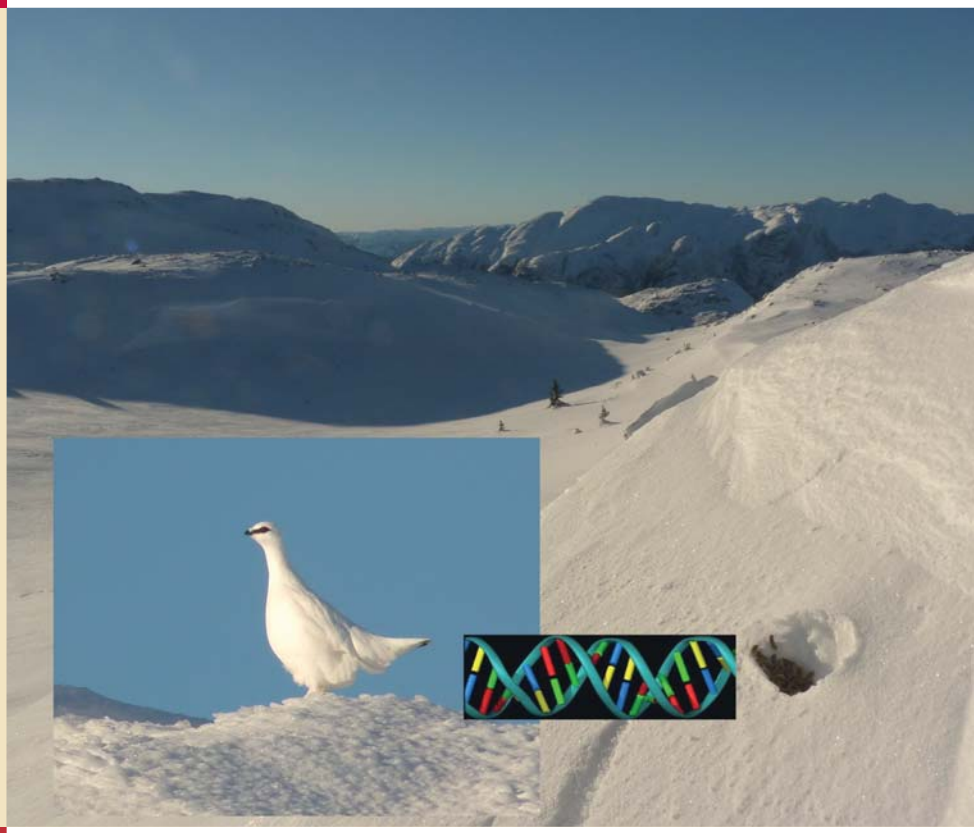
Master Thesis

Frode Bergan

Evaluation of in-field DNA degradation of mitochondrial- and genomic DNA in snowsampled faecal pellets from rock ptarmigan *(Lagopus muta)*



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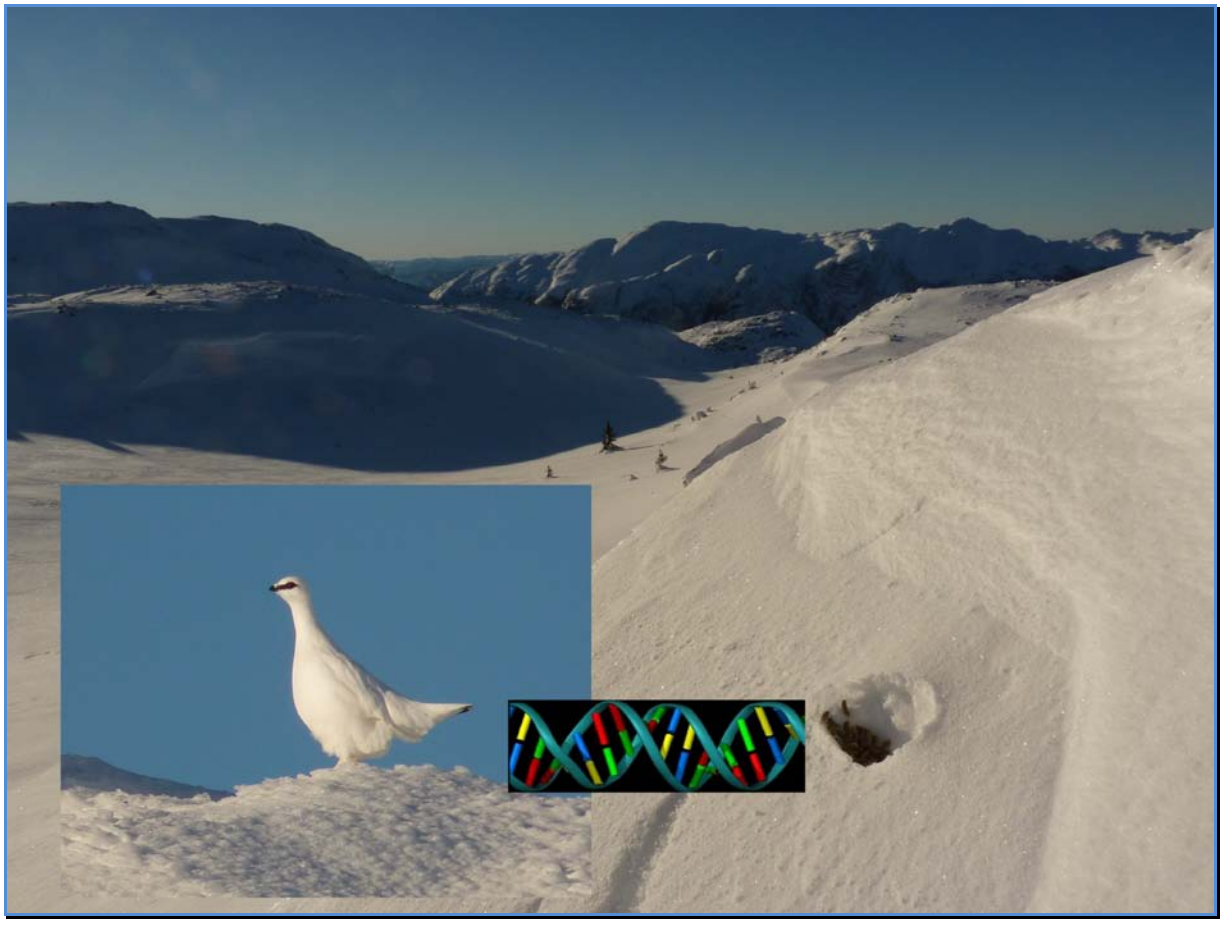

Høgskolen i Telemark
Fakultet for allmennvitenskapelige fag

Høgskolen i Telemark 2011
Fakultet for allmennvitenskapelige fag
Institutt for natur-, helse- og miljøvernfag
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Trykket ved Høgskolens kopisenter i Bø
Omslagsfoto Frode Bergan

Preface

This master thesis is a result of my study from 2009 to 2011 at Telemark University College, Department of Environmental and Health Studies were I also am employed as an engineer. I plan to submit this thesis to the journal *Conservation Genetics* for publication and have therefore followed this journal´s style througout the thesis. This study was performed on a limited budget. Therefore, multiple analyses of both DNA extraction and genotyping, which are quite costly, were not performed, and my results may be conservative when it comes to duration and validity of FP DNA.

I would like to thank my supervisors Howard Parker and Mona Sæbø for their inspiration and excellent guidance. I would also like to thank Harald Klempe for providing with GIS expertise, Per Christian Hagen for statistical help as well as Inger Hanssen-Bauer for help with the meteorological data. Thanks to Elin Marie Hasti Sveinhaug and Nils Henning Melby who helped with the fieldwork and to Live Semb Vestgarden for valuable comments on the manuscript. Thanks to the staff at the University College Library in Bø for supplying me with literature and to Lars Erik Brynjulvsrud for grouse feathers. The head of my department, Tone Jøran Oredalen and my colleagues have been very flexible and supporting. Thank you! Frøydis and Linnéa have been supporting and patient when husband or father have spent much of his spare time collecting shit, doing labwork or writing. Thanks!

Telemark University College, 28. september 2011

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Frode Bergan

Aim of the study

The aim of this study was to evaluate methods for genetic monitoring by duration and degradation of faecal pellet DNA as noninvasive genetic material collected on snow. As target species, I found the rock ptarmigan (*Lagopus muta*) a highly relevant bird to study. First, since it is the world´s most extreme year round alpine and polar bird, and second because rock ptarmigan habitat increasingly is becoming more fragmented in our changing

world. The following methods for collecting and analysing rock ptarmigan DNA have been assessed during the study:

- the practicality of collecting faecal pellets from rock ptarmigan snow roosts from winter to early summer;
- extraction of faecal pellet DNA using three different methods in initial trials and one method in subsequent extractions;
- extraction of DNA from feathers using Qiamp blood and tissue kit;
- measurement of DNA concentration in faecal pellet DNA templates by OD 260 nm spectrophotometry;
- PCR amplification of mitochondrial DNA (mtDNA), and genomic DNA (nDNA);
- evaluation of mtDNA in species determination by conventional gel electrophoresis;
- evaluation of nDNA microsatellite genotyping, and sex dermination by capillary electrophoresis and conventional gel electrophoresis respectively;
- evaluation of the duration of mtDNA and three nDNA microsatellites in faecal pellets from repeated sampling during the study period from week 2 to 27 in 2010;
- monitoring of climatic factors by temperature logging in each snow roost, combined with observations of precipitation data from a nearby weather station.

The thesis is planned to be submitted as a manuscript for the journal *Conservation Genetics*. Hence the style of the journal is followed in general througout the thesis. Allthough all the tasks above were carried out as a natural part in the thesis, the main focus has been to produce a manuscript for the journal with focus on the duration and degradation of faecal pellet DNA.

Evaluation of in-field DNA degradation of mitochondrial- and genomic DNA in snowsampled faecal pellets from rock ptarmigan (*Lagopus muta*)

Frode Bergan

Abstract

The use of non invasive genetic sampling (NGS) have become increasingly relevant in genetic studies applied to ecology. Reliable methods should be tailored to species and environments. Rock ptarmigan (*Lagopus muta*) spend their entire lifecycle in the alpine region and roost in snow holes to save energy and avoid predation. Faecal pellets (FP) were collected from 20 individual rock ptarmigan snow roosts in the Lifjell mountain area in Telemark, Norway repeatedly between 17 january and 7 july, 2010. DNA was extracted from 146 samples, and degradation evaluated in PCR products of mitochondrial DNA (mtDNA), and 3 genomic DNA (nDNA) microsatellites. For mtDNA, the mean duration in 20 roosts was 73.8 and 59.4 days respectively in a best and worst case scenario. For the 3 nDNA microsatellites, mean duration times varied between 38.8 - 58.1 and 20.9 - 45.8 days respectively in a best and worst case scenario. MtDNA had a significantly longer duration than nDNA, and duration times between individual nDNA microsatellites varied significantly. Overall PCR error rates in the study period were 0.48 for mtDNA and 0.53 - 0.68 for 3 nDNA microsatellites. DNA degradation appeared to accelerate considerably when frozen, snow covered FPs eventually became exposed to higher temperatures and precipitation during the spring snow-melt. FPs from rock ptarmigan snow roosts serves as a promising DNA source in genetic studies, and method may be applied to other snowroosting grouse.

