Chapter 2

Materials and Methods

2.1 Animal Housing and Photic Treatment

Animals were maintained and sacrificed at the Nicolaus Copernicus University in Torun, Poland and procedures were approved by the Local Committee for Ethics in Animal Research in Bydgoszcz, Poland (decision #52/2020). For tissue collection, 72 Siberian hamsters out of 293 were sacrificed and others were sent back to breeding colony after the short-day acclimation. All individuals were maintained in standard laboratory cages (1245; Tecniplast) with deciduous wood chips as bedding material and paper tubes as nesting material. Whole litter shared one cage until a month and offspring were ablactated at postnatal day 18.

Animals were maintained individually or in same-sex pairs under long photoperiod (LD; 16:8) at 20° ± 2°C and fed with standard rodent food with higher protein and fat until the second month of life (Labofeed H Standard, Morawski, Kcynia, Poland) and afterwards with standard maintenance food (Labofeed B standard, Morawski, Kcynia, Poland). At age of 2 to 3.5 months, 54 animals were transferred to short photoperiod (SD; 8:16) to induce winter response. For control, 17 individuals remained in the long photoperiod. To determine phenotype of individuals body mass changes, moulting and torpor were used

during the acclimation. Hamsters were weighed (Scout Pro 200, OHAUS) every week. Fur colour was assessed weekly during weighting, based on Figala metric (Figala et al. 1973) where 1 represents the darkest pelage which is observed in summer conditions and 6 represents the lightest pelage which is observed in winter. After 12 weeks of SD treatment, 48 individuals were chosen and three groups which differed in propensity to torpor use and moulting stage were created. Upon transferring hamsters from LD to SD, a subset of the population exhibited significant changes in body mass and fur colour, categorizing them as responders (PP and WP). Specifically, these responders showed a drastic decrease in body mass and developed a winter coat with lighter fur color. In contrast, non-responders or summer phenotype (SP) maintained a relatively stable body mass and retained their summer coat coloration despite the shortened photoperiod. Additionally, within the responder group, a notable proportion of hamsters entered a state of torpor, further indicating a robust physiological adaptation to the SD conditions and categorized as fully responders or winter phenotype (WP). The hamsters that did not engage in torpor were categorized as partially responders or partial phenotype (PP). For control group, 12 individuals were chosen from the LD acclimation. After 14 weeks of acclimation, animals were sacrificed by cervical dislocation, regardless of light regime. Liver tissues were covered within RNALater and freezing medium (Tissue-Tek OCT, Compound; Sakura Finetek) on dry ice and then stored at -80°C for later procedure.

2.2 Molecular Extraction Protocols

2.2.1 DNA Extraction

Genomic DNA was extracted from liver tissues of Siberian Hamsters using DNeasy Blood & Tissue (Qiagen; catalog #69504). Liver tissues were weighed to be 25 mg and cut into 2-3 mm pieces on dry ice and transferred into 1.5 ml Lo-Bind Eppendorf tubes. For lysis, 180 µL of ATL and 20 µL proteinase K were added into each tube. Samples

were incubated at 56 °C and vortexed every 10 minutes until they are completely lysed. For each sample 200 µL AL: 100% Ethanol (1:1) were premixed and added into tubes. Lysates were vortexed for 15 seconds and transferred into columns within collection tubes. Tubes were centrifuged for 1 minute at 7500xg. Collection tube and flow-through were discarded. Into each column, 500 µL of AW1 was added and centrifuged for 1 minute at 7500xg. Flow-through and collection tube were discarded. For second wash, 500 µL of AW2 was added into each column and centrifuged for 3 minutes at 20000xg. Columns were carefully transferred to avoid contamination into new 1.5 ml Lo-Bind tubes. To elute the DNA, 200 µL of AE was added into each tube. Incubated at room temperature (RT) for 1 minute. After incubation, samples were centrifuged for 1 minute at 7500xg. Initial quantity was determined using Nanodrop spectrophotometry (Thermo Fisher Scientific). Samples were then aliquoted as 20 µL and sent for Qubit measurement. Aliquots (20 µL) were stored at –80°C for downstream analyses. to be used in genomic analyses.

