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Evaluation of in-field stability of mitochondrial and nuclear DNA in snow sampled fecal pellets from Rock ptarmigan (*Lagopus muta*)

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Abstract. Non-invasive genetic sampling (NGS) is highly relevant in genetic studies applied to ecology. Methods should be tailored to species and environments. In this context, the in-field stability of fecal DNA is essential. Here we describe a method to test the stability of DNA in fecal pellets (FPs) of rock ptarmigan (*Lagopus muta*). Rock ptarmigan spend their entire lifecycle in the alpine region and roost in individual snow holes to save energy and avoid predation. Roosting birds commonly deposit FPs and individual snow roosts are not reused. FPs (n=146) were collected from 20 individual rock ptarmigan snow roosts in the Lifjell Mountains in Telemark County, southeast Norway between 17 January and 7 July, 2010. DNA was extracted from the 146 samples and we tested in-field temporal stability in mitochondrial control region DNA, nuclear DNA, chromo-helicase-DNA binding (CHD) gene and three microsatellites (msats). MtDNA and CHD gene were scored on polyacrylamide gel while msats were sized by capillary electrophoresis. For mtDNA, the mean stability in 20 roosts was 74 and 59 days respectively in a best and worst case scenario. For the CHD gene, the mean stability was 122 and 88 days and for the 3 msats, mean stability varied between 39 - 58 and 21 - 46 days respectively in the two scenarios. The CHD gene had the highest stability. MtDNA had significantly higher stability than msats and between individual msats stability varied significantly. 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 appear to be a reliable DNA source when analyzing mtDNA (species determination) and nDNA (sex determination and msat analysis). These methods may also be applicable to other snow-roosting grouse.

Key words: *Lagopus muta*, noninvasive genetic sampling, DNA stability, microsatellite, mitochondrial DNA, sexing, snow

Introduction

Obtaining reliable DNA sources in genetic studies of ecologically significant traits in wild vertebrates can be challenging. In elusive or protected species, DNA is often obtainable only by non-invasive 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 feces, and there are several problems involved with genotyping and population genetics based on these DNA sources (Broquet and Petit 2004; Waits and Paetkau 2005). Rock ptarmigan (*Lagopus muta*) roost in snow holes whenever possible to reduce heat loss and predation (Watson and Moss 2008). While in the roost, birds usually deposit a pile of fecal pellets (FPs) on the bottom (Fig. 1). 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, storage techniques and routines. The feasibility of NGS has been studied in avian species by Segelbacher and Steinbruck (2001) who found that sexing and msat analysis from feces-based DNA gave the same results as feather or blood DNA. Regnaut *et al.* (2006b) found that DNA degradation in feces from capercaillie (*Tetrao urogallus*) was mainly caused by free DNases in the collect-

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

ed material. Maudet *et al.* (2004) collected feces for DNA analysis in ungulates and showed that winter samples had longer stability than those collected in spring. All grouse species and some passerines (*Passeriformes*) roost in snow (Watson and Moss 2008) which suggests that feces collected from snow roosts could potentially be an important source of NGS for many bird species. The rate of DNA degradation in noninvasively collected sources varies 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 are unusual among arctic and alpine birds in spending their whole lifecycle primarily above tree line from 83˚ N on Greenland to 35˚ N in Japan (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 (*L. 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). Presently, the conservation status for rock ptarmigan worldwide according to IUCN (http://www.iucnredlist.org/) version 2012.2 is rated "least concern" with population trend "decreasing", for reasons unknown. In Fennoscandia, the rock ptarmigan can legally be hunted. In a recent study Lehikoinen *et al.* (2013) found that 9 of 14 common montane birds, including rock ptarmigan, showed a declining trend over a 10-year period from 2002 to 2012. Some local populations, as in Japan, may even 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 depend upon a sufficient snow layer in which to build snow roosts for insulation and protection from predators (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; Imperio *et al.* 2013). Smaller populations may in turn lead to increased subpopulation isolation, genetic drift, and inbreeding.

The main objective of this study was to investigate whether FPs collected from natural snow roosts of rock ptarmigan are a reliable source of mtDNA and nDNA for the determination of species, sex and genetic population structure measured by msat markers. The impact of temperature and precipitation on DNA degradation is also assessed. To our knowledge this is the first study to assess validity of DNA extracted from avian feces collected from snow roosts. These results should also be relevant for the 18 other snow-roosting grouse species (Watson and Moss 2008).

Fig. 2. Location of the study area in the Lifjell Mountains, Telemark County, Norway (N59˚ 30,000´ E8˚ 58,810´).

Material and Methods

Field sampling

In January and February 2010, recently abandoned roosts with FPs were located by cross-country skiing through an alpine area of approximately 3 km^2 in 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 ≤ 2 days when first found. All roosts were located between 1,020 - 1,100 m a.s.l. A roost typically contained 20 to 50 FPs. The minimum distance between sampled roosts was 2 meters.

Roosts typically occurred 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 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 if the initially exposed roost should later become buried in snow. Roost positions were stored on a handheld GPS recorder. Temperature loggers were situated adjacent to the collection of FPs in each pipe (Fig. 3) and temperatures 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 identified. Precipitation data were obtained from a standard Norwegian Meteorological Institute weather station located approximately 6 km south of the central study area at 360 m a.s.l. One FP was collected from each of the 20 roosts

3
In-field stability of DNA from fecal pellets of Rock ptarmigan (Lagopus muta)

Fig. 3. The collection of fecal 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 overheating. A temperature logger, model KTL-108 from Keytag 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.

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 (week numbers refer to week of the year). If a roost was covered by more than 50 cm of snow at a later sampling, the roost was not sampled. Roosts covered by less snow were exposed by digging with a spade and the snow replaced after sampling. Pellets were collected randomly by rearranging the pile of FPs at each collection and then selecting one pellet. FPs were collected in individual 15 ml polycarbonate containers and stored at -20˚ C prior to DNA extraction performed within one week after collection.

To establish a control set of sexed individuals, 22 rock ptarmigan of known sex, based on gonadal inspection, were obtained from a local hunter and the liver excised for CHD analysis.

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 scalpel blade to obtain a homogenous solution with the lysis buffer. DNA was eluted in 200 µl elution buffer.

Primers designed by Nystrom *et al.* (2006) were used to assess stability in mtDNA control region and to remove possible erroneous sampling of willow ptarmigan roosts. This primer complex consisted of one general forward primer, Lagsp3F 5´-CATACATTATGGTACCGGTAC-3´ and the two species-specific reverse primers, Lag3R 5´-TGGTGGAC GGTCGATTGTAG-3´ and Mut3R 5´-GGGTAGGCAGGTATTTATAGT-3´ identifying willow- or rock ptarmigan respectively. The mtDNA PCR reagents were mixed in a 25 µl volume solution containing 2 µl template DNA corresponding to approximately 10 ng, 0.2 mM of each dNTP, 1x PCR gold buffer, 10 pmol 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 and sized on polyacrylamide gel, identifying 212 bp band for rock ptarmigan or possible 154 bp for willow ptarmigan.

Stability of nDNA was evaluated by two separate analyses by first determining sex of the birds by detecting size difference in the chromosome-specific intron in the sex-linked CHD gene. Male birds are monogametic in having two CHD chromosomes (zz) while females are heterogametic (wz) (Griffiths *et al.* 1996). To establish a control set of birds of known sex, 22 shot rock ptarmigan were dissected and sexed by visual examination for the presence of testes or left ovary. Approximately 5g of liver from each individual was excised and stored in 70% ethanol. DNA was extracted from 10 mg liver samples using Qiamp® DNA blood and tissue kit from QIAGEN following manufacturer's protocol. Target fragment of CHD gene was amplified in PCR reactions with a primer pair designed for capercaillie (*Tetrao urogallus*) by Perez *et al.* (2011). The primers were forward primer P8mod (5´ - TCCCAAGGATGAGRAAYTGTG - 3´) and reverse primer PU (5'- AAAGCTGATCTGGAATTTCAG – 3´). PCR reagents setup was as for MtDNA. The amplification was performed in a thermocycler with an initial 3 min denaturation at 94˚ C followed by 60 cycles of 94˚ C for 30 s, 53˚ C annealing for 90 s and 72˚ C extension for 60 s. Finally, a 72˚ C extension was performed for 30 min before conservation at 4˚ C. The amplified products were electrophoresed as for mtDNA above. Scoring was performed by identifying one single 189 bp band for rock ptarmigan males or with additionally one 214 bp band for females.

