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Investigation of the role of insulin receptor
genes in wing polyphenism using gene knock
down and differential gene expression analysis
in the non-model organism *Gerris buenoi*

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Abstract

Investigation of the role of insulin receptor genes in wing polyphenism using gene knock down and differential gene expression analysis in the non-model organism *Gerris buenoi*

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Wing polyphenism is a type of phenotypic plasticity present in several insect species whereby a genotype have the ability to develop alternative wing morphs when exposed to different environmental cues. One organism demonstrating a clear case of wing polyphenism is the water strider species, *Gerris buenoi*, which develop long- or short wings depending on exposure to different photoperiods (the time the organism is exposed to light during a 24 h period). The molecular mechanism behind wing polyphenism in insects in general, and in water striders in particular, is largely unknown. From a study on wing polyphenism in the Brown planthopper (*Nilaparvata lugens*), some candidate genes have been identified and include two insulin genes and the Forkhead transcription factor (FOXO). Since these genes have been demonstrated to affect wing polyphenism in Brown planthopper (BPH) and since *G. buenoi* contains an additional insulin receptor homolog, the potential role of these genes in regulating wing polyphenism in *G. buenoi* have in this project been investigated. The functional genetic technique RNA interference (RNAi) was used to evaluate the function of the genes. This method knock down gene expression in the genes mentioned above, one at a time, to investigate if they have a function in wing polyphenism in *G. buenoi*. DsRNA with specific homology to each target gene was successfully produced. However, when attempting to inject the dsRNA through micro injection all injected liquid leaked out from the body cavity, and the RNAi was therefore not successful. Further optimisation of the injection protocol has to be done to be able to perform RNAi properly in the future. Thereafter, RT-qPCR was used to evaluate whether the insulin receptor genes and FOXO are differentially expressed between the two photoperiods giving rise to the different wing morphs. The differential gene expression experiment showed differences between the mRNA levels of all target genes between *G. buenoi* being reared in the two different photoperiods. More specific upregulation of the genes FOXO and insulin receptor 2 in short winged *G. buenoi* were demonstrated. Further, insulin receptor 1-like, was also demonstrated to be upregulated in the short winged morph. Results presented in this project are in line with the previously identified regulation pattern in BPH, still the results need further evaluation. Since gene expression differences were present for all candidate genes between *G. buenoi* reared in the different photoperiods, these's genes could still be seen as potential candidate genes in wing polyphenism in water striders.

Sammanfattning

Förmågan hos en organism att kunna anpassa sig till förändringar i omgivningen är positivt ur överlevnadssynpunkt. Vilka egenskaper och utseende en organism innehar beror till stor del på en organisms gener. Trots detta, finns det arter, som utifrån samma gener utvecklar helt olika egenskaper och utseenden. Detta fenomen kallas inom vetenskapen för fenotypisk plasticitet, och bidrar till exempel till att en organism kan anpassa sig till rådande omständigheter.

Ett speciellt fall av fenotypisk plasticitet inom insekter är vingpolyfenism, vilket innebär att en insekts vingutseende påverkas av speciella miljöförhållanden. *Gerris buenoi* är en art inom släktet skräddare, som utvecklar långa eller korta vingar beroende på dagslängd och är därför en plastisk art. Utvecklar skräddaren långa vingar, innebär det också för skräddaren en förmåga att kunna flyga och förflytta sig till bättre levnadsförhållanden. Korta vingar innebär istället att skräddaren blir oförmögen till att förflytta sig men kommer istället hinna lägga flera ägg. Flera forskargrupper försöker idag ta reda på hur regleringen för vingpolyphenism styrs.

I det här projektet har fyra gener undersökts hos *G. buenoi* för att ta reda på om dessa styr val av vingutseende. Tre av dessa gener ger upphov till insulinreceptorer. Tanken är att dessa receptor på något vis leder vidare yttre miljöfaktorer, i detta fall dagslängd, till signaler i insektens celler som påverkar vingutvecklingens fortsatta gång.

För att undersöka geners funktioner kan olika biotekniska verktyg användas. I detta projekt gjordes försök att tysta generna hos *G. buenoi*, för att se hur utseendet hos skräddaren påverkades när generna inte längre var närvarande. Under en gentystning så injiceras labbtillverkat RNA in i skräddarens blod. RNA:t tas sedan upp av skräddarens celler och binder specifikt till skräddarens RNA. Tack vare olika naturligt förekommande reglermekanismer i celler, så kommer detta aktivera olika typer av proteinkomplex och därmed bryta ner det RNA som bundits in. Resultat i detta projekt visade att mer optimering krävs vid själva injiceringen, då den vätska som RNA:t lösts i gärna läckte ut genom injiceringshålet istället för att tas upp av blodet.

Som nämnts innan så utvecklar *G. buenoi* korta eller långa vingar beroende på dagslängd. 12 timmars dagslängd ger långa vingar medan 18 timmars dagslängd ger korta vingar. Därför undersökte jag också i det här projektet om generna uttrycktes olika mycket i de olika miljöerna. Om generna uttrycks olika i de två miljöerna, kan ledtrådar ges om huruvida generna är involverade i vingpolyphenism. Experimentet utfördes genom att rena fram RNA från skräddarna från de två miljöerna och sedan jämföra RNA-nivåerna för de specifika generna med hjälp av olika biotekniska verktyg. När detta utfördes sågs en viss skillnad mellan de olika miljöernas RNA-nivåer. Forskare har tidigare sett att dessa gener påverkar vingpolyphenism i en annan insektsart. Resultat från detta projekt visar alltså på att detta även kan vara fallet i skräddare.

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Abbreviations

AE	amplification efficiency
BPH	brown planthopper
cDNA	complementary DNA
Ct	threshold cycle
Cq	quantification cycle
FOXO	forkhead transcription factor
INR1	insulin receptor 1
INR2	insulin receptor 2
INR1-like	insulin receptor 1-like
RNAi	RNA interference
RT	reverse transcription
RT-qPCR	quantitative reverse transcription polymerase chain reaction
siRNA	silencing RNA

1 Introduction

Phenotypic plasticity is a phenomenon, describing the ability of an organism to alter its phenotype depending on environmental cues. That is to say, the organism's capacity to produce several phenotypes based on the same genotype. To possess the feature of phenotypic plasticity is an advantage when living in an environment of change. Wing polyphenism is a special case of phenotypic plasticity that gives rise to discrete wing morphs in a population, with no or very reduced levels of continuous variation (Hayes *et al.* 2019).

The water strider species *Gerris buenoi* is wing polymorphic meaning that it can either develop long or short wings. Since this species possess, like several other insects, the feature of wing polyphenism (as a result of phenotypic plasticity), the wing development is influenced by environmental cues during the organism's developmental stages (Hayes *et al.* 2019). Known cues to influence wing polyphenism in insects are nymphal density, photoperiod, and temperature (Hayes *et al.* 2019). Yet today the underlying molecular mechanism behind plasticity in wing polyphenism is still largely unknown (Wang *et al.* 2015). Several genes are known to be involved in insect wing development like *aristaless*, *distal-less* and *vestigial* in *Drosophila* (Chen *et al.* 2016), and *Tribolium castaneum* (Beermann *et al.* 2001, Linz *et al.* 2018). Xu *et al.* (2015) published a study proposing a model of how wing polyphenism is partly controlled by two insulin receptors in the brown planthopper (BPH), *Nilaparvata lugens*. They showed how Insulin Receptor 2 (INR2) could physically interact with Insulin Receptor 1 (INR1). INR2 had a suppressing effect on INR1, leading to activation of the forkhead transcription factor (FOXO), resulting in the development of short-winged morphs. Long-winged morphs, however, is induced by a signalling cascade through INR1 and the Akt pathway. With this, they showed for the first time a binary control of different wing morph trajectories during development (Xu *et al.* 2015).

Three genes encoding insulin receptor genes are present in the genome of *G. buenoi* (Armisen *et al.* 2018), whereas most arthropods only have one or two copies of these genes. When analysing several species within the Gerromorpha (semiaquatic bugs, infraorder of the Hemiptera), it was shown that this is not unique for *G. buenoi* and is instead common within the infraorder (Armisen *et al.* 2018). The third insulin receptor has been given the name Insulin Receptor 1-like (INR1-like), since it was shown to be a retrocopy in INR1, containing no introns (Armisen *et al.* 2018). Since insulin receptor genes and FOXO are involved in the molecular mechanism of wing polyphenism in BPHs, and since water striders have a third insulin receptor gene, I aimed to evaluate the role of the insulin receptor genes and FOXO in wing polyphenism in *G. buenoi*. This was done using RNA interference (RNAi) and quantitative reverse transcription PCR (RT-qPCR). One environmental cue controlling wing

polyphenism in *G. buenoi* is photoperiod. *G. buenoi* development of long wings is induced in a light:dark cycle of 12:12 and short wings in a cycle of 18:6 (Gudmunds *et al.* unpublished observations). According to the results from this project, a potential difference between mRNA expression levels for the target genes could be seen between the *G. buenoi* reared in the two different photoperiods.

2 Material & methods

Resources used in this project can be seen in Appendix A.

2.1 Cultivation of *G. buenoi*

The *G. buenoi* water striders population used in this project was given to Arild Husby and his group in November 2018, and have been cultivated in the lab for an unknown number of generations. The water striders were kept in plastic boxes filled with tap water and were fed with frozen crickets. Pieces of Styrofoam were placed in the boxes for rest and oviposition. The water striders were kept in two different photoperiods during this project, 12 h daylight and 18 h daylight. The two different environments were created in walk-in chambers which were kept at 25 °C, with relative humidity at 65 % and light intensity at $0.00085 \mu E m^{-2} s^{-1}$.