Key words: *Lagopus muta*, noninvasive genetic sampling, nuclear DNA, mitochondrial DNA, DNA degradation, snow

Introduction

Genetic studies of ecologically significant traits in wild vertebrates are highly dependent on reliable DNA sources. In elusive or protected species, DNA sources are often obtainable only by noninvasive genetic sampling (NGS) i.e. sampling of DNA from sources left behind by the

animal, thereby eliminating the need for capture (Taberlet et al. 1999). The main sources of NGS are hair, feathers and faeces. The problems involved with genotyping and population genetics based on NGS have recently received considerable focus (Broquet and Petit 2004; Waits and Paetkau 2005).

Field-collected genetic material will, to some extent, always be degraded before DNA is extracted in the laboratory. This degradation may implement allele dropouts (ADO) or false alleles (FA), and should be minimized as much as possible by optimizing sampling and storage techniques and routines. The feasibility of NGS have been studied in avian species by e.g. Regnaut et al (2006) who found that DNA degradation in faeces from capercaillie (*Tetrao urogallus*) was mainly caused by free DNases in the collected material and that considerations regarding sample preservation as well as polymerase chain reaction (PCR) multitube analysis were factors that brought the genotyping reliability up to 98 % in 11 microsatellites.

Two different NGS sources in birds, faeces and feathers have given the same results in sex determination and microsatellite analysis for several avian species (Segelbacher and Steinbruck 2001).

In addition to choice of material for genetic analysis, the duration of DNA in NGS have also been addressed. (Kovach et al. 2003) studied the validity of faecal DNA in three lagomorph species in eastern USA and they obtained high quality DNA, with reduced quality over time from all three species. Seasonal collection of faeces for DNA analysis in ungulates showed that winter samples had longer duration than those collected in spring (Maudet, Luikart et al. 2004).

The rate of DNA degradation in noninvasively collected sources vary with environmental factors and between taxa. Hence it is important to establish species and environment specific methods for NGS that can be applied to small, and/or vulnerable populations (Taberlet et al. 1999; Lucchini et al. 2002).

Rock ptarmigan (*Lagopus muta*) are unusual among arctic and alpine birds in spending their whole lifecycle above treeline from 83°N on Greenland to 38°N in Tadjikistan (Holder and Montgomerie 1993; Watson and Moss 2008). In Norway, the rock ptarmigan altitudinal range overlaps somewhat with that of the closely related willow ptarmigan (*Lagopus lagopus*) that normally breeds at somewhat lower altitudes (Kvasnes et al. 2010). These two species are

known to interbreed in the wild and to produce viable young (Quintela et al. 2010). As many as 22-30 subspecies are described for rock ptarmigan based on morphology (Browning 1979; Storch 2000), though little genetic information exists for these. Presently, the conservation status for rock ptarmigan worldwide according to International Union for Conservation of Nature (IUCN) (http://www.iucnredlist.org/) in 2009 was "least concern" though local populations, as in Japan, may be endangered (Murata et al. 2007). Elevated tree line and changes in the composition of alpine flora have been reported in southern Norway (Odland et al. 2010). An increasingly warmer climate may gradually reduce the area of alpine habitat available to alpine species in general and rock ptarmigan in particular, leading to more fragmented alpine landscapes and more isolated populations. Increased hybridization between rock- and willow ptarmigan may result with increased habitat overlap in a warmer climate (Quintela et al. 2010). A reduced snow cover in arctic and alpine areas (Post et al. 2009) may affect the winter survival of rock ptarmigan which highly depend upon a sufficient snow layer in which to build snow roosts for insulation (Watson and Moss 2008). Both climate change and increased human activity may lead to reduced rock ptarmigan population sizes in alpine areas (Watson and Moss 2004). Smaller populations may in turn lead to increased sub-population isolation, genetic drift, and inbreeding. Under such possible threats it seems appropriate to be one step ahead and develop methods useful in genetic and genomic conservation (Ouborg et al. 2010).

Rock ptarmigan roost in the snow whenever possible to reduce heat loss and predation (Watson and Moss 2008). While in the roost, birds usually deposit a pile of faecal pellets (FP) on the bottom (Fig. 1). Roosts that are vacated shortly after snowfall contain day-fresh FPs. The resulting holes and tracks in the snow are readily located at considerable distances by scanning mountain slopes with binoculars. Roosts that become covered with snow, however, can first be located in spring when they emerge as the snow thaw.

The main objective of this study was to investigate whether FPs collected from snow roosts of rock ptarmigan are a reliable source of mtDNA and nDNA, and the durability measured as degradation of FP DNA lying in snow roosts over time. Additionally, the sex determination capability of nDNA extracted from FPs using primers developed for grouse was evaluated as was the impact of temperature and precipitation on DNA degradation. To my knowledge this is the first study to assess validity of DNA extracted from avian faeces collected from snow roosts.

Fig. 1 A recently abandoned rock ptarmigan snow roost containing a small number of faecal pellets. This snow roost was dug in hard-packed snow and only partially covered the bird. A clump of caecal faeces, deposited just after leaving the roost, are visible in the upper picture. (Photo Frode Bergan)

Material and methods

Field sampling

In January and February 2010 recently abandoned roosts with FPs were searched up by crosscountry skiing through an alpine area of approx 3 km^2 within the Lifjell mountains in Telemark county, southeast Norway (N59°30,000´ E8°58,810´) (Fig. 2). Roosts (n = 20) were located shortly after a snowfall to insure that their age was $\leq$ 2 days when first found. All roosts were located between 1020 – 1100 m above sea level (mean = 1073). A roost typically contained 20 to 50 FPs. The minimum distance between sampled roosts was 2 meters, and the maximum 1.6 km.

Roosts typically occured in clusters, and to avoid spreading of FPs and possible mixing with pellets from adjacent roosts, pellets were confined by placing a perforated piece of pvc pipe (length approximately 20 cm, diameter 15 cm) around the FPs in each roost (Fig. 3). The pipe was perforated and painted white to minimize the heating of FPs to above ambient

temperature. Each roost was marked with a thin wooden pole attached to the pipe to enable their location once buried in snow. Roost positions were stored on a handheld GPS recorder. A keytag temperature logger (mod. KTL-108 from Keylog Recorders, The Netherlands) wrapped in beige-colored paper was situated adjacent to the collection of FPs in each pipe (Fig. 3). Temperatures were recorded at 3-hour intervals. In this manner, the date on which each roost collection of FPs emerged upon the snow surface during snow melting, thereby becoming exposed to the elements, could be recorded. This date was indicated by a change from near stable temperatures to clearly alternating temperatures between day and night. Precipitation data were obtained from a standard Norwegian Meteorological Institute weather station located at N59°27,265´ E9°02,007´ - approximately 6 km south of the central study area.

One FP was collected from each of the 20 roosts the first time the roost was discovered between week 2 and 6, and thereafter usually at 2-3 week intervals (depending on the weather) until week 27 of the sampling period. If a roost was covered by more than 50 cm of snow at a later sampling, the roost was not sampled due to the time and labour involved. Roosts covered by less snow were exposed by digging with a spade, and snow replaced after sampling. Pellets were collected randomly by rearranging the pile of FPs at each collection and then simply picking one pellet. FPs were not touched with bare hands and care was taken not to transfer material from one roost to another. FPs were collected in individual 15 ml polycarbonate containers and stored at -20°C prior to DNA extraction performed within one week after collection.

Feathers from 2 willow ptarmigan (*Lagopus lagopus*), 3 rock ptarmigan (*L. muta*), 10 black grouse (*Tetrao tetrix*) and 4 capercaillies (*T. urogallus*) of known sex (determined by plumage or gonadal inspection) were collected to check for correct sexing before possibly applying method to FP DNA.

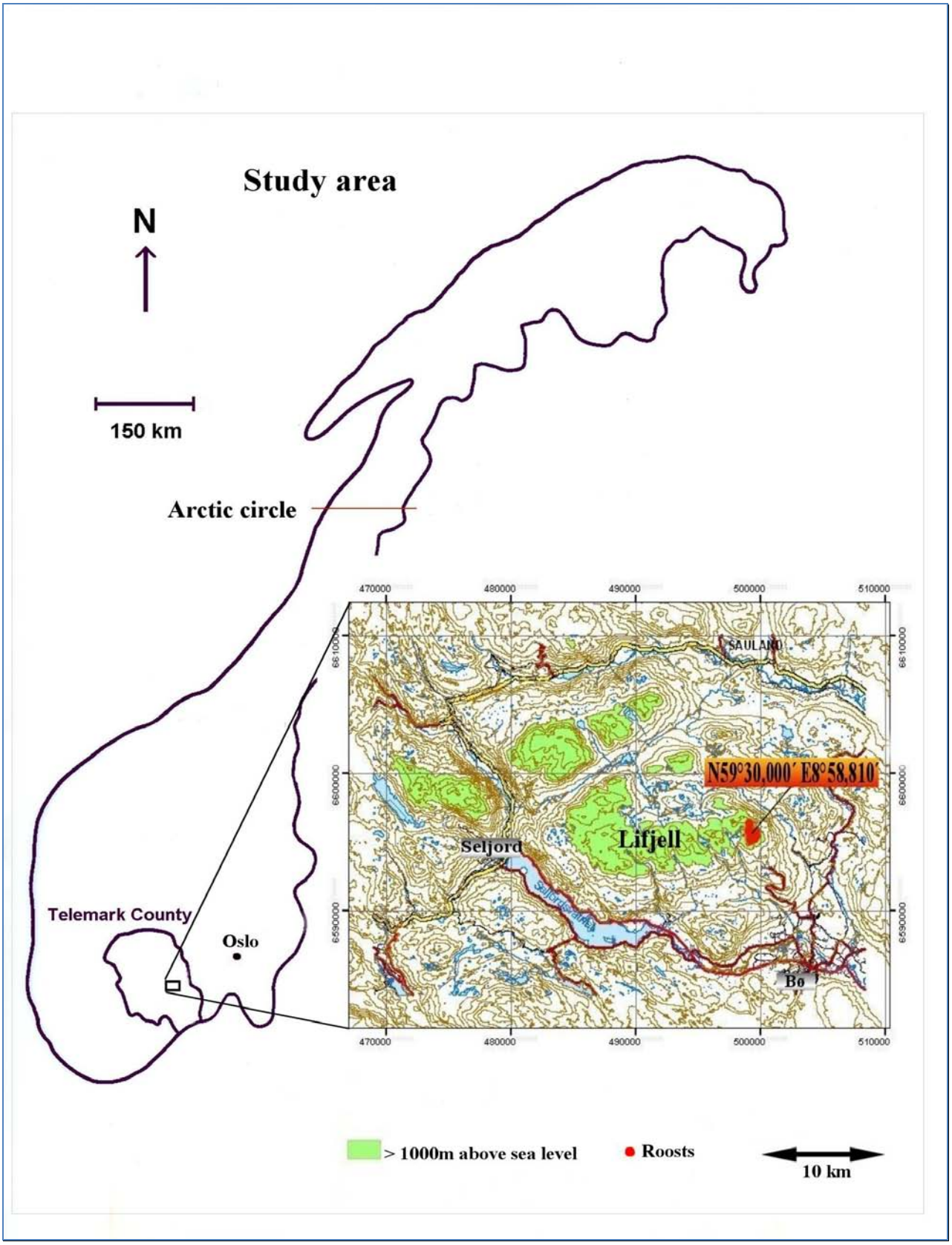

Fig. 2 Map of the Lifjell mountains, Telemark County, southeast Norway showing roost sampling locality.