DNA from the 146 field collected fecal samples was examined for sex using the same protocol as for the control set above. Stability of nDNA was also evaluated by measuring the degradation in three msat loci, TTT2 and TTD6 (Caizergues *et al.* 2001), and TUT1 (Segelbacher *et al.* 2000) (Table 1).

The PCR reagent concentrations were as for mtDNA above. Each forward primer was marked with fluorescent dye and msats 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. Genemapper 4.0 software from Applied Biosystems was used to visualize and size fragments.

Negative controls were implemented in all PCR amplification setups by replacing template DNA with distilled water in one tube. All am-

		Locus		
		TTT2	TTD6	TUT1
Primers	F	GTGAATGGATGGATGTATGAA	GGACTGCTTGTGATACTTGCT	GGTCTACATTTGGCTCTGACC
(5´-3´)	R	GTCTGTCAATGAACTTCTTGG	CATGCAGATGACTTTCAGCA	ATATGGCATCCCAGCTATGG
Size-range		168-184 [1)]	106-139 [2)]	153-279 [3)]
Nr. of alleles		5 [4)]	11 [5)]	5 [6)]

Table 1. Primer sequences for each loci. Observed microsatellite size range in basepairs (bp) and number 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. [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).

plifications were performed only once, but 10% of the 146 samples were randomly reanalyzed.

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. GE for the CHD gene was defined as products gaining one gel band (male to female) or losing one (female to male) and for msats 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 stability 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 stability of individual msat loci; and between mtDNA, CHD gene and msats; parametric two-way ANOVA was used, as values were normally distributed. Probability p = 0.05 was used as the significance cut-off level. Other analyses were descriptive.

Results

Annual Positive PCR amplifications were obtained from all of the first reference samples except for one roost (roost 15). From this roost caecal feces was erroneously sampled and hence the second sampling was used as ref.

All 20 roosts contained FPs from rock ptarmigan, of which 16 contained male DNA and 4 female DNA. The dinucleotide msat TTD6 gave 11 different alleles as scored by capillary electrophoresis, whereas the tetranucleotid TUT1 and the combined tri- and tetranucleotid TTT2 both gave 5 different alleles.

In both scenarios, mtDNA represented by the species-specific primers showed a higher stability than any of the msats (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 stability than TTD6 and TUT1 in scenario 1 (F = 6.3

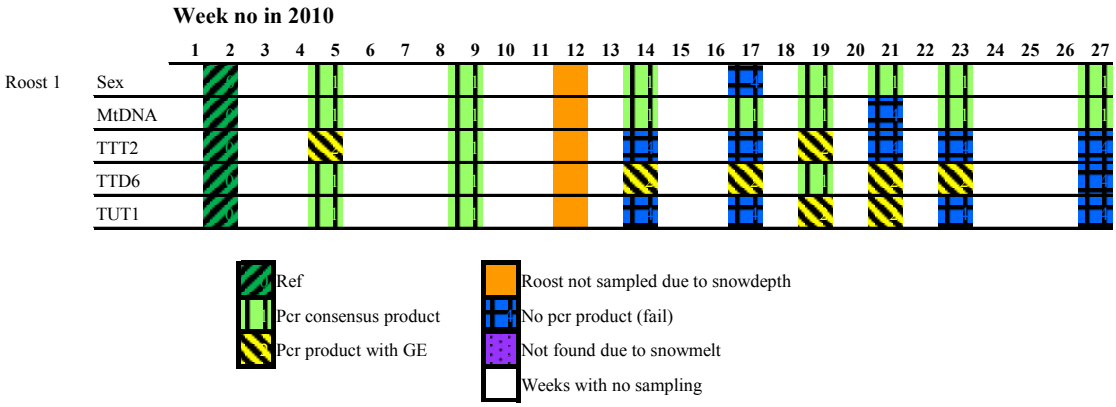

Fig. 4. An example (roost 1) of the sampling scheme recorded for each snow roost showing the stability of mitochondrial DNA and genomic DNA microsatellites in fecal pellets. **Ref** = the first sample from each roost to which all following samples are compared to when evaluating species identification, sex or genotype; **PCR consensus product** = sample gives the same species identification, sex or the same genotype as ref.; **PCR product with GE** = mtDNA with fragment size different from 212 bp, for CHD gene as products gaining one gel band (male to female) or losing one (female to male) or 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 and CHD by conventional gel electrophoresis or microsatellites by capillary electrophoresis; **Not found due to snow melt** = all snow had melted and no FPs were found (not valid for roost 1); **Weeks with no sampling** = interval between samplings.

		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)	
mtDNA		0-128	59 ± 41	0-170	74 ± 47
CHD		0-154	88 ± 47	4-170	122 ± 33
msat	TTT2	0-77	21 ± 28	0-122	38 ± 40
	TTD6	0-111	44 ± 35	0-137	58 ± 14
	TUT1	0-111	46 ± 32	0-137	53 ± 40

Table 2. Summary of stability in fecal pellet mtDNA, CHD gene and 3 microsatellites from 20 snow roosts of rock ptarmigan, Lifjell Mountains, Telemark County, southeast Norway. **[1)] GE (genotyping error)** = For mtDNA: PCR product with fragment size different from 212 bp, for CHD gene as products gaining one gel band (male to female) or losing one (female to male), for msats: false allele (FA) or allelic dropout (ADO).

p = 0.0044). For scenario 2 the difference was not significant. CHD gene stability was even higher than mtDNA. The difference was significant in scenario 2 (F = 24.8 p = 0.0001). Stability of mtDNA, and nDNA was highly variable between roosts for both scenarios (Table 2).

Twelve of the roosts eventually emerged from the melting snow, as shown by a suddenly alternating day/night temperature (example Fig. 5 for roost 8) 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 generally low, and fails and GEs high in these samples. The PCR amplifications of the randomly picked 10% of 146 samples were all in agreement with the first analyses.

Discussion

Compared to NGS in moist or temperate environments or seasons, fecal sampling under cold, dry conditions aids in avoiding enzymatic DNA degradation. 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 ≥ 50 cm (Watson and Moss 2008). Therefore fecal samples from each roost are believed to be from one individual only. In this study the feasibility of using rock ptarmigan FPs as a DNA source for genetic testing was evaluated. Two scenarios were constructed in order to address the assumption that individual FPs in the roost may have been subject to different degrees of degradation due to unequal freezing and thawing, as well as friction in compressed snow. Different amounts of epithelial cells in freshly deposited FPs may also have resulted in a collection of pellets from individual roosts with highly variable contents of DNA. The first, or worst case scenario gave a minimum mean temporal stability for FP DNA from 21 days in msats (TTT2) to 88 days in CHD. The equivalent for the second, or best case scenario was 38 days to 122 days. The range, however, starts at 0 days in both scenarios for all msats and 14 days for CHD best case. Zero days are conservative, but there is no evidence of stability longer than 0 days as long as a fail or GE occurs in the first sampling after the reference. Despite these 0 values, the stability of FP DNA seems relatively high. In contrast, Panasci *et al.* (2011) found