2.2 RNAi

2.2.1 Designing of PCR primers for generation of a DNA template for dsRNA synthesis

The *G. buenoi* genome dataset and gene prediction data were downloaded from National Agricultural Library (United States Department of Agriculture 2018). The genome data set used was GBUE_BCM_assembly_v1-0 and the curated gene prediction dataset used was OGSv1.0 created by Armisen *et al.* (2018). Target gene names in the gff file, GBUE_OGSv1-0.gff, were used to identify gene-ID, scaffold number, and exon positions (Table 1). The target gene sequences were located by gene ID in the coding sequence data file, (GBUE_OGSv1-0_CDS.fa) and in the transcription data file (GBUE_OGSv1-0_trans.fa). To visualize exon and intron data for each gene, and potential 5' and 3' UTR sequences, all target genes coding sequence and transcription sequence data, were imported into snapGene (version 4.3.8.1) and mapped to the scaffolds.

Table 1. Table presenting gene ID, annotation and scaffold number of genes used in the project.

Gene ID	Annotation	Scaffold
GBUE020965-RA	INR1	66
GBUE020810-RA	INR1	13662
GBUE009720-RA	INR2	758
GBUE021108-RA	INR2	798
GBUE019859-RA	INR1-like	4241
GBUE004076-RA	Distal-less	185
GBUE021071-RA	FOXO	501
GBUE010321-RA	ovo/shavenbaby	721

ik5@workspace website provides a BLAST algorithm for *G. buenoi* genome-, transcript- and coding sequence. BLAST was performed between the coding sequence of each target gene to the total coding sequences of *G. buenoi*, to identify non-homologous exon sequences between the different candidate genes tested here. Primers were designed from one of these non-homologous sequences.

To check for potential non-specific PCR products, forward and reverse primers were BLASTed against the *G. buenoi* genome. Primers were chosen by the criteria of having a low risk of self-dimer and primer-dimer formations which were evaluated by Eurofin oligo analysis tool (Eurofin Genomics 2019). Another criterion for the primers, were to not anneal perfect to another target in the 3' end of the primer (Simsek *et al.* 2000). Other criteria to fulfil were to have GC-content of 40-60%, and a T_m of 52-58 °C (Lorenz 2012). The dsRNA sequence was designed with the criterion of not having more than 18 nt perfect homology to another RNA sequence (Paim *et al.* 2013).

All primers were flanked by the T7 RNA polymerase promoter sequence: 5'taatagcactcactataggagaccac3' at the 5' end and were synthesized by Eurofins Genomics. The Thermo Fisher scientific T_m calculation tool (Thermo Fisher Scientific 2019) was used for determination of primer specific T_m for the Phusion DNA polymerase that was used.

2.2.2 DNA extraction

Total DNA extractions were performed on *G. buenoi* with NucleoSpin DNA Insect kit according to the manufacturer's protocol (Macherey-Nagel 2019). Before extraction, the

insects were added to 99.9 % ethanol. Agitation was performed with Vortex 2 from MO BIO Laboratories, Inc, during 1 h and 40 minutes, and an elution volume of 100 µL was used. NanoDrop™ 2000 was used to evaluate the purity (260/280) and to estimate the DNA concentration. The DNA was analysed on a 1% agarose gel using GelRed™ Biotium.

2.2.3 PCR

All PCR reactions were performed with Phusion High-Fidelity DNA Polymerase (according to the protocol in Appendix B) and genomic DNA was used as template in the reactions. Published primers for the gene *shavenbaby* (*svb*) was used as a positive control when performing the PCR reactions (Ray *et al.* 2019), to confirm intact DNA template. The colony PCR was performed by picking a single bacterial colony from an agar plate and adding this to 30 µL milliQ water. This solution was then used as a template in PCR reactions performed with the same conditions as described above. The PCR products were analysed on 1% agarose based gel electrophoresis, using GelRed™ Biotium and GeneRuler™ 100 bp Plus DNA Ladder. Purification of PCR products was performed with GeneJET Gel extraction and DNA Clean Up Micro kit according to manufacturer's protocol (Thermo Fisher Scientific 2013a). Concentration and purity measurements were made using NanoDrop™ 2000. PCR products were sequenced using the Eurofin sequencing service for Sanger sequencing.

2.2.4. dsRNA synthesis

dsRNA synthesis was performed with TranscriptAid T7 High Yield Transcription Kit according to manufacturer's protocol (Thermo Fisher Scientific 2014a). The reaction incubation time used was 6 hours at 37°C for dsRNA segments up to 600 bp, and longer segments were incubated for 3 h. Removal of DNA template from the *in vitro* transcription products was performed by DNase I treatment. 2 µL DNase, RNase-free were added to each reaction tube, incubated at 37°C for 15 minutes, followed by the addition of 2 µL 0.5 M EDTA (pH 8.0) and incubation for 10 minutes at 65°C.

The dsRNA products were cleaned with GeneJet RNA purification kit according to the manufacturer's protocol (Thermo Fisher Scientific 2014b), except for an extra 5 minutes incubation time before the eluting step, which was performed in 50 µL Spradling buffer (5 mM KCl, 10 mM NaH₂PO₄ (pH 7.8)) instead of water.

2.2.5. Injection of dsRNA in *G. buenoi* using CellTram Oil

Injection needles were constructed using Narishige PC-10 Pipette Puller. Two different injection techniques were evaluated. Injection with microneedles attached to a Hamilton Syringe and injection with CellTram Oil. Prior to injection, the water striders were anaesthetized with 7 L/min for 30-60 seconds, using Benchtop Flow Buddy System. Injection protocols were optimised with different water strider species and injection position. After

injection, the water strider rested on wet paper in 1 h before returning to the water. Needles with different thicknesses were evaluated.

2.3. Differential gene expression

2.3.1 RNA extraction

Samples for RNA extraction were placed in 300 μ L RNA later supplemented with 1% Tween 20 and stored at room temperature. Total RNA extraction was performed with GeneJET RNA purification kit, using the manufacturer's protocol (Thermo Fisher Scientific 2014b). Stored insects were added to 40-60 μ L Lysis buffer supplemented with 20 μ L 2 M DTT (per 200 μ L Lysis buffer) and were grinded thoroughly with a sterile pestle. Supplemented lysis buffer was then added up to 300 μ L. The lysate was pipetted up and down for homogenization purposes and subsequent steps were done according to protocol. Elution was performed in 50 μ L nuclease-free water. RNA integrity was assessed with Agilent 2100 Bioanalyzer and genomic DNA were removed with DNase I treatment.

2.3.2 qPCR primers

Primers for RT-qPCR were designed to generate an amplicon of near 150 nt in length, with a T_m between 58-60°C. The NCBI primer design tool (National Center of Biotechnology Information 2019) was used to generate primers to choose among. The primers were BLAST:ed against the *G. buenoi* transcriptome at ik5@workspace to make sure they were specific against the target region and primers with least homology to other regions were chosen.

2.3.3 RT-qPCR

The RevertAid First Strand cDNA Synthesis Kit was used for cDNA synthesis using oligo dT primers (Thermo Fisher Scientific 2013b) and 0.608 μ g of total RNA as template. Thereafter, Luminaris Color HiGreen Master Mix was used according to manufacturer's protocol (Thermo Fisher Scientific 2012) together with CFX96 Touch Real-Time PCR Detection System for the RT-qPCR. A three-step cycling protocol was used, where the protocol was 50 UDG pre-treatment, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, and 60°C and 72°C for 30 s. The qPCR was performed in 20 μ L reactions, using a final concentration of 0.3 μ M of forward and reverse primer in each reaction. To investigate whether multiple PCR products, primer-dimers or self dimers were formed during the thermal cycling, a 2% agarose based gel electrophoresis was run using GelRed TM Biotium. Ribosomal 18S rRNA was used as a reference gene for normalization of Ct values.

3 Theory

3.1 Gene knockdown using RNA interference

RNAi is a natural post-transcriptional gene regulatory mechanism and occurs in most eukaryotic organisms (Tenea & Burlibasa 2012). The biological function can favourably be utilized experimentally within a functional genomic context, where the molecular cascade can be triggered by the introduction of exogenous dsRNA within cells. The RNAi technique offers gene-specific silencing of transcriptionally active genes since dsRNA can be designed towards specific regions of target genes to cause degradation of mRNA. The technique can be used experimentally both in cell cultures and *in vivo*, which makes it appropriate for studies of gene function (Paim *et al.* 2013).

When dsRNA is in contact with the endonuclease Dicer within the cytoplasm, the RNAi-processes is initiated and the dsRNA is fragmented into pieces of 21-25 nt (Paim *et al.* 2013). These fragments, called small interfering RNA:s, form along with cellular proteins the so-called RNA-induced silencing complex (RISC). A protein included in the RISC opens the siRNA while double stranded and binds the antisense strand while the sense strand is degraded. The anti-sense strand within the RISC can then base pair with complementary RNA:s. A nuclease in the RISC thereafter degrades the target RNA and consequently knock-down of the targeted gene has occurred (Paim *et al.* 2013).