Fig. 3 The collection of faecal pellets from each roost was arranged in a 20 cm long and 15 cm diameter piece of pvc pipe, perforated and painted white to eliminate over-heating. A temperature logger wrapped in beige-colored paper was situated adjacent to the pipe in order to record when the collection of pellets emerged at the snow surface (indicated by alternating day/night temperature) during the spring snow melt (photo Frode Bergan).

Laboratory methods

DNA from 146 FP samples was extracted using Qiamp® DNA stool kit from QIAGEN. Extraction was performed according to manufacturer´s protocol with minor modifications. A fragment amounting to approximately one third of a pellet was used in each extraction. Weight of fragments was from 60 to 300 mg dependent on water content. The pellet fragment was finely sliced with a small scalpel blade to obtain a homogenous solution with the lysis buffer. DNA was eluated in 200 ul eluation buffer. DNA yield in extracts was found by measuring optical density at 260 nm (OD260) with picodrop spectrophotometer. A standard solution of OD260 50 ng/µl was regularly implemented for calibration. Extractions and DNA yield measurements were performed in a laboratory designed for minimum contamination and DNA degradation risk. FP DNA was also extracted from 21 samples using FastDNA® spin kit for soil and 3 samples using Dynabeads® DNA DIRECT™, but continued extractions were done using only Qiamp® which seemed to give best results. Only results from Qiamp® method are shown.

MtDNA primers designed by Nystrom et al. (2006) were used to assess duration and degradation in mtDNA and to remove possible erroneous sampling of willow ptarmigan roosts. This species specific primer complex consisted of one forward primer; Lagsp3F 5´-CATACATTATGGTACCGGTAC-3´ and the two reverse primers; Lag3R 5´-TGGTGGAC GGTCGATTGTAG-3´ and Mut3R 5´-GGGTAGGCAGGTATTTATAGT-3´. The mtDNA PCR reagents were mixed in a 25 µl volume solution containing 2 µl template DNA, 0.2 mM of each dNTP, 1x PCR gold buffer, 0.4 mM of each primer, 2.5 mM of $MgCl_2$, 0.1 µg/µl of bovine serum albumin (BSA) and 1 unit of enzyme amplitaq gold ld (low yield) polymerase. The amplification was performed in a thermocycler with an initial 10 min denaturation at 94°C followed by 35 cycles of 94°C for 20 s, 56°C annealing for 30 s and 72°C extension for 15 s. Finally, a 72°C extension was performed for 7 min before conservation at 4°C. The amplified products were electrophoresed for 45 min/150 volts in 6 % polyacrylamide gel and stained in 3% GelRed™ from biotium. A standard ladder was applied to gels for sizing of fragments, and gels were exposed to ultraviolet light. Scoring was performed by identifying 212 bp bands for rock ptarmigan or possible 154 bp for willow ptarmigan.

To assess duration and degradation of nDNA three microsatellite loci were amplified; TTT2 and TTD6 (Caizergues et al. 2001), and TUT1 (Segelbacher al. 2000) (Table 1).

Table 1 Description of primers and microsatellites used in assessing nDNA degradation

Locus	Primer sequence (5´-3´)	Repeat
TTT2	**F:** GTGAATGGATGGATGTATGAA	$(GATA)_{11}(GAT)(GATA)_{14}$
	R: GTCTGTCAATGAACTTCTTGG	
TTD6	**F:** GGACTGCTTGTGATACTTGCT	$(CA)_{17}$
	R: CATGCAGATGACTTTCAGCA	
TUT1	**F:** GGTCTACATTTGGCTCTGACC	$(CTAT)_{12}$
	R: ATATGGCATCCCAGCTATGG	

The PCR reagent mix concentration was as for mtDNA above. Each forward primer was marked with fluorescent dye and microsatellites amplified separately at an initial 15 min

denaturation at 95°C followed by 40 cycles of 94°C for 30 s, 60°C annealing for 90 s and 72°C extension for 60 s. Finally, a 72°C extension was performed for 10 min before conservation at 4°C. One µl of each of the amplified samples was mixed with 12 µl Hi-Di™ formamide and 0.5 µl ROX™ standard, briefly centrifuged, and denatured at 94°C. Samples were finally injected on capillary electrophoresis sequencer ABI 3130xl, and Genemapper 4.0 software was used to visualize and size fragments.

To determine the sex of birds from feather DNA as a pretest to possibly evaluate the duration of sexing capabilities in FP DNA, DNA from 19 feather samples was extracted using Qiamp® DNA blood and tissue kit from QIAGEN following manufacturers protocol. Target nDNA was amplified in PCR reactions with two reverse primers TuWR (5´-TAATCAGAGCAA CCTGAATGC-3´) and TuZR (5´- GGAATGTTAACATACTCCTT CACA-3´) designed for capercaillie by Regnaut et al. (2006), and forward primer 2550F (5´- GTTACTG ATTCGTCTACGAGA-3´) (Fridolfsson and Ellegren 1999). The PCR reagent mix concentration was as for mtDNA above. Thermocycler setup was 96°C for 3 min followed by 10 cycles of touch-down from 60°C to 50°C, then followed by 40 cycles of 94°C for 45 s, 53°C for 45 s and 72°C for 45 s. Finally, an extension of 72°C for 7 min and conservation at 4°C was conducted. Amplified products were electrophoresed in 6% polyacrylamide gel as for mtDNA above. Scoring was done by identifying a single z band in males or dual bands wz, in females (Regnaut et al. 2006).

To check for contamination negative controls were implemented both for mtDNA and nDNA PCR´s by replacing template DNA with destilled water in one tube along with all PCR amplification setups.

Data analysis

Genotyping error (GE) in FP DNA from samples collected after the first reference collection from each roost was for mtDNA defined as PCR products with sizes different from 212bp, and for nDNA microsatellites as PCR products with false alleles (FA) or allelic dropouts (ADO). The first roost samplings were regarded as the reference (ref) to which the later samples were compared to when analyzing and evaluating data. As an example, the sampling scheme for roost 1 (Fig. 4) is shown to illustrate the sampling flow. Two scenarios were looked at to evaluate the duration of FPs as a valid DNA source for both mtDNA and nDNA; 1) the minimum number of days from collection of reference FPs to first PCR fail or GE, and

2) the minimum number of days from collection of reference FPs to last consensus sample. "Analyse-it" software was used for statistical analysis. To test for differences in the duration of individual nDNA microsatellite loci, and between mtDNA and nDNA microsatellites, parametric two-way anova was used as values were normally distributed. Other analysis were descriptive.

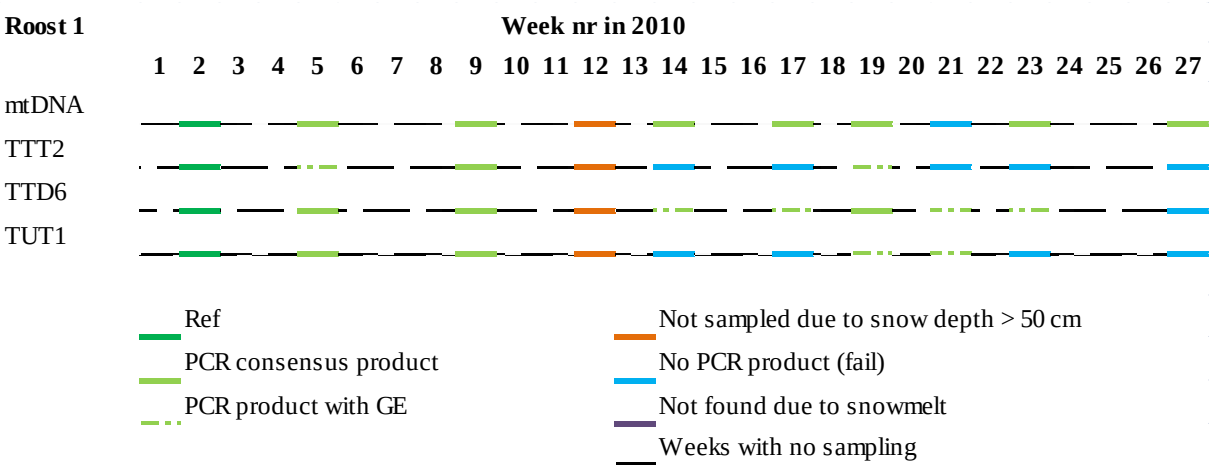

Fig. 4 An example (roost 1) of the sampling scheme recorded for each snow roost showing the duration of mitochondrial DNA and genomic DNA microsatellites in faecal pellets. **Ref =** the first sample from each roost to which all following samples are compared to when evaluating genotype or species identification; **PCR consensus product =** sample gives the same species identification or the same genotype as ref.; **PCR product with GE =** mtDNA with fragment size different from 212 bp or nDNA microsatellites with missing alleles, false alleles or both compared to ref.**; Not sampled due to snow depth > 50 cm =** The roost was visited but no sample removed due to deep snow; **No PCR product (fail) =** no detectable PCR product from mtDNA by conventional gel electrophoresis or nDNA microsatellites by capillary electrophoresis; **Not found due to snow melt =** all snow had melted and no FPs were found; **Weeks with no sampling =** interval between samplings.

Results

Reference sampling

Positive PCR amplifications were obtained from all of the first reference samples except from roost 15 (Appendix a). From this roost caecal faeces were erroneously sampled and hence the second sampling was used as reference.

Species identification

All 20 roosts contained FPs from rock ptarmigan (with fragmentlength 212 bp).

Duration of FP as DNA source

All roost sampling schemes are shown in Appendix a. In both scenarios, mtDNA represented by the species specific primers showed a higher durability than any of the nDNA microsatellite markers (Table 2). The difference, however was only significant between mtDNA and TTT2 (scenario 1: F=13.6 p=0.015; scenario 2: F=7.3 p=0.014. TTT2 had a significantly lower duration than TTD6 and TUT1 in scenario 1 (F=6.3 p=0.0044). For scenario 2 the difference was not significant. Duration of mtDNA and nDNA was highly variable between roosts for both scenarios (Table 3).

Table 2 Summary of duration in faecal pellet mitochondrial DNA and 3 genomic DNA microsatellites from 20 snow roosts of rock ptarmigan, Lifjell mountains, Telemark County, southeast Norway.

		Minimum number of days to first PCR fail or GE[1)] (scenario 1)		Minimum number of days to last consensus sample (scenario 2)	
		(Range	mean ± SD)	(Range	mean ± SD)
mtDNA		0-128	59.4 ± 40.6	0-170	73.8 ± 47.4
nDNA	TTT2	0-77	20.9 ± 27.7	0-122	38.2 ± 40.4
	TTD6	0-111	44.1 ± 34.8	0-137	58.1 ± 13.9
	TUT1	0-111	45.8 ± 31.7	0-137	52.7 ± 40.3

1) GE (genotyping error) = For mtDNA: PCR product with fragment size different from 212 bp. For nDNA: False allele (FA) or Allelic dropout (ADO).