2.2.2 RNA Extraction

To extract RNA, we used hybrid extraction protocol which is using TRIzol plus column purification. RNA were extracted from liver tissues of Siberian hamsters using RNeasy Plus Mini Kit (Qiagen; catalog #74134). Liver tissues (N=24) were weighed to be 50 mg and cut into <2 mm^3 pieces on dry ice and transferred into 1.5 ml Lo-Bind Eppendorf tubes. After adding 1 ml/50 mg TRIzol, samples were homogenized using Polytron. Samples were incubated on ice for 5 minutes to let nucleoprotein complex dissociate. 200 µL chloroform was added each tube and mixed thoroughly. Tubes were centrifuged at 1200xg for 15 minutes at +4 C. Aqueous phase was transferred into new clean 1.5 ml tube. Freshly prepared 1:1 (v:v) 80% EtOH was added into each tube. From each sample, 500 µL was transferred onto RNA-specific spin columns and centrifuged at 8000xg for 30 seconds. This step was repeated twice. 700 µL RW1 buffer was added into each sample and centrifuged at 8000xg for 15 seconds. To wash the column, 500 µL RPE was added and centrifuged at 8000xg for 15 seconds. Then 500 µL RPE was added and centrifuged at

Reagent	6-10 rxns	3-5 rxns
Stock Enzymatic Shearing Cocktails (2x10^4 u/ml	2 µL	1 µL
50% Glycerol	198 µL	99 µL
Final extension	65	6 min

Table 2.1: Working stock enzyme preperation

8000xg for 2 minutes. Columns were transferred to new 1.5 ml tubes. To elute the RNA, 15 µL RNase free water was added and incubated for 1 minute. At the end of incubation, tubes were centrifuged at 8000xg for 1 minute. Initial quantity was determined using Nanodrop spectrophotometry (Thermo Fisher Scientific). This aliquotes were stored in -20 °C to be used in transcriptomic analyses.

2.2.3 Methylated Chromatin Extraction

Frozen liver tissues (n=24) were cut into small pieces (1-3 mm^3) a petri dish resting on a block of dry ice. Chopped tissues were weighed in a clear 15 ml falcon tube. Cross-linking solution was prepared in fume hood by using 10 ml PBS per gram and addition of 1.5% formaldehyde to the final concentration. Tubes were rotated for 15 minutes at room temperature. To stop cross-linking, 0.125 M glycine was added and kept rotating for 5 minutes at RT. Samples were centrifuged for 5 minutes at 720 rpm at +4 C. Supernatant was discarded and sample was washed with 10 ml ice cold PBS. Tubes were centrifuged for 5 minutes at 720 rpm at +4 C and wash buffer was discarded. Next, the tissue chunks were resuspended in 1 ml of lysis buffer, 5 µL PIC and 5 µL PMSF. MP beads were used to homogenize tissues. Tissues were incubated on ice for 30 minutes. After homogenization, 10 µL of supernatant was monitored under microscope. This step was repeated again until there were no more non-disrupted cells. Lysates were transferred into a 1.7 ml microcentrifuge tube and centrifuged for 10 minutes at 5000 rpm at +4 C to pellet nuclei. Supernatants were carefully discarded. Pellets were resuspended in 350 µL

digestion buffer (supplemented with 1.75 µL PIC and 1.75 µL PMSF) and incubated for 5 minutes at 37 C. Fifty µL of the resuspension was added into 4 clean microcentrifuge tubes and incubated at 37 C for 2 minutes. Resuspensions were optimized for shearing conditions using enzymatic digestion, with brief vortex in every 2 minutes.

- 50 µL chromatin + 2.5 µL dH_2O (without enzyme). Incubated for 10 minutes at 37 C.
- 50 µL chromatin + 2.5 µL working stock enzyme. Incubated for 5 minutes at 37 C.
- 50 µL chromatin + 2.5 µL working stock enzyme. Incubated for 10 minutes at 37 C.
- 50 µL chromatin + 2.5 µL working stock enzyme. Incubated for 15 minutes at 37 C.

The remaining chromatin was placed at -80 C. Reactions were stopped by adding 1 µL ice-cold 0.5 M EDTA to each tube and incubation on ice for 10 minutes. Centrifuged the sheared chromatin samples for 10 minutes at 15000 rpm at +4 C. Supernatants were collected. Then 150 µL dH_2O and 10 µL 5 M NaCl were added to each tube. Samples were incubated at 65 C using a thermocycler fr overnight to reverse cross-linking. After the overnight incubation, 1 µL RNase A was added and incubated for 15 minutes at 37 C. To each sample, 10 µL of Proteinase K was added and incubated at 42 C for 1.5 hours. Two hundred µL 1:1 phenol:chloroform (TE pH=8) was added to each sample, vortexed and centrifuged for 5 minutes at maximum rpm. Aqueous phase was transferred into clean tubes, then 20 µL 3 M sodium acetate (pH = 5.2) and 500 µL 100% EtOH were added. Mixture was vortexed and placed at -80 C for 1 hour. At the end of the incubation, samples were centrifuged at maximum speed for 10 minutes at +4 C. Supernatant was carefully removed. Addeed 500 µL 70% ice cold EtOH, spun for 5 minutes at maximum speed at +4 C, removed the supernatant and air-dried the pellet. Pellet was resuspended in 30 µL dH_2O and by using NanoDrop DNA quantification was performed. Samples were run on agarose gel. Ten µL of sample was transferred into new tube and immunoprecipitation solution was added. Samples were incubated at +4 C for overnight. Next day, tubes were