RNAi is a highly valuable technique within the field of functional genomics (McGinnis 2010, Heigwer *et al.* 2018). Several RNAi studies have been performed on arthropods for assessing gene function (Paim *et al.* 2013, Crumière and Khila 2019, Heigwer *et al.* 2018). For example, in water striders, RNAi was used together with gene expression analysis to evaluate the role of the gene *Ultrabithorax* (a homeobox gene) in leg-size control (Khila *et al.* 2009).

3.2 dsRNA synthesis

T7 RNA-polymerase, originally from the T7-bacteriophage, can be used in *in vitro* synthesis of RNA. To synthesize the desired dsRNA, a DNA-template is constructed with perfect sequence homology to the gene of interest, flanked by the T7 promoter sequence in both ends. Since a T7 promotor is present on both DNA strands in a direction facing each other, both strands of DNA is transcribed into RNA, and the annealing of the sense-and antisense strand is an automatic process (Khila *et al.* 2009).

When using dsRNA molecules of size 200 to 500 bp, there will be an effective uptake of the exogenous RNA to the cells, where smaller-sized molecules will have a decreased efficiency. Using sizes up to 1000 bp is still effective (Vogel *et al.* 2019). In a review article from 2013 discussing RNAi in the Triatominae (order Hemiptera), normal lengths of dsRNA used were around 500 bp (Paim *et al.* 2013). Crumière and Khila (2019), used dsRNA lengths of approximately 320 and 530 bp when performing gene-knock down in water striders.

3.3 Delivery of dsRNA to the cells during RNAi

To introduce the dsRNA to the cells, a suitable delivery method is needed. Two potential methods are injection and ingestion, where microinjection in the hemolymph is the more commonly used method in insect studies (Paim *et al.* 2013). In a study by Khila *et al.* (2009), an injection volume of 1.5-2 μ L dsRNA with a concentration of 2 μ g/ μ L gave a good result when injecting adult stage water striders. In Triatominae, injection quantities with effective knock-down spanned between 2 to 30 μ g dsRNA (Paim *et al.* 2013). Further, the RNAi experiment performed in BPH used smaller quantities of dsRNA in range 25 -150 ng depending on the developmental stage (Xu *et al.* 2015).

4. Results

4.1 Knock-down of insulin receptor genes and FOXO using RNAi

To be able to investigate whether the different insulin receptors and the transcription factor FOXO play an important role in wing polyphenism in *G. buenoi*, an RNAi experiment was conducted. Previous results have demonstrated that photoperiod is the main factor regulating the decision of wing morph in *G. buenoi* and therefore photoperiod was used as treatment during the RNAi experiment. Since photoperiods of 12 and 18 hours lead to predominant long-winged morphs respectively short-winged morphs (Gudmunds *et al.* unpublished observations), the effect of silencing of different genes can easily be investigated as a reduction of a particular wing morph in a particular treatment.

First, to be able to perform gene silencing against the target genes (INR1, INR2, INR1-like and FOXO), dsRNA had to be produced. And, for the production of dsRNA, DNA templates containing segments from the target genes flanked by the T7 RNA promoter sequence had to be constructed during PCR.

4.1.1 Amplification of target genes flanked with T7 RNA promoter sequence

PCR reactions were performed for all insulin genes and FOXO where two different amplicons per gene were produced. The primer set used can be seen in Table 2. In addition to the genes of interest, PCR reactions were performed on *svb* and colony PCR was performed on a bacterial strain carrying a plasmid encoding Green Fluorescent Protein (GFP). The use of *svb* is to show that the DNA template is not degraded since the primers have previously shown to work in *G. buenoi* (Ray *et al.* 2019). GFP, however, will work as a negative control in the RNAi experiment.

As DNA template in the PCR reactions, extracted genomic DNA from *G. buenoi* were used. Two DNA extractions were performed, where one sample consisted of 5 stored individuals and the other of two fresh randomly chosen individuals of respective sex. The concentration and purity measurements of the extracted DNA was assessed by NanoDrop™ 2000 (Table 3). To investigate how fragmented the DNA was after extraction, gel electrophoresis was performed, see Figure 1. Two strong bands could be seen in the sample containing extracted DNA from the fresh water striders. Additional smear flanking the strong bands could also be seen in the gel picture. Since samples from stored individuals showed a lot of fragmentation, the genomic DNA from the fresh individuals was used as a template in all PCR reactions.

To examine whether the PCR primers would successfully produce DNA of correct size, pilot reactions of small volumes were performed for each amplicon. All primer pairs were shown to work successfully, meaning that they produce only one PCR product and thus several reactions of larger volume were performed to gain a sufficient amount of DNA templates for the dsRNA synthesis. The PCR products were analyzed with agarose gel electrophoresis to investigate the size of the product and to visualize potential off-target PCR products (Figure 2). All PCR:s produced single bands of correct sizes indicating that the correct product had been obtained without any off-target amplification. Yields received when using normal PCR with genomic DNA as template spanned between 690 to 1350 ng (Figure 2). The colony PCR gave a higher total yield of 3060 ng. Some presence of primer dimers can be seen in most of the PCR products (less than 100 bp), but not in great extent. The sequence of all PCR products were determined by Sanger sequencing and the results confirmed the right sequence for all PCR products.

Table 2. Primer pairs used in PCR. Forward and reverse primers for the genes INR1, INR2, INR1-like and FOXO can be seen in the table together with amplicon length. The capital letters in the primer sequences are the annealing part of the primers, while lowercase letters stands for the T7 RNA polymerase promoter sequence.

Gene	GeneID		Forward	Reverse	Amplicon length [bp]
Insulin receptor 1	GBUE02096 5-RA	Primer set 1	taatacgactcactatagggagaccacTTCG TACACGACCAACGCATAG	taatacgactcactatagggagaccacGTTGAGCTT CAAGGATGCAAAGTC	285
	GBUE02081 0-RA	Primer set 2	taatacgactcactatagggagaccacACTC CAAAGCCTTCTACACC	taatacgactcactatagggagaccacCCTGATAAC TGTCCAGACACTC	446
Insulin receptor 2	GBUE00972 0-RA	Primer set 1	aatacgactcactatagggagaccacATGA CGTGGGTTGTGATGGTG	taatacgactcactatagggagaccacTATTAAAGA GGGCGACAGATCC	532
	GBUE02110 8-RA	Primer set 2	taatacgactcactatagggagaccacACGA CCGTTCTCAATGGTGAAG	taatacgactcactatagggagaccacTTCTGGTGC ATGTCGCAAAGTG	457
Insulin receptor 1-like	GBUE01985 9-RA	Primer set 1	taatacgactcactatagggagaccacCCGT GTAGAAGTTCAAGCATG	taatacgactcactatagggagaccacGAAGAACTC GCTCTCACAC	423
	GBUE01985 9-RA	Primer set 2	taatacgactcactatagggagaccacTGCT CGGTGGTCTGAAGGATAC	taatacgactcactatagggagaccacCAGCACGTA GTCTCGTTGTAACAG	551
FOXO	GBUE02107 1-RA	Primer set 1	taatacgactcactatagggagaccacCGGG GAAGAGATCTAAGGATTC	taatacgactcactatagggagaccacATAAGGCAT AACCTCAGTTTGC	310
	GBUE02107 1-RA	Primer set 2	taatacgactcactatagggagaccacACCC AAGAATGACCGGAG	taatacgactcactatagggagaccacGTCAAAACG GAGCCAGAAG	308
Distal-less	GBUE00407 6-RA	Primer set 1	taatacgactcactatagggagaccacATAT CAAGTGTCCTTCAAGCAG	taatacgactcactatagggagaccacTCGTTTTTA GGCGGGAATACG	221
	GBUE00407 6-RA	Primer set 2	taatacgactcactatagggagaccacGGAC GACAAATCCAAGAGAGGAAG	taatacgactcactatagggagaccacGTAGGTGTT GTGGTGACAGGC	321
Shavenbaby	GBUE01032 1-RA	Primer set 1	GGTGGCCAACAAAGTTCCCT	CCTGTGTTGAAGA ACGCCAA	192
GFP		Primers	taatacgactcactatagggagacca	taatacgactcactatagggagacca	

Table 3. Concentrations and 260/280 purity ratio of nucleic acid isolated from *G. buenoi* stored (sample 1) and fresh (sample 2) individuals using NanoDrop. The measurements were done in replicates of 3.

Sample	nucleic acid [ng/ μ L]	Total yield [ng]	260/280
1.	56.1	5610	1.71
2.	42.1	4210	1.92

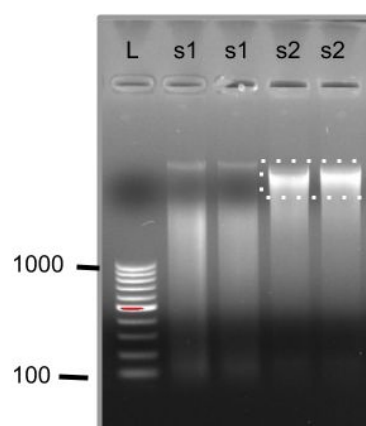


Figure 1. Gel analysis of whole DNA extraction from *G. buenoi*. L is an abbreviation for the ladder, which spans between 100 to 1000 bp. S1 and S2 indicates stored and fresh samples respectively, two replicates of each are present. The dotted line in s2 lanes marks the DNA bands.

