167	1,000000	0,002797	1,000000	1,000000	0,004227	0,000099		0,912737	0,168901	0,000063
9	1,000000	1,000000	0,500144	1,000000	1,000000	1,000000	0,020330	0,912737		1,000000	0,023088
10	1,000000	1,000000	1,000000	1,000000	1,000000	1,000000	0,169243	0,168901	1,000000		0,277077
11	1,000000	0,008909	1,000000	1,000000	0,025824	1,000000	1,000000	0,000063	0,023088	0,277077	



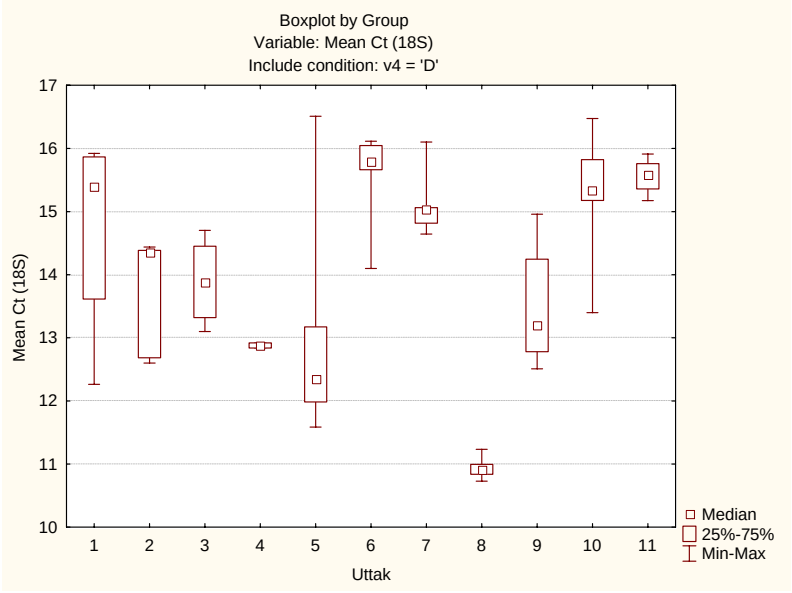
Kruskal-Wallis test of 18S mean Ct vs. sampling no/date, diet group C:

Depend.: Mean Ct (18S)	Multiple Comparisons p values (2-tailed); Mean Ct (18S) (StAR utregning threshold 20 april) Independent (grouping) variable: Uttak Kruskal-Wallis test: H (10, N= 107) =52,85646 p =,0000 Include condition: v4 = 'C'										
	1 R:59,833	2 R:53,600	3 R:84,750	4 R:56,250	5 R:30,889	6 R:64,615	7 R:80,167	8 R:18,000	9 R:38,903	10 R:72,714	11 R:100,33
1		1,000000	1,000000	1,000000	1,000000	1,000000	1,000000	0,579411	1,000000	1,000000	1,000000
2	1,000000		1,000000	1,000000	1,000000	1,000000	1,000000	1,000000	1,000000	1,000000	0,708516
3	1,000000	1,000000		1,000000	0,213034	1,000000	1,000000	0,018937	0,298222	1,000000	1,000000
4	1,000000	1,000000	1,000000		1,000000	1,000000	1,000000	1,000000	1,000000	1,000000	1,000000
5	1,000000	1,000000	0,213034	1,000000		0,670939	0,142306	1,000000	1,000000	0,088386	0,001197
6	1,000000	1,000000	1,000000	1,000000	0,670939		1,000000	0,029254	0,668621	1,000000	1,000000
7	1,000000	1,000000	1,000000	1,000000	0,142306	1,000000		0,007927	0,157857	1,000000	1,000000
8	0,579411	1,000000	0,018937	1,000000	1,000000	0,029254	0,007927		1,000000	0,002024	0,000026
9	1,000000	1,000000	0,298222	1,000000	1,000000	0,668621	0,157857	1,000000		0,039342	0,000499
10	1,000000	1,000000	1,000000	1,000000	0,088386	1,000000	1,000000	0,002024	0,039342		1,000000
11	1,000000	0,708516	1,000000	1,000000	0,001197	1,000000	1,000000	0,000026	0,000499	1,000000	



Kruskal-Wallis test of 18S mean Ct vs. sampling no/date, diet group D:

Depend.: Mean Ct (18S)	Multiple Comparisons p values (2-tailed); Mean Ct (18S) (StAR utregning threshold 20 april) Independent (grouping) variable: Uttak Kruskal-Wallis test: H (10, N= 86) =60,24839 p =,0000 Include condition: v4 = 'D'										
	1 R:57,250	2 R:38,600	3 R:43,500	4 R:29,000	5 R:30,333	6 R:72,750	7 R:63,200	8 R:6,0000	9 R:35,269	10 R:67,727	11 R:69,750
1		1,000000	1,000000	1,000000	1,000000	1,000000	1,000000	0,024164	1,000000	1,000000	1,000000
2	1,000000		1,000000	1,000000	1,000000	0,904188	1,000000	0,852251	1,000000	1,000000	1,000000
3	1,000000	1,000000		1,000000	1,000000	1,000000	1,000000	0,555904	1,000000	1,000000	1,000000
4	1,000000	1,000000	1,000000		1,000000	1,000000	1,000000	1,000000	1,000000	1,000000	1,000000
5	1,000000	1,000000	1,000000	1,000000		0,091225	1,000000	1,000000	1,000000	0,174359	0,795588
6	1,000000	0,904188	1,000000	1,000000	0,091225		1,000000	0,000000	0,011281	1,000000	1,000000
7	1,000000	1,000000	1,000000	1,000000	1,000000	1,000000		0,001191	1,000000	1,000000	1,000000
8	0,024164	0,852251	0,555904	1,000000	1,000000	0,000000	0,001191		0,061508	0,000000	0,000675
9	1,000000	1,000000	1,000000	1,000000	1,000000	0,011281	1,000000	0,061508		0,016584	0,557608
10	1,000000	1,000000	1,000000	1,000000	0,174359	1,000000	1,000000	0,000000	0,016584		1,000000
11	1,000000	1,000000	1,000000	1,000000	0,795588	1,000000	1,000000	0,000675	0,557608	1,000000	



Appendix 6

Raw data.

***Quantification of StAR, showing fish number (fish no), Ct values (18S/StAR Ct1, Ct2 and mean Ct), standard deviations of the mean Ct's (18S/StAR st.d), standard deviation^2 of the mean Ct's (18S/StAR S2), ΔCt (dCt), ΔΔCt (ddCt) and fold change/relative Q (rel Q)*:**

Fish no	18S Ct 1	18S Ct 2	18S mean Ct	18S st.d.	18S S2	StAR Ct 1	StAR Ct 2	StAR mean Ct	StAR st.d.	StAR S2	dCt	St.d. (dCt)	cal.	ddCt	(-)ddCt	rel Q
24	14,28	14,23	14,26	0,03	0,00	34,00	33,76	33,88	0,17	0,03	19,62	0,18		-1,18	1,18	2,27
27	14,30	14,32	14,31	0,02	0,00	33,95	33,95	33,95	0,00	0,00	19,64	0,02		-1,17	1,17	2,25
28	12,55	12,64	12,60	0,07	0,00	33,54	33,26	33,40	0,20	0,04	20,80	0,21		0,00	0,00	1,00
34	13,15	12,90	13,02	0,18	0,03	27,95	28,19	28,07	0,17	0,03	15,05	0,24		-5,76	5,76	54,07
38	12,93	13,10	13,02	0,12	0,02	34,71	34,60	34,66	0,08	0,01	21,64	0,15		0,84	-0,84	0,56
40	14,64	14,62	14,63	0,01	0,00	33,89	33,69	33,79	0,14	0,02	19,16	0,14		-1,65	1,65	3,13
43	14,64	14,64	14,64	0,00	0,00	33,25	33,22	33,24	0,03	0,00	18,60	0,03		-2,21	2,21	4,62
48	13,97	13,98	13,97	0,00	0,00	34,71	34,82	34,76	0,08	0,01	20,79	0,08		-0,02	0,02	1,01
49	13,50	13,51	13,50	0,01	0,00	29,74	29,70	29,72	0,03	0,00	16,22	0,04		-4,59	4,59	24,05
50	12,58	12,57	12,57	0,01	0,00	33,43	33,27	33,35	0,11	0,01	20,78	0,11		-0,03	0,03	1,02
51	14,16	14,15	14,15	0,01	0,00	33,88	34,07	33,98	0,14	0,02	19,82	0,14		-0,98	0,98	1,98
53	14,33	14,33	14,33	0,00	0,00	35,95	35,78	35,86	0,12	0,02	21,54	0,12		0,73	-0,73	0,60
55	16,39	16,42	16,41	0,02	0,00	37,14	37,19	37,17	0,03	0,00	20,76	0,04		-0,05	0,05	1,03
60	14,94	14,99	14,96	0,03	0,00	34,09	34,29	34,19	0,14	0,02	19,23	0,15		-1,58	1,58	2,99
62	15,81	15,82	15,81	0,01	0,00	33,87	33,71	33,79	0,12	0,01	17,98	0,12		-2,83	2,83	7,10
63	15,94	15,91	15,92	0,02	0,00	36,90	36,81	36,85	0,06	0,00	20,93	0,06		0,13	-0,13	0,92
66	12,26	12,27	12,26	0,00	0,00	29,73	29,58	29,65	0,10	0,01	17,39	0,10		-3,42	3,42	10,67
68	12,60	12,53	12,56	0,05	0,00	34,93	34,71	34,82	0,15	0,02	22,26	0,16		1,45	-1,45	0,36
70	14,45	14,44	14,44	0,01	0,00	34,67	34,53	34,60	0,10	0,01	20,15	0,10		-0,65	0,65	1,57
71	14,38	14,40	14,39	0,01	0,00	33,01	33,00	33,01	0,01	0,00	18,62	0,01		-2,19	2,19	4,56
73	14,19	14,18	14,18	0,00	0,00	34,00	33,81	33,91	0,14	0,02	19,72	0,14		-1,08	1,08	2,12
74	14,13	14,11	14,12	0,02	0,00	34,54	34,53	34,53	0,00	0,00	20,41	0,02		-0,39	0,39	1,31
75	14,35	14,34	14,35	0,01	0,00	35,50	35,26	35,38	0,17	0,03	21,03	0,17		0,23	-0,23	0,85
77	14,16	14,20	14,18	0,03	0,00	34,05	34,05	34,05	0,00	0,00	19,87	0,03		-0,93	0,93	1,91
78	13,09	12,85	12,97	0,17	0,03	33,78	33,91	33,85	0,10	0,01	20,87	0,20		0,07	-0,07	0,96
82	11,75	11,71	11,73	0,03	0,00	33,35	33,50	33,42	0,11	0,01	21,70	0,12		0,89	-0,89	0,54
83	13,91	13,97	13,94	0,04	0,00	34,32	34,36	34,34	0,03	0,00	20,40	0,05		-0,40	0,40	1,32
86	11,82	11,81	11,82	0,01	0,00	34,41	34,53	34,47	0,09	0,01	22,66	0,09		1,85	-1,85	0,28
87	11,72	11,68	11,70	0,03	0,00	33,03	33,23	33,13	0,14	0,02	21,43	0,14		0,63	-0,63	0,65
88	12,33	12,24	12,29	0,07	0,00	33,42	33,53	33,47	0,08	0,01	21,19	0,10		0,38	-0,38	0,77
89	12,22	12,42	12,32	0,14	0,02	33,20	33,14	33,17	0,04	0,00	20,85	0,15		0,05	-0,05	0,97