spun briefly to collect liquid from the cap and placed on magnetic stand to pellet beads on the side. Supernatant was removed. Beads were washed once with 800 µL ChIP Buffer I. Then were washed with 800 µL ChIP Buffer II. After the final wash, supernatant was removed without disturbing the beads.

Reagent	**1x Reaction (< 60 µL)**	**1x Reaction (> 60 µL)**
Protein G magnetic beads	25 µL	25 µL
ChIP Buffer I	10 µl	20 µL
Sheared chromatin (7-25 µg)	20-60 µL	61-10 µL
PIC	1 µL	2 µL
dH_2O	up to 100 µL	up to 200 µL
Mecp2 antibody	1-3 µg	1-3 µg
Sample	10 µL	10 µL

Table 2.2: Immunoprecipitation solution

Beads were resuspended with 50 µL EB AM2. Incubated for 15 minutes at room temperature on rotator. Tubes were spun to collect the liquid from the caps. Fifty µL Reverse Crosslink Buffer was added to eluted chromatin and mixed by pipetting up and down. Tubes were placed on magnetic stand and beads were pelleted. Supernatant which contains the chromatin was transferred into clean tubes. An input DNA control was prepared by combining 10 µL of input DNA with 88 µL of ChIP Buffer 2 and 2 µL of 5M NaCl, bringing the final volume to 100 µL.

Proteinase K (2 µL) was added and samples were incubated for 1 hour at 37 C. During the incubation, proteinase K stop solution was placed at RT for 30 minutes. To stop the activity, 2 µL proteinase K stop solution was added. Tubes were centrifuged and stored at -20 C until sequencing. The sequencing was done by Polyomics group.

2.3 Library Preparation and Sequencing

2.3.1 Whole Genome Sequencing of Liver Tissues Chapter

DNA samples were sequenced on Oxford Nanopore Technologies GridION platform using SQK-LSK110 Ligation Sequencing Kit with EXP-PBC001 PCR Barcoding Kit. DNA samples were prepared to be 1000ng in 45 µL nuclease-free water. For each sample, in 0.2 ml flat-capped PCR tubes 7 µL Ultra II End Prep Reaction Buffer (NEBNext; cat #E7647), 3 µL Ultra II End Prep Enzyme Mix (NEBNext; cat #E7646) and 45 µL of DNA sample were mixed. Volume was topped up to 60 µL with nuclease free water. Samples were incubated for 5 minutes at 20 °C and 65 °C, respectively and transferred into 1.5 ml Lo-Bind Eppendorf tubes. Into each tube 60 µL resuspended beads (Agencourt AMPure XP Beads; catalog #A63881) were added. Samples were incubated on rotator mixer for 5 minutes at RT. After incubation, tubes were placed on magnetic rack and beads were pelleted. Without disturbing, beads were washed within 180 µL 70% Ethanol, spun down, placed back to pellet and ethanol was discarded. This washing was done twice. Upon second wash ethanol was discarded and beads were allowed to air-dry briefly (30 seconds). Beads were resuspended in 30 µL nuclease free water and incubated at RT for 2 minutes. To retain, samples were placed back to magnetic rack and elute was transferred into new tube. For ligating barcode adapter, in a 0.2 ml PCR tube, each 30 µL DNA sample was mixed within 20 µL Barcode Adapter (BCA, from PBC-001) and Blunt/TA Ligase Master Mix (NEBNext; catalog #M0367). Incubated for 10 minutes at RT. Beads were resuspended by vortexing and 40 µL of resuspended beads were added and placed to rotator mixer for 5 minutes at RT. Samples were spun down, placed to magnetic rack and supernatant was discarded. Beads were washed within 200 µL of 70% Ethanol, twice. Residual ethanol was discarded, and beads were let dry. To resuspend, 25 µL nuclease free water was added and incubated for 2 minutes at RT. Beads were pelleted on magnetic rack and 15 µL elute was retained. For barcoding step, 2 µL of each sample was mixed within 2 µL of barcodes (1-12), 50 µL Long Taq 2X Master Mix (NEBNext; catalog #M0287) and 46 µL

nuclease free water. Barcoding was performed using SimpliAmp Thermal Cycler (Applied Biosystems, Thermo Fisher Scientific). Each sample was transferred into 1.5 ml Lo-Bind