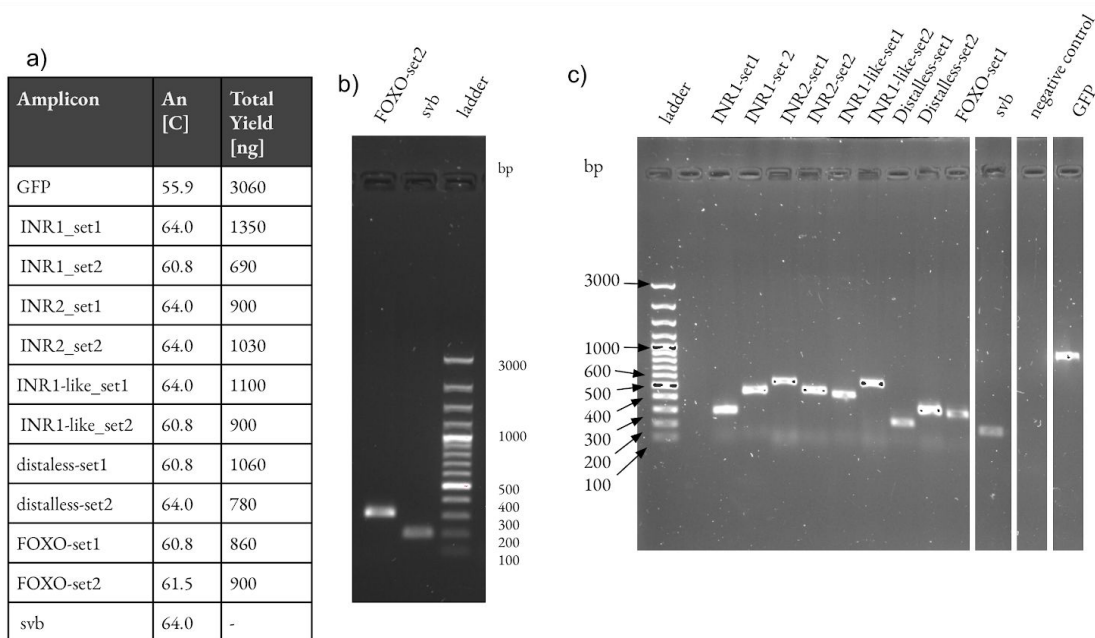


Figure 2. Results from PCR experiments a) Annealing temperatures for single PCR products and the highest yields received when performing a 50 μ L reaction, using 2 μ L DNA template and measuring concentration with NanoDrop TM 2000, b) and c) showing PCR products for the different amplicons using the GeneRuler TM 100 bp Plus DNA as Ladder. *svb* was used as a positive control in the PCR reaction. Gel image in c) have been cut, for the full gel image see appendix C.

4.1.2 Synthesis of dsRNA to be used in gene knockdown

dsRNA synthesis was performed by *in vitro* transcription using T7 RNA polymerase, using one amplicon produced per gene as DNA template. The chosen amplicons were INR1-set2, INR2-set2, INR1-like-set1, distal-less-set1 and FOXO-set1. A GFP dsRNA synthesis was also included. After DNase I treatment to remove template DNA and purification of the dsRNA, the dsRNA was analysed on a gel to examine the presence of RNA of expected sizes. All RNA products had the expected size when compared to the Run RiboRuler RNA Ladder, High Range (Figure 3). Several vague bands were also visible in most of the lanes, probably consisting of dsRNA segments sticking together. Comparison of starting DNA template quantity and final concentrations of dsRNA can be seen in table 4, where the dsRNA concentrations were measured with both NanoDrop TM 2000 and Qubit. dsRNA concentrations differed a lot between NanoDrop TM 2000 and Qubit, where Qubit showed substantially lower concentrations for all samples (Table 4). Since the two methods utilize different techniques when measuring RNA concentration, different results could be expected, since other components in the samples could interfere with the results. Since Qubit uses fluorescence chemistry, with fluorochromes binding only to RNA, I chose to trust the concentrations derived with Qubit more than NanoDrop. NanoDrop reports nucleic acid concentration from RNA, DNA and free nucleotides, where the presence of proteins also can

contribute to the results, why NanoDrop can present higher dsRNA concentrations than Qubit (Ponti *et al.* 2018).

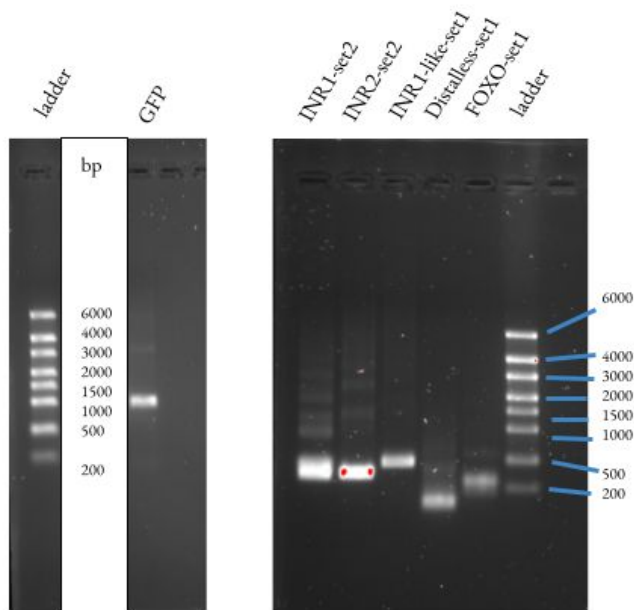


Figure 3. 1% agarose gel electrophoresis results showing dsRNA produced by *in vitro* transcription. Relevant gel lanes is presented in this gel image, for the full gel image see Appendix C.

Table 4. Table showing template DNA concentration for each gene in the *in vitro* transcription reactions. DsRNA concentration after the reactions measured by NanoDrop™ 2000 and Qubit is also included in the table, as well as the calculated total yields.

Gene	DNA template concentration of each PCR product measured with NanoDrop™ 2000	DsRNA concentration measured with NanoDrop™ 2000	Total yield calculated from NanoDrop™ 2000 results	DsRNA concentration measured with Qubit	Total yield calculated from Qubit results
INR1_set1	414 ng	883 ng/μL	44.2 μg	388 ng/μL	19.4 μg
INR2_set1	623 ng	1060 ng/μL	53.0 μg	290 ng/μL	14.5 μg
INR1-like_set1	659 ng	2210 ng/μL	111 μg	378 ng/μL	18.9 μg
distal-less-set2	640 ng	416 ng/μL	20.8 μg	not measured	-
FOXO_set1	540 ng	2850 ng/μL	143 μg	830 ng/μL	41.5 μg
GFP	612 ng	3530 ng/μL	177 μg	716 ng/μL	35.8 μg

4.1.3 Injection of dsRNA in *G. buenoi* to induce gene silencing of target genes

Prior to the injection of dsRNA, the injection protocol was optimised on several different water strider species, including *G. buenoi*. First, an injection experiment was performed on *Aquarius najas* in the thoracic region using microneedles attached to a Hamilton Syringe. An injection volume of 0.5 to 2 μL was evaluated. The result showed that a maximum volume of 1 μL could be injected. When trying a larger injection volume, the water started to leak out from the hole made by the needle. Microneedles attached to a Hamilton Syringe did not work successfully on *G. lacustris* and thus Cell Tram oil was used instead. Several injection positions and needle thicknesses were evaluated. Needles were filled with liquid by sucking up liquid through the needle tip. It was not possible to inject *G. lacustris* with water, while after a performed injection all liquid leaked out from the puncture hole. A test was performed to make sure the needle was not under low pressure. After filling the needle with liquid, the needle tip was dipped into water. The needle should not proceed to be filled with water if the pressure was adjusted. When adapting this procedure before injection, the injections were still not possible.

4.2 Evaluating differential gene expression between water striders living in 12h and 18h photoperiod

Instead of proceeding with the optimisation of the micro injections, differential gene expression between *G. buenoi* reared in the two photoperiods was conducted. If the insulin receptor genes and *FOXO* would have a role in wing polyphenism, the gene expression between water striders reared in the different photoperiods could potentially be different. Therefore, a gene expression analysis between *G. buenoi* kept in 12h and 18h day length was performed, comparing target gene mRNA levels between the photoperiods.

4.2.1 Total RNA extraction of *G. buenoi*

Total RNA extractions were performed on nymphs of instar 1 to 5 (developmental stages) from the different photoperiods (12 and 18 hours). The number of insects used in each sample can be seen in Table 5. Several insects were used in the RNA extractions to make sure enough RNA was extracted for subsequent experiments. After extraction, a DNase I treatment was performed on all RNA samples, but prior to this an RNA integrity analysis was performed. This analysis showed sharp 18S and 28S rRNA bands for all samples (Figure 4 and 5).

However, a lot of smearing were visible in all samples (Figure 4). When treating the samples with DNase I, a lot of smear, that could potentially be genomic DNA contaminations, were still left, (Figure 6). When performing RT-qPCR, it is important not to have DNA contaminated sample since DNA contaminated samples can interfere with the expression analysis.

Table 5. Number of insects used in total RNA extractions. I1-I5 stands for instar 1 to 5.