90	12,47	12,21	12,34	0,18	0,03	33,97	34,06	34,02	0,07	0,00	21,68	0,20		0,87	-0,87	0,55
92	14,08	14,09	14,08	0,01	0,00	33,98	34,04	34,01	0,04	0,00	19,93	0,04		-0,88	0,88	1,84
97	11,94	12,15	12,04	0,15	0,02	33,16	32,97	33,07	0,13	0,02	21,02	0,19		0,22	-0,22	0,86
98	14,09	14,09	14,09	0,00	0,00	32,86	32,66	32,76	0,14	0,02	18,67	0,14		-2,13	2,13	4,39
99	14,02	14,02	14,02	0,00	0,00	34,72	34,60	34,66	0,08	0,01	20,64	0,08		-0,16	0,16	1,12
101	14,27	14,25	14,26	0,01	0,00	33,92	34,04	33,98	0,08	0,01	19,72	0,08		-1,08	1,08	2,12
104	14,38	14,39	14,38	0,00	0,00	35,12	35,31	35,21	0,14	0,02	20,83	0,14		0,02	-0,02	0,98
107	14,33	14,39	14,36	0,05	0,00	35,18	34,93	35,05	0,17	0,03	20,69	0,18		-0,11	0,11	1,08
108	14,45	14,43	14,44	0,01	0,00	35,73	35,70	35,72	0,02	0,00	21,28	0,03		0,47	-0,47	0,72
113	12,72	12,64	12,68	0,06	0,00	35,50	35,30	35,40	0,14	0,02	22,72	0,16		1,91	-1,91	0,27
115	12,59	12,61	12,60	0,02	0,00	34,10	34,13	34,11	0,03	0,00	21,51	0,03		0,71	-0,71	0,61
123	13,21	13,33	13,27	0,08	0,01	34,72	34,81	34,77	0,06	0,00	21,50	0,10		0,69	-0,69	0,62
125	14,64	14,62	14,63	0,01	0,00	35,69	35,69	35,69	0,00	0,00	21,06	0,02		0,25	-0,25	0,84
129	12,95	13,09	13,02	0,09	0,01	34,97	34,76	34,87	0,15	0,02	21,85	0,17		1,04	-1,04	0,49
131	16,07	15,94	16,01	0,09	0,01	39,65	39,68	39,67	0,03	0,00	23,66	0,09		2,86	-2,86	0,14
132	14,66	14,67	14,66	0,00	0,00	35,44	35,53	35,48	0,06	0,00	20,82	0,06		0,01	-0,01	0,99
134	13,39	13,24	13,32	0,10	0,01	35,60	35,66	35,63	0,04	0,00	22,32	0,11		1,51	-1,51	0,35
135	15,55	15,35	15,45	0,14	0,02	35,02	35,26	35,14	0,17	0,03	19,69	0,22		-1,11	1,11	2,16
136	15,31	15,35	15,33	0,03	0,00	34,40	34,17	34,28	0,16	0,03	18,95	0,16		-1,85	1,85	3,61
143	15,58	15,52	15,55	0,04	0,00	33,78	33,92	33,85	0,10	0,01	18,30	0,11		-2,50	2,50	5,68
145	13,80	13,85	13,83	0,04	0,00	35,21	35,09	35,15	0,09	0,01	21,32	0,10		0,52	-0,52	0,70
147	15,49	15,45	15,47	0,02	0,00	36,95	36,95	36,95	0,00	0,00	21,48	0,02		0,67	-0,67	0,63
151	14,66	14,66	14,66	0,00	0,00	34,74	34,91	34,83	0,12	0,01	20,17	0,12		-0,64	0,64	1,56
155	13,00	13,20	13,10	0,14	0,02	34,21	34,11	34,16	0,07	0,00	21,06	0,15		0,26	-0,26	0,84
157	13,42	13,67	13,54	0,18	0,03	34,60	34,75	34,67	0,11	0,01	21,13	0,21		0,32	-0,32	0,80
160	14,71	14,70	14,70	0,01	0,00	34,51	34,74	34,63	0,16	0,03	19,92	0,16		-0,88	0,88	1,84
161	14,20	14,20	14,20	0,00	0,00	33,86	33,85	33,86	0,01	0,00	19,66	0,01	20,81	-1,15	1,15	2,21
166	13,83	13,93	13,88	0,07	0,00	33,75	33,62	33,68	0,09	0,01	19,80	0,12		-1,00	1,00	2,01
167	13,10	13,14	13,12	0,03	0,00	35,21	35,42	35,31	0,15	0,02	22,19	0,15		1,39	-1,39	0,38
171	14,55	14,55	14,55	0,01	0,00	34,38	34,12	34,25	0,19	0,03	19,70	0,19		-1,10	1,10	2,15
172	14,56	14,56	14,56	0,00	0,00	34,94	34,74	34,84	0,14	0,02	20,28	0,14		-0,52	0,52	1,44
174	12,88	12,87	12,87	0,01	0,00	33,79	33,87	33,83	0,05	0,00	20,95	0,05		0,15	-0,15	0,90
176	12,94	13,05	12,99	0,08	0,01	33,95	34,09	34,02	0,10	0,01	21,03	0,12		0,22	-0,22	0,86
179	14,57	14,62	14,59	0,03	0,00	34,97	34,84	34,90	0,09	0,01	20,31	0,10		-0,50	0,50	1,41
186	12,43	12,57	12,50	0,10	0,01	33,90	34,07	33,99	0,12	0,01	21,48	0,16		0,68	-0,68	0,62
193	12,76	12,88	12,82	0,09	0,01	34,44	34,58	34,51	0,09	0,01	21,69	0,13		0,88	-0,88	0,54
199	14,19	14,18	14,19	0,00	0,00	34,25	34,32	34,29	0,05	0,00	20,10	0,05		-0,70	0,70	1,63
203	12,93	12,75	12,84	0,13	0,02	33,58	33,58	33,58	0,00	0,00	20,74	0,13		-0,06	0,06	1,05
204	12,99	12,85	12,92	0,10	0,01	33,48	33,41	33,45	0,05	0,00	20,53	0,11		-0,28	0,28	1,21
212	14,27	14,31	14,29	0,03	0,00	34,31	34,48	34,40	0,12	0,01	20,11	0,12		-0,70	0,70	1,62
213	14,09	14,10	14,09	0,01	0,00	35,25	35,31	35,28	0,04	0,00	21,18	0,04		0,38	-0,38	0,77
214	14,38	14,40	14,39	0,02	0,00	32,31	32,38	32,35	0,05	0,00	17,96	0,05		-2,85	2,85	7,19
220	11,91	12,07	11,99	0,11	0,01	33,08	33,01	33,05	0,05	0,00	21,06	0,12		0,25	-0,25	0,84