Table 3 Summary of FPs genomic DNA duration (n = 3 microsatellites pooled) for 20 rock ptarmigan roosts, Lifjell mountains, Telemark county, southeast Norway.

	Minimum number of days to first PCR fail or GE[1)] (scenario 1) (Range mean ± SD)		Minimum number of days to last consensus sample (scenario 2) (Range mean ± SD)	
Roost1	0-47	31.3 ± 27.1	15-11	60.0 ± 52.7
Roost 2A	0-15	10.0 ± 8.7	0-15	10.0 ± 8.7
Roost 2B	15-15	15.0 ± 0.0	15-15	15.0 ± 0.0
Roost 3	0-47	20.7 ± 24.0	0-47	20.7 ± 24.0
Roost 4	0-15	5.0 ± 8.7	0-15	5.0 ± 8.7
Roost 5	15-82	37.3 ± 38.7	15-82	37.3 ± 38.7
Roost 6	76-111	99.3 ± 20.2	111-122	114.7 ± 6.4
Roost 7	0-41	16.7 ± 21.5	0-122	54.3 ± 62.1
Roost 8	9-76	53.7 ± 38.7	76-137	96.3 ± 35.2
Roost 9	41-76	64.3 ± 20.2	41-76	64.3 ± 20.2
Roost 10	9-76	53.7 ± 38.7	76-76	76.0 ± 0.0
Roost 11	41-41	41.0 ± 0.0	41-137	73.0 ± 55.4
Roost 12	0-78	40.7 ± 39.1	0-119	54.3 ± 60.2
Roost 13	0-0	0.0 ± 0.0	0-0	0.0 ± 0.0
Roost 14	0-36	19.3 ± 18.1	0-58	31.3 ± 29.3
Roost 15	36-78	56.7 ± 21.0	36-78	56.7 ± 21.0
Roost 16	0-92	37.7 ± 48.2	57-115	88.0 ± 29.2
Roost 17	0-77	51.3 ± 44.5	0-77	51.3 ± 44.5
Roost 18	22-77	58.7 ± 31.8	22-77	58.7 ± 31.8
Roost 19	0-56	25.7 ± 28.3	0-56	26.0 ± 28.8

1) GE (genotyping error) = For mtDNA: PCR product with fragment size different from 212 bp. For nDNA: False allele (FA) or Allelic dropout (ADO).

Overall PCR fails and error rates

The TTT2 microsatellite stands out as the nDNA marker with the highest degradation with a PCR fail rate of 0.68 (Table 4). The fail rate was lower in mtDNA (0.48) than in any of the nDNA microsatellites (0.68 - 0.53).

Table 4 Error rates for 3 genomic DNA microsatellite loci, and the number of failed mitochondrial DNA and genomic DNA PCR´s for the entire sampling period (weeks 2 - 27 in 2010).

	mtDNA	nDNA			Mean
		TTT2	TTD6	TUT1	
nPCR	146	146	146	146	
Fail	0.48	0.68	0.55	0.53	0.56
ADO		0.35	0.44	0.39	0.39
FA		0.35	0.23	0.25	0.28
Tot GE		0.48	0.45	0.38	0.43

nPCR = total number of PCR´s conducted **Fail** = number of failed PCRs divided by nPCR **ADO** = number of samples showing allelic dropout divided by number of positive PCR products **FA** = number of samples showing false allele(s) divided by number of positive PCR products **Tot GE** = number of samples showing ADO and/or FA divided by number of positive PCR products

Roost emergence in the course of snow melting

Twelve of the roosts emerged from the snow, as shown by a suddenly alternating day/night temperature (Fig. 5 for roost 8, and Appendix c for all roosts) and were thereby exposed to sun and weather around week 20-22. This coincided in general with the first major rainfalls in the sampling period (Fig. 5). Three samplings were done from the 20 roosts after this coincidence in weeks 21, 23 and 27, during which time the PCR success was in general low, and fails and GEs high in these samples (Appendix a).

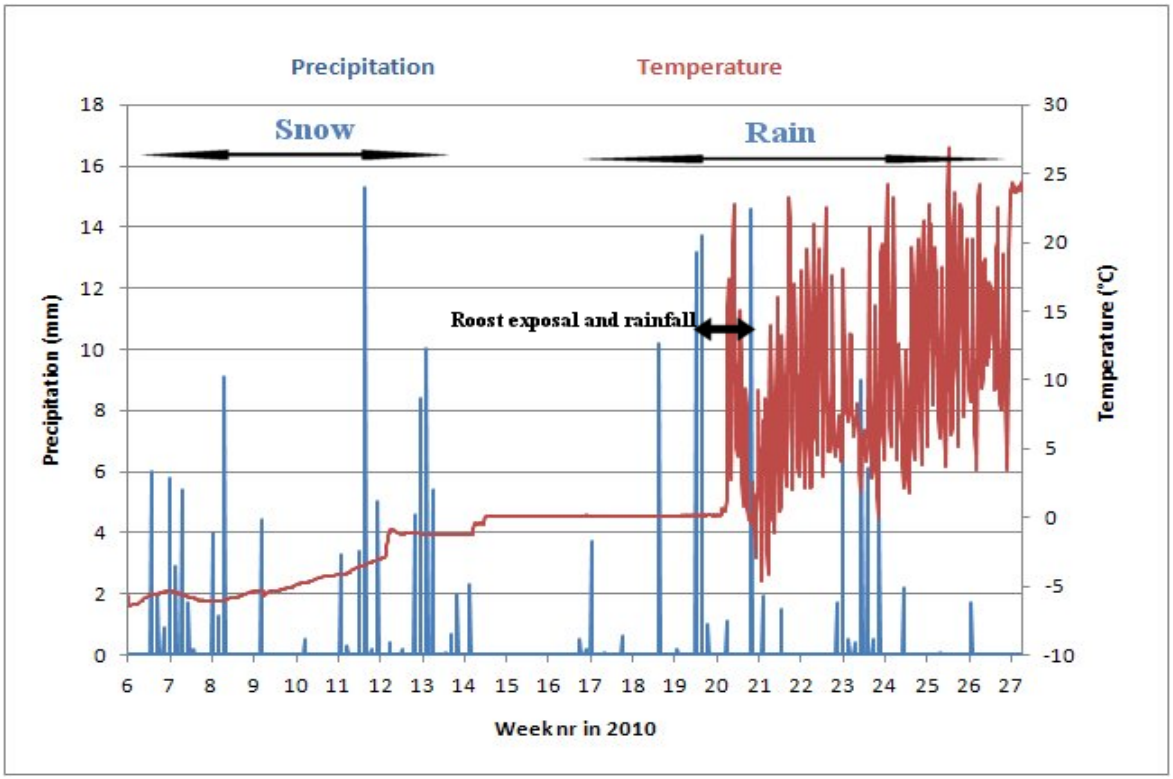

Fig. 5 Coincidence between roost emergence from snow during the spring melt (week 20) shown by suddenly fluctuating temperature (red) and first major rainfalls (blue peaks) in weeks 19-20 of the study period. PCR success for faecal pellet DNA was generally low in roosts after week 20. Example is from roost 8 temperature logger.

DNA concentration in extracted samples

Measurement of OD260 showed highly inconsistent results varying from -10 to 51,4 ng/µl with mean value 7.2 ng/µl in the 146 samples. There was considerable variation within the same sample when doing repeated measurements. Measurement of 50ng/µl standard gave consistent results within specified limits. Data from measurement of DNA concentration are not shown.

Sex determination

Only 74 % (14/19) PCR amplifications of nDNA z and w CHD genes from grouse feathers of known species and sex, using reverse primers designed for capercaillie, scored correctly in the initial test. Controls were established as known species and gender based on the morphology of dead birds. Amplification of rock ptarmigan FP DNA with the same primers was therefore not performed.

Genotyping

The dinucleotide microsatellite TTD6 gave 11 different alleles as scored by capillary electrophoresis, whereas the tetranucleotid TUT1 and combined tri- and tetranucleotid TTT2 both gave 5 different alleles (Table 5). Allele definitions and genotyping of individual roost are shown in Appendix b.

Table 5 Observed microsatellite size range in basepairs (bp) and numbe. of alleles for the three genomic DNA microsatellites found in the reference samples of 20 rock ptarmigan roosts sampled in the Lifjell mountains, Telemark County, southeast Norway. Scoring performed by genemapper software after capillary electrophoresis.

	Locus		
	TTT2	TTD6	TUT1
Size-range (bp)	168.3-184.30 **1)**	106.1-139.0 **2)**	152.8-278.7 **3)**
Nr. of alleles	5 **4)**	11 **5)**	5 **6)**

1) 179 bp in capercaillie (Caizergues et al. 2001) **2)** 131 bp in capercaillie (Caizergues et al. 2001) **3)** 217 bp in capercaillie (Segelbacher et al. 2000) **4)** 8 alleles in rock ptarmigan (Caizergues et al. 2001) **5)** 6 alleles in rock ptarmigan (Caizergues et al. 2001) **6)** 8 alleles in capercaillie (Segelbacher et al. 2000)

Discussion

In this study the feasibility of using rock ptarmigan faecal pellets as a DNA source was evaluated. Two scenarios were used in describing the duration of FP DNA. This was done to meet the assumption that individual FPs in the roost may have been subject to different degrees of degradation due to unequal freezing and thawing as wel as friction in compressed snow. Different amounts of epithelical cells in fresh deposited FP may also have resulted in an inhomogenous collection of FPs in the roost when it comes to DNA content in individual pellets. The first, or worst case scenario gave a minimum mean lifetime for FP DNA from 20.9 days in nDNA (TTT2) to 59.4 in mtDNA. The equivalent for the second, or best case scenario was 38.2 days to 73.8 days. The range though, starts at 0 days in both scenarios for all nDNA microsatellites and for mtDNA. Zero days are conservative, but there is no evidence of duration longer than 0 days as long as a fail or GE occurs in the first sampling after the reference. Despite these 0 values, the duration of FP DNA seems relatively high. In contrast Panasci et al. (2011) found that scat DNA from coyote was highly degraded already after 5 days after deposition, probably because of differences in digestion system, diet as well as environmental and climatic factors. The success of the method in my study may in part be due to the low water and high fiber content of the rock ptarmigan winter diet (Watson and Moss 2008), whose undigested remains tend to scrape a large number of epithelial cells from

the intestinal wall, ultimately ending up in the fecal pellets. The less fiberous summer diet may be a less reliable source of DNA.

The sensitivity of the method used when scoring amplified mtDNA is lower compared to nDNA (conventional gel electrophoresis vs capillary electrophoresis of fluorescence marked products). Therefore the reported differences in duration between mtDNA and nDNA, with mtDNA having the best duration, probably are smaller than if capillary electrophoresis had been used on both. In addition mitochondrial DNA exists in multiple copies in each mitochondria and many mitochondria are present in each cell, whereas genomic DNA exists as a single copy in the core of each cell. MtDNA should therefore give a higher PCR success than nDNA, which is shown in this study.