Instar stage	Photoperiod	Number of individuals used
I1	12	10
	18	10
I2	12	5
	18	4
I3	12	5
	18	5
I4	12	5
	18	5
I5	12	1
	18	1

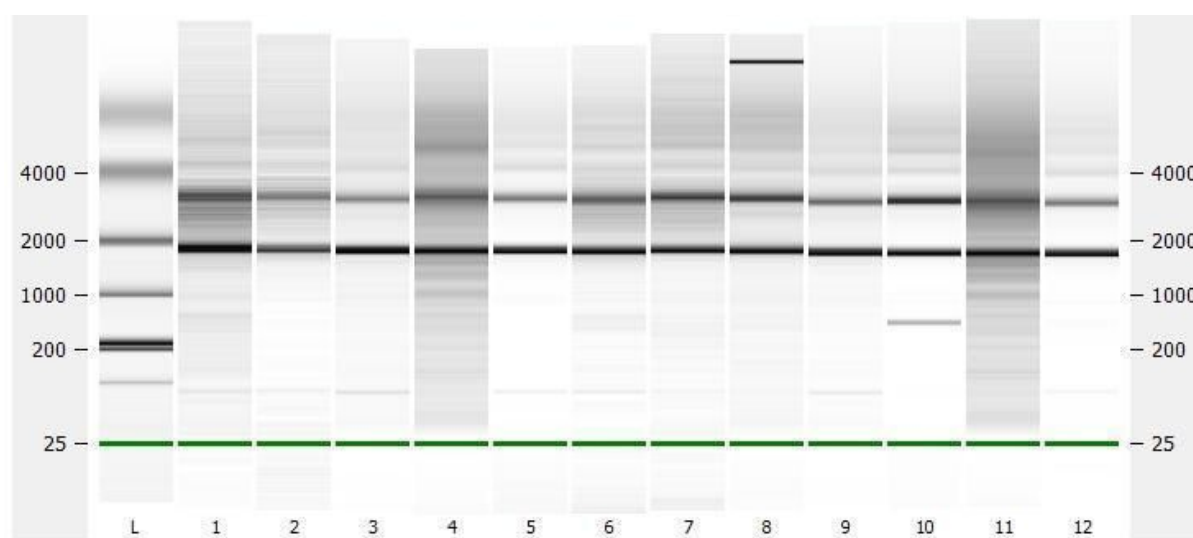


Figure 4. Gel picture of RNA integrity analysis using Agilent 2100 bioanalyzer before DNase I treatment. Abbreviation L stands for ladder. Sample 1- 5 (sample of instar 1 to 5) contains total RNA extracted from the photoperiod of 12 h, sample 6-10 (sample of instar 1 to 5) contains total RNA extracted from the photoperiod of 18 h, and sample 11 and 12 are replicates of sample 4 and 5. The size of the bands is measured in nucleotides.

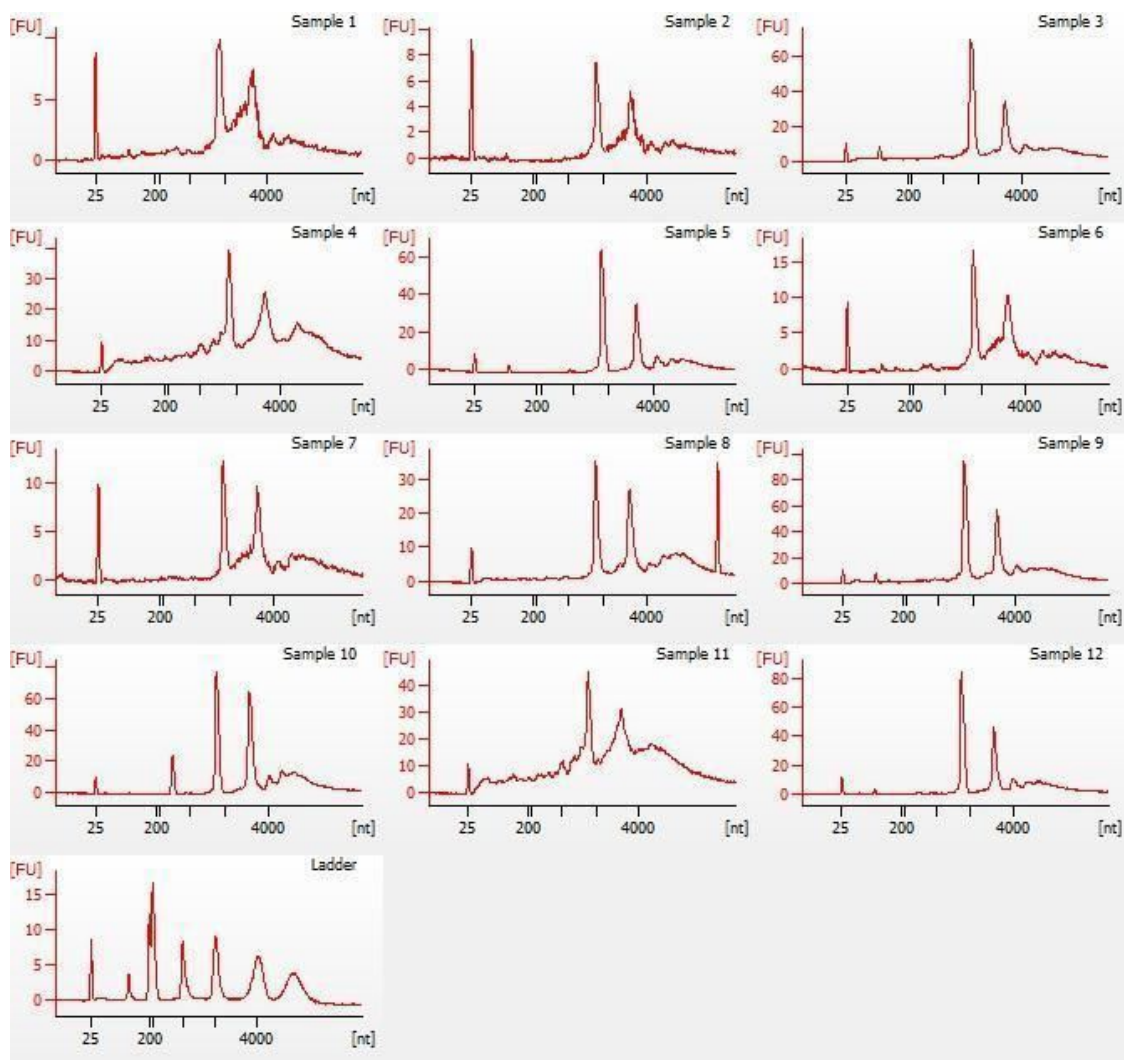


Figure 5. Electropherogram from RNA integrity analysis using Agilent 2100 Bioanalyzer before DNase I treatment. Sample 1- 5 (sample of instar 1 to 5) contains total RNA extracted from photoperiod of 12 h, sample 6- 10 (sample of instar 1 to 5) contains total RNA extracted from photoperiod of 18 h , and sample 11 and 12 are replicates of sample 4 and 5.

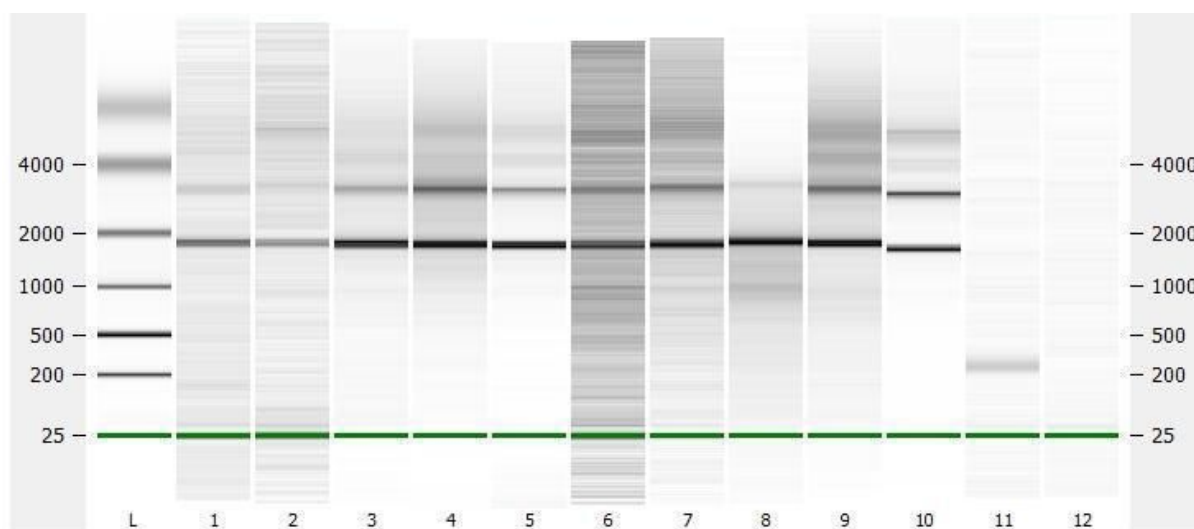


Figure 6. Gel picture of RNA integrity analysis using Agilent 2100 bioanalyzer after DNase I treatment. Abbreviation L stands for ladder and nt stands for nucleotides. Sample 1- 5 (sample of instar 1 to 5 nymphs) contains total RNA extracted from treatment 12 h daylight and sample 6- 10 (sample of instar 1 to 5) contains total RNA extracted from treatment 18 h daylight. The size of the bands is measured in nucleotides. Sample 11 and 12 does not belong to this experiment.

4.2.2 Differential gene expression on 5 instar nymphs using RT-qPCR

The differential gene expression experiment proceeded with already extracted total RNA from 5th instar male nymphs from the different photoperiods. Total RNA had prior been extracted with Trizol extraction of 3 biological replicates from each photoperiod. RNA integrity and DNA contamination analyze results of the RNA can be seen in Appendix D.