224	11,57	11,64	11,61	0,04	0,00	32,96	33,13	33,05	0,12	0,02	21,44	0,13		0,63	-0,63	0,64
225	14,09	14,11	14,10	0,01	0,00	36,16	36,18	36,17	0,02	0,00	22,07	0,02		1,27	-1,27	0,42
226	14,61	14,58	14,59	0,02	0,00	34,82	34,76	34,79	0,04	0,00	20,20	0,04		-0,61	0,61	1,53
227	11,52	11,37	11,45	0,11	0,01	33,76	34,02	33,89	0,18	0,03	22,45	0,21		1,64	-1,64	0,32
228	12,14	11,85	11,99	0,20	0,04	34,24	34,00	34,12	0,17	0,03	22,13	0,26		1,32	-1,32	0,40
232	11,78	11,77	11,78	0,01	0,00	33,79	33,63	33,71	0,11	0,01	21,93	0,11		1,13	-1,13	0,46
233	11,76	11,94	11,85	0,13	0,02	32,80	32,80	32,80	0,00	0,00	20,95	0,13		0,15	-0,15	0,90
235	14,20	14,20	14,20	0,01	0,00	33,90	33,59	33,74	0,22	0,05	19,54	0,22		-1,26	1,26	2,40
239	13,96	13,96	13,96	0,00	0,00	32,35	32,19	32,27	0,11	0,01	18,31	0,11		-2,50	2,50	5,65
242	11,83	11,80	11,81	0,02	0,00	33,12	33,09	33,10	0,03	0,00	21,29	0,03		0,48	-0,48	0,71
243	11,98	11,88	11,93	0,07	0,01	32,29	32,50	32,39	0,15	0,02	20,47	0,17		-0,34	0,34	1,27
244	11,80	11,65	11,72	0,10	0,01	33,74	33,62	33,68	0,08	0,01	21,96	0,13		1,15	-1,15	0,45
250	11,64	11,72	11,68	0,06	0,00	32,97	32,97	32,97	0,00	0,00	21,29	0,06		0,49	-0,49	0,71
253	11,87	12,01	11,94	0,10	0,01	32,66	32,90	32,78	0,17	0,03	20,84	0,20		0,04	-0,04	0,97
254	11,70	11,61	11,66	0,07	0,00	33,06	33,09	33,07	0,02	0,00	21,42	0,07		0,61	-0,61	0,66
255	12,52	12,54	12,53	0,02	0,00	34,26	34,21	34,24	0,03	0,00	21,71	0,04		0,90	-0,90	0,54
259	13,75	13,89	13,82	0,09	0,01	34,15	34,32	34,24	0,12	0,01	20,42	0,15		-0,39	0,39	1,31
264	13,06	13,02	13,04	0,03	0,00	33,54	33,71	33,63	0,12	0,01	20,59	0,12		-0,22	0,22	1,16
266	11,87	11,67	11,77	0,14	0,02	33,63	33,47	33,55	0,11	0,01	21,77	0,18		0,97	-0,97	0,51
269	13,12	13,08	13,10	0,03	0,00	34,24	34,08	34,16	0,11	0,01	21,07	0,11		0,26	-0,26	0,84
270	13,48	13,54	13,51	0,04	0,00	34,21	34,52	34,36	0,22	0,05	20,85	0,22		0,05	-0,05	0,97
272	13,60	13,36	13,48	0,17	0,03	33,47	33,36	33,41	0,08	0,01	19,94	0,19		-0,87	0,87	1,83
273	13,56	13,48	13,52	0,06	0,00	33,09	33,32	33,20	0,16	0,03	19,68	0,17		-1,12	1,12	2,18
274	13,16	13,19	13,17	0,02	0,00	34,43	34,31	34,37	0,08	0,01	21,19	0,09		0,39	-0,39	0,76
275	11,89	12,08	11,98	0,13	0,02	33,74	33,92	33,83	0,12	0,02	21,84	0,18		1,04	-1,04	0,49
278	11,86	12,10	11,98	0,17	0,03	33,22	33,16	33,19	0,04	0,00	21,21	0,18		0,40	-0,40	0,76
286	12,74	12,68	12,71	0,04	0,00	33,23	33,03	33,13	0,14	0,02	20,42	0,15		-0,38	0,38	1,30
291	16,50	16,52	16,51	0,02	0,00	36,01	35,86	35,94	0,10	0,01	19,42	0,10		-1,38	1,38	2,60
292	11,61	11,56	11,59	0,04	0,00	33,46	33,56	33,51	0,08	0,01	21,92	0,08		1,12	-1,12	0,46
294	12,21	12,07	12,14	0,10	0,01	33,89	33,94	33,92	0,03	0,00	21,78	0,10		0,97	-0,97	0,51
298	13,95	13,96	13,96	0,01	0,00	33,72	33,93	33,83	0,15	0,02	19,87	0,15		-0,94	0,94	1,92
299	14,91	15,02	14,97	0,08	0,01	35,53	35,49	35,51	0,03	0,00	20,54	0,08		-0,26	0,26	1,20
301	15,69	15,65	15,67	0,03	0,00	36,36	36,37	36,37	0,01	0,00	20,69	0,03		-0,11	0,11	1,08
303	12,27	12,28	12,28	0,01	0,00	34,80	34,47	34,64	0,23	0,05	22,36	0,23		1,55	-1,55	0,34
304	14,24	14,23	14,23	0,00	0,00	34,95	35,00	34,97	0,04	0,00	20,74	0,04		-0,07	0,07	1,05
306	14,33	14,29	14,31	0,03	0,00	35,14	34,87	35,01	0,19	0,03	20,69	0,19		-0,11	0,11	1,08
309	14,23	14,25	14,24	0,02	0,00	35,00	35,09	35,04	0,07	0,00	20,80	0,07		0,00	0,00	1,00
310	12,85	12,79	12,82	0,04	0,00	34,33	34,64	34,49	0,22	0,05	21,67	0,23		0,86	-0,86	0,55
311	12,64	12,58	12,61	0,04	0,00	34,61	34,59	34,60	0,01	0,00	21,99	0,04		1,18	-1,18	0,44
315	13,10	13,12	13,11	0,01	0,00	35,78	35,86	35,82	0,06	0,00	22,71	0,06		1,90	-1,90	0,27
317	14,67	14,61	14,64	0,04	0,00	35,61	35,67	35,64	0,04	0,00	21,00	0,06		0,19	-0,19	0,87
318	13,32	13,53	13,43	0,14	0,02	35,91	36,03	35,97	0,09	0,01	22,54	0,17		1,74	-1,74	0,30
321	14,34	14,36	14,35	0,01	0,00	36,55	36,52	36,54	0,02	0,00	22,18	0,02		1,38	-1,38	0,38

324	15,04	15,07	15,06	0,02	0,00	33,79	33,76	33,77	0,03	0,00	18,72	0,04		-2,09	2,09	4,25
325	14,10	14,13	14,11	0,02	0,00	34,11	34,08	34,09	0,02	0,00	19,98	0,03		-0,82	0,82	1,77
326	13,96	13,99	13,98	0,02	0,00	33,39	33,70	33,54	0,22	0,05	19,57	0,22		-1,24	1,24	2,36
327	15,50	15,52	15,51	0,01	0,00	35,16	35,00	35,08	0,12	0,01	19,57	0,12		-1,24	1,24	2,36
328	13,98	13,95	13,96	0,02	0,00	32,90	32,91	32,90	0,01	0,00	18,94	0,03		-1,86	1,86	3,64
329	13,95	13,95	13,95	0,00	0,00	32,99	33,04	33,01	0,04	0,00	19,06	0,04		-1,75	1,75	3,36
330	11,97	11,90	11,93	0,05	0,00	33,25	33,29	33,27	0,03	0,00	21,33	0,06		0,53	-0,53	0,69
332	12,88	12,96	12,92	0,06	0,00	33,84	33,55	33,69	0,21	0,04	20,77	0,22		-0,03	0,03	1,02
334	14,73	14,72	14,72	0,01	0,00	33,80	33,98	33,89	0,12	0,01	19,17	0,12		-1,64	1,64	3,11
335	14,43	14,34	14,39	0,06	0,00	34,82	34,88	34,85	0,04	0,00	20,46	0,07		-0,34	0,34	1,27
336	15,51	15,54	15,53	0,02	0,00	35,15	35,14	35,14	0,01	0,00	19,61	0,02		-1,19	1,19	2,28
339	14,80	14,67	14,73	0,09	0,01	35,37	35,29	35,33	0,05	0,00	20,60	0,11		-0,20	0,20	1,15
340	12,89	13,14	13,01	0,18	0,03	33,88	33,88	33,88	0,00	0,00	20,87	0,18		0,06	-0,06	0,96
343	14,50	14,54	14,52	0,03	0,00	34,27	34,02	34,14	0,18	0,03	19,62	0,18		-1,18	1,18	2,27
345	12,40	12,27	12,34	0,09	0,01	33,68	33,90	33,79	0,15	0,02	21,45	0,18		0,65	-0,65	0,64
347	14,25	14,25	14,25	0,00	0,00	33,89	33,58	33,74	0,22	0,05	19,49	0,22		-1,32	1,32	2,50
348	14,22	14,21	14,22	0,00	0,00	33,26	33,48	33,37	0,15	0,02	19,15	0,15		-1,65	1,65	3,14
349	14,27	14,28	14,28	0,01	0,00	33,47	33,71	33,59	0,16	0,03	19,31	0,16		-1,49	1,49	2,82
350	12,68	12,70	12,69	0,02	0,00	33,93	33,79	33,86	0,10	0,01	21,17	0,11		0,37	-0,37	0,78
351	14,32	14,31	14,32	0,00	0,00	34,04	34,18	34,11	0,10	0,01	19,79	0,10		-1,01	1,01	2,02
353	13,59	13,57	13,58	0,01	0,00	34,53	34,49	34,51	0,03	0,00	20,93	0,03		0,13	-0,13	0,91
354	14,04	14,16	14,10	0,09	0,01	35,35	35,24	35,30	0,08	0,01	21,20	0,12		0,39	-0,39	0,76
358	15,72	15,75	15,73	0,02	0,00	35,06	35,17	35,12	0,08	0,01	19,38	0,08		-1,42	1,42	2,68
359	16,08	16,15	16,11	0,05	0,00	35,92	35,90	35,91	0,01	0,00	19,80	0,05		-1,01	1,01	2,01
360	15,83	15,86	15,85	0,03	0,00	34,94	34,63	34,79	0,22	0,05	18,94	0,23		-1,87	1,87	3,65
361	16,12	16,08	16,10	0,03	0,00	35,54	35,48	35,51	0,04	0,00	19,42	0,05		-1,39	1,39	2,62
363	16,02	15,97	16,00	0,04	0,00	35,44	35,41	35,42	0,02	0,00	19,43	0,04		-1,38	1,38	2,60
365	15,60	15,59	15,59	0,01	0,00	35,51	35,39	35,45	0,09	0,01	19,86	0,09		-0,95	0,95	1,93
366	15,76	15,73	15,75	0,02	0,00	37,01	36,75	36,88	0,18	0,03	21,14	0,18		0,33	-0,33	0,80
379	14,60	14,61	14,60	0,01	0,00	35,66	35,39	35,52	0,19	0,04	20,92	0,19		0,11	-0,11	0,93
380	15,48	15,46	15,47	0,01	0,00	35,73	35,59	35,66	0,10	0,01	20,19	0,10		-0,62	0,62	1,53
383	15,01	14,69	14,85	0,23	0,05	36,59	36,51	36,55	0,06	0,00	21,70	0,24		0,89	-0,89	0,54
388	15,70	15,97	15,83	0,19	0,04	36,21	36,05	36,13	0,11	0,01	20,29	0,22		-0,51	0,51	1,43
391	15,99	15,98	15,98	0,00	0,00	34,95	34,73	34,84	0,16	0,02	18,85	0,16		-1,95	1,95	3,87
394	15,58	15,63	15,60	0,03	0,00	35,33	35,34	35,34	0,01	0,00	19,73	0,03		-1,07	1,07	2,10
404	16,14	16,14	16,14	0,00	0,00	34,77	34,97	34,87	0,14	0,02	18,74	0,14		-2,07	2,07	4,20
406	16,56	16,42	16,49	0,10	0,01	36,00	35,95	35,97	0,04	0,00	19,49	0,10		-1,32	1,32	2,50
410	16,32	16,32	16,32	0,00	0,00	37,11	37,13	37,12	0,02	0,00	20,80	0,02		0,00	0,00	1,00
419	16,60	16,60	16,60	0,00	0,00	35,17	34,90	35,03	0,19	0,04	18,43	0,19		-2,38	2,38	5,20
421	13,40	13,46	13,43	0,05	0,00	33,01	33,20	33,11	0,13	0,02	19,68	0,14		-1,13	1,13	2,19
424	14,64	14,58	14,61	0,05	0,00	34,03	34,22	34,13	0,14	0,02	19,52	0,14		-1,29	1,29	2,45
427	14,55	14,74	14,65	0,13	0,02	33,82	33,67	33,75	0,11	0,01	19,10	0,17		-1,70	1,70	3,26
428	14,54	14,66	14,60	0,09	0,01	32,65	32,81	32,73	0,11	0,01	18,13	0,14		-2,67	2,67	6,38