Step	Temperature (°C)	Duration
Initial denaturation	95	3 min
Denaturation	95	15 sec
Annealing	62	15 sec
Extension	62	1 kb/min
Final extension	65	1 kb/min
Cycles	12–15 cycles	

Table 2.3: Thermal cycler conditions for PCR barcoding in WGS

Eppendorf tubes and mixed with 60 µL resuspended beads. Samples were incubated on rotator mixer for 5 minutes at RT. Then samples were spun down and pelleted the beads on magnetic rack and supernatant was pipetted off. Beads were washed within 200 µL 70% Ethanol twice without disturbing. Ethanol was discarded and beads were allowed to air-dry briefly (30 seconds). Samples were resuspended in 10 µL nuclease free water and incubated for 2 minutes at RT. Next, placed back on magnetic rack and 10 µL elute was retained. Concentrations were measured using Nanodrop. Barcodes were mixed in a separate tube to be total 1000 ng in 47 µL. For DNA repair and end prep steps, 47 µL of each DNA was mixed within 1 µL DCS, 3.5 µL FFPE Repair Buffer (NEBNext; catalog #M6630), 2 µL FFPE Enzyme Mix (NEBNext; catalog #M6630), 3.5 µL Ultra II End Prep Reaction Buffer and 2 µL Ultra II End Prep Enzyme Mix. Mixture was incubated for 5 minutes at 20°C and 65 °C, respectively, using thermal cycler. Afterwards samples were mixed with 60 µL resuspended beads and incubated on rotator mixer for 5 minutes at RT. At the end of incubation, tubes were placed on magnetic rack and pelleted the beads. Supernatant was discarded and beads were washed within 200 µL 70% Ethanol, twice. After discarding residual ethanol, allowed to air-dry briefly (30 seconds). Beads were resuspended in 60 µL nuclease free water and incubated at RT for 2 minutes. Pelleted the beads on magnetic rack and retained 60 µL elute. For clean-up, 60 µL of each sample was mixed within 10 µL Quick T4 Ligase (NEBNext; catalog #M2200), 25 µL LNB, 5 µL AMX-F. Tubes were incubated at RT for 10 minutes. Next, 40 µL resuspended beads were added and incubated on rotator mixer for 5 minutes at RT. Samples were spun down and pelleted on magnet. Supernatant was discarded. Beads were washed within 250 µL

LFB, flicked to mix, incubated at RT for 30 seconds and spun down, twice. After spinning down, residual buffer was discarded, and beads were resuspended in 15 µL Elution Buffer and incubated at 37 °C for 10 minutes. Samples were placed on magnetic rack and 15 µL of elute was retained. To sequence the samples, FLO-MIN106D flowcells were primed for loading. To prepare priming buffer 30 µL of FLT was mixed within FB and vortexed. The yellow buffer was discarded from priming port on flowcell using P1000, carefully. From priming port, 800 µL priming buffer was added and incubated for 5 minutes at RT. Meanwhile, 37.5 µL Sequencing Buffer and 25.5 µL Loading Beads were mixed within 12 µL library. Next, 200 µL priming buffer was added through priming port. Library was immediately added through sample port dropwise manner. On MinKNOW Software, the ligation and barcoding kits were selected and started sequencing.

2.3.2 Transcriptome Sequencing of Liver Tissues

RNA samples were sequenced on Oxford Nanopore Technologies GridION platform using SQK-PCB111.24 kit. To perform reverse transcription and strand switching, 10 µL of 200 ng RNA samples were mixed with 1 µL CRTA and 1 µL AB in a 0.2 ml PCR tube. Tubes were incubated for 5 minutes at 60 C using thermal cycler and cooled down at room temperature (RT) for 10 minutes. To each tube 3.6 µL QLB, 1.4 µL T4 DNA Ligase and 1 µL RNase Out were added. Mixed thoroughly and incubated for 10 minutes at RT. At the end of incubation, 1 µL Lambda exonuclease and 1 µL USER were added and mixed. Using the thermal cycler, samples were incubated for 15 minutes at 37 C. Each sample was transferred into 1.5 ml tubes and 36 µL RNase-free XP Beads were added. Incubated on rotator mixer for 5 minutes at RT. Samples were spun down, placed on magnetic rack and supernatant was discarded. Beads were washed within 100 µL SFB, twice. Tubes were spun down and placed back to magnetic rack and the residual SFB was let dry. Beads were resuspended in 12 µL nuclease-free water, incubated for 10 minutes at RT and elutes were retained. Into each tube, 1 µL RTP and 1 µL dNTP (10 mm) mix

were added. Incubated for 15 minutes at RT. 4.5 µL Maxima H Minus 5x Buffer, 1 µL RNase Out, 2 µL SSPII were mixed with RT-primed sample and incubated in thermal cycler for 2 minutes at 42 C. 1 µL Maxima H Minus RT Enzyme was added into each tube and mixed. Reverse transcription was performed using a thermal cycler:

Step	Temperature (C)	Duration	#Cycles
RT and Strand Switching	42	90 min	1
Heat inactivation	85	5 min	

Table 2.4: Reverse Transcription Steps

To select full-length transcripts 5 µL of each sample was transferred into new 0.2 ml PCR tubes. To each tube, 0.75 µL Barcode (1-24), 6.75 µL nuclease-free water, 12.5 µL 2X LongAMP Hot Start Taq Master Mix were added and mixed. Barcoding and selection were performed:

Step	Temperature (C)	Duration	#Cycles
Initial denaturation	95	30 sec	1
Denaturation	95	15 sec	14
Annealing	62	15 sec	
Extension	65	2 min	
Final extension	65	6 min	1

Table 2.5: Barcoding and selection of transcripts

At the end of this step, samples were incubated for 37 C and 80 C for 15 minutes, respectively. Each sample was transferred into new 1.5 ml tube. 20 µL AMPure XP Beads was added into each tube, rotated for 5 minutes at RT. Tubes were spun down, placed to magnetic rack. Beads were washed with 200 µL 70% EtOH, twice. Residual EtOH was removed, and beads were allowed to air-dry briefly (30 seconds). 12 µL EB was added to resuspend the beads and incubated for 10 minutes at RT. Retained 12 µL of sample into new tube. Quantities and purities were measured by using NanoDrop 2000. Samples were pooled into one tube to have 22 ng or barcoded samples in 11 µL. To add the adapters, 0.6 µL RAPT and 3.4 µL RDP were mixed and 1 µL of mixture was added to the pooled samples and incubated for 5 minutes. Library was prepared by mixing 12 µL of adapter-ligated pooled samples, 37.5 µL SBII and 25.5 µL LBII. Flowcell was primed and loaded to GridION.

2.3.3 ChIP Sequencing

ChIP-seq libraries were constructed using the NEBNext Ultra II DNA Library Prep Kit for Illumina, following the manufacturer's protocol. Sequencing was performed on the Illumina NextSeq 2000 platform in paired-end mode with 100 base pair reads.

2.4 Bioinformatic and Statistical Analyses

2.4.1 Genome Assembly and Annotation

To assemble the genome, total DNA from 46 liver samples (n_{female}=26,n_{male}=20) was extracted and sequenced. Following sequencing, barcodes were trimmed and categorised using *porechop* and *guppy*. A *de novo* genome assembly was performed with *Flye* using the *–nano-raw* and *–genome-size 2.1g* flags. Gaps in the resulting assembly were then patched by aligning reads to a previous short-read sequence with *minimap2*. A subsequent polishing step was carried out using *racon* (*-m 8 -x 6 -g -8 -w 500*) and *medaka* to improve base accuracy. Raw reads totalled 15,205,279 across libraries, comprising 9.777 Gb of sequence (genome size 2.1 Gb), yielding an estimated nominal coverage of 4.66x (Table 2.6). For genome annotation, the following software and databases were employed: *hisat2*, *maker3*, *EVidenceModeler*, *Augustus*, *RepeatModeler*, *tRNAscan-SE*, *AGAT*, *InterProScan*, *Blast*, *UniProt*, and *StringTie2*. Transcriptome data from nine endocrine tissues (Stewart et al. 2022, n_{female}=3,n_{male}=3) were mapped to the assembly using *hisat2* and *stringtie2*, thereby facilitating the identification of coding regions. Repeat sequences were identified and masked by running *RepeatModeler* twice using the mouse genome as a reference. Transfer RNA (tRNA) genes were annotated with *tRNAscan-SE*. For gene prediction, *Augustus* was run with training sets derived from the Chinese hamster (taxid: 10029, *Cricetulus griseus*), Desert hamster (taxid:109678, *Phodopus roborovskii*),

and house mouse (taxid:10090, *Mus musculus*) genomes. The resulting predictions, along with the transcriptomic evidence, were combined in *maker3* to generate a preliminary annotation. This annotation was refined by integrating it into *EVidenceModeler* (*EVM*) and re-running the pipeline twice to improve gene model accuracy. Functional annotation of the predicted genes was conducted via *UniProt* and *InterProScan*, and *BlastN* was used to further characterise the annotated regions. Finally, *AGAT* commands were applied to remove redundant entries and produce a polished annotation. The assembly and annotation are stored in NCBI BioProject #PRJNA1029001.