To construct a cDNA library, cDNA synthesis was performed on each biological replicate using Oligo (dT)18 primers. Prior to synthesis, the quantity of RNA introduced in the cDNA synthesis was normalised between all replicates, by adding the same amount of RNA to all reaction tubes. Three control reactions were also included in the experiment; no template, no reverse transcriptase -, and positive control (GAPDH) included in the kit. cDNA synthesis experiments were run twice, where the first try did not include any disruption of secondary structure of the template RNA. In the second run, after mixing template RNA with primers and water, a 5 minutes incubation time in 65°C was conducted on all samples to disrupt secondary structures. Incubation temperature for the cDNA synthesis at the second run was also raised 3°C compared to the first run temperature, where 42°C was used. The created cDNA library was then used for RT-qPCR experiments.

Table 6. All RT-qPCR primers used in this project

Gene	GeneID		Forward	Reverse	Amplicon length [bp]
Insulin receptor 1	GBUE020 810-RA	qPCR Primer set	TAAGGTTGAGAAGC TAATGTGGC	TTCGTTGGATGG CACCTGAAT	112
Insulin receptor 2	GBUE021 108-RA	qPCR Primer set	ATCACAGGTGTTGG TGCAGAC	GAAGCAGAATGC AACGAGCAAT	111
Insulin receptor 1-like	GBUE019 859-RA	qPCR Primer set	ACTTACGTGTACGC AGGGTG	CCTGCTCGTGGT AACCGAAT	136
FOXO	GBUE021 071-RA	qPCR Primer set	GTGGGTAGGCGAAA CCAGAG	GCAGCCTCGAAG AGACGATG	132
Ribosomal 18s rRNA reference gene	Scaffold 1750	qPCR Primer set	CGCTACTACCGATT GAA	GGAAACCTTGTT ACGACTT	129

Two qPCR experiments were performed, where the qPCR primers used can be seen in Table 6. The purpose of the first experiment was to determine the amplification efficiency (AE) of the target genes and compare that AE to the chosen reference gene for in-between sample normalization. Ten-fold cDNA template dilution series (1:1 to 1:10 000) were performed for all genes (INR1, INR2, INR1-like, FOXO and Ribosomal 18s rRNA) in replicates of three. Further 1:1 template dilution was performed on the three controls from the cDNA synthesis. For each gene, a plot comparing the Cq values for each dilution and the log template concentration was constructed, and the slope was calculated (Figure 7).

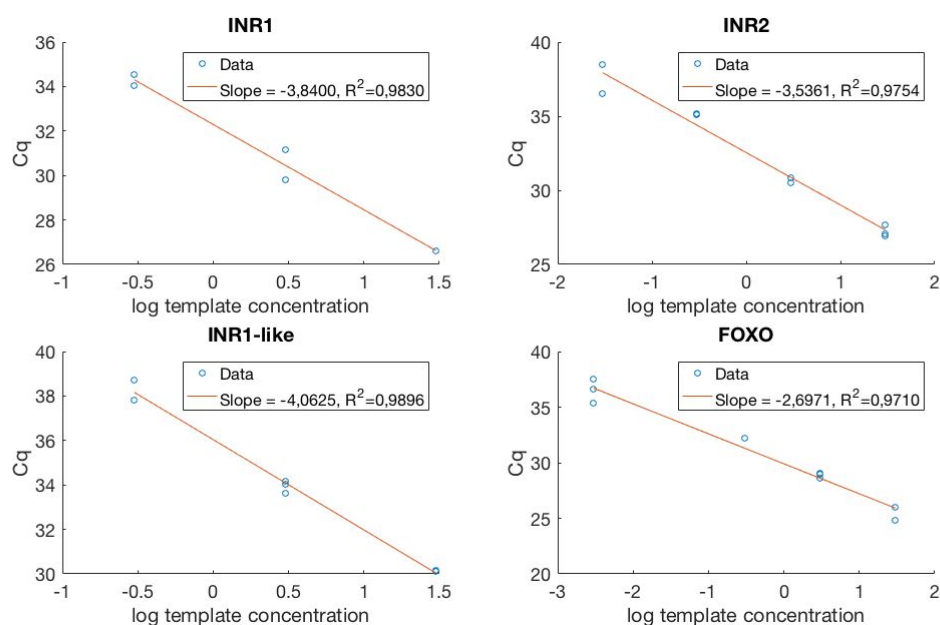


Figure 7. Standard curves for a) INR1, b) INR2, c) INR1-like and d) FOXO

The AE was then estimated for each gene (Table 7), where an accepted AE range is between 90-110%. AE for INR2 was qualified to be in accepted range. To investigate whether multiple PCR products have been formed during the thermal cycling or if primer-dimers or self-dimers were formed, an agarose based gel electrophoresis was run (Figure 8). Formation of primer-dimers, self-dimers or multiple PCR products would lead to mis-interpreting in the RT-qPCR since SYBR green binds to all dsDNA in the reactions. INR1 and INR2 amplicon should span segments of 112 respectively 111 bp, and showed broad clear bands between 100 and 200 bp. INR1-like and FOXO amplicons should span segments of 136 bp respectively 132 bp and showed broad clear bands near 200 bp in the gel. No presence of primer-or self dimers were visible in the gel.

Table 7. Table showing the AE for the different target genes. AE for INR2 was qualified to be in accepted AE range (90-110%).

Gene	AE [%]
INR1	82.1
INR2	91.8
INR1-like	76.3
FOXO	135

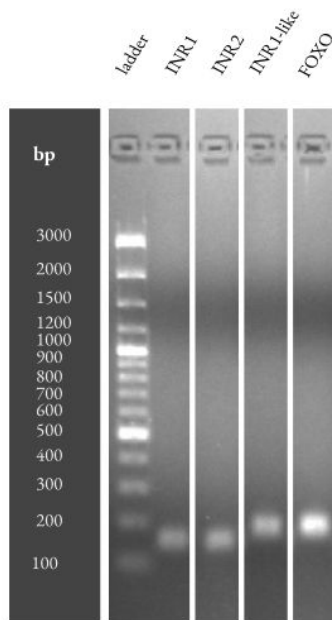


Figure 8. Gel electrophoresis result for the first qPCR run. The 1:10 dilutions are analysed in these lanes, from left, GeneRuler 100 bp Plus DNA Ladder, INR1, INR2, INR1-like and FOXO. Relevant gel lanes is presented in this image, for the full gel image see Appendix C.

The reference gene had no amplification, so the RT- qPCR experiment was repeated. In the second attempt, the second round of produced cDNA was used instead of the first, where secondary structures had been reduced by heat denaturation. Since no amplification of the reference gene was visible in none of the dilutions in the first qPCR experiment, a second try with the same dilution series as last was performed for Ribosomal 18s rRNA gene. Further, in this experiment, cDNA from all 3 biological replicates from each photoperiod was analysed, targeting the INR1, INR2, INR1-like and FOXO genes. Three technical replicates for each reaction was used and the chosen dilution for all reactions was set to 1:10. As previous, all controls from the cDNA synthesis were included in the experiment. Neither “no template” nor “reverse transcriptase” controls showed any amplification in the first or the second run, meanwhile the positive control showed amplification. The reference gene had no amplification in the second run either, meaning no sample normalisation could be performed. Nevertheless, relative expression was calculated for the targets, comparing mRNA levels of each target between the 18h and 12h photoperiod.

Relative gene expression was calculated according to Livak & Schmittgen (2001) using the relative $\Delta\Delta C_t$ method. For each target three biological replicates were used together with three technical replicates of each. The mean C_t value of the technical replicates was first calculated. Thereafter the mean C_t value of the three biological replicates were calculated

together with the standard deviations. If normalise against a reference, the target Ct value is subtracted by the reference CT value, according to the equation,

$$\Delta Ct = Ct_{\text{target gene}} - Ct_{\text{reference gene}}$$

Thereafter, the $\Delta\Delta Ct$ value is calculated by,

$$\Delta\Delta Ct = \Delta Ct_{\text{target sample}} - \Delta Ct_{\text{calibration sample}}$$

Since no normalisation step was done, the $\Delta\Delta Ct$ value was calculated directly according to,

$$\Delta\Delta Ct = Ct_{18h} - Ct_{12h}$$

The fold change was then calculated according to

$$FC = 2^{\Delta\Delta Ct},$$

where the standard deviation was incorporated in the fold change, and is presented between brackets (Table 8).

Potential result for *G. buenoi* reared in 18h photoperiod, would be to have an upregulation of the gene FOXO compared to 12h photoperiods reared *G. buenoi*. The hypothesis is based on that Xu *et al.* (2015) saw that a high INR1 signalling activity inhibited FOXO, which led to long wings. Therefore FOXO, could be upregulated in the 18 h photoperiod, in the short-winged morph. Calculations from RT-qPCR demonstrated an upregulation of FOXO in the 18h photoperiod reared *G. buenoi*, which was shown by a fold change of 1.53 (1.31-1.80) (Table 8).

Difference in gene expression between INR1, INR2 and INR1-like could be seen as well when evaluating the RT-qPCR data. According to Xu *et al.* (2015), INR2 has a suppressing effect on INR1, leading to activation of FOXO, inducing short-winged morphs. INR2 would therefore potentially be upregulated in short-winged 18h photoperiod reared *G. buenoi*. The RT-qPCR results showed an upregulation of INR2 in the 18h photoperiod, with a fold change of 1.25 (1.12-1.39). A slight downregulation of INR1 with a fold change of 0.56 (0.38-0.82) could be seen in the short-winged morphs (18h photoperiod). INR1-like had instead an upregulation in the short-winged morphs (18h photoperiod), with a fold change of 1.93 (1.31-1.80) (Table 8).