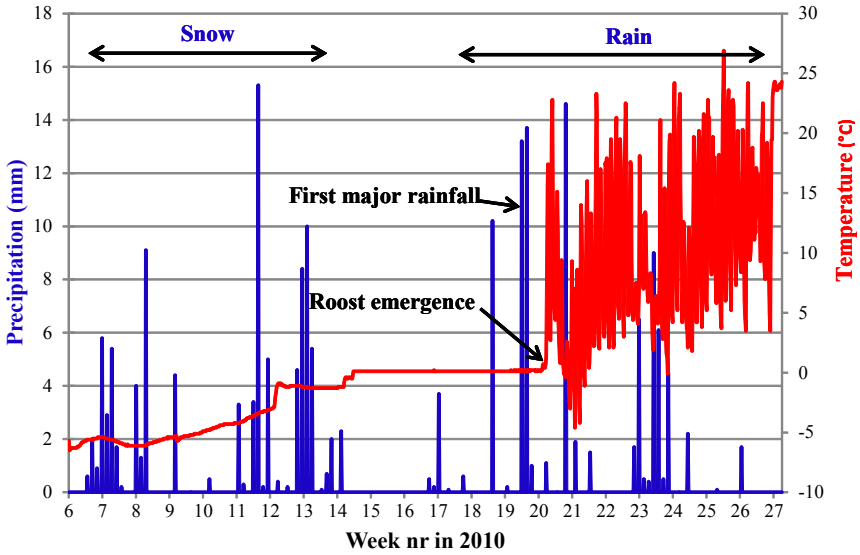

Fig. 5. Coincidence between roost emergence from snow during the spring melt (week 20) shown by suddenly fluctuating temperature (red) and first major rainfalls (blue peaks) in weeks 19-20 of the study period. PCR success for fecal pellet DNA was generally low in roosts after week 20. Example is from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

that scat DNA from coyote (*Canis latrans*) was highly degraded only 5 days following deposition, probably due to different digestive systems, diets, and environmental factors. The success of the method in our 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 may tend to scrape a large number of epithelial cells from the intestinal wall, ultimately ending up in the fecal pellets. The less fiber-rich summer diet may be a less reliable source of DNA.

The sensitivity of the method used when scoring amplified mtDNA and CHD genes is lower compared to msats were fluorescence marked primers are used. Also, when comparing results from various PCR amplification setups, the variation observed may be inherent in the methods as significant differences in specificity and sensitivity. For this reason, it is difficult to conclude from our data that one type of DNA is more stable than the other in genetic testing. However the results indicate that the nDNA (used in msats and sex determination) is no less stable than mtDNA, even though mtDNA exists in multiple copies in multiple mitochondria in each cell.

One factor in DNA degradation may be the successive freezing and thawing of fecal samples. However, preservation of FPs by silica desiccation or ethanol storage as tested in studies of gorillas and chimpanzees by Nsubuga *et al.* (2004) was considered not essential in this study since most samples were collected frozen and DNA extraction was performed shortly after collection. Number of alleles from the 3 msats (TTT2; 5, TTD6; 11 and TUT1; 5) were somewhat different from numbers found by Caizergues *et al.* (2001) in 10 rock ptarmigan from the southern French alps (TTT2 and TTD6) and Segelbacher *et al.* (2000) in 20 capercaillie (TUT1) (8, 6 and 8 alleles respectively) (Table 1). All three msats were relatively short (less than 200 bp). Only one allele was more than 200 bp (278.7 bp for TUT1 in roost 7). The results here suggest that more complex msats as TTT2 ((GATA)11(GAT)(GATA)14) are more likely to be degraded than simpler di- or tetranucleotid repeats, though this could be due to design of primers and PCR conditions. Additional msats 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 msats 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.

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. Gonadal inspection of the 22 shot birds gave the same sex result as the genetic molecular method, which suggests the latter is reliable. The ratio of male to female FP DNA from the 20 roosts seemed high (4:1). However, small flocks of 3-7 males were commonly observed while making the FP field collections, suggesting a dominance of males on the study area at this time. Flocks of male rock ptarmigan were also observed by Cotter (1999), though closer to breeding time. The complex of template DNA CHD gene and the new primers P8mod and PU (Perez *et al.* 2011) amplify shorter fragments than other bird-sexing primers and seem to be robust and convenient for sexing rock ptarmigan. This was relevant in this study of NGS, which often may give degraded DNA. Initially, primers designed by Regnaut *et al.* (2006a) and Fridolfsson and Ellegren (1999) for sexing capercaillie were tested but did not give consistent results, and hence not followed up.

Weather induced factors such as thawing/freezing and out-washing are known to cause degradation in DNA, as free DNases are more active under moist conditions (Regnaut *et al.* 2006b; Brinkman *et al.* 2010). Our results cannot pinpoint an exact window of time when rapid degradation occurs, but suggest that rock ptarmigan FPs, as well as fecal pellet feces of other snow-roosting species, should be collected when snow covers the ground and preferably before the spring melt, in order to obtain best results. We suspect that more data collected from the roosts in the weeks preceding 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 FP exposure.

Preferably, future genetic studies of rock ptarmigan or other grouse species based on NGS using FPs from snow roosts should start with assessing genotyping errors in a number of msat 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.

Conclusions

Fecal pellets from rock ptarmigan snow roosts appear to be a promising NGS source for mtDNA, and nDNA. Samples are relatively easy to obtain during suitable weather conditions immediately following a recent snowfall and are often obtainable in large amounts from each individual. Deposition of FPs in snow during 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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References

Brinkman, T.J., Schwartz, M.K., Person, D.K., Pilgrim, K.L. and Hundertmark, K.J. 2010: Effects of time and rainfall on PCR success using DNA extracted from deer fecal pellets. *Conservation Genetics*, **11**: 1547-1552.

Broquet, T. and Petit, E. 2004: Quantifying genotyping errors in noninvasive population genetics. *Molecular Ecology*, **13**: 3601-3608.

Caizergues, A., Dubois, S., Loiseau, A., Mondor, G. and Rasplus, J.Y. 2001: Isolation and characterization of microsatellite loci in black grouse (*Tetrao tetrix*). *Molecular*

Ecology Notes, **1**: 36-38.

Cotter, R.C. 1999: The reproductive biology of rock ptarmigan (*Lagopus mutus*) in the central Canadian Arctic. *Arctic*, **52**: 23-32.

Fridolfsson, A.K. and Ellegren, H. 1999: A simple and universal method for molecular sexing of non-ratite birds. *Journal of Avian Biology*, **30**: 116-121.

Griffiths, R., Daan, S. and Dijkstra, C. 1996: Sex identification in birds using two CHD genes. *Proceedings of the Royal Society of London Series B-Biological Sciences*, **263**: 1251-1256.

Holder, K. and Montgomerie R. 1993: Rock Ptarmigan (*Lagopus mutus*). The Academy of Natural Sciences; Washington, D.C.: The American Ornithologists´ Union., Philadelphia, Pa.

Hughes-Stamm, S.R., Ashton, K.J. and van Daal, A. 2011: Assessment of DNA degradation and the genotyping success of highly degraded samples. *International Journal of Legal Medicine*, **125**: 341-348.

Imperio, S., Bionda, R., Viterbi, R. and Provenzale, A. 2013: Climate Change and Human Disturbance Can Lead to Local Extinction of Alpine Rock Ptarmigan: New Insight from the Western Italian Alps. *PloS ONE*, **8**.e81598.

Kvasnes, M.A.J., Storaas, T., Pedersen, H.C., Bjork, S. and Nilsen, E.B. 2010: Spatial dynamics of Norwegian tetraonid populations. *Ecological Research*, **25**: 367-374.

Lehikoinen, A., Green, M., Husby, M., Kålås, J.A. and Lindstrøm, Å. 2013: Common montane birds are declining in northern Europe. *Journal of Avian Biology*, **44**: 1-12.

Lucchini, V., Fabbri, E., Marucco, F., Ricci, S., Boitani, L. and Randi, E. 2002: Noninvasive molecular tracking of colonizing wolf (*Canis lupus*) packs in the western Italian Alps. *Molecular Ecology*, **11**: 857-868.

Maudet, C., Luikart, G., Dubray, D., Von Hardenberg, A. and Taberlet, P. 2004: Low genotyping error rates in wild ungulate faeces sampled in winter. *Molecular Ecology Notes*, **4**: 772-775.

Murata, K., Tamada, A., Ichikawa, Y., Hagihara, M., Sato Y., Nakamura, H., Nakamura, M., Sakanakura, T. and Asakawa, M. 2007: Geographical distribution and seasonality of the prevalence of leucocytozoon lovati in Japanese rock ptarmigans (*Lagopus mutus japonicus*) found in the alpine regions of Japan. *Journal of Veterinary Medical Science*, **69**: 171-176.