Table 2.6: Per-library sequencing quality metrics for raw FASTQ files used in the genome build.

File	Reads	Bases (Gb)	Avg len (bp)	N50 (bp)	GC (%)	Q30 (%)	Cov (X)
10	509,939.000	0.293	574.500	636.000	45.640	7.010	0.140
11	264,632.000	0.206	779.200	789.000	46.620	8.650	0.098
12	503,762.000	0.326	647.300	655.000	43.080	12.170	0.155
13	339,449.000	0.225	663.900	679.000	44.320	11.020	0.107
14	326,187.000	0.208	637.000	698.000	47.490	6.230	0.099
15	23,390.000	0.082	3,508.900	4,151.000	40.880	13.740	0.039
16	562,615.000	0.244	433.500	517.000	46.150	11.050	0.116
17	522,298.000	0.228	436.100	522.000	45.230	11.330	0.108
18	321,001.000	0.154	480.800	550.000	43.860	12.650	0.073
1	664,647.000	0.391	587.600	573.000	44.880	11.080	0.186
20	48,781.000	0.135	2,767.800	3,480.000	40.940	13.530	0.064
21	242,707.000	0.173	714.400	736.000	44.200	11.830	0.083
22	563,517.000	0.350	620.200	671.000	44.460	12.530	0.166
23	64,057.000	0.089	1,394.800	2,025.000	41.220	12.650	0.043
24	484,868.000	0.332	684.000	736.000	43.170	12.630	0.158
25	36,475.000	0.104	2,863.100	3,532.000	40.590	13.650	0.050
27	811,630.000	0.512	631.000	661.000	46.200	10.580	0.244
28	17,555.000	0.052	2,955.200	3,711.000	41.030	13.560	0.025
2	258,031.000	0.130	504.800	560.000	43.820	11.980	0.062
30	899,241.000	0.485	538.800	570.000	45.020	11.390	0.231
32	459,462.000	0.198	431.100	519.000	45.120	13.100	0.094

Continued on next page

File	Reads	Bases (Gb)	Avg len (bp)	N50 (bp)	GC (%)	Q30 (%)	Cov (X)
34	664,492.000	0.438	658.800	655.000	44.980	13.520	0.208
35	44,246.000	0.140	3,158.700	3,908.000	40.860	13.530	0.067
36	13,602.000	0.038	2,825.200	3,540.000	40.980	13.500	0.018
38	135,064.000	0.088	651.900	732.000	45.810	9.470	0.042
39	30,892.000	0.096	3,093.700	3,872.000	40.920	13.500	0.046
3	311,606.000	0.184	589.200	628.000	43.820	8.350	0.087
42	24,000.000	0.073	3,022.500	3,823.000	41.010	13.700	0.035
46	29,050.000	0.092	3,159.900	4,029.000	41.090	13.680	0.044
49	365,785.000	0.160	436.700	506.000	44.600	12.600	0.076
4	732,351.000	0.371	506.800	496.000	45.120	7.570	0.177
51	2,145.000	0.007	3,263.400	4,222.000	40.800	9.080	0.003
55	680,714.000	0.376	552.400	582.000	44.450	8.100	0.179
56	868,327.000	0.438	504.100	520.000	44.070	8.050	0.208
57	436,264.000	0.313	716.900	814.000	46.370	6.410	0.149
58	241,091.000	0.116	480.300	571.000	44.790	12.820	0.055
59	269,577.000	0.137	507.800	567.000	44.590	10.970	0.065
5	44,436.000	0.030	664.300	2,254.000	42.700	12.800	0.014
61	271,876.000	0.210	773.000	860.000	43.830	13.180	0.100
62	788,551.000	0.490	620.900	665.000	44.980	11.240	0.233
63	23,935.000	0.088	3,686.800	4,637.000	40.820	13.730	0.042
64	53,312.000	0.158	2,960.200	3,604.000	40.980	13.470	0.075
66	200,638.000	0.110	547.800	616.000	44.560	12.490	0.052
67	30,903.000	0.098	3,156.400	3,917.000	40.970	13.520	0.046
6	621,834.000	0.333	535.700	582.000	44.790	7.450	0.159
9	396,344.000	0.279	704.100	813.000	46.200	6.920	0.133
TOTAL	**15205279**	**9.777**					**4.654**

All scripts and detailed pipeline information are available in the project's GitHub repository (`https://github.com/iremdnzl/GenomeAssemblyAnnotation.git`).