Table 8. Calculated fold changes between photoperiod 12h and 18h for INR, INR2, INR1-like and FOXO.

Gene	Group	Average Ct of three biological replicates used	S.D	$\Delta\Delta Ct = Ct_{18h} - Ct_{12h}$	Fold difference of 18h relative 12h photoperiod $FC = 2^{\Delta\Delta Ct}$
INR1	12h	28.92	2.03	0.8361	0.56 (0.38-0.82)
INR1	18h	29.75	0.55	0.8306	0.56 (0.38-0.82)
INR2	12h	30.67	0.93	-0.3202	1.25 (1.12-1.39)
INR2	18h	30.35	0.16	-0.3202	1.25 (1.12-1.39)
INR1-like	12h	35.67	2.02	-0.9506	1.93 (1.31-2.85)
INR1-like	18h	34.72	0.56	-0.9506	1.93 (1.31-2.85)
FOXO	12h	29.41	0.70	-0.6202	1.53 (1.31-1.80)
FOXO	18h	28.79	0.23	-0.6202	1.53 (1.31-1.80)

5 Discussion

5.1 RNAi experiment

Xu *et al.* (2015) proposed a model for wing polyphenism in brown planthopper, where the insulin receptor genes and FOXO were proposed as regulator genes. In this project, an approach to perform gene-knockdown experiments in the water strider species, *G. buenoi* has been conducted, using RNAi against the target genes INR1, INR2, INR1-like and FOXO.

dsRNA was successfully synthesized for INR1, INR2, INR1-like and FOXO and injection attempts in *G. buenoi* were performed with Cell Tram Oil. When performing injections, all the injected liquid leaked out from the puncture hole and therefore no gene-knockdown could be performed during this project. Further optimisation work has to be done to be able to inject the dsRNA into *G. buenoi*. Since the liquid leaked out when removing the needle, the hole created by it could have been too big, meaning a too thick needle could potentially be the error. If using a finer needle, which would just create a small hole, together with a very slow

injection speed, injection could potential have worked. Better adjustment of pressure, or the use of other injection positions are other optimisation possibilities.

5.2 Differential gene expression in instar 5 nymphs

A second approach to investigate the insulin genes and FOXO potential role in plastic wing polyphenism was also performed, where differential gene expression between the two photoperiods were evaluated. When doing RT-qPCR on total RNA extracted from 5th instar nymphs, fold-change between the mRNA levels between the two photoperiods could be calculated for each gene.

The first question to evaluate was, whether there were differences between the target genes mRNA levels when comparing the two photoperiods. The next question would then be to see in which photoperiod there is a down- or up regulation, and whether it is consistent with Xu *et al.*'s (2015) results. The mRNA levels of INR1, INR2, INR1-like and FOXO were quantified during this experiment, comparing mRNA levels between 18h and 12h photoperiods. Two qPCR experiments were performed. In the first experiment, dilution series of different template concentrations were performed for each target to gain standard curves and to assess the slopes for each target. An optimal slope is -3.33, which gives an AE of 100%. INR2 had an AE of approximately 92%. The other targets were too high or too low. Potential deviation for not having an AE in the accepted range of 90%-110% could be due to pipetting errors during the RT-qPCR set up. Every part in the RT-qPCR reaction was pipetted separately and no master mix was performed meaning this could potentially have impacted the results. Another potential error could be the presence of PCR inhibitors in the template samples (Bar *et al.* 2012). The reference gene gave no amplicon at all, neither in the first experiment nor the second, even though secondary structures in the RNA had been taken care of. The explanation for this is that only Oligo-dT primers were used in the RT-reaction, which do not reverse transcribe rRNA since it does not contain any poly(A)-tail (Jain *et al.* 2006). The use of randomized hexamers would have amplified rRNA. Instead of using an rRNA based reference gene, a mRNA based reference gene would have been preferred instead.

No accurate or reliable result is possible without the use of one or several reference genes. The reference gene has the potential to eliminate variations in cDNA concentration between samples. Studies have shown that, even though using a common reference gene, significant variations could occur during normalisation, since the reference is really relying on stable expression. Several potential reference genes should optimally be evaluated by for example GeNorm and a GeNorm pilot study (Hellemans *et al.* 2014). Even though normalisation against a reference gene was left out, RT-qPCR calculations were performed. These calculations could still give an approximation about in which direction the results from an

additional experiment would have. Further, results showing both up- and down regulations would imply real differences between the two photoperiods.

Calculations from RT-qPCR demonstrated an upregulation of FOXO in the 18h photoperiod reared *G. buenoi*. This result means a similar potential regulation mechanism as in BPH, using FOXO as an inhibitor for wing development could be present in *G. buenoi*. Further, the calculated upregulations of INR2 and downregulation of INR1 in the 18h photoperiod reared *G. buenoi*, was shown to be in line with the regulation patterns seen in BPH by Xu *et al.* (2015). Results also showed that INR1-like had an upregulation in the short-winged morphs (18h photoperiod). Differential gene expression of INR1-like, between the two photoperiods, could potentially mean INR1-like have a role in wing polyphenism in *G. buenoi*. Knock-down of the gene, which was intended to be done in this project, could have shown if the INR1-like gene has a crucial part in the molecular mechanism involved in wing polyphenism.

If these results are significant, it could mean that this mechanism potentially is conserved in the order Hemiptera, and could be present in several wing polyphenic insect species. An RNA extraction was performed on instar 1 to 5 nymphs, with the purpose to perform RT-qPCR experiments in the different developmental stages. Information regarding if and when the target gene's expression start to deviate between the photoperiods would have showed when the wing morph determination takes place.

Together with gene knock-down of the target genes, answers regarding the more precise function of the genes could in the future be found. Information regarding the mechanism behind wing polyphenism sheds light on the role of phenotypic plasticity in evolution.

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8 Supplements

8.1 Appendix A - Important resources

Table A-1. Table containing resources used during the project, with source and catalogue number if present.

Resource	Source	Identifier
Genome		
GBUE_BCM_assembly_v1-0	Armisen <i>et al.</i> (2018)	
GBUE_OGSv1	Armisen <i>et al.</i> (2018)	
Commercial kits		
NucleoSpin DNA Insect	MACHEREY-NAGEL	REF#740470.10
Phusion High-Fidelity DNA Polymerase	ThermoFisher Scientific	Cat# F530S
GeneJET Gel extraction and DNA Clean Up Micro kit	ThermoFisher Scientific	Cat#K0831
GeneJET RNA Purification Kit	ThermoFisher Scientific	Cat#K0731
TranscriptAid T7 High Yield Transcription Kit	ThermoFisher Scientific	Cat#0441
RevertAid First Strand cDNA Synthesis Kit	ThermoFisher Scientific	Cat#K1622
Luminaris Color HiGreen Master Mix	ThermoFisher Scientific	Cat#K0391
Technical supply		
NanoDrop™ 2000 Spectrophotometers	Thermo Scientific	
Qubit Fluorometric Quantification	Thermo Scientific	
Agilent 2100 Bioanalyzer	Agilent	
CFX96 Touch Real-Time PCR Detection System	BIO-RAD	
Flystuff (59-122BCU) Benchtop Flow Buddy System with Ultimate Fly Pad and Gun		

8.2 Appendix B - PCR

Table B-1. 50 μ L PCR reactions were carried out using the following composition.

Chemical	Volume	Final concentration
H ₂ O	up to 50 μ L	-
5X Phusion HF Buffer	10 μ L	-
10 mM dNTPs	1 μ L	200 μ M each
10 mM forward primer	2.5 μ L	0.5 μ M
10 mM reverse primer	2.5 μ L	0.5 μ M
Template DNA	2 μ L	around 40 ng/ μ L
Phusion DNA Polymerase	0.2 μ L	0.02 U/ μ L

Table B-2. Following PCR setting was used during PCR

Step	$^{\circ}$ C	time	cycles
Denaturation	98.0	0:30 s	1
Denaturation	98.0	0:10 s	33
Annealing	see each genes An.	0:30 s	
Extension	72.0	0:30 s	
Final extension	72.0	10:00 min	1

8.3 Appendix C - full gel images

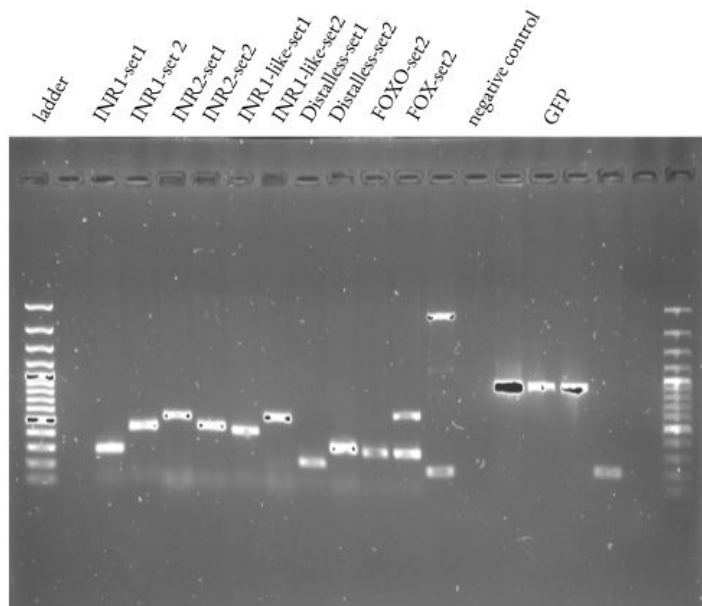


Figure C-1. Uncut gel image of the gel electrophoresis analysis of PCR products.