Nsubuga, A.M., Robbins, M.M., Roeder, A.D., Morin, P.A., Boesch, C. and Vigilant, L. 2004: Factors affecting the amount of genomic DNA extracted from ape faeces and the identification of an improved sample storage method. *Molecular Ecology*, **13**: 2089-2094.

Nystrom, J., Dalen, L., Hellstrom, P., Ekenstedt, J., Angleby, H. and Angerbjorn, A. 2006: Effect of local prey availability on gyrfalcon diet: DNA analysis on ptarmigan remains at nest sites. *Journal of Zoology*, **269**: 57-64.

Odland, A., Hoitomt, T. and Olsen, S.L. 2010: Increasing Vascular Plant Richness on 13 High Mountain Summits in Southern Norway since the Early 1970s. *Arctic Antarctic and Alpine Research*, **42**: 458-470.

Panasci, M., Ballard, W.B., Breck S., Rodriguez D., Densmore L.D., Wester D.B. and Baker R.J. 2011: Evaluation of Fecal DNA Preservation Techniques and Effects of Sample Age and Diet on Genotyping Success. *Journal of Wildlife Management*, **75**: 1616-1624.

Perez, T., Vazquez, J.F., Quiros, F. and Dominguez, A. 2011: Improving non-invasive genotyping in capercaillie (*Tetrao urogallus*): redesigning sexing and microsatellite primers to increase efficiency on faeces samples. *Conservation Genetics Resources*, **3**: 483-487.

Post, E., Forchhammer, M.C., Bret-Harte, M.S., Callaghan, T.V., Christensen, T.R., Elberling, B., Fox, A.D., Gilg, O., Hik, D.S., Hoye, T.T., Ims, R.A., Jeppesen, E., Klein, D.R., Madsen, J., McGuire, A.D., Rysgaard, S., Schindler, D.E., Stirling, I., Tamstorf, M.P., Tyler, N.J.C., van der Wal, R., Welker, J., Wookey, P.A., Schmidt, N.M. and Aastrup, P. 2009: Ecological Dynamics Across the Arctic Associated with Recent Climate Change. *Science*, **325**: 1355-1358.

Quintela M., Thulin C.G. and Hoglund J. 2010: Detecting hybridization between willow grouse (*Lagopus lagopus*) and rock ptarmigan (*L. muta*) in Central Sweden through Bayesian admixture analyses and mtDNA screening. *Conservation Genetics*, **11**: 557-569.

Regnaut, S., Christe, P., Chapuisat, M. and Fumagalli, L. 2006a: Genotyping faeces reveals facultative kin association on capercaillie's leks. *Conservation Genetics*, **7**: 665-674.

Regnaut S., Lucas F.S. and Fumagalli L. 2006b: DNA degradation in avian faecal samples and feasibility of non-invasive genetic studies of threatened capercaillie populations. *Conservation Genetics*, **7**: 449-453.

Segelbacher, G., Paxton, R.J., Steinbruck, G., Trontelj, P. and Storch, I. 2000: Characterization of microsatellites in capercaillie *Tetrao urogallus* (AVES). *Molecular Ecology*, **9**: 1934-1935.

Segelbacher, G. and Steinbruck, G. 2001: Bird faeces for sex identification microsatellite analysis. *Die Vogelwarte*, **41**: 139-142.

Taberlet, P., Waits, L.P. and Luikart, G. 1999: Noninvasive genetic sampling: look before you leap. *Trends in Ecology & Evolution*, **14**: 323-327.

Waits L.P. and Paetkau D. 2005: Noninvasive genetic sampling tools for wildlife biologists: A review of applications and recommendations for accurate data collection. *Journal of Wildlife Management*, **69**: 1419-1433.

Watson, A. and Moss, R. 2004: Impacts of ski-development on ptarmigan (*Lagopus mutus*) at Cairn Gorm, Scotland. *Biological Conservation*, **116**: 267-275.

Watson, A. and Moss, R. 2008: Grouse. HarperCollins Publishers, London.

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 species identification, sex or genotype

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

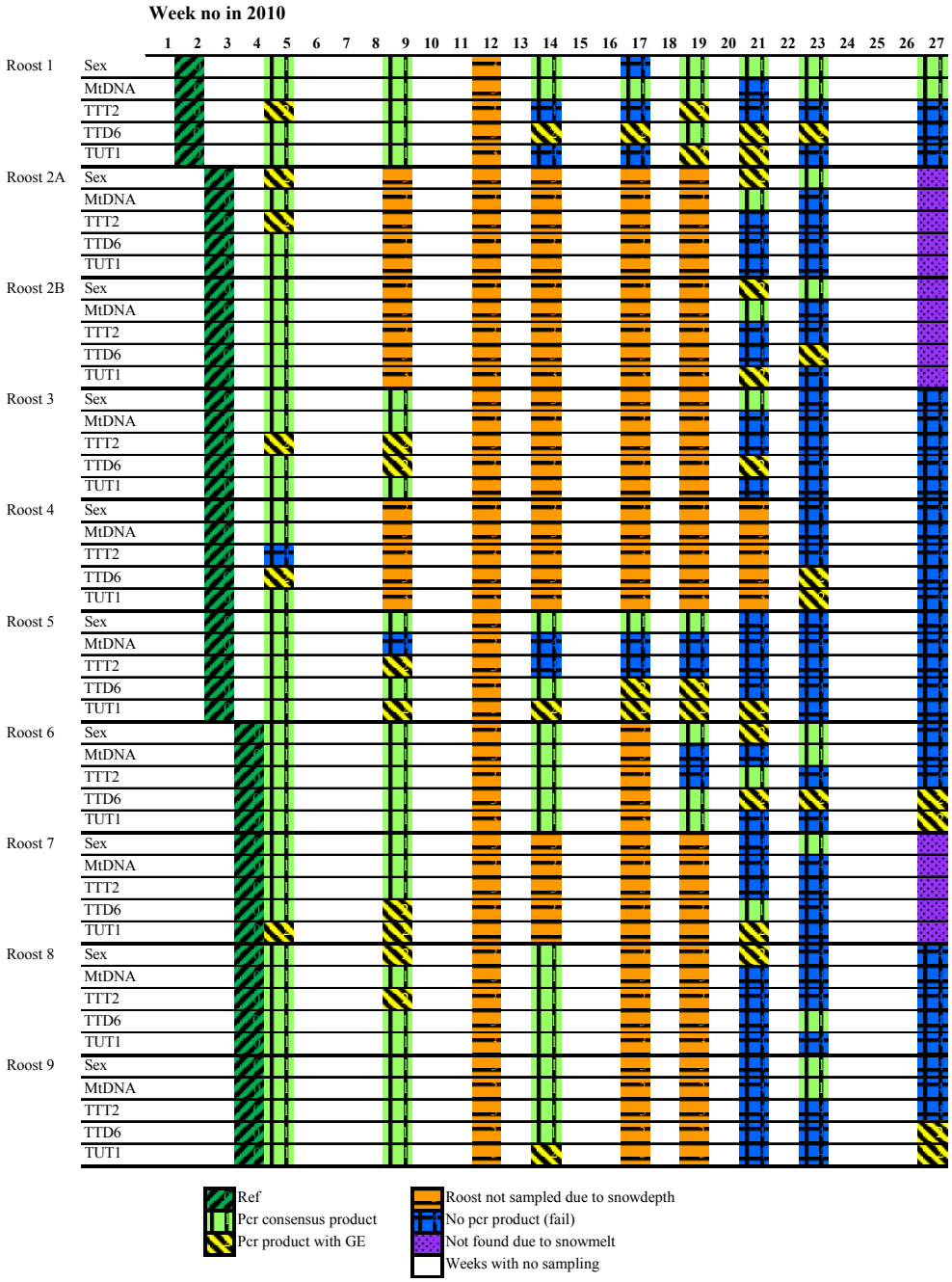
PCR product with GE = mtDNA with fragment size different from 212 bp, CHD gene as products gaining one gel band (male to female) or losing one (female to male), or microsatellites with missing alleles, false alleles or both compared to ref.