430	14,57	14,38	14,48	0,14	0,02	34,64	34,69	34,67	0,03	0,00	20,19	0,14		-0,61	0,61	1,53
441	15,07	15,05	15,06	0,02	0,00	34,72	34,73	34,73	0,01	0,00	19,66	0,02		-1,14	1,14	2,21
445	14,82	14,82	14,82	0,00	0,00	34,01	34,16	34,08	0,11	0,01	19,27	0,11		-1,54	1,54	2,90
446	16,09	16,11	16,10	0,01	0,00	36,80	36,76	36,78	0,03	0,00	20,68	0,03		-0,13	0,13	1,09
447	14,95	15,10	15,03	0,11	0,01	34,52	34,33	34,43	0,14	0,02	19,40	0,17		-1,40	1,40	2,64
450	14,55	14,74	14,64	0,13	0,02	34,35	34,11	34,23	0,17	0,03	19,59	0,22		-1,22	1,22	2,33
455	15,35	15,39	15,37	0,03	0,00	33,60	33,78	33,69	0,13	0,02	18,32	0,13		-2,49	2,49	5,60
458	15,07	15,02	15,05	0,04	0,00	33,32	33,33	33,33	0,01	0,00	18,28	0,04		-2,53	2,53	5,77
460	15,23	15,41	15,32	0,13	0,02	33,40	33,18	33,29	0,16	0,02	17,97	0,20		-2,84	2,84	7,15
462	15,07	15,06	15,07	0,00	0,00	33,82	34,04	33,93	0,15	0,02	18,86	0,15		-1,94	1,94	3,85
463	15,40	15,38	15,39	0,02	0,00	33,13	33,14	33,13	0,00	0,00	17,74	0,02		-3,06	3,06	8,35
465	15,34	15,30	15,32	0,03	0,00	33,09	32,82	32,96	0,19	0,04	17,63	0,19		-3,17	3,17	9,02
467	15,45	15,33	15,39	0,09	0,01	34,92	35,08	35,00	0,11	0,01	19,61	0,14		-1,19	1,19	2,29
471	11,21	11,19	11,20	0,01	0,00	33,09	32,92	33,01	0,12	0,02	21,81	0,12		1,00	-1,00	0,50
472	11,30	11,28	11,29	0,01	0,00	31,24	31,18	31,21	0,04	0,00	19,92	0,05		-0,88	0,88	1,84
473	10,94	10,86	10,90	0,05	0,00	31,54	31,58	31,56	0,03	0,00	20,66	0,06		-0,14	0,14	1,10
474	13,74	13,75	13,75	0,01	0,00	32,41	32,64	32,52	0,17	0,03	18,78	0,17		-2,03	2,03	4,08
475	11,35	11,02	11,18	0,23	0,05	32,46	32,36	32,41	0,07	0,00	21,23	0,24		0,42	-0,42	0,75
477	13,72	13,73	13,73	0,01	0,00	32,36	32,39	32,37	0,02	0,00	18,65	0,02		-2,16	2,16	4,46
478	11,27	11,28	11,27	0,01	0,00	31,47	31,59	31,53	0,08	0,01	20,26	0,08		-0,55	0,55	1,46
479	11,38	11,16	11,27	0,15	0,02	31,88	32,02	31,95	0,10	0,01	20,68	0,18		-0,13	0,13	1,09
481	11,19	11,26	11,22	0,05	0,00	32,46	32,36	32,41	0,06	0,00	21,19	0,08		0,38	-0,38	0,77
484	11,36	11,33	11,34	0,03	0,00	29,47	29,42	29,44	0,04	0,00	18,10	0,05		-2,71	2,71	6,52
485	11,30	11,33	11,32	0,02	0,00	33,13	33,26	33,20	0,09	0,01	21,88	0,10		1,08	-1,08	0,47
492	11,06	11,16	11,11	0,07	0,00	32,49	32,79	32,64	0,21	0,04	21,53	0,22		0,73	-0,73	0,60
493	11,46	11,25	11,35	0,15	0,02	30,72	30,96	30,84	0,17	0,03	19,49	0,23		-1,31	1,31	2,49
494	11,42	11,47	11,45	0,04	0,00	32,53	32,53	32,53	0,00	0,00	21,09	0,04		0,28	-0,28	0,82
498	11,41	11,38	11,39	0,02	0,00	29,67	29,71	29,69	0,03	0,00	18,30	0,03		-2,51	2,51	5,69
500	11,44	11,29	11,36	0,11	0,01	32,71	33,04	32,87	0,23	0,05	21,51	0,25		0,70	-0,70	0,61
502	13,88	13,88	13,88	0,00	0,00	32,23	32,24	32,24	0,01	0,00	18,36	0,01		-2,44	2,44	5,44
503	13,94	13,92	13,93	0,01	0,00	31,73	31,60	31,66	0,09	0,01	17,73	0,09		-3,08	3,08	8,43
504	11,83	11,62	11,73	0,15	0,02	32,88	32,77	32,83	0,08	0,01	21,10	0,17		0,29	-0,29	0,82
511	11,41	11,28	11,34	0,10	0,01	29,29	29,42	29,36	0,10	0,01	18,01	0,14		-2,79	2,79	6,93
512	10,82	10,74	10,78	0,06	0,00	31,77	31,57	31,67	0,14	0,02	20,89	0,15		0,09	-0,09	0,94
513	10,79	10,89	10,84	0,07	0,00	32,63	32,79	32,71	0,11	0,01	21,87	0,13		1,07	-1,07	0,48
515	10,97	10,85	10,91	0,08	0,01	31,18	31,40	31,29	0,15	0,02	20,38	0,17		-0,42	0,42	1,34
517	10,81	10,99	10,90	0,12	0,02	30,74	30,73	30,73	0,01	0,00	19,83	0,12		-0,97	0,97	1,96
518	10,71	10,75	10,73	0,02	0,00	31,91	32,17	32,04	0,19	0,03	21,31	0,19		0,50	-0,50	0,71
519	11,01	10,87	10,94	0,10	0,01	31,91	31,91	31,91	0,00	0,00	20,97	0,10		0,16	-0,16	0,89
522	10,94	11,05	10,99	0,08	0,01	31,58	31,34	31,46	0,17	0,03	20,47	0,18		-0,34	0,34	1,26
523	10,85	11,02	10,94	0,12	0,01	32,58	32,54	32,56	0,03	0,00	21,62	0,12		0,82	-0,82	0,57
527	10,94	11,21	11,07	0,19	0,04	33,01	32,90	32,95	0,08	0,01	21,88	0,20		1,07	-1,07	0,48
529	11,18	11,29	11,23	0,08	0,01	32,51	32,48	32,49	0,02	0,00	21,26	0,09		0,45	-0,45	0,73