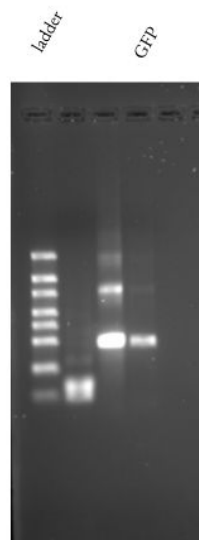


Figure C-2. Uncut gel image of the gel electrophoresis analysis of dsRNA products.

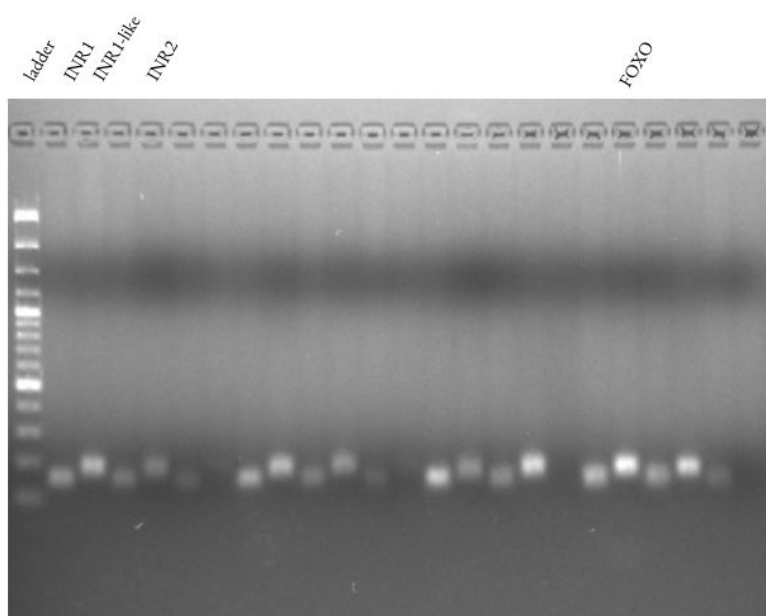


Figure C-3. Uncut gel images from gel electrophoresis analysis of RT-qPCR products.

8.4 Appendix D - Agilent 2100 Bioanalyzer

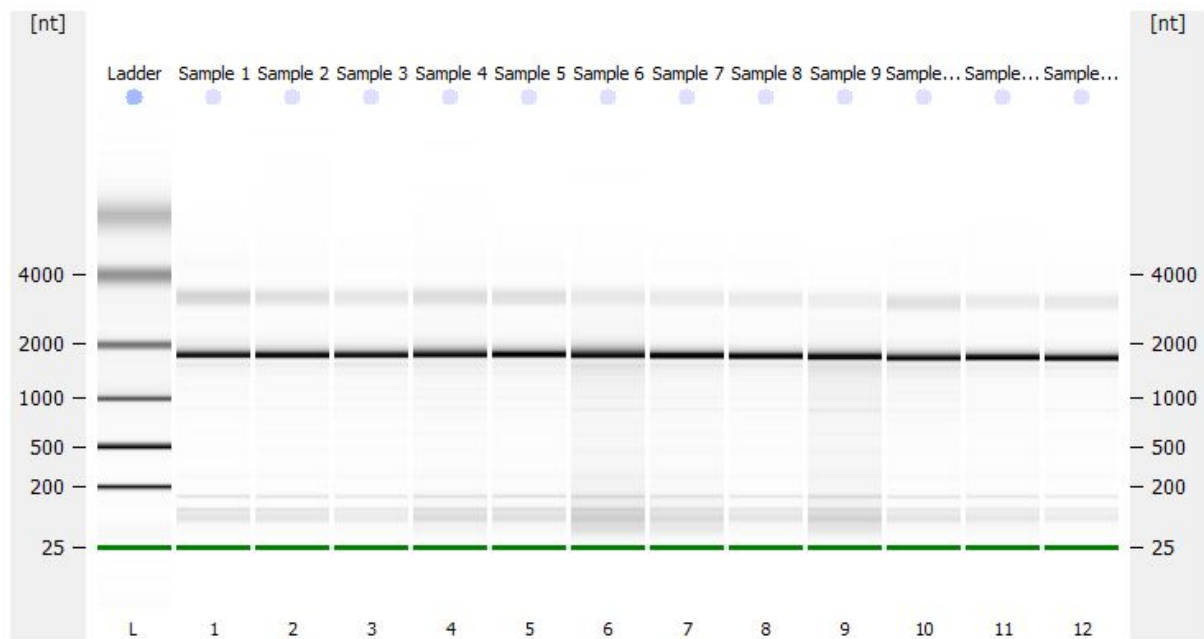


Figure D-1. Gel picture of RNA integrity analysis using Agilent 2100 bioanalyzer. L stands for ladder and sample 1-12 are 12 biological replicates. Biological replicate 1, 3, 8, 6, 13 and 15 were used in this study. Replicates 1, 3 and 8 belong to treatment 12 h daylight and replicates 6, 13 and 15 belong to treatment 18 h daylight.

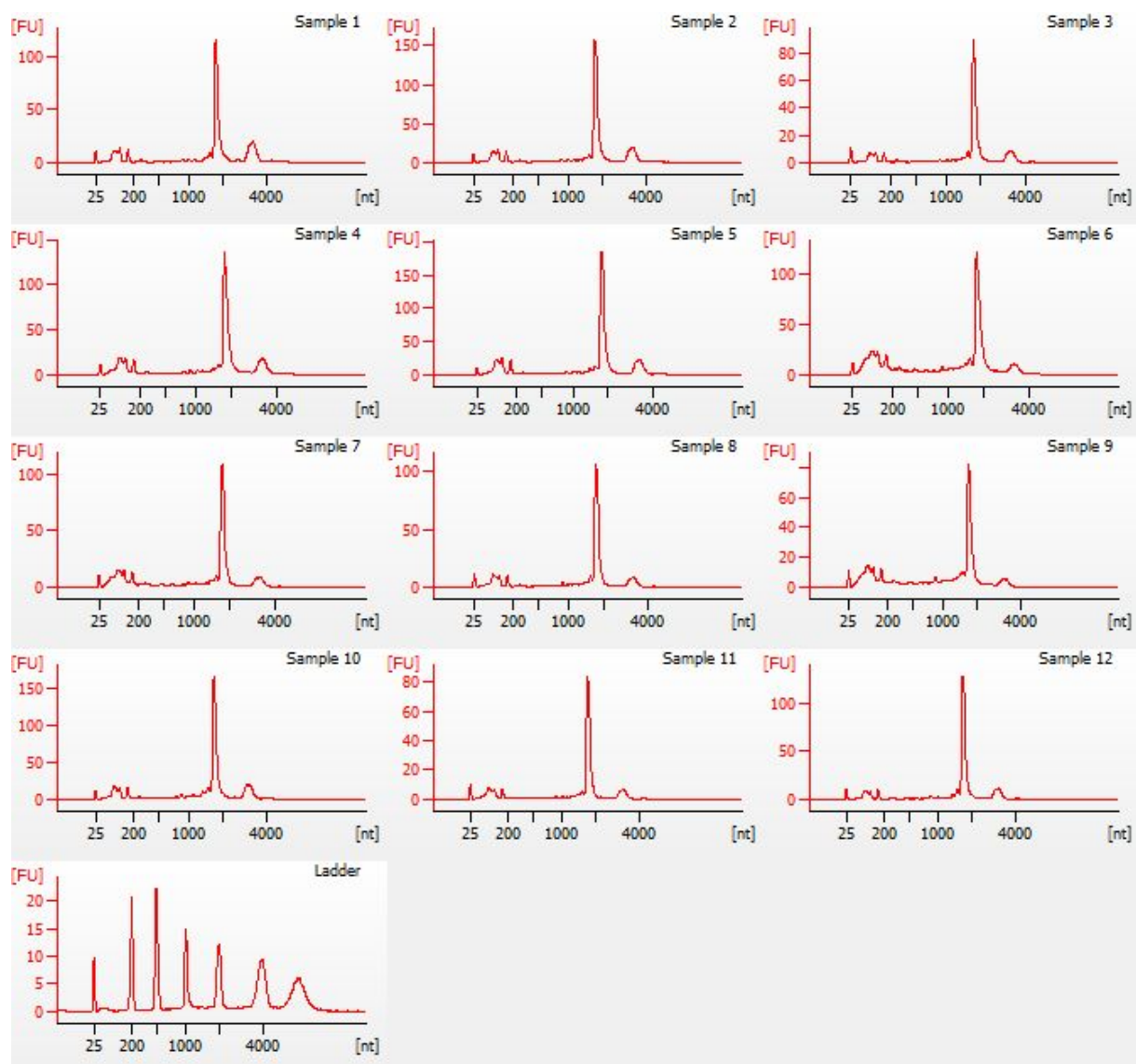


Figure D-2. Electropherogram from RNA integrity analysis using Agilent 2100 Bioanalyzer. Biological replicate 1, 3, 8, 6, 13 and 15 were used in this study. Replicates 1, 3 and 8 belong to treatment 12 h daylight and replicates 6, 13 and 15 belong to treatment 18 h daylight.