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 or CHD gene by conventional gel electrophoresis, or microsatellites by capillary electrophoresis was obtained

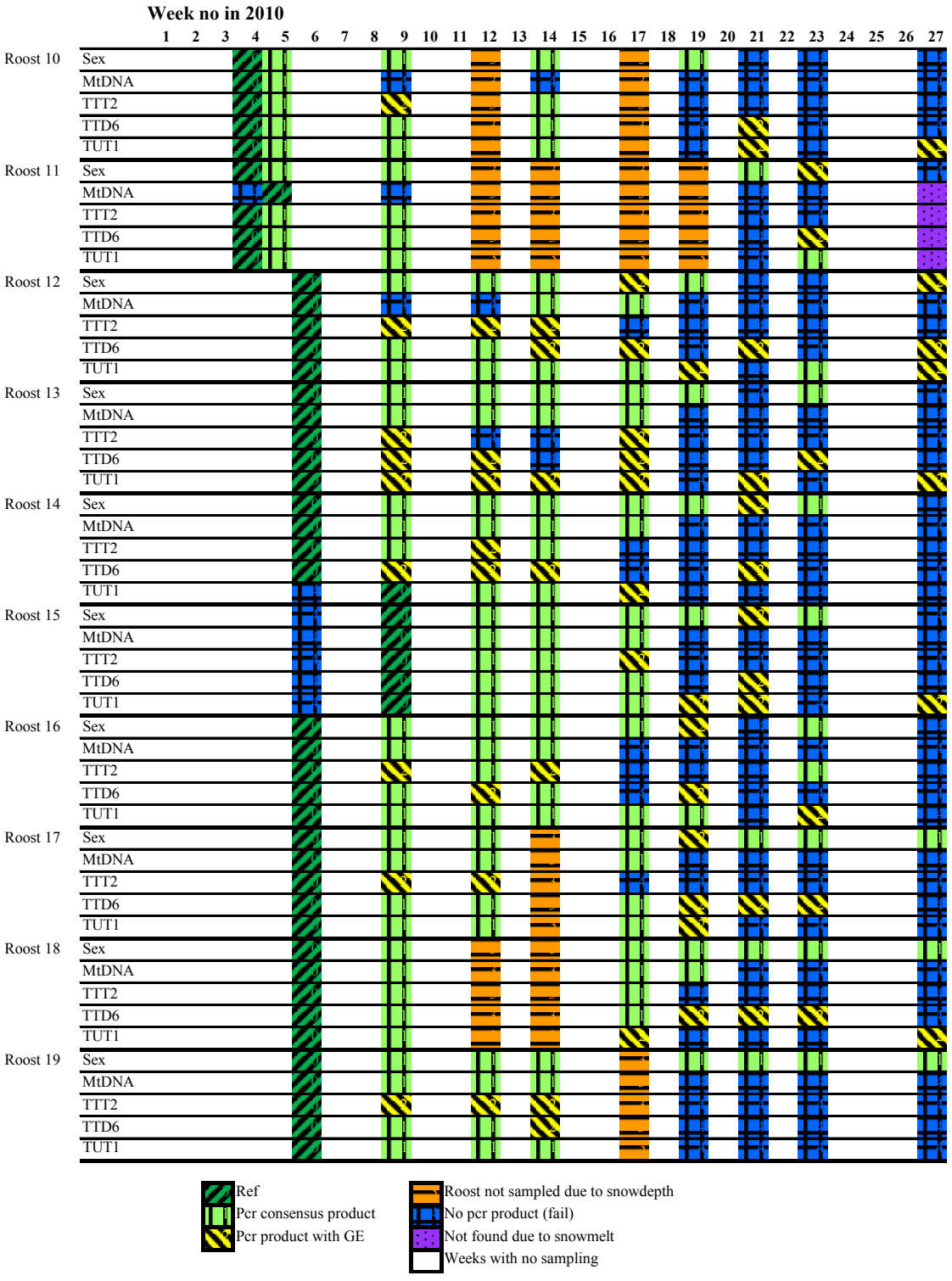
Not found due to snowmelt = the FPs in the roost had either disappeared in the vegetation or eroded away after all snow had melted

Weeks with no sampling = interval between samplings



9

In-field stability of DNA from fecal pellets of Rock ptarmigan (Lagopus muta)



APPENDIX B

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

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 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.

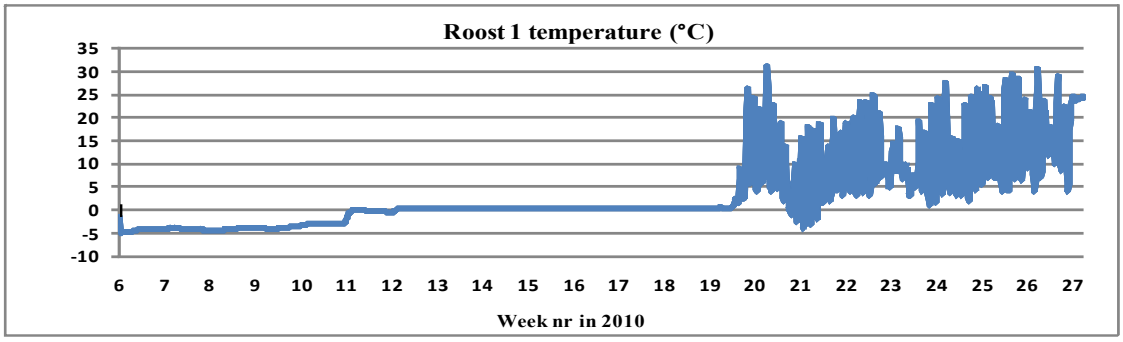
	Locus		
Roost	TTT2	TTD6	TUT1
1	C-E	A-E	B-D
2A	C	F	D
2B	C	F-J	B-D
3	C	A-K	B-D
4	D	I	B
5	E	A-F	A-B
6	D	C-H	B
7	C-E	E	D-E
8	B-D	A	B
9	D	F-J	C-D
10	A	D	D
11	A	D	D
12	C	A-I	D
13	C-D	A-F	B-C
14	C	A-C	B-C
15	C-D	A-F	B-C
16	B	A-B	B
17	B-C	C-G	B-D
18	C	A-C	B-D
19	C-D	A-C	B-D
nHet	7	14	12

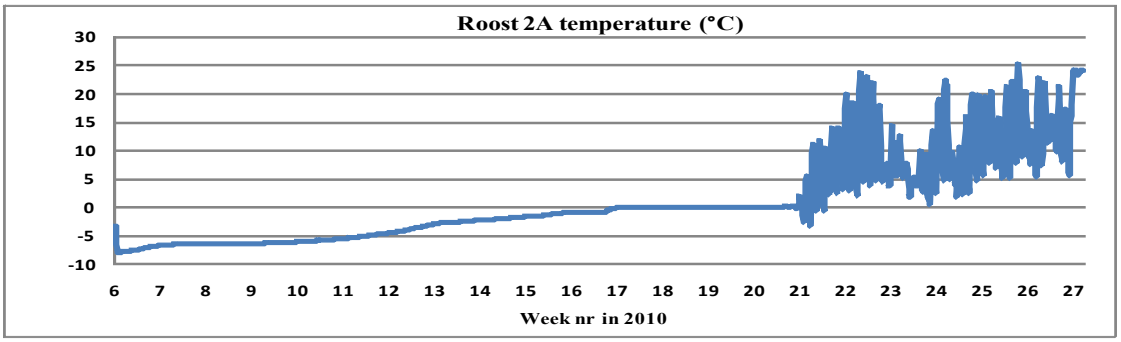
nHet = number of heterozygote roost ref samples

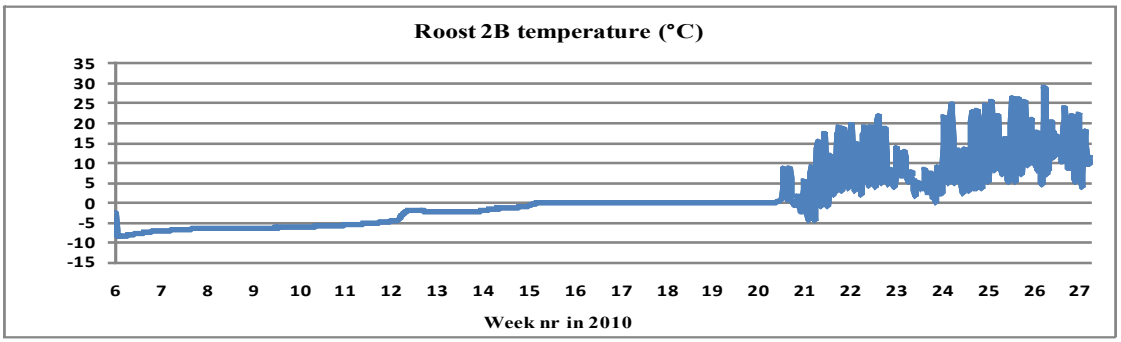
Table b. Genotyping of reference samples from each of 20 roosts. Alleles are defined in table a.