2.4.2 Genome-Wide Analyses

To investigate genome-wide patterns of variation, I first generated genotype likelihoods from low-coverage reads in *ANGSD* (v*0.94*) (Korneliussen et al. 2014) and used these likelihoods for population summaries. Minor-allele frequency (MAF), the site-frequency spectrum (SFS), Tajima's D, and runs of homozygosity were computed from genotype likelihoods without hard genotype calls. Variant discovery used the SAMtools likelihood model (`-GL 1`) with major/minor inference and MAF estimation (`-doMajorMinor 1 -doMaf 1`), posterior recalibration (`-doPost 1`), and conservative base and mapping quality filters (`-minMapQ 30 -minQ 20`), while requiring representation across individuals (`-minInd 30`) and no cohort-depth caps (`-setMinDepth`/`-setMaxDepth` not used). To define the association panel, I applied a discovery threshold of `-SNP_pval 1e-4`, exported the SNP list, and retained sites with intermediate frequency (MAF ≥ 0.05) for downstream analyses. Where needed for interoperability, I emitted genotype-posterior BCF and BEAGLE files (`-doGeno 32 -doBcf 1 -doGlf 2`).

For genotype–phenotype association, I used *ANGSD* EM regression (`-doAsso 4`) model on photoresponsiveness index (PI) (Lynch et al. 1989) (Suppl. Table A.1). This model operates on genotype probabilities and maintains power and type-I error control at low sequencing depth (Korneliussen et al. 2014). Association runs used likelihood-appropriate filters (`-minMapQ 30 -minQ 20 -minMaf 0.05 -minInd 30`), omitted ultra-low-coverage-sensitive read-count and cohort-depth cut-offs (`-setMinDepth`, `-setMaxDepth`, `-minCount`, `-minHigh`), and retained posterior recalibration (`-doPost 1`). Population-structure covariates (PC1–PC2) were derived from the same genotype-likelihood set and passed via `-cov`. P-values were written with `-Pvalue 1`.

To control multiple testing, I defined the per-trait test burden m as the number of loci entering the EM regression (unique sites in the `.lrt0` output for that trait). Genome-wide significance was set by Bonferroni correction $\left(\alpha\text{Bonf} = 0.05/\text{m}\right)$, and I additionally controlled the false discovery rate at 5% using the Benjamini–Hochberg procedure applied to the same p-value vector. I also report a suggestive threshold of $P<1x10^{-5}$. Genomic calibration was evaluated with quantile–quantile plots and the genomic control factor λ computed from the median test statistic; λ values near 1 indicate well-calibrated tests. Final result tables (per trait) report m, αBonf, numbers of loci surpassing Bonferroni and 5% FDR, and top loci with effect size (beta), standard error, and nearest gene annotation ($\pm$ 50 kb).

All scripts and detailed pipeline information are available in the project's GitHub repository (`https://github.com/iremdnzl/GenomeWideAnalyses.git`).

2.4.3 Differential Expression Analyses

All bioinformatic procedures were performed in *RStudio* within a *Conda* environment to ensure consistent software dependencies and reproducibility. Raw nanopore data were demultiplexed and basecalled using *Guppy, super accuracy model* 4.2.1, generating FASTQ files from the original FAST5 reads. Adapter sequences were then removed with *Porechop* v0.2.4, and only high-quality reads of at least 25 bases, with a mean Q score of at least 9, were retained using *Filtlong* v0.2 (`github.com/rrwick/Filtlong`).

Subsequently, the filtered reads were aligned to the Siberian hamster transcriptome and assembled genome (#PRJNA1029001) using *Minimap2*. Quantification of transcript abundance was carried out with *Salmon* v0.14.2, which exploits a lightweight alignment-based method to estimate transcript-level expression. Normalisation and statistical analyses for differentially expressed genes were conducted in *edgeR* v3.24.3, implementing empirical Bayes methods to account for biological variation and to control the false discovery rate.

To facilitate visual exploration of expression patterns, log-transformed expression levels were clustered by partitioning around medoids (PAM) using the *cluster* package, and heatmaps were generated with the *ComplexHeatmap* package.

2.4.4 ChIP-Seq Data Processing

Raw sequencing reads were processed to remove adapter sequences and low-quality bases using FastP (Chen 2023), with trimming of bases having an average Phred score below 15. Read quality was assessed both before and after trimming using FastQC (`http://www.bioinformatics.babraham.ac.uk/projects/fastqc/`). To detect potential cross-species contamination, reads were screened for contamination against a panel of commonly sequenced reference genomes using FastQ Screen (Wingett and Andrews 2018), as implemented by the MVLS Shared Research Facility.