Successive freezing and thawing of faecal samples may degrade DNA. Therefore it cannot be ruled out that DNA degradation to some degree has occurred in the process between collection and DNA extraction of FPs. However, the effect was likely small since most FPs were collected frozen. All samples have also undergone the same treatment between collection and freezing at the laboratory. Preservation of FPs by silica desiccation or ethanol storage as tested in studies of gorillas and chimpanzees (Nsubuga et al. 2004; Roeder et al. 2004) was considered not essential in this study since most samples were collected frozen and DNA extraction was performed shortly after collection.

Rock ptarmigan often roost in small groups, but are not known to share the same roost or to reuse roosts, and distance between individual roosts is usually $\geq$ 50 cm (Watson and Moss 2008). Therefore samples from each roost are believed to be from one individual only.

All three microsatellites were relatively short (less than 200 bp). Only one allele was more than 200 bp (278.7 bp for TUT1 in roost 7). My results suggests that more complex microsatellites as TTT2 ($(GATA)_{11}(GAT)(GATA)_{14}$) are more likely to be degraded than simpler di- or tetranucleotid repeats. Additional microsatellites should be tested on FP DNA before conducting a larger scale study of e.g. population genetics in the rock ptarmigan. The use of single nucleotide polymorfisms (SNPs) in even shorter fragments than most microsatellites may be more robust in genotyping minute amounts of DNA from highly degraded samples and analyzed with realtime PCR (Hughes-Stamm et al. 2011). The latter method should also be tested on NGS in rock ptarmigan.

Spectrophotometric measurement of OD260 in this study does not seem to be stable and sensitive enough to give reliable results, for either absolute nor for relative values. Thus quantification of DNA should be obtained by other methods such as quantitative PCR as used in alpine ibex (Hausknecht et al. 2009).

Sex determination is important when studying population structure (Ito et al. 2003). Sexing may be an easy task on captured birds with external sexually dimorphic characters, but when collecting FPs molecular methods are needed. Therefore it is important to implement methods that enable the sex determination of individual birds when working with NGS. Male birds are monogametic in having two chromodomain-helicase-DNA (CHD) z chromosomes (zz) while females are heterogametic (wz) (Griffiths et al. 1996). Sex determination in this study was based on scoring samples from gel electrophoresis as heterogametic (female) or homogametic (male) based on two bands or one band respectively. The primers used for sex determination was designed in a study of capercaillie, a member of the tetraonidae as is the rock ptarmigan (Regnaut et al. 2006). Unfortunately, the authors did not report the observed fragmentsizes of scored products from males and females, and hence the results from my study fail to give correct sexing since there was inconsistencies in the size of products representing the two fragments.

Weather induced factors such as thawing/freezing and outwashing are known to cause degradation in DNA, as free DNases are more active under moist conditions (Regnaut et al. 2006; Brinkman et al. 2010). My results cannot pinpoint an exact window of time when rapid degradation occurs, but rather roughly suggest when rock ptarmigan FPs should be collected during the time of year when snow covers the ground, and in particular during the spring melt, in order to obtain best results. My results may be representative for other snow-roosting species living under similar climatic conditions. I speculate that more data from the roosts in the preceding weeks of the first major rainfalls, when many roosts were not sampled due to snow depth, would have confirmed an increased DNA degradation synchronous with rain and FPs exposure.

I suggest that future genetic studies of rock ptarmigan or other grouse species, based on NGS using FPs from snow roosts, start with assessing genotyping errors in a number of microsatellite loci using all FPs found in each freshly-made roost. Multitube PCR reactions should then be performed to obtain consensus genotyping over a minimum number of

replicates. Further analysis should be done on nDNA from rock ptarmigan blood or tissue samples and faecal pellet samples to establish reliable sex determination methods.

Conclusions

Faecal pellets from rock ptarmigan snow roosts appear to be a promising source for mtDNA and nDNA microsatellites in NGS. Samples are relatively easy to obtain during suitable weather conditions and are often obtainable in large amounts from each individual. Deposition of FPs in snow and cold, dry weather probably ensures low DNA degradation. The collection of FPs in winter from newly used roosts appears to be a suitable method for NGS in rock ptarmigan as well as other snow roosting grouse.

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Appendix a

Sampling scheme with PCR results from all 20 roosts (following pages). Explanation of the acronyms used here:

Ref = the first sample from each roost to which the following samples are compared to when evaluating genotype or species identification

PCR consensus product = sample gives the same species identification or the same genotype as ref.

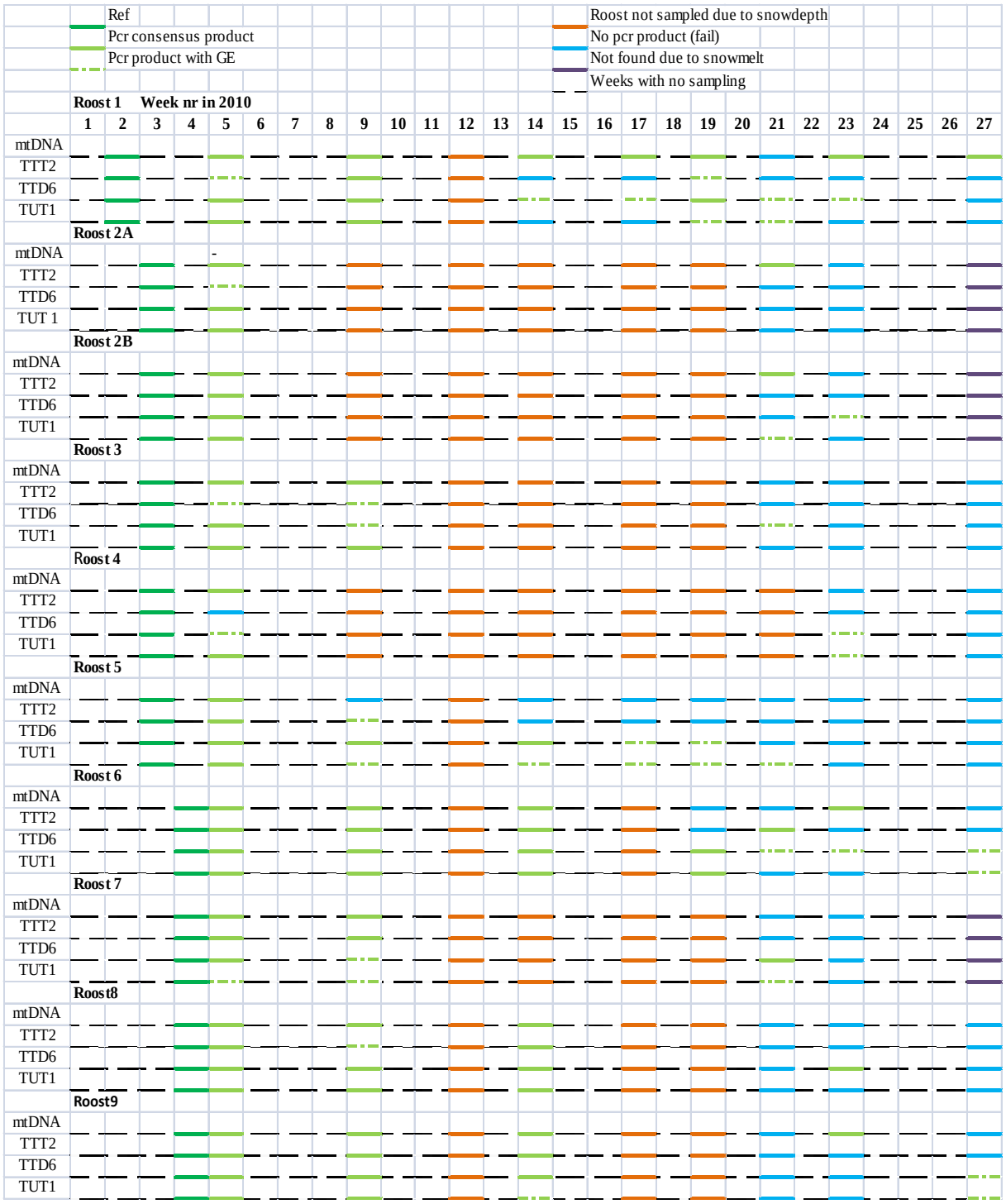
PCR product with GE = mtDNA with fragment size different from 212 bp or nDNA microsatellites with missing alleles, false alleles or both compared to ref.

Not sampled due to snow depth = The roost was visited, but not sampled due to the dificulty of digging through deep snow

No PCR product (**fail**) = no detectable PCR product from mtDNA by conventional gel electrophoresis or nDNA microsatellites by capillary electrophoresis was obtained

Not found due to snowmelt = the FPs in the roost had either disappeared in the vegetation or eroded away after all snow had melted

Weeks with no sampling = interval between samplings


Ref
Pcr consensus product
Pcr product with GE
Roost not sampled due to snowdepth
No pcr product (fail)
Not found due to snowmelt
Weeks with no sampling
Roost 1 Week nr in 2010
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27
mtDNA
TTT2
TTD6
TUT1
Roost 2A
mtDNA
TTT2
TTD6
TUT 1
Roost 2B
mtDNA
TTT2
TTD6
TUT1
Roost 3
mtDNA
TTT2
TTD6
TUT1
Roost 4
mtDNA
TTT2
TTD6
TUT1
Roost 5
mtDNA
TTT2
TTD6
TUT1
Roost 6
mtDNA
TTT2
TTD6
TUT1
Roost 7
mtDNA
TTT2
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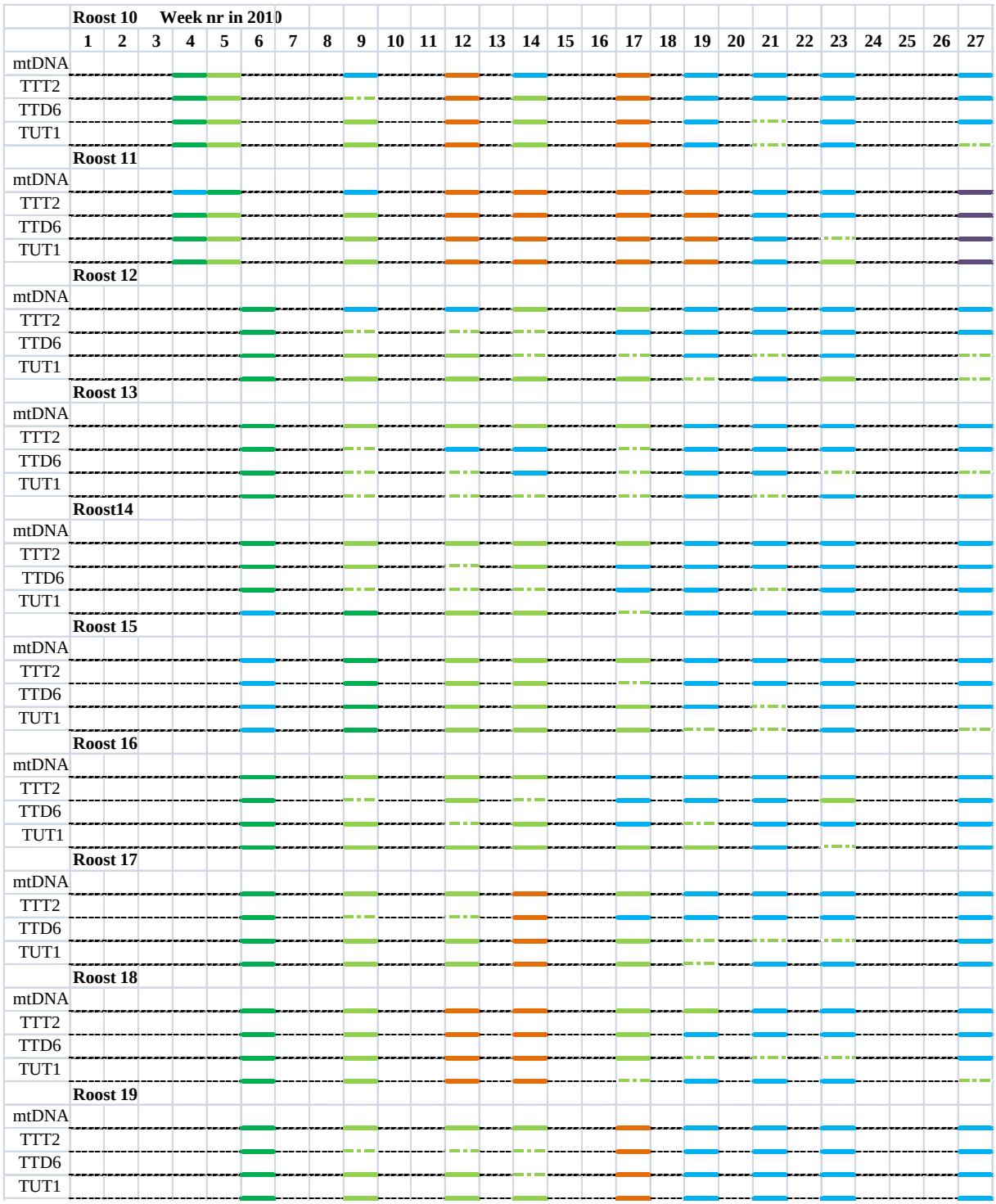
TUT1
Roost9
mtDNA
TTT2
TTD6
TUT1