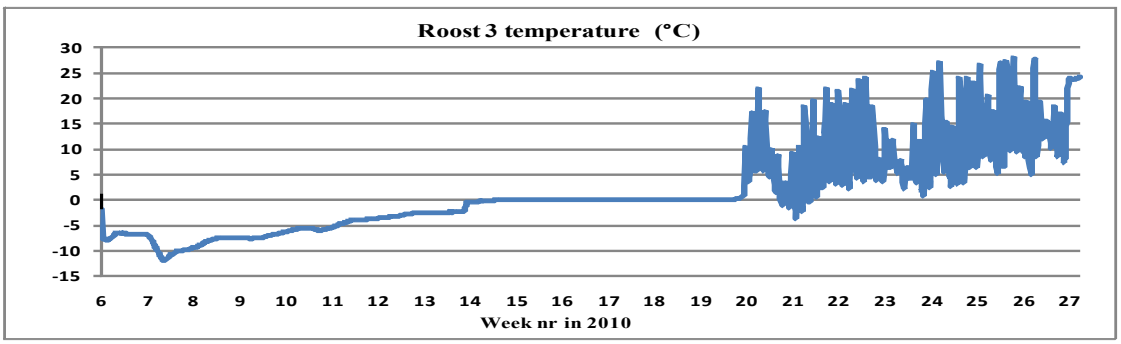
APPENDIX C

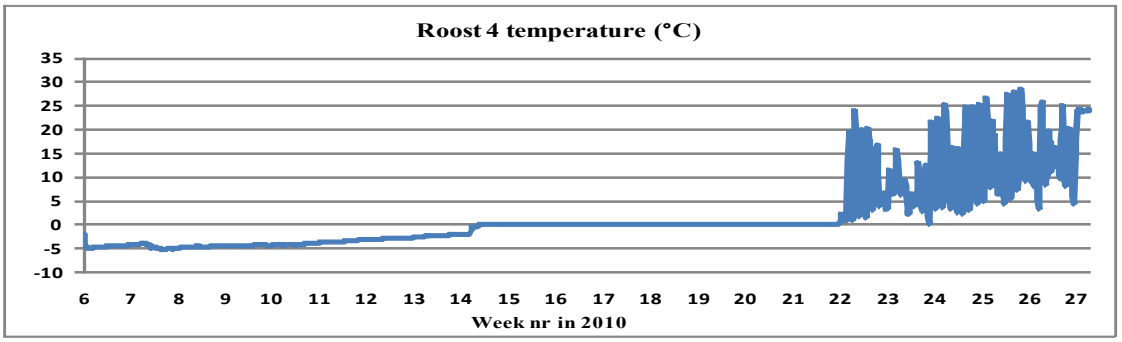
Temperature logger readings from all 20 roosts (following pages). Stable temperatures at 0˚ C or slightly below means that roost are under snow surface. Alternating roost temperatures means that roost FPs are more affected by precipitation and radiation.