Quality-filtered reads were aligned to the *Phodopus sungorus* reference genome using Bowtie2 (Langmead and Salzberg 2012). Duplicate reads were identified and removed using the MarkDuplicates tool from the Picard suite (Picard, 2019). Peak calling was performed with SICER2 (Zhang et al. 2021), and resulting peaks were annotated using the ChIPpeakAnno package (Zhu et al. 2010).

2.4.5 Testicular Methylation Analysis

This methods section is adapted from my previously published article Denizli et al. 2025

2.4.5.1 Animal Housing and Photic Treatment

Three to eight-month-old male Siberian hamsters were chosen from a colony housed at the University of Aberdeen. In polypropylene cages, the hamsters were housed under a long photoperiod (LD) (15L:9D). Cotton nesting material was given, as well as unlimited food and water. The study was carried out under a Home Office license that was accepted (70/7917), and all protocols were authorized by the University of Aberdeen's Animal Welfare and Ethics Review Board.

Twelve adult male hamsters were employed in this investigation. Six male hamsters were chosen at random from the colony and placed in cabinets for 8 weeks to be treated with short photoperiod (Arrowmight; SD 9D:15 L) as the experimental group. The photoperiodic control group consisted of six male hamsters housed in the long-day colony room. The animals were sacrificed by cervical dislocation following ethical guidelines, and aeADAM scales (Adam Equipment PGL2002) were used to measure the testicular mass to within ±0.1 g. Tissues were kept at -80 °C after being frozen in dry ice powder.

2.4.5.2 DNA Extraction

Using Qiagen QIAmp DNA Mini Kit (Qiagen; catalog #51304 and #51306) and following the manufacturer's protocol genomic DNA was extracted from >30 mg of testes tissue. Genomic DNA was purified with NaAcetate (3M, pH 5.2) and precipitated with EtOH. The quantity and 280/260 values were determined using NanoDrop.

2.4.5.3 Whole Genome Sequencing

The Oxford Nanopore minION and SQK-LSK109 ligation sequencing kit were used to sequence the extracted genomic DNA. To identify individual samples, EXP-NBD104 native barcoding was used. Initially, 1.5–3 µg of gDNA were made in nuclease-free water. Each barcode was combined in equimolar levels to create a 700 ng pooled library. Library of twelve samples, including DNA from six hamster testes treated with LD and six treated with SD, were loaded onto the minION platform using the primed Flowcell (FLO-MIN106D). Sequencing was carried out according to the manufacturer's instructions. After 72 hours of longread sequencing at a voltage of -180 mV, *fast5* files were produced to make further analysis easier.

We first used *guppy* to basecall the readings from FAST5 files in preparation for bioinformatic analysis. *Porechop* was used to reduce the barcode (Wick et al. 2017). Using *minimap2* (Li 2018), we mapped reads to the *Phodopus sungorus* reference genome (SUB13765567). Each file was then indexed and used using *NanoPolish* for methylation calling (Loman et al. 2015), where the SD group served as the "Case" data and the LD group as the "Control." Chromosome-aligned DNA fragments were not included in subsequent analyses. The

log_lik_ratio values were used to filter methylated regions. Methylation is shown by likelihood values greater than 0, while unmethylation is indicated by lower values. On the recommendation of the developers on GitHub, we employed *log_lik_ratio*>2 to have solid proof for methylation.

Moore and colleagues' study provided the chromosomal data (Moore et al. 2022). We visualized the genome-wide distribution of differentially methylated areas using the R tools *tidyr* and *dplyr*. Using the *AnnotationDbi* (Hervé Pagès, Marc Carlson, Seth Falcon, Nianhua Li 2017) packages of R, we were able to annotate the gene structures (gene body, exon, intron, and promoter) with the *Phodopus sungorus* annotation and analyze the methylation pattern variations in each area. The *ggplot2* software for R and *Venn diagrams* (`https://bioinformatics.psb.ugent.be/webtools/Venn/`) were utilized to illustrate the results. Lastly, we performed Gene Ontology (GO) enrichment analysis on the regions that were discovered using *ShinyGO* 0.76 (Ge et al. 2020). The study's code is available on github at `https://github.com/iremdnzl/TestesMethylation.git`.

The variaton in testes mass throughout several photoperiods was examined using principal component analysis (PCA). The *FactomineR* library in R (R version 4.4.1) was used to do the PCA, and the *ggplot2* and stats packages were used for visualization.

2.4.6 Analysis of statistics

R statistical program was used to conduct the statistical analysis. Read counts were compared using t-tests. The package contains the *stat_compare_means()* statistical comparison tool, which by default employs two-tailed t-tests.