531	10,73	10,88	10,81	0,11	0,01	30,79	30,71	30,75	0,05	0,00	19,94	0,12		-0,86	0,86	1,82
532	10,86	10,94	10,90	0,05	0,00	32,19	32,29	32,24	0,07	0,00	21,34	0,09		0,53	-0,53	0,69
553	13,61	13,64	13,62	0,02	0,00	30,38	30,42	30,40	0,03	0,00	16,77	0,03		-4,03	4,03	16,36
554	13,68	13,71	13,69	0,02	0,00	30,75	30,99	30,87	0,17	0,03	17,18	0,18		-3,63	3,63	12,38
555	10,89	11,14	11,01	0,18	0,03	31,18	31,36	31,27	0,13	0,02	20,26	0,22		-0,54	0,54	1,46
556	11,14	11,36	11,25	0,16	0,02	30,80	30,61	30,70	0,14	0,02	19,45	0,21		-1,36	1,36	2,56
557	11,22	11,22	11,22	0,00	0,00	28,08	28,13	28,11	0,03	0,00	16,89	0,03		-3,92	3,92	15,14
558	10,94	11,21	11,07	0,19	0,04	31,48	31,38	31,43	0,07	0,00	20,36	0,20		-0,45	0,45	1,36
559	11,29	11,37	11,33	0,06	0,00	30,83	30,76	30,80	0,05	0,00	19,47	0,08		-1,34	1,34	2,53
560	13,69	13,71	13,70	0,01	0,00	30,89	31,05	30,97	0,11	0,01	17,26	0,11		-3,54	3,54	11,65
561	13,66	13,68	13,67	0,01	0,00	31,54	31,57	31,55	0,02	0,00	17,88	0,02		-2,92	2,92	7,59
562	11,46	11,36	11,41	0,07	0,01	27,99	28,02	28,01	0,02	0,00	16,60	0,07		-4,21	4,21	18,50
563	11,26	11,37	11,32	0,07	0,01	28,19	28,14	28,16	0,04	0,00	16,85	0,08		-3,96	3,96	15,54
564	11,45	11,31	11,38	0,10	0,01	31,37	31,43	31,40	0,05	0,00	20,02	0,11		-0,78	0,78	1,72
565	11,53	11,74	11,63	0,15	0,02	28,12	27,93	28,03	0,14	0,02	16,39	0,20		-4,41	4,41	21,32
566	11,48	11,69	11,58	0,15	0,02	27,33	27,39	27,36	0,04	0,00	15,78	0,15		-5,03	5,03	32,59
567	11,46	11,73	11,60	0,20	0,04	28,47	28,37	28,42	0,07	0,00	16,82	0,21		-3,98	3,98	15,82
568	11,89	11,94	11,92	0,04	0,00	30,97	31,05	31,01	0,06	0,00	19,10	0,07		-1,71	1,71	3,27
569	13,93	13,93	13,93	0,00	0,00	31,24	31,24	31,24	0,00	0,00	17,31	0,00		-3,50	3,50	11,30
570	11,78	11,89	11,83	0,08	0,01	26,26	26,27	26,27	0,01	0,00	14,43	0,08		-6,38	6,38	83,01
571	11,85	11,72	11,79	0,09	0,01	30,63	30,77	30,70	0,10	0,01	18,91	0,13		-1,89	1,89	3,71
572	13,93	13,97	13,95	0,03	0,00	31,58	31,40	31,49	0,12	0,01	17,54	0,13		-3,26	3,26	9,60
573	11,85	11,72	11,78	0,10	0,01	29,87	29,79	29,83	0,06	0,00	18,04	0,11		-2,76	2,76	6,79
574	12,05	12,27	12,16	0,15	0,02	31,30	31,51	31,40	0,15	0,02	19,24	0,22		-1,56	1,56	2,95
575	11,67	11,92	11,80	0,18	0,03	32,35	32,34	32,34	0,00	0,00	20,55	0,18		-0,26	0,26	1,20
576	12,07	11,87	11,97	0,14	0,02	30,63	30,46	30,54	0,12	0,01	18,57	0,19		-2,24	2,24	4,72
577	13,94	13,93	13,94	0,01	0,00	29,56	29,42	29,49	0,09	0,01	15,55	0,09		-5,25	5,25	38,11
578	13,94	13,97	13,96	0,02	0,00	30,93	30,62	30,77	0,22	0,05	16,82	0,22		-3,99	3,99	15,89
579	11,85	11,70	11,78	0,10	0,01	29,86	29,90	29,88	0,02	0,00	18,10	0,10		-2,70	2,70	6,51
580	12,38	12,38	12,38	0,01	0,00	30,27	30,24	30,25	0,02	0,00	17,87	0,03		-2,93	2,93	7,63
581	11,81	11,89	11,85	0,05	0,00	29,98	30,08	30,03	0,06	0,00	18,18	0,08		-2,63	2,63	6,17
582	13,95	13,97	13,96	0,01	0,00	32,13	32,19	32,16	0,05	0,00	18,20	0,05		-2,61	2,61	6,09
583	11,88	12,08	11,98	0,14	0,02	30,06	30,00	30,03	0,04	0,00	18,05	0,14		-2,75	2,75	6,74
584	11,80	11,99	11,90	0,14	0,02	28,84	29,09	28,97	0,18	0,03	17,07	0,22		-3,74	3,74	13,33
585	12,06	12,02	12,04	0,03	0,00	31,12	31,22	31,17	0,07	0,01	19,13	0,08		-1,67	1,67	3,19
593	12,28	12,18	12,23	0,07	0,00	32,17	32,05	32,11	0,09	0,01	19,88	0,11		-0,93	0,93	1,90
595	13,60	13,83	13,72	0,17	0,03	32,95	33,21	33,08	0,19	0,03	19,37	0,25		-1,44	1,44	2,71
596	11,36	11,36	11,36	0,00	0,00	31,08	30,95	31,01	0,09	0,01	19,65	0,09		-1,15	1,15	2,22
597	12,94	13,06	13,00	0,08	0,01	32,06	32,09	32,08	0,03	0,00	19,08	0,08		-1,73	1,73	3,32
598	14,43	14,45	14,44	0,02	0,00	31,97	31,94	31,96	0,02	0,00	17,52	0,02		-3,29	3,29	9,78
599	14,65	14,65	14,65	0,00	0,00	32,36	32,36	32,36	0,01	0,00	17,71	0,01		-3,10	3,10	8,56
600	13,14	13,13	13,14	0,00	0,00	30,46	30,36	30,41	0,08	0,01	17,27	0,08		-3,53	3,53	11,57
601	13,61	13,48	13,55	0,09	0,01	31,51	31,32	31,41	0,14	0,02	17,87	0,17		-2,94	2,94	7,66

602	13,42	13,27	13,34	0,11	0,01	30,09	30,19	30,14	0,07	0,00	16,80	0,13		-4,01	4,01	16,12
603	13,64	13,67	13,66	0,02	0,00	29,73	29,52	29,62	0,15	0,02	15,97	0,15		-4,84	4,84	28,65
604	14,61	14,62	14,61	0,01	0,00	31,85	31,55	31,70	0,21	0,04	17,09	0,21		-3,72	3,72	13,15
613	11,43	11,25	11,34	0,13	0,02	29,49	29,78	29,64	0,21	0,04	18,30	0,24		-2,51	2,51	5,69
614	11,33	11,29	11,31	0,02	0,00	30,43	30,54	30,49	0,08	0,01	19,18	0,08		-1,63	1,63	3,09
615	11,40	11,40	11,40	0,00	0,00	30,18	30,21	30,19	0,02	0,00	18,79	0,02		-2,01	2,01	4,04
616	11,26	11,25	11,26	0,00	0,00	31,12	30,83	30,97	0,20	0,04	19,72	0,20		-1,09	1,09	2,13
617	11,31	11,38	11,35	0,05	0,00	27,41	27,45	27,43	0,03	0,00	16,08	0,06		-4,72	4,72	26,43
618	11,37	11,48	11,43	0,07	0,01	30,92	31,00	30,96	0,05	0,00	19,53	0,09		-1,27	1,27	2,42
619	13,83	13,86	13,84	0,02	0,00	29,61	29,67	29,64	0,04	0,00	15,79	0,05		-5,01	5,01	32,25
621	11,49	11,33	11,41	0,12	0,01	28,62	28,62	28,62	0,00	0,00	17,21	0,12		-3,59	3,59	12,06
623	11,55	11,48	11,52	0,05	0,00	30,14	30,04	30,09	0,07	0,01	18,57	0,09		-2,23	2,23	4,70
624	11,34	11,50	11,42	0,11	0,01	32,44	32,38	32,41	0,05	0,00	20,99	0,12		0,18	-0,18	0,88
626	11,45	11,46	11,46	0,01	0,00	30,66	30,61	30,64	0,03	0,00	19,18	0,03		-1,63	1,63	3,09
627	12,20	11,97	12,09	0,16	0,03	28,68	28,85	28,77	0,12	0,02	16,68	0,20		-4,13	4,13	17,47
629	12,49	12,34	12,41	0,10	0,01	27,92	28,04	27,98	0,08	0,01	15,57	0,13		-5,24	5,24	37,78
631	14,21	14,20	14,21	0,00	0,00	31,60	31,72	31,66	0,08	0,01	17,45	0,08		-3,35	3,35	10,22
632	13,78	13,78	13,78	0,00	0,00	32,02	32,00	32,01	0,01	0,00	18,23	0,01		-2,58	2,58	5,97
633	13,22	12,93	13,07	0,21	0,04	30,54	30,65	30,59	0,08	0,01	17,52	0,22		-3,29	3,29	9,77
634	13,20	13,36	13,28	0,11	0,01	28,73	28,85	28,79	0,08	0,01	15,52	0,14		-5,29	5,29	39,10
635	13,57	13,52	13,54	0,04	0,00	31,52	31,38	31,45	0,10	0,01	17,90	0,11		-2,90	2,90	7,47
636	13,99	13,88	13,94	0,08	0,01	30,38	30,58	30,48	0,14	0,02	16,54	0,16		-4,26	4,26	19,21
637	13,65	13,72	13,69	0,05	0,00	30,46	30,57	30,51	0,08	0,01	16,83	0,10		-3,98	3,98	15,76
638	13,53	13,30	13,41	0,16	0,03	31,16	31,24	31,20	0,05	0,00	17,79	0,17		-3,02	3,02	8,11
640	14,60	14,55	14,57	0,03	0,00	33,14	32,82	32,98	0,23	0,05	18,41	0,23		-2,40	2,40	5,27
641	14,14	13,88	14,01	0,19	0,04	33,09	33,17	33,13	0,06	0,00	19,12	0,20		-1,69	1,69	3,22
642	14,16	14,23	14,19	0,05	0,00	34,09	34,34	34,22	0,18	0,03	20,02	0,18		-0,78	0,78	1,72
643	13,97	14,01	13,99	0,03	0,00	30,87	30,70	30,78	0,12	0,02	16,80	0,13		-4,01	4,01	16,11
644	14,35	14,31	14,33	0,03	0,00	32,82	32,93	32,88	0,08	0,01	18,55	0,09		-2,26	2,26	4,78
645	14,06	14,03	14,04	0,03	0,00	30,81	30,93	30,87	0,08	0,01	16,83	0,09		-3,98	3,98	15,78
647	14,39	14,32	14,35	0,05	0,00	30,84	30,69	30,77	0,11	0,01	16,41	0,12		-4,39	4,39	20,98
650	14,33	14,57	14,45	0,17	0,03	30,13	30,14	30,13	0,01	0,00	15,68	0,17		-5,12	5,12	34,87
651	14,09	13,90	14,00	0,14	0,02	33,12	33,32	33,22	0,14	0,02	19,22	0,20		-1,59	1,59	3,00
652	14,50	14,40	14,45	0,07	0,00	31,89	31,86	31,88	0,02	0,00	17,42	0,07		-3,38	3,38	10,43
653	14,52	14,56	14,54	0,03	0,00	30,96	30,98	30,97	0,01	0,00	16,43	0,03		-4,37	4,37	20,71
654	14,95	14,96	14,96	0,01	0,00	30,84	30,60	30,72	0,17	0,03	15,77	0,17		-5,04	5,04	32,90
656	12,79	12,77	12,78	0,02	0,00	32,43	32,41	32,42	0,01	0,00	19,64	0,02		-1,17	1,17	2,25
657	14,28	14,28	14,28	0,00	0,00	31,27	30,99	31,13	0,20	0,04	16,85	0,20		-3,96	3,96	15,51
658	13,31	13,33	13,32	0,01	0,00	29,81	29,81	29,81	0,00	0,00	16,49	0,01		-4,32	4,32	19,93
659	14,31	14,39	14,35	0,05	0,00	31,77	31,74	31,76	0,02	0,00	17,41	0,06		-3,40	3,40	10,54
661	12,99	13,15	13,07	0,12	0,01	27,67	27,40	27,53	0,19	0,04	14,46	0,22		-6,35	6,35	81,36
662	13,20	13,21	13,21	0,01	0,00	29,38	29,19	29,29	0,13	0,02	16,08	0,13		-4,73	4,73	26,49
663	13,27	13,29	13,28	0,01	0,00	29,63	29,71	29,67	0,06	0,00	16,39	0,06		-4,41	4,41	21,30