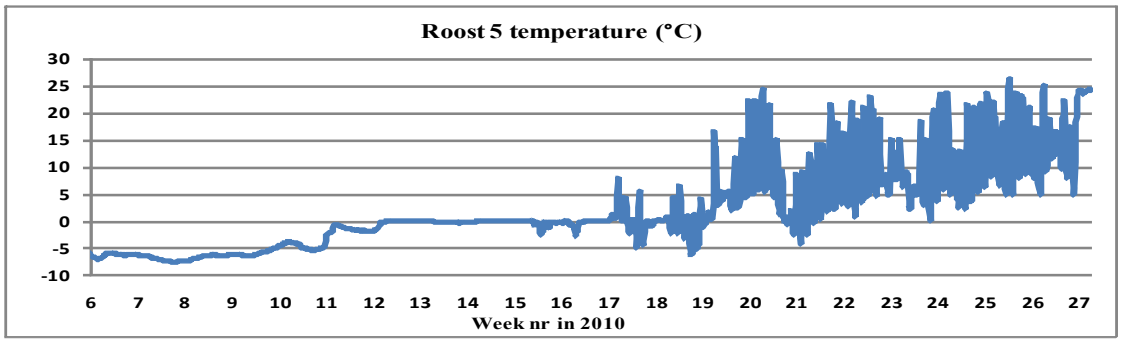


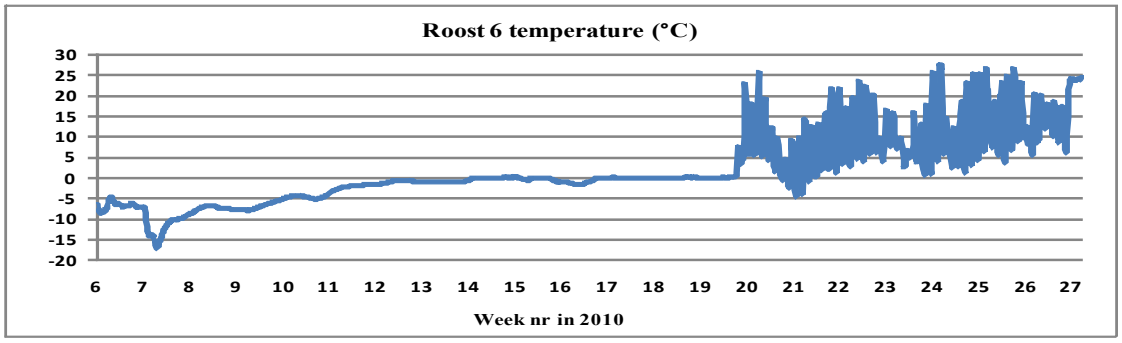


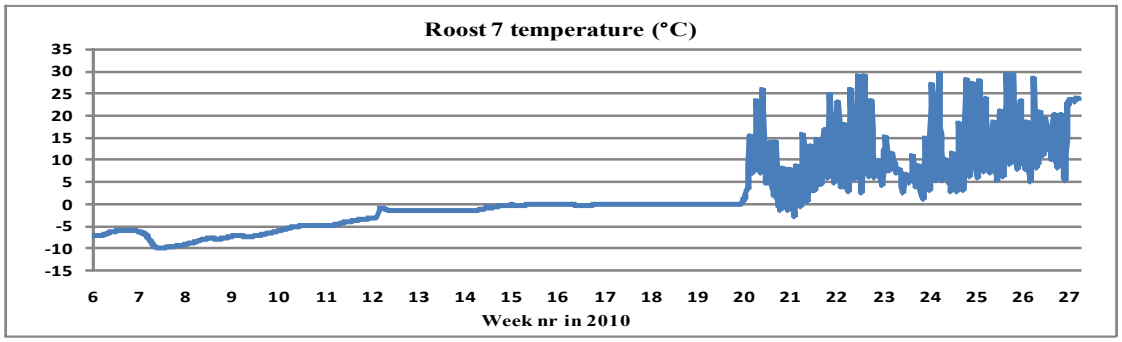


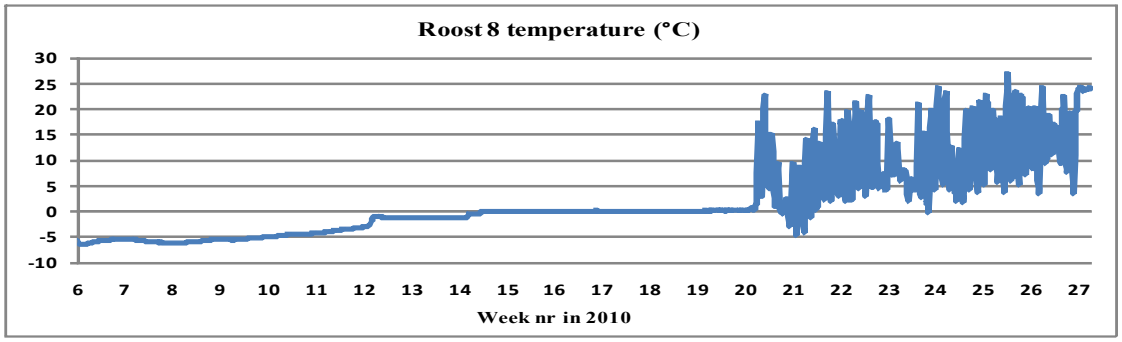


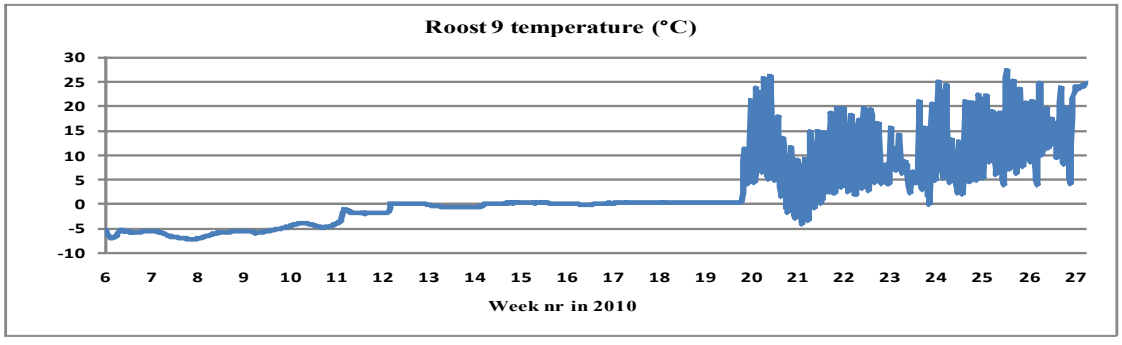



Roost 5 temperature (°C)
30
25
20
15
10
5
0
-5
-10
6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27
Week nr in 2010

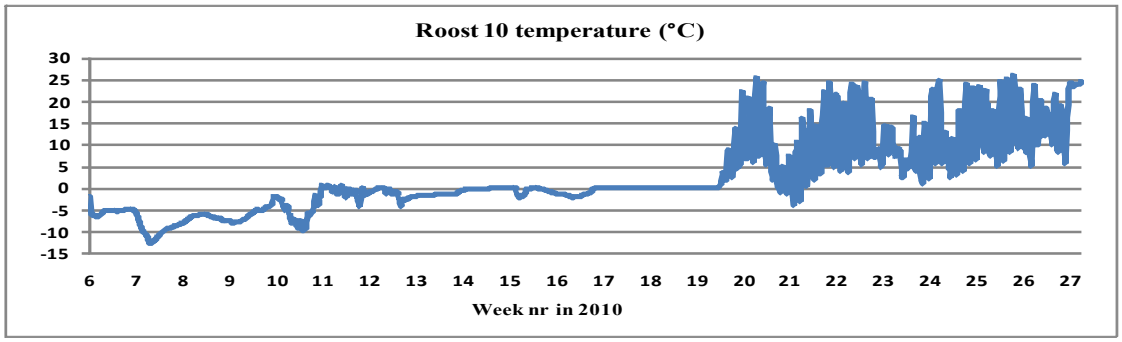

Roost 6 temperature (°C)
30
25
20
15
10
5
0
-5
-10
-15
-20
6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27
Week nr in 2010

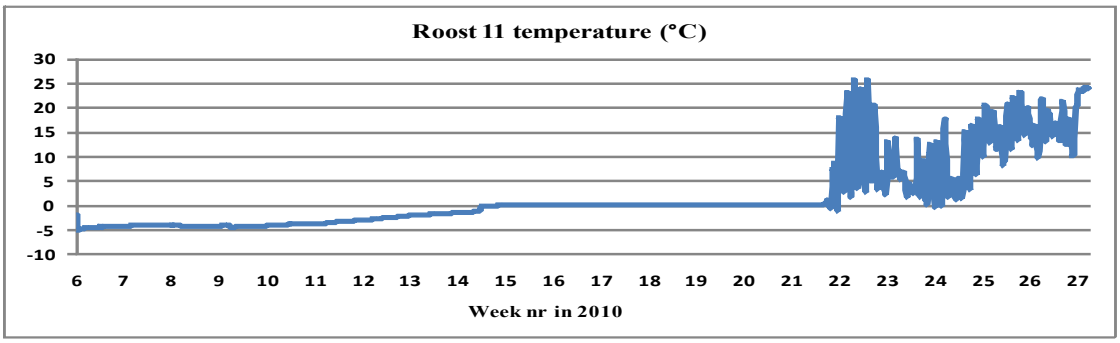

Roost 7 temperature (°C)
35
30
25
20
15
10
5
0
-5
-10
-15
6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27
Week nr in 2010

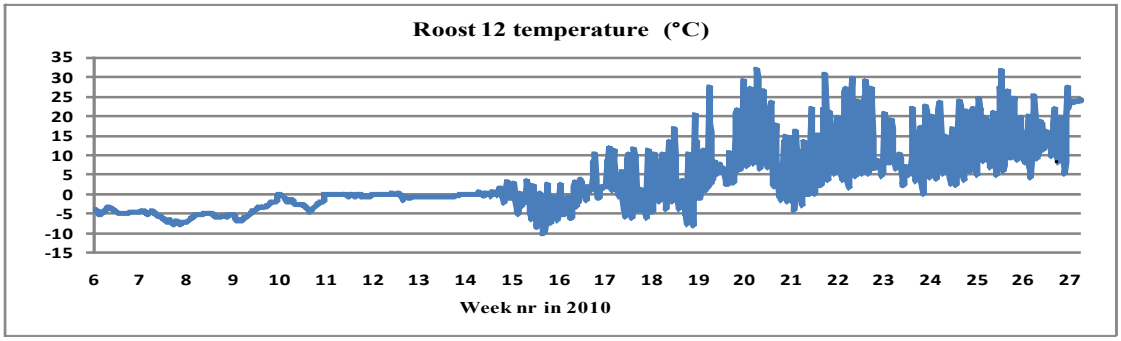

Roost 8 temperature (°C)
30
25
20
15
10
5
0
-5
-10
6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27
Week nr in 2010

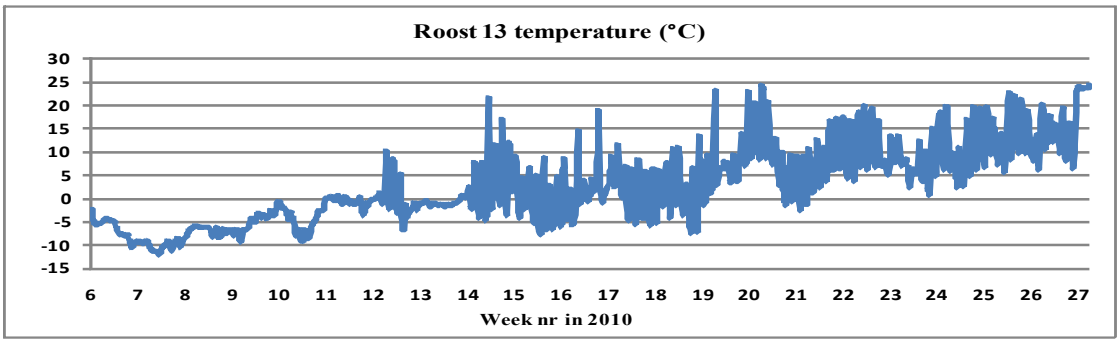

Roost 9 temperature (°C)
30
25
20
15
10
5
0
-5
-10
6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27
Week nr in 2010