Roost 10 Week nr in 2010
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27
mtDNA
TTT2
TTD6
TUT1
Roost 11
mtDNA
TTT2
TTD6
TUT1
Roost 12
mtDNA
TTT2
TTD6
TUT1
Roost 13
mtDNA
TTT2
TTD6
TUT1
Roost14
mtDNA
TTT2
TTD6
TUT1
Roost 15
mtDNA
TTT2
TTD6
TUT1
Roost 16
mtDNA
TTT2
TTD6
TUT1
Roost 17
mtDNA
TTT2
TTD6
TUT1
Roost 18
mtDNA
TTT2
TTD6
TUT1
Roost 19
mtDNA
TTT2
TTD6
TUT1

Appendix B

Definition of alleles based on scoring of signalsize from capillary electrophoresis (Table a). And genotyping of FP DNA ref sample from each roost (Table b).

Table a Definition of alleles from scoring of signal size in fluorescence marked primer products in capillary electrophoresis of FP DNA from 20 rock ptarmigan roosts. Observed number of alleles shown in brackets.

Allele definitions					
TTT2		TTD6		TUT1	
Size (bp)	Allele	Size (bp)	Allele	Size (bp)	Allele
168.3-168.4	A (2)	106.1-107.0	A (11)	152.8-152.8	A (1)
172.0-172.2	B (3)	109.0-109.0	B (1)	164.2-164.7	B (14)
175.9.-177.6	C (12)	115.1-115.6	C (5)	184.3-184.7	C (4)
179.9-180.4	D (7)	117.4-117.5	D (2)	188.5-189.0	D (12)
184.0-184.3	E (3)	119.4-119.6	E (2)	278.7-278.7	E (1)
		121.4-122.0	F (6)		
		123.8-123.8	G (1)		
		126.0-126.0	H (1)		
		128.0-128.1	I (2)		
		134.6-134.7	J (2)		
		139.0-139.0	K (1)		

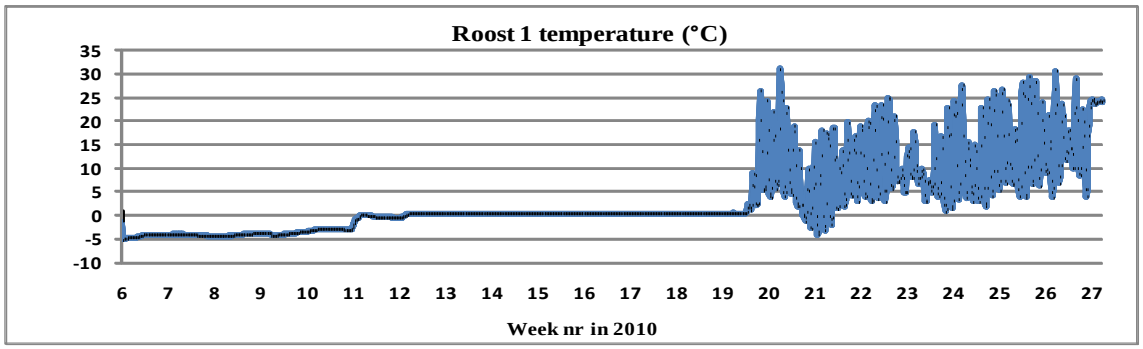
Table b Genotyping of reference samples from each of 20 roosts. Alleles are defined in table a.

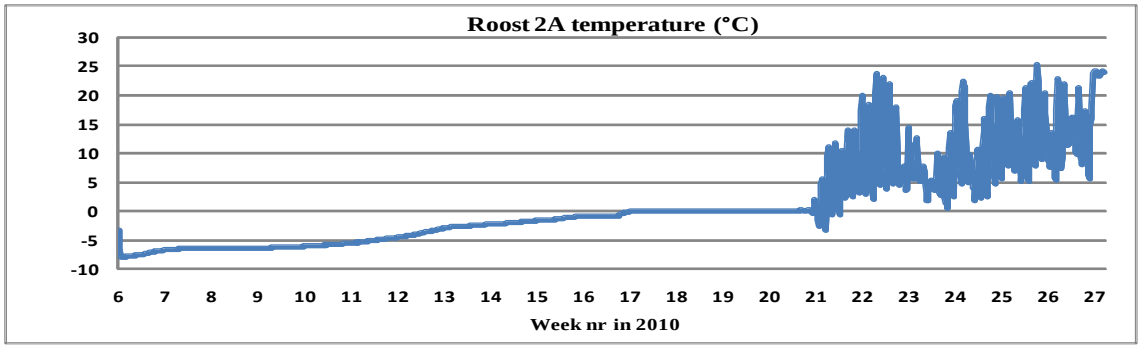
	Locus		
Roost	TTT2	TTD6	TUT1
1	C-E	A-E	B-D
2A	C	F	D
2B	C	F-J	B-D
3	C	A-K	B-D
4	D	I	B
5	E	A-F	A-B
6	D	C-H	B
7	C-E	E	D-E
8	B-D	A	B
9	D	F-J	C-D
10	A	D	D
11	A	D	D
12	C	A-I	D
13	C-D	A-F	B-C
14	C	A-C	B-C
15	C-D	A-F	B-C
16	B	A-B	B
17	B-C	C-G	B-D
18	C	A-C	B-D
19	C-D	A-C	B-D
nHet	7	14	12

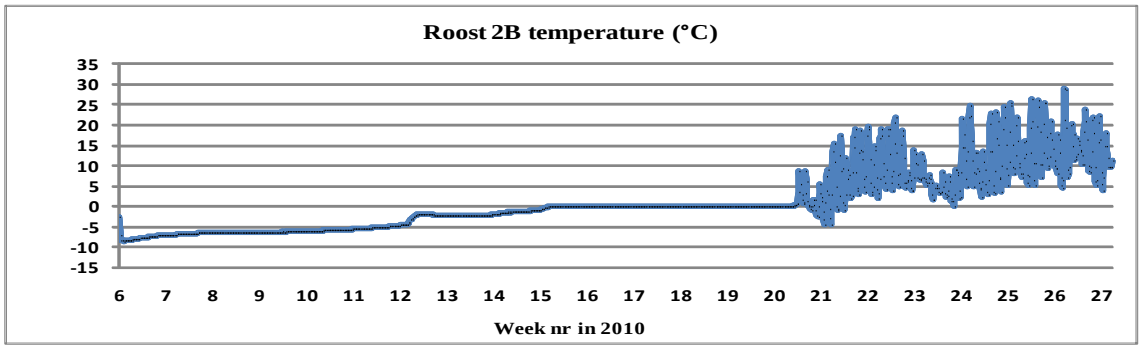
nHet = number of heterozygote roost ref samples

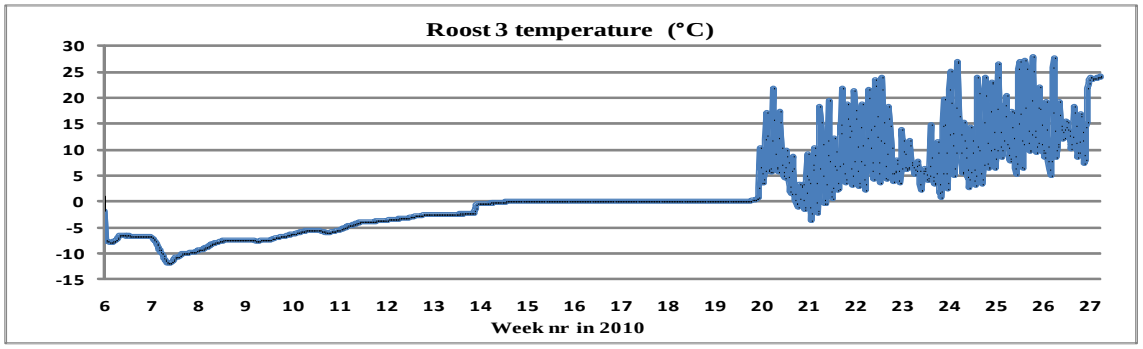
Appendix c

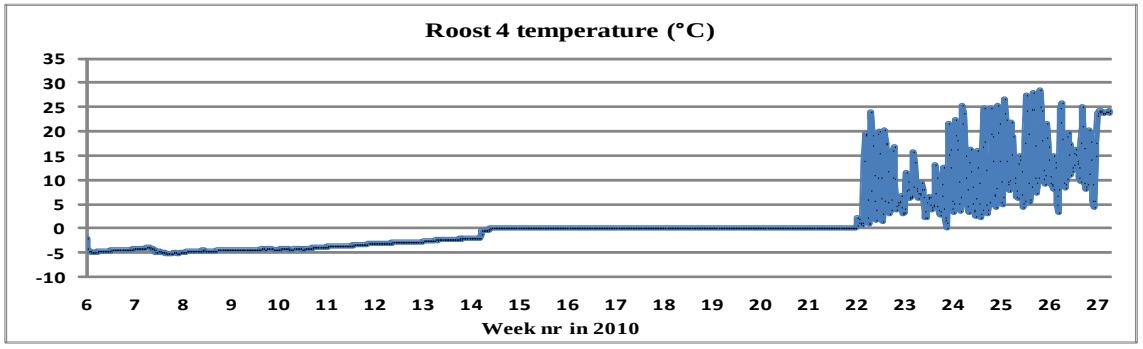
Temperaturelogger readings from all 20 roosts (following pages). Stable temperatures at 0°C or slightly below means that roost are under snow surface. Alternating roost temperatures means that roost FPs are more affected by precipitation and radiation.