667	13,26	13,53	13,40	0,20	0,04	31,78	31,89	31,83	0,08	0,01	18,44	0,21		-2,37	2,37	5,16
668	14,45	14,47	14,46	0,01	0,00	32,18	32,02	32,10	0,12	0,01	17,64	0,12		-3,17	3,17	8,99
672	13,07	13,32	13,19	0,18	0,03	28,89	28,79	28,84	0,07	0,00	15,65	0,19		-5,16	5,16	35,63
673	13,61	13,72	13,66	0,07	0,01	28,97	29,14	29,06	0,12	0,02	15,39	0,14		-5,41	5,41	42,63
674	12,57	12,62	12,60	0,04	0,00	28,05	27,87	27,96	0,12	0,02	15,36	0,13		-5,44	5,44	43,50
675	12,54	12,47	12,51	0,05	0,00	31,05	31,01	31,03	0,02	0,00	18,52	0,06		-2,28	2,28	4,87
678	12,51	12,55	12,53	0,03	0,00	32,22	32,49	32,35	0,19	0,04	19,82	0,19		-0,98	0,98	1,98
679	12,49	12,55	12,52	0,04	0,00	30,48	30,66	30,57	0,13	0,02	18,05	0,14		-2,75	2,75	6,73
682	12,68	12,49	12,58	0,14	0,02	29,94	30,10	30,02	0,12	0,01	17,44	0,18		-3,37	3,37	10,32
683	12,64	12,97	12,80	0,24	0,06	29,67	29,75	29,71	0,05	0,00	16,90	0,24		-3,90	3,90	14,95
684	12,89	12,77	12,83	0,09	0,01	31,62	31,86	31,74	0,17	0,03	18,91	0,19		-1,89	1,89	3,72
685	14,25	14,24	14,25	0,01	0,00	31,52	31,72	31,62	0,14	0,02	17,38	0,14		-3,43	3,43	10,78
687	12,81	12,92	12,87	0,08	0,01	29,98	30,15	30,07	0,12	0,01	17,20	0,14		-3,61	3,61	12,18
688	12,73	12,82	12,77	0,06	0,00	28,62	28,56	28,59	0,05	0,00	15,82	0,07		-4,99	4,99	31,78
689	13,12	12,88	13,00	0,17	0,03	28,29	28,39	28,34	0,07	0,00	15,34	0,18		-5,46	5,46	44,14
690	14,30	14,02	14,16	0,20	0,04	32,52	32,70	32,61	0,13	0,02	18,45	0,24		-2,35	2,35	5,11
692	14,84	14,84	14,84	0,00	0,00	32,95	33,06	33,01	0,07	0,01	18,16	0,07		-2,64	2,64	6,24
693	14,81	14,76	14,79	0,03	0,00	32,41	32,53	32,47	0,08	0,01	17,68	0,09		-3,12	3,12	8,71
695	15,20	15,12	15,16	0,05	0,00	32,62	32,63	32,63	0,01	0,00	17,47	0,05		-3,34	3,34	10,11
697	14,51	14,56	14,54	0,03	0,00	33,32	33,39	33,35	0,05	0,00	18,82	0,06		-1,99	1,99	3,97
698	14,23	14,34	14,28	0,08	0,01	31,37	31,37	31,37	0,00	0,00	17,08	0,08		-3,72	3,72	13,19
700	14,34	14,27	14,30	0,05	0,00	30,94	31,13	31,03	0,13	0,02	16,73	0,14		-4,08	4,08	16,86
702	14,95	14,79	14,87	0,11	0,01	33,58	33,58	33,58	0,00	0,00	18,71	0,11		-2,10	2,10	4,27
703	15,17	15,39	15,28	0,16	0,02	34,10	34,05	34,08	0,04	0,00	18,80	0,16		-2,01	2,01	4,03
704	15,30	15,31	15,31	0,01	0,00	34,13	34,05	34,09	0,05	0,00	18,78	0,05		-2,03	2,03	4,07
706	15,63	15,61	15,62	0,01	0,00	32,27	32,04	32,15	0,16	0,03	16,53	0,16		-4,27	4,27	19,35
707	15,12	15,23	15,18	0,08	0,01	31,94	32,02	31,98	0,05	0,00	16,80	0,09		-4,00	4,00	16,01
708	15,24	15,43	15,34	0,14	0,02	34,11	34,07	34,09	0,03	0,00	18,75	0,14		-2,06	2,06	4,16
712	12,27	12,41	12,34	0,10	0,01	32,80	32,80	32,80	0,00	0,00	20,46	0,10		-0,35	0,35	1,27
713	12,45	12,24	12,35	0,15	0,02	30,13	30,40	30,26	0,19	0,03	17,92	0,24		-2,89	2,89	7,40
714	14,12	14,14	14,13	0,01	0,00	30,26	30,17	30,21	0,06	0,00	16,08	0,07		-4,72	4,72	26,42
716	12,50	12,46	12,48	0,02	0,00	29,32	29,33	29,33	0,00	0,00	16,85	0,02		-3,96	3,96	15,55
717	13,61	13,63	13,62	0,01	0,00	32,38	32,51	32,45	0,09	0,01	18,83	0,09		-1,98	1,98	3,94
718	12,62	12,48	12,55	0,10	0,01	30,59	30,57	30,58	0,01	0,00	18,02	0,10		-2,78	2,78	6,89
719	12,71	12,78	12,74	0,05	0,00	30,77	30,96	30,86	0,13	0,02	18,12	0,14		-2,68	2,68	6,43
720	12,69	12,72	12,71	0,02	0,00	28,60	28,69	28,64	0,06	0,00	15,94	0,06		-4,87	4,87	29,22
721	13,55	13,33	13,44	0,16	0,02	28,30	28,04	28,17	0,19	0,04	14,73	0,24		-6,08	6,08	67,64
722	14,61	14,62	14,61	0,01	0,00	28,93	28,71	28,82	0,16	0,03	14,20	0,16		-6,60	6,60	97,10
723	13,18	13,40	13,29	0,16	0,03	32,18	32,46	32,32	0,20	0,04	19,03	0,26		-1,78	1,78	3,44
724	14,61	14,56	14,58	0,03	0,00	31,44	31,71	31,57	0,19	0,04	16,99	0,20		-3,82	3,82	14,09
725	13,86	13,89	13,88	0,02	0,00	30,78	30,55	30,67	0,16	0,03	16,79	0,16		-4,02	4,02	16,20
726	13,69	13,80	13,75	0,08	0,01	28,95	28,84	28,89	0,08	0,01	15,15	0,11		-5,66	5,66	50,57
729	14,67	14,69	14,68	0,01	0,00	31,07	31,32	31,20	0,18	0,03	16,51	0,18		-4,29	4,29	19,58

730	15,04	15,14	15,09	0,07	0,00	29,88	29,99	29,94	0,08	0,01	14,84	0,11		-5,96	5,96	62,42
731	13,89	13,79	13,84	0,07	0,00	30,69	30,62	30,65	0,05	0,00	16,81	0,08		-4,00	4,00	15,96
733	14,78	14,78	14,78	0,00	0,00	30,86	30,75	30,80	0,08	0,01	16,02	0,08		-4,78	4,78	27,54
734	14,93	14,93	14,93	0,00	0,00	31,90	31,75	31,83	0,11	0,01	16,90	0,11		-3,90	3,90	14,98
735	13,98	13,96	13,97	0,01	0,00	29,59	29,77	29,68	0,12	0,02	15,71	0,12		-5,10	5,10	34,29
737	14,91	14,97	14,94	0,05	0,00	32,84	32,82	32,83	0,01	0,00	17,89	0,05		-2,91	2,91	7,53
738	14,32	14,21	14,27	0,07	0,01	29,55	29,65	29,60	0,07	0,00	15,33	0,10		-5,47	5,47	44,37
741	14,50	14,54	14,52	0,03	0,00	31,91	32,12	32,01	0,15	0,02	17,49	0,15		-3,31	3,31	9,93
743	13,29	13,26	13,28	0,02	0,00	31,23	31,14	31,19	0,06	0,00	17,91	0,07		-2,89	2,89	7,43
744	13,45	13,58	13,51	0,09	0,01	29,88	29,69	29,79	0,14	0,02	16,28	0,16		-4,53	4,53	23,10
745	13,35	13,19	13,27	0,11	0,01	30,69	30,82	30,75	0,09	0,01	17,48	0,14		-3,32	3,32	10,00
747	14,52	14,48	14,50	0,03	0,00	31,65	31,89	31,77	0,17	0,03	17,27	0,17		-3,54	3,54	11,64
748	13,49	13,60	13,55	0,08	0,01	31,70	31,89	31,79	0,13	0,02	18,25	0,15		-2,56	2,56	5,89
750	13,52	13,69	13,61	0,12	0,01	29,88	29,97	29,92	0,06	0,00	16,31	0,14		-4,49	4,49	22,49
751	13,46	13,68	13,57	0,16	0,02	32,00	31,99	31,99	0,01	0,00	18,43	0,16		-2,38	2,38	5,20
752	13,71	13,59	13,65	0,09	0,01	31,02	31,09	31,05	0,05	0,00	17,40	0,10		-3,40	3,40	10,57
753	16,13	16,00	16,07	0,09	0,01	33,04	33,10	33,07	0,04	0,00	17,00	0,10		-3,80	3,80	13,96
756	15,64	15,62	15,63	0,02	0,00	33,47	33,36	33,41	0,08	0,01	17,78	0,08		-3,03	3,03	8,14
757	15,85	15,80	15,82	0,03	0,00	34,42	34,47	34,44	0,04	0,00	18,62	0,05		-2,18	2,18	4,54
758	16,45	16,50	16,47	0,04	0,00	33,03	33,23	33,13	0,14	0,02	16,65	0,15		-4,15	4,15	17,79
762	13,42	13,38	13,40	0,03	0,00	28,78	28,85	28,82	0,05	0,00	15,42	0,05		-5,39	5,39	41,93
763	14,21	14,45	14,33	0,17	0,03	29,52	29,58	29,55	0,04	0,00	15,22	0,18		-5,58	5,58	47,94
764	14,16	14,37	14,26	0,15	0,02	30,04	30,04	30,04	0,00	0,00	15,78	0,15		-5,03	5,03	32,66
765	15,26	15,17	15,22	0,06	0,00	31,28	31,22	31,25	0,04	0,00	16,03	0,07		-4,77	4,77	27,36
766	15,13	15,20	15,16	0,05	0,00	30,60	30,44	30,52	0,11	0,01	15,35	0,12		-5,45	5,45	43,77
768	11,68	11,67	11,67	0,00	0,00	30,40	30,34	30,37	0,04	0,00	18,70	0,04		-2,11	2,11	4,32
770	13,95	13,95	13,95	0,00	0,00	31,49	31,63	31,56	0,10	0,01	17,61	0,10		-3,19	3,19	9,16
771	12,27	12,35	12,31	0,05	0,00	31,39	31,27	31,33	0,08	0,01	19,02	0,10		-1,78	1,78	3,44
772	12,24	12,34	12,29	0,07	0,01	30,26	30,16	30,21	0,07	0,00	17,92	0,10		-2,88	2,88	7,37
773	15,23	15,30	15,27	0,05	0,00	34,68	34,52	34,60	0,11	0,01	19,33	0,12		-1,48	1,48	2,78
776	14,71	14,94	14,83	0,16	0,03	35,47	35,32	35,40	0,10	0,01	20,57	0,19		-0,24	0,24	1,18
780	14,50	14,76	14,63	0,18	0,03	33,96	33,73	33,85	0,16	0,03	19,21	0,25		-1,59	1,59	3,02
783	14,77	14,75	14,76	0,02	0,00	35,67	35,91	35,79	0,17	0,03	21,03	0,17		0,22	-0,22	0,86
787	15,17	15,27	15,22	0,07	0,00	35,14	35,01	35,07	0,09	0,01	19,86	0,11		-0,95	0,95	1,93
789	14,69	14,54	14,61	0,11	0,01	34,97	35,18	35,07	0,15	0,02	20,46	0,19		-0,35	0,35	1,27
791	15,14	15,10	15,12	0,03	0,00	35,72	35,46	35,59	0,19	0,03	20,47	0,19		-0,34	0,34	1,26
796	14,96	14,91	14,93	0,03	0,00	36,30	36,08	36,19	0,16	0,02	21,25	0,16		0,45	-0,45	0,73
800	15,35	15,32	15,33	0,02	0,00	35,37	35,52	35,44	0,11	0,01	20,11	0,11		-0,69	0,69	1,62
805	14,82	15,00	14,91	0,12	0,02	33,55	33,29	33,42	0,18	0,03	18,51	0,22		-2,30	2,30	4,91
806	15,24	15,27	15,25	0,02	0,00	35,46	35,74	35,60	0,20	0,04	20,35	0,20		-0,46	0,46	1,37
810	15,00	14,76	14,88	0,18	0,03	34,39	34,45	34,42	0,04	0,00	19,54	0,18		-1,27	1,27	2,40
811	15,07	15,09	15,08	0,01	0,00	34,22	34,04	34,13	0,13	0,02	19,05	0,13		-1,76	1,76	3,38
813	15,28	15,23	15,25	0,03	0,00	34,56	34,38	34,47	0,13	0,02	19,22	0,13		-1,59	1,59	3,01