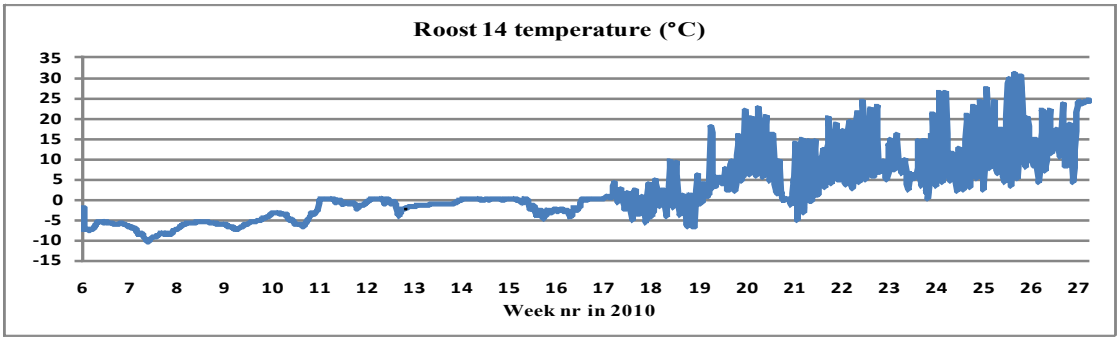
13
In-field stability of DNA from fecal pellets of Rock ptarmigan (Lagopus muta)

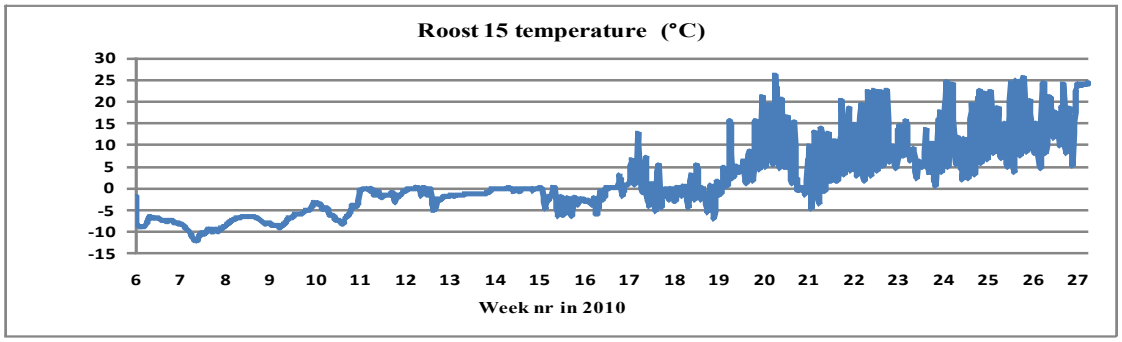


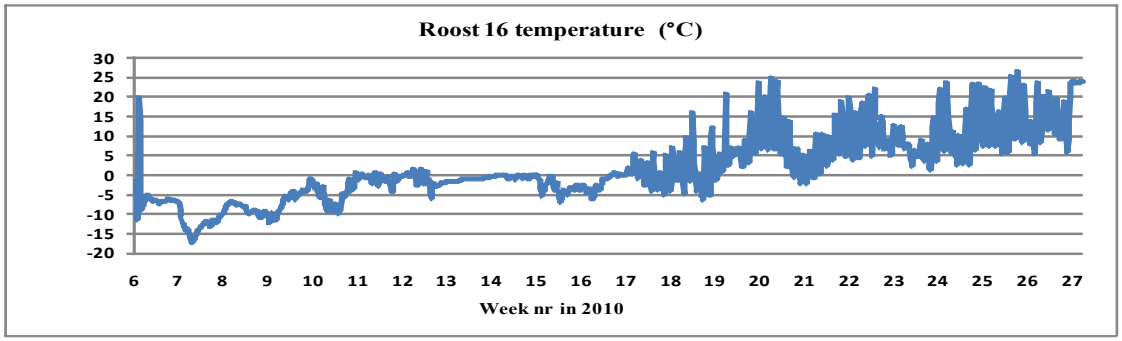


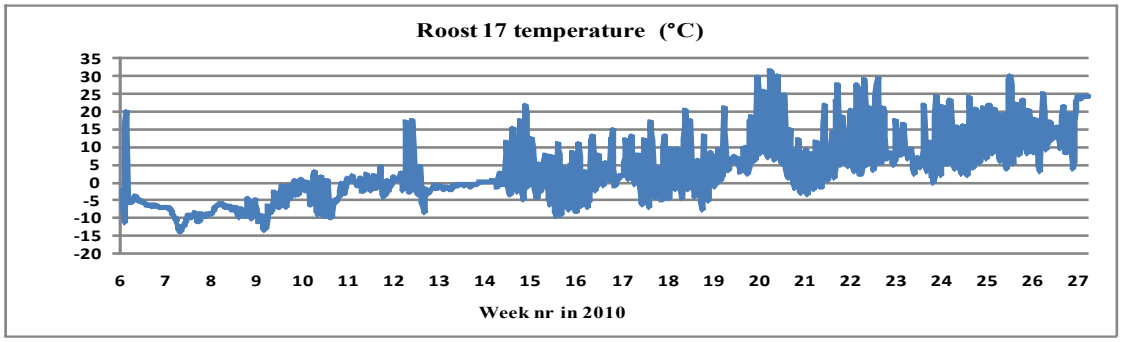


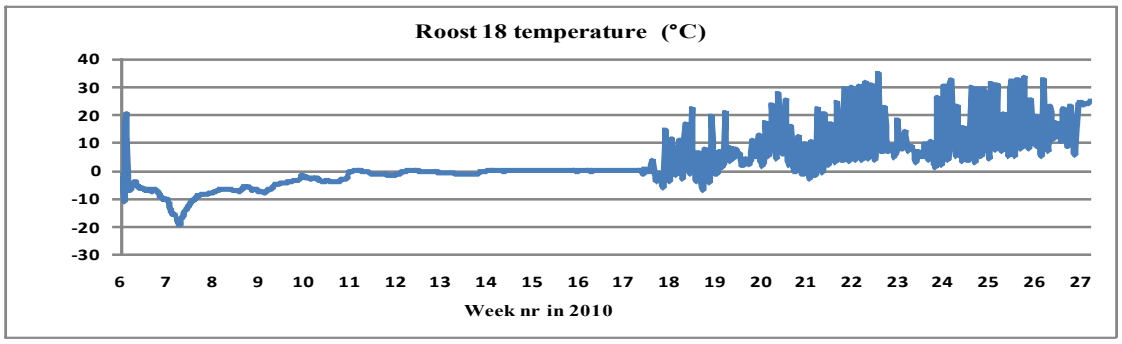


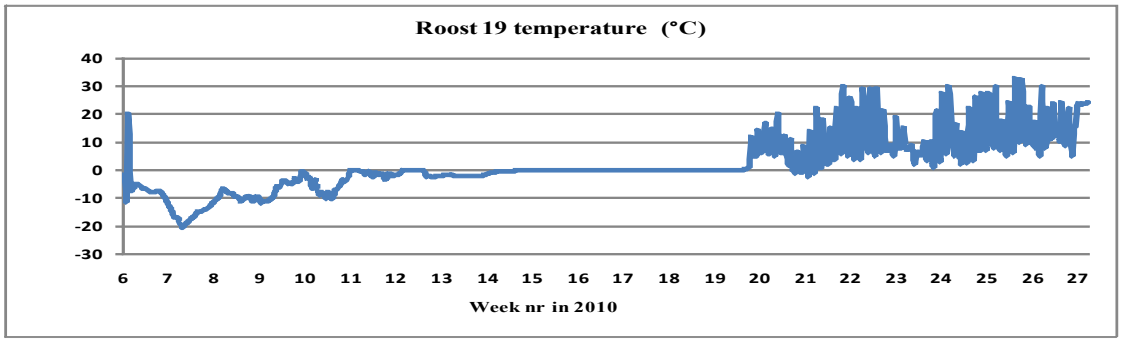



Roost 15 temperature (°C)
30 25 20 15 10 5 0 -5 -10 -15
6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27
Week nr in 2010


Roost 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