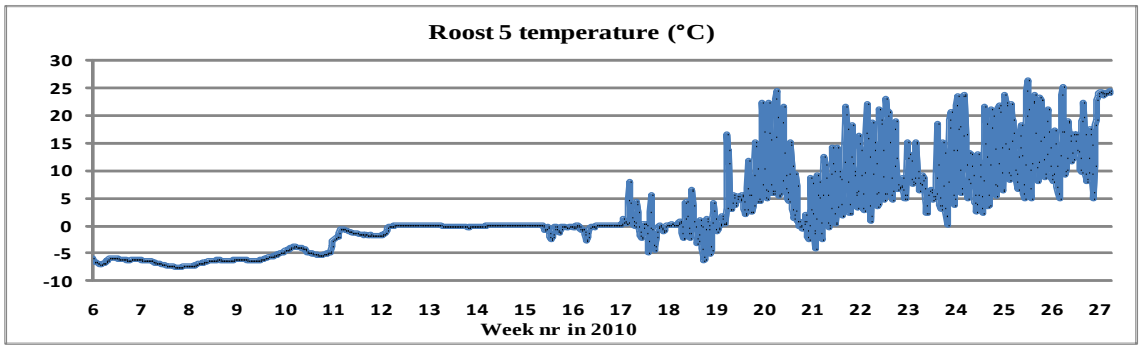

Roost 1 temperature (°C)
35 30 25 20 15 10 5 0 -5 -10
6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27
Week nr in 2010

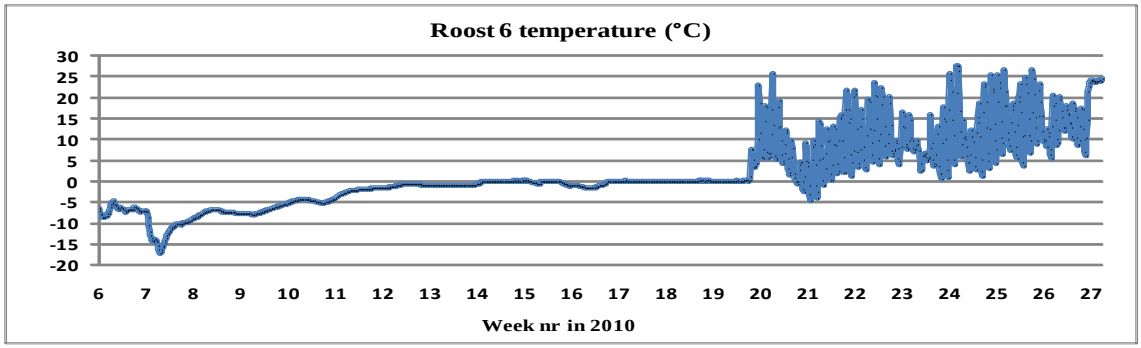

Roost 2A temperature (°C)
30 25 20 15 10 5 0 -5 -10
6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27
Week nr in 2010


Roost 2B temperature (°C)
35 30 25 20 15 10 5 0 -5 -10 -15
6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27
Week nr in 2010


Roost 3 temperature (°C)
30 25 20 15 10 5 0 -5 -10 -15
6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27
Week nr in 2010


Roost 4 temperature (°C)
35 30 25 20 15 10 5 0 -5 -10
6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27
Week nr in 2010


Roost 5 temperature (°C)
30 25 20 15 10 5 0 -5 -10
6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27
Week nr in 2010


Roost 6 temperature (°C)
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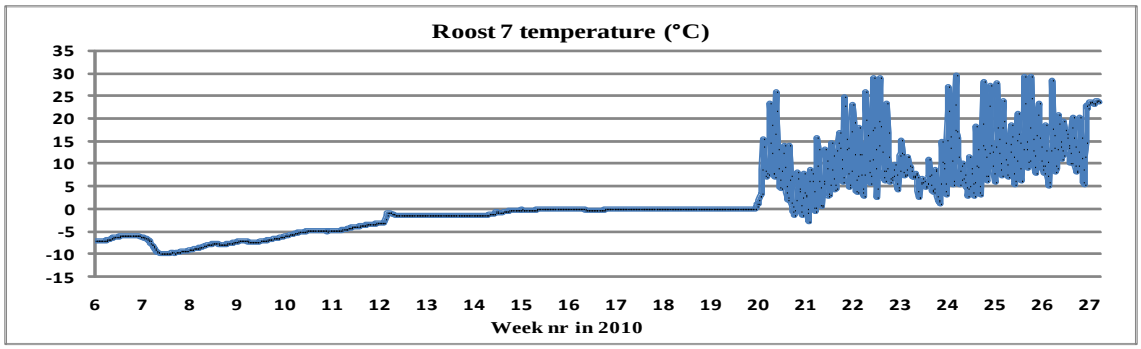
6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27
Week nr in 2010


Roost 7 temperature (°C)
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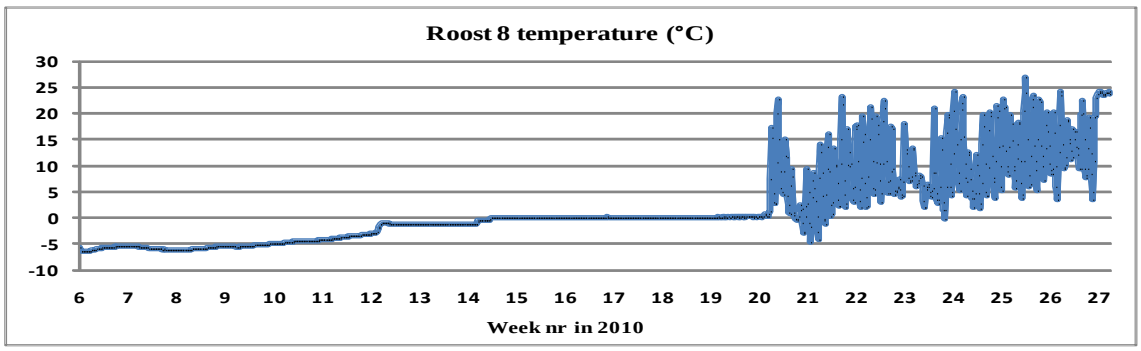
6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27
Week nr in 2010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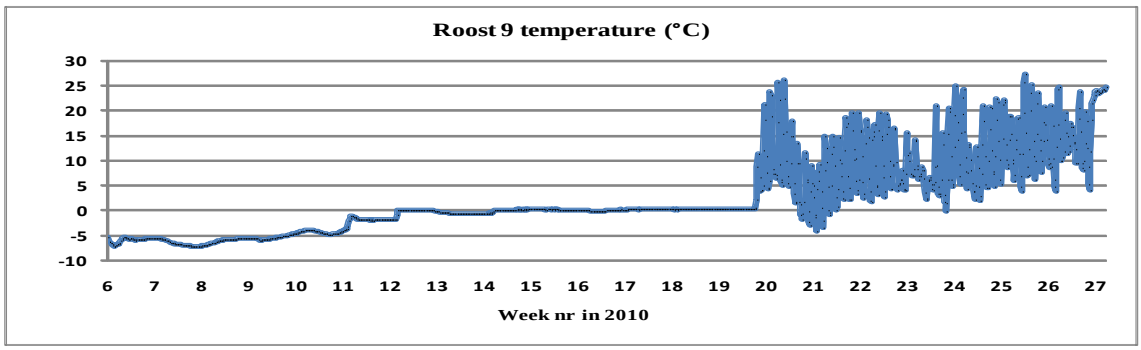
6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27
Week nr in 2010