817	15,20	15,33	15,26	0,09	0,01	33,47	33,55	33,51	0,06	0,00	18,25	0,11	-2,56	2,56	5,90
825	14,90	15,05	14,98	0,11	0,01	35,38	35,67	35,53	0,21	0,04	20,55	0,24	-0,26	0,26	1,20
829	15,72	15,77	15,74	0,04	0,00	34,76	34,80	34,78	0,02	0,00	19,04	0,04	-1,77	1,77	3,41
831	15,27	15,38	15,32	0,08	0,01	34,42	34,22	34,32	0,14	0,02	19,00	0,16	-1,81	1,81	3,50
832	15,73	15,64	15,69	0,06	0,00	35,49	35,24	35,36	0,17	0,03	19,68	0,18	-1,13	1,13	2,19
837	15,49	15,60	15,54	0,08	0,01	33,73	34,03	33,88	0,21	0,04	18,34	0,23	-2,47	2,47	5,53
838	15,95	15,87	15,91	0,06	0,00	35,54	35,70	35,62	0,11	0,01	19,71	0,13	-1,09	1,09	2,13
840	15,56	15,65	15,60	0,07	0,00	35,90	36,12	36,01	0,16	0,02	20,41	0,17	-0,40	0,40	1,32
852	15,17	15,18	15,17	0,01	0,00	37,13	37,22	37,17	0,06	0,00	22,00	0,06	1,19	-1,19	0,44

***GSI and E2 data, shown together with fish number (fish no) and date of sampling*:**

Fish No	Date	GSI	Estradiol (ng/ml)
24	13.06.2005	1,3	0,800
27	13.06.2005	1,3	0,360
28	13.06.2005	1,4	0,450
34	13.06.2005	4,7	0,440
38	13.06.2005	2,2	0,230
40	13.06.2005	1,2	0,130
43	13.06.2005	1,3	0,150
48	13.06.2005	1,8	0,620
49	13.06.2005	9,2	3,880
50	13.06.2005	1,2	0,310
51	13.06.2005	1,8	1,590
53	13.06.2005	2,0	1,040
55	13.06.2005	1,1	0,330
60	13.06.2005	1,4	0,170
62	13.06.2005	20,5	2,460
63	13.06.2005	1,2	2,130
66	13.06.2005	22,9	10,590
68	11.07.2005	1,1	0,030
70	11.07.2005	1,1	0,710
71	11.07.2005	1,6	0,490
73	11.07.2005	1,3	0,210
74	11.07.2005	3,4	0,180
75	11.07.2005	1,1	0,300
77	11.07.2005	10,0	0,630
78	11.07.2005	2,2	0,220
82	11.07.2005	5,0	0,260
83	11.07.2005	1,5	1,300
86	11.07.2005	2,6	1,330

87	11.07.2005	10,3	1,300
88	11.07.2005	1,2	1,180
89	11.07.2005	1,4	0,710
90	11.07.2005	1,3	0,530
92	11.07.2005	1,1	0,590
97	11.07.2005	2,2	0,270
98	11.07.2005	3,8	0,420
99	11.07.2005	3,2	0,830
101	11.07.2005	1,1	0,350
104	11.07.2005	0,9	0,430
107	11.07.2005	4,3	1,230
108	11.07.2005	1,6	0,170
113	11.07.2005	1,6	0,190
115	11.07.2005	4,6	0,070
123	17.08.2005	1,0	0,560
125	17.08.2005	0,9	1,080
129	17.08.2005	1,4	1,310
131	17.08.2005	1,9	0,340
132	17.08.2005	1,4	0,800
134	17.08.2005	1,6	0,810
135	17.08.2005	0,9	0,900
136	17.08.2005	1,6	0,460
143	17.08.2005	0,9	2,340
145	17.08.2005	2,2	2,110
147	17.08.2005	4,3	0,060
151	17.08.2005	1,8	0,690
155	17.08.2005	1,1	2,090
157	17.08.2005	1,0	0,430
160	17.08.2005	1,3	0,700
161	17.08.2005	5,3	4,030
166	19.09.2005	2,0	0,990
167	19.09.2005	1,4	1,320
171	19.09.2005	1,5	2,890
172	19.09.2005	2,2	2,400
174	19.09.2005	1,1	2,230
176	19.09.2005	1,2	0,770
179	19.09.2005	1,5	1,200
186	19.09.2005	1,5	5,520
193	19.09.2005	3,2	0,700
199	19.09.2005	3,0	1,510
203	19.09.2005	1,4	1,890
204	19.09.2005	3,7	0,250
212	19.09.2005	1,5	
213	19.09.2005	1,7	
214	17.10.2005	2,0	2,690

220	17.10.2005	1,5	5,010
224	17.10.2005	2,9	4,080
225	17.10.2005	2,4	1,230
226	17.10.2005	4,3	2,060
227	17.10.2005	3,3	2,530
228	17.10.2005	2,9	8,180
232	17.10.2005	2,7	1,720
233	17.10.2005	1,6	2,250
235	17.10.2005	1,9	1,110
239	17.10.2005	2,0	2,580
242	17.10.2005	2,1	3,160
243	17.10.2005	2,2	1,860
244	17.10.2005	2,9	2,290
250	17.10.2005	3,7	6,050
253	17.10.2005	2,3	4,200
254	17.10.2005	3,0	3,750
255	17.10.2005	1,4	2,670
259	17.10.2005	2,8	3,920
264	17.10.2005	1,3	3,040
266	17.10.2005	3,0	
269	17.10.2005	3,1	3,840
270	17.10.2005	2,1	1,490
272	17.10.2005	2,2	1,420
273	17.10.2005	1,6	0,760
274	17.10.2005	3,1	1,340
275	17.10.2005	1,9	1,010
278	17.10.2005	1,8	0,690
286	17.10.2005	1,3	2,570
291	17.10.2005	2,5	1,250
292	17.10.2005	2,7	0,930
294	21.11.2005	5,7	2,600
298	21.11.2005	8,3	1,980
299	21.11.2005	4,3	1,390
301	21.11.2005	5,1	1,540
303	21.11.2005	4,2	1,310
304	21.11.2005	5,4	3,410
306	21.11.2005	3,8	3,240
309	21.11.2005	7,1	3,030
310	21.11.2005	4,5	2,090
311	21.11.2005	5,9	1,940
315	21.11.2005	5,2	4,270
317	21.11.2005	3,8	2,010
318	21.11.2005	3,3	4,010
321	21.11.2005	4,6	4,210
324	21.11.2005	4,2	1,880