Roost 9 temperature (°C)
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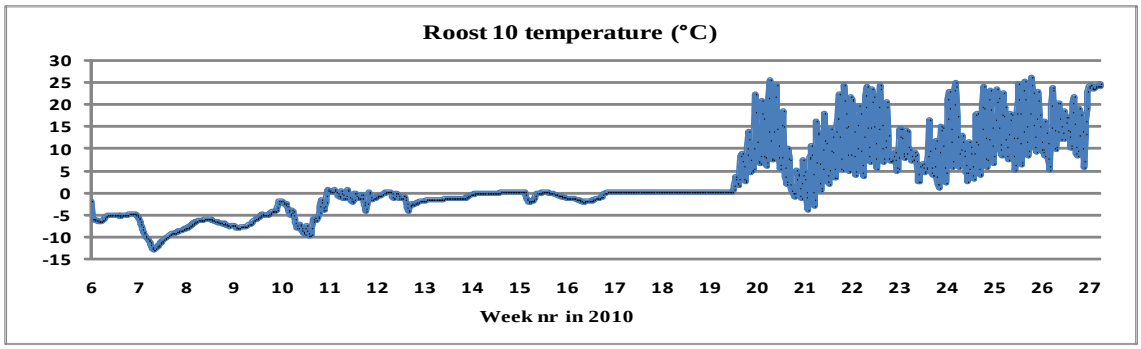
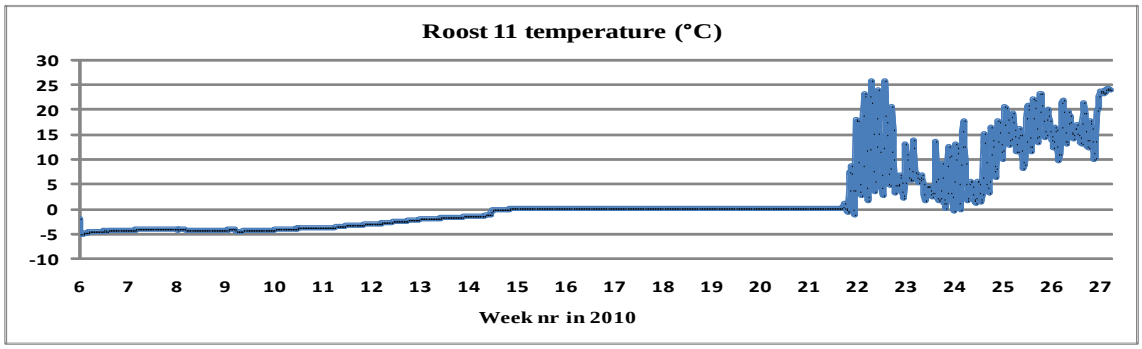
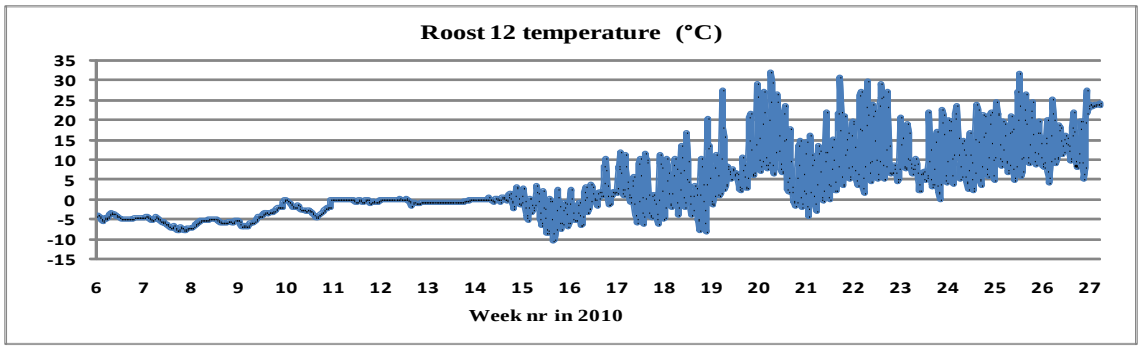
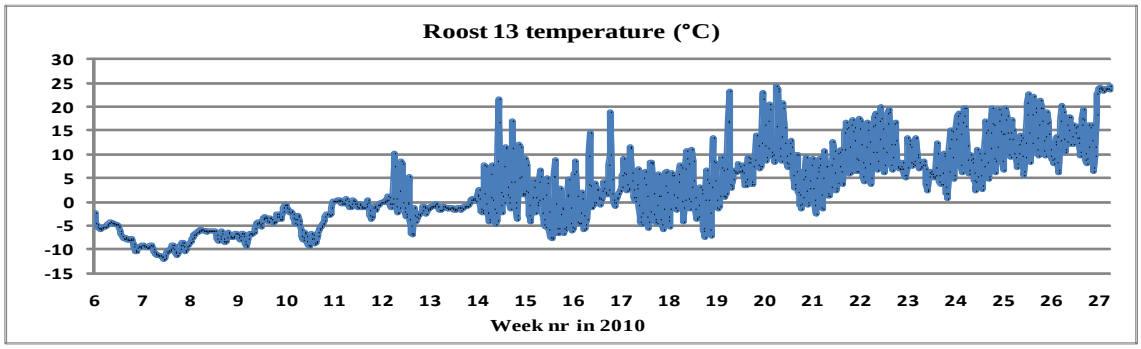
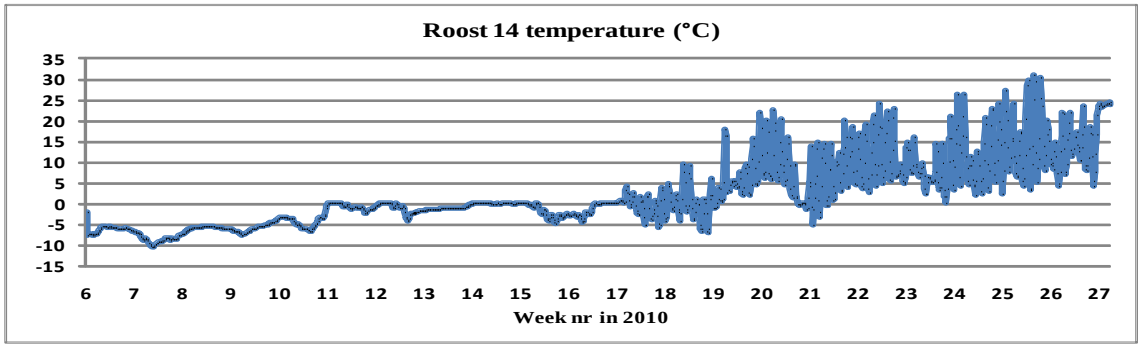
6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27
Week nr in 2010


Roost 10 temperature (°C)
30 25 20 15 10 5 0 -5 -10 -15
6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27
Week nr in 2010


Roost 11 temperature (°C)
30 25 20 15 10 5 0 -5 -10
6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27
Week nr in 2010


Roost 12 temperature (°C)
35 30 25 20 15 10 5 0 -5 -10 -15
6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27
Week nr in 2010


Roost 13 temperature (°C)
30 25 20 15 10 5 0 -5 -10 -15
6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27
Week nr in 2010


Roost 14 temperature (°C)
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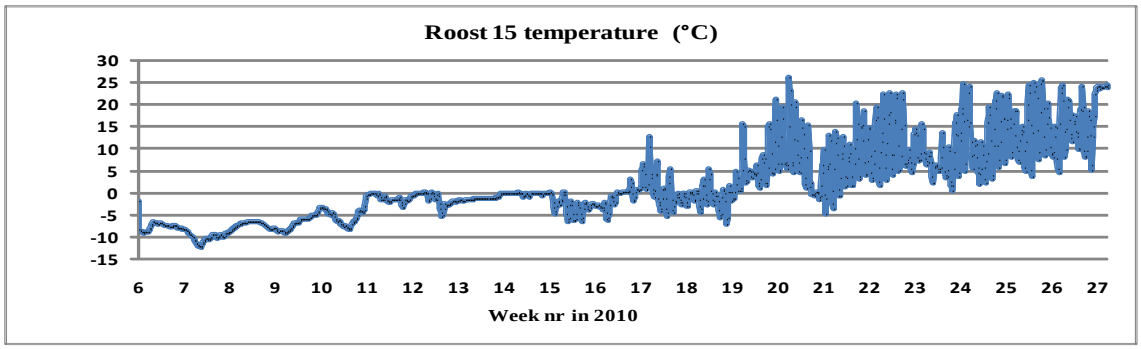
Week nr in 2010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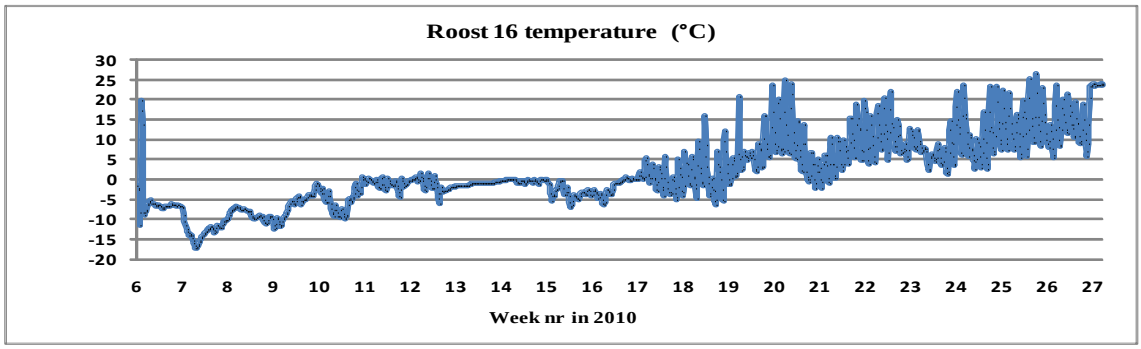
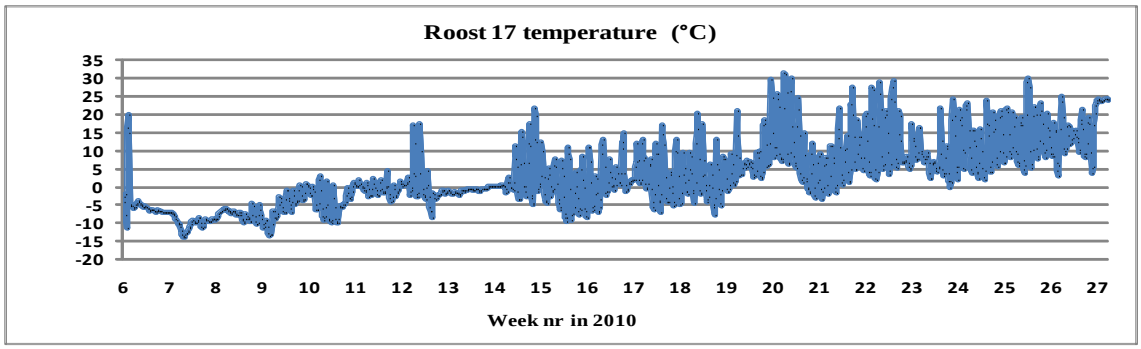
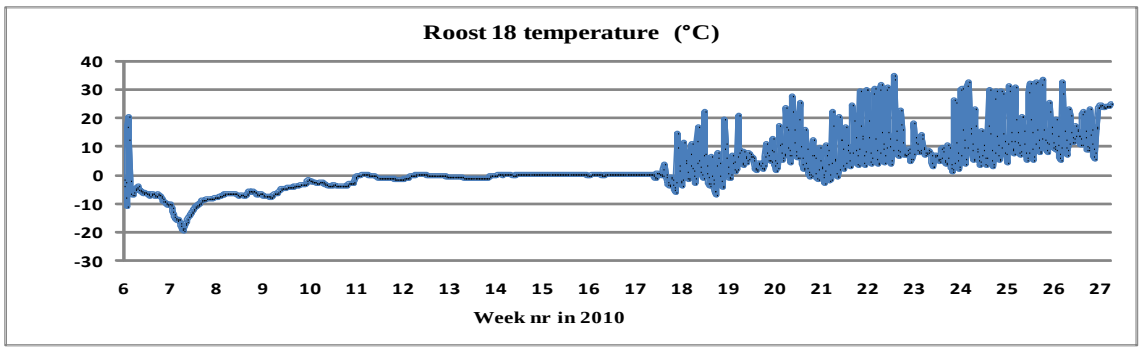
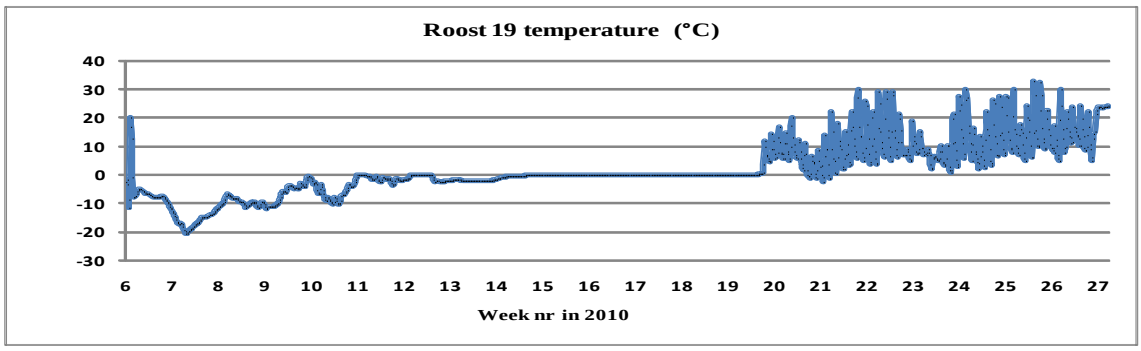
30 25 20 15 10 5 0 -5 -10 -15
6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27
Week nr in 2010


Roost 16 temperature (°C)
30 25 20 15 10 5 0 -5 -10 -15 -20
6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27
Week nr in 2010


Roost 17 temperature (°C)
35 30 25 20 15 10 5 0 -5 -10 -15 -20
6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27
Week nr in 2010


Roost 18 temperature (°C)
40 30 20 10 0 -10 -20 -30
6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27
Week nr in 2010


Roost 19 temperature (°C)
40 30 20 10 0 -10 -20 -30
6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27
Week nr in 2010