325	21.11.2005	6,1	2,620
326	21.11.2005	3,9	1,490
327	21.11.2005	4,3	1,890
328	21.11.2005	7,1	3,710
329	21.11.2005	4,5	2,380
330	21.11.2005	5,3	2,140
332	21.11.2005	6,1	4,010
334	21.11.2005	3,7	2,590
335	21.11.2005	3,7	5,100
336	21.11.2005	4,0	4,780
339	21.11.2005	10,0	3,140
340	21.11.2005	5,9	2,310
343	21.11.2005	8,5	3,020
345	21.11.2005	4,0	4,310
347	21.11.2005	6,1	11,480
348	21.11.2005	8,8	9,630
349	21.11.2005	5,3	5,540
350	21.11.2005	4,0	2,060
351	21.11.2005	5,5	2,780
353	21.11.2005	6,4	7,060
354	21.11.2005	3,6	3,670
358	21.11.2005	7,5	2,380
359	21.11.2005	6,7	5,060
360	21.11.2005	4,9	2,440
361	21.11.2005	6,6	4,460
363	21.11.2005	2,6	3,390
365	21.11.2005	5,5	6,230
366	21.11.2005	4,5	5,330
379	19.12.2005	8,0	7,090
380	19.12.2005	8,5	6,150
383	19.12.2005	11,1	6,540
388	19.12.2005	10,8	3,770
391	19.12.2005	6,8	4,300
394	19.12.2005	6,9	7,800
404	19.12.2005	9,8	5,110
406	19.12.2005	9,9	12,590
410	19.12.2005	11,9	6,560
419	19.12.2005	9,0	15,610
421	19.12.2005	12,9	8,010
424	19.12.2005	11,2	5,170
427	19.12.2005	10,6	6,100
428	19.12.2005	0,2	2,560
430	19.12.2005	9,6	5,280
441	19.12.2005	6,9	5,270
445	19.12.2005	10,6	9,060

446	19.12.2005	8,7	8,640
447	19.12.2005	9,9	8,380
450	19.12.2005	11,6	4,140
455	23.01.2006	16,6	13,240
458	23.01.2006	16,2	31,050
460	23.01.2006	8,8	5,820
462	23.01.2006	10,6	3,280
463	23.01.2006	11,6	7,080
465	23.01.2006	20,5	0,000
467	23.01.2006	14,0	16,660
471	23.01.2006	12,4	15,890
472	23.01.2006	12,7	20,150
473	23.01.2006	18,6	15,090
474	23.01.2006	9,0	7,820
475	23.01.2006	19,0	16,580
477	23.01.2006	14,2	12,740
478	23.01.2006	20,6	22,240
479	23.01.2006	18,8	20,010
481	23.01.2006	16,3	33,470
484	23.01.2006	17,2	34,510
485	23.01.2006	17,5	15,730
492	23.01.2006	15,7	18,660
493	23.01.2006	21,7	55,420
494	23.01.2006	11,6	14,270
498	23.01.2006	13,6	9,540
500	23.01.2006	15,1	15,510
502	23.01.2006	14,1	16,430
503	23.01.2006	14,8	25,870
504	23.01.2006	18,3	12,390
511	23.01.2006	25,1	8,450
512	23.01.2006	15,9	18,530
513	23.01.2006	13,6	6,090
515	23.01.2006	12,3	13,470
517	23.01.2006	14,4	12,740
518	23.01.2006	17,3	17,750
519	23.01.2006	16,1	7,520
522	23.01.2006	13,7	0,250
523	23.01.2006	11,5	16,830
527	23.01.2006	17,3	19,720
529	23.01.2006	15,0	0,350
531	23.01.2006	14,5	14,160
532	23.01.2006	14,3	19,640
553	21.02.2006	26,2	25,550
554	21.02.2006	31,0	11,320
555	21.02.2006	31,6	20,440

556	21.02.2006	28,4	13,960
557	21.02.2006	23,0	11,320
558	21.02.2006	24,0	11,840
559	21.02.2006	25,2	30,890
560	21.02.2006	27,9	4,420
561	21.02.2006	32,2	8,870
562	21.02.2006	31,1	32,180
563	21.02.2006	31,4	28,760
564	21.02.2006	30,1	31,740
565	21.02.2006	26,9	10,120
566	21.02.2006	23,7	11,900
567	21.02.2006	17,9	17,370
568	21.02.2006	22,0	16,810
569	21.02.2006	31,9	17,090
570	21.02.2006	10,0	0,000
571	21.02.2006	32,2	27,740
572	21.02.2006	29,9	18,230
573	21.02.2006	24,0	2,770
574	21.02.2006	17,4	22,450
575	21.02.2006	17,8	22,060
576	21.02.2006	25,1	25,130
577	21.02.2006	26,2	0,000
578	21.02.2006	28,5	12,210
579	21.02.2006	26,9	8,190
580	21.02.2006	22,0	27,290
581	21.02.2006	30,0	19,770
582	21.02.2006	18,2	27,600
583	21.02.2006	22,3	14,100
584	21.02.2006	26,8	21,350
585	21.02.2006	28,0	12,200
593	21.02.2006	37,0	25,020
595	21.02.2006	18,5	23,580
596	21.02.2006	26,1	
597	21.02.2006	25,5	19,380
598	21.02.2006	26,5	32,230
599	21.02.2006	22,9	14,850
600	21.02.2006	30,4	11,200
601	21.02.2006	34,2	6,380
602	21.02.2006	27,6	7,410
603	21.02.2006	25,3	47,680
604	21.02.2006	29,2	9,860
613	21.02.2006	22,0	9,660
614	21.02.2006	27,2	13,390
615	21.02.2006	29,3	11,030
616	21.02.2006	23,1	16,450

617	21.02.2006	28,3	17,230
618	21.02.2006	35,4	19,760
619	21.02.2006	23,8	16,250
621	21.02.2006	24,9	17,990
623	21.02.2006	21,9	11,980
624	21.02.2006	23,9	0,000
626	21.02.2006	28,6	12,800
627	21.02.2006	25,4	7,640
629	21.02.2006	28,7	15,700
631	21.02.2006	1,2	0,020
632	21.02.2006	26,2	13,990
633	21.02.2006	21,5	14,130
634	21.02.2006	27,1	15,690
635	21.02.2006	22,2	14,790
636	21.02.2006	35,8	1,440
637	21.02.2006	3,2	1,610
638	21.02.2006	28,1	14,160
640	21.02.2006	29,9	17,660
641	21.02.2006	21,8	44,300
642	21.02.2006	19,6	15,620
643	21.02.2006	24,2	17,160
644	21.02.2006	30,5	12,190
645	21.02.2006	24,4	23,690
647	21.02.2006	26,8	9,650
650	21.02.2006	24,3	23,660
651	21.02.2006	25,6	19,730
652	21.02.2006	22,8	25,200
653	21.02.2006	22,8	37,590
654	21.02.2006	25,4	12,800
656	21.02.2006	22,9	39,330
657	21.02.2006	13,4	21,510
658	21.02.2006	18,3	14,520
659	21.02.2006	31,0	2,860
661	21.02.2006	25,1	18,060
662	21.02.2006	22,2	27,360
663	21.02.2006	23,0	32,540
667	21.02.2006	23,5	13,580
668	21.02.2006	31,2	23,000
672	21.02.2006	25,5	30,360
673	21.02.2006	29,4	19,760
674	21.02.2006	22,2	43,210
675	21.02.2006	2,9	42,730
678	21.02.2006	11,7	14,530
679	21.02.2006	27,1	45,830
682	21.02.2006	21,3	32,010

683	21.02.2006	27,1	22,890
684	21.02.2006	34,0	35,710
685	21.02.2006	24,4	18,150
687	21.02.2006	22,3	32,530
688	21.02.2006	27,3	19,830
689	21.02.2006	31,0	47,890
690	21.02.2006	28,8	
692	21.02.2006	25,8	30,860
693	21.03.2006	31,1	32,550
695	21.03.2006	31,7	36,050
697	21.03.2006	26,0	32,530
698	21.03.2006	26,1	9,360
700	21.03.2006	27,1	21,010
702	21.03.2006	26,3	24,950
703	21.03.2006	24,9	48,690
704	21.03.2006	28,1	30,930
706	21.03.2006	29,1	
707	21.03.2006	21,5	16,800
708	21.03.2006	23,8	3,440
712	21.03.2006	27,9	27,680
713	21.03.2006	12,1	4,670
714	21.03.2006	28,3	5,560
716	21.03.2006	18,4	13,700
717	21.03.2006	28,1	
718	21.03.2006	26,4	1,550
719	21.03.2006	17,1	9,580
720	21.03.2006	23,5	7,210
721	21.03.2006	16,4	13,190
722	21.03.2006	19,8	15,510
723	21.03.2006	25,4	6,970
724	21.03.2006	26,7	
725	21.03.2006	24,8	4,780
726	21.03.2006	14,9	11,670
729	21.03.2006	41,0	32,900
730	21.03.2006	18,4	3,040
731	21.03.2006	23,4	7,500
733	21.03.2006	23,3	32,530
734	21.03.2006	21,3	
735	21.03.2006	18,0	6,370
737	21.03.2006	19,8	10,970
738	21.03.2006	25,0	12,380
741	21.03.2006	24,1	20,010
743	21.03.2006	24,8	2,350
744	21.03.2006	24,0	6,080
745	21.03.2006	24,4	7,550

747	21.03.2006	25,0	5,850
748	21.03.2006	27,8	6,750
750	21.03.2006	26,3	14,490
751	21.03.2006	29,5	3,250
752	21.03.2006	24,2	7,700
753	21.03.2006	19,3	3,170
756	21.03.2006	29,0	10,760
757	21.03.2006	27,1	11,910
758	21.03.2006	23,6	6,850
762	21.03.2006	19,3	21,480
763	21.03.2006	20,0	4,610
764	21.03.2006	24,5	14,910
765	21.03.2006	23,1	14,960
766	21.03.2006	22,3	11,510
768	21.03.2006	23,8	9,740
770	21.03.2006	24,2	10,080
771	21.03.2006	18,0	3,840
772	21.03.2006	20,9	9,820
773	15.05.2006	1,7	0,020
776	15.05.2006	2,7	0,020
780	15.05.2006	1,5	0,020
783	15.05.2006	3,7	0,040
787	15.05.2006	3,6	0,020
789	15.05.2006	2,8	0,020
791	15.05.2006	6,3	0,020
796	15.05.2006	9,8	0,020
800	15.05.2006	2,4	0,020
805	15.05.2006	3,5	0,020
806	15.05.2006	2,6	0,020
810	15.05.2006	5,5	0,020
811	15.05.2006	7,4	0,020
813	15.05.2006	2,3	0,020
817	15.05.2006	2,4	0,310
825	15.05.2006	3,1	0,020
829	15.05.2006	2,8	0,020
831	15.05.2006	2,5	0,020
832	15.05.2006	2,9	0,020
837	15.05.2006	2,0	0,020
838	15.05.2006	2,8	0,020
840	15.05.2006	2,8	0,020
852	15.05.2006	10,0	0,020