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Uncovering thermoregulation in laboratory mice

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Summary

Altricial mammals are generally born hairless and have trouble maintaining a homeothermic endothermic physiology, usually relying on their progenitors or nest to keep a high and stable body temperature, until having around half of their adult size. Mouse pups are no exception and are born poikilothermic, unable to maintain a high and stable body temperature by themselves, and thus relying on the environment for their temperature regulation. Meaning that they experience a shift from poikilothermic to endothermic physiology during their development. Nevertheless, the adult *Mus musculus* has been described as a facultative heterotherm. There is still an open discussion on how the endothermy evolved, how the ancestral mammal first transitioned to a stable endothermic homeothermy and what genes are involved in it. Also, it remains unknown why other species retain signs of heterothermy in their adult lives, especially benefiting from it in times of challenging environmental conditions, such as low food availability. Many studies have focused on this evolutionary question, yet a clear answer is lacking for many species, especially regarding its genetic regulation. Therefore, especially in a context of ongoing climate changes, understanding how (genetically) and when (at what age) this transition occurs during a mammal's life is key, not only to answer this question, but also to better understand the mechanisms of thermoregulation in a changing habitat, within the ongoing climate changes, offering new insight of value for conservation efforts. To do so, we plan to study thermoregulation changes under controlled conditions, namely by following the first 40 days of postnatal development of laboratory mice, namely *Mus musculus,* C57BL/6J strain. In order to do so, we will regularly measure and compare the acute and short-term surface (Ts) and internal (Tb) body temperature loss in pups, both individually, by short-term separation of each pup from their litter, during their active (night) and inactive (day) periods; and within each litter; and monitor and compare Ts and Tb in juveniles. For the latter, which already have fur, we will induce, every two days, from the P22 until P40, a 60 min nest restriction, as an account for nesting insulation on these measurements after weaning and throughout their development until 40 days of age. To identify the ontogenic onset of endothermy, two organ samples (Brain in hippocampus and amygdala, and liver) will be taken before weaning and two afterwards, in four spaced time points of the pup development (P4, P15, P23 and P40). The hypothesis we wish to test is that the onset of endothermy in mice occurs after weaning.

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1. General Information

Keywords

Gene expression, mice, thermoregulation, evolution, endothermy

Funding sources

2021.04866.BD FCT grant

International code of classification

none

2. Study design

Introduction

From an evolutionary point of view, the endothermic homeothermic thermoregulation in small altricial mammals, like rodents, develops at around one third to a half of the animal's adult size [8]. By having a small hairless body, pups are constantly losing heat, having to manage a higher and unbalanced energetic challenge, relying on adult parents or nests to keep a high and stable body temperature. To overcome this challenge, they usually resort to heterothermic physiology. This way, during their development, pups can have a controlled reduction of their metabolism and body temperature, saving energy that is crucial for them to grow [8].

Although this heterothermic physiology is very likely to be employed by many species, until the present date, it has only been reported in 4 bird orders and 1 marsupial order. In these two cases, the hatchlings are poikilothermic at birth following a heterothermic thermoregulation afterwards, during cold exposure. By this, it is possible to infer that daily heterothermy is phylogenetically old and likely plesiomorphic. Besides birds and marsupials, daily heterothermy in placental mammals has only been described in two orders, Insectivora and Rodentia. However, juveniles from other orders, like bats, are known to enter a torpor state [9]. Yet, no studies were published on detailed developmental sequences, identifying the transition between poikilothermy and heterothermy or endothermy. The main difference between the studied marsupials and the placentals is that, in the latter, poikilothermy at birth is followed by a homeothermic phase after which the endothermic thermoregulation is established, meaning that the ability to employ daily heterothermy appears later in life (i.e. poikilothermy–endothermy–heterothermy) [8] This suggests that in placentals, daily heterothermy is a trait that evolved secondarily after a homeothermic phase, in response to energetic

challenges, probably due to climate change [5]. As mammals and birds arose from different reptilian lineages, it is likely that endothermy emerged from two separate classes, and daily heterothermy seems to have evolved at least three times, given the differences in developmental sequence between marsupials and placentals [8].

On an ontogenetic point of view, a recent study compared pups (0-4 days) from four different rodent species, ranging in different heterothermy levels as adults [10]. They tested the adaptation to cold during hypoxia conditions, concluding that heterotherms retain traits that are common to all newborn mammals [11], including high tolerance to body temperature decreases. Even as newborns, the heterotherms species were more tolerant of cold temperatures and O2 limitation than homeotherms [12,13]. Additionally, from the tested species, the rat was the one showing the greatest thermogenic response, followed by the facultative heterotherm species, and lastly by the hibernator (squirrel) with almost no response. These differences were coherent with the ones seen on the same adult species [14], indicating that the adult phenotype characteristics are already present at birth. This was the first comparative study that attempted to test a link between pup and adult stages in different species [10], concerning the heterothermy and endothermy physiologies, studying some environmental parameters (temperature and O2 levels). Nonetheless, the question of when (ontogenetically) is the homeothermic stage achieved and how (which genes trigger it), still remains unanswered. For this project we will test whether the endothermic regulation starts around weaning age. Specifically, we aim to answer the following questions: 1) is endothermy developed gradually, rather than abruptly?; 2) is endothermy a pleiotropic trait, where previously studied metabolic control and temperature-related genes take part?; and 3) is a high level of gene expression after weaning indicative of emergence of endothermy?. We will use an inbred *Mus musculus* strain (C57BL/6J) in a laboratory setting as a model for a facultative heterotherm species [15] as adults. To test our main hypothesis and answer our additional experimental questions, we will reproduce the laboratory mice in-house. Firstly, we will measure and study the relation between acute and short-term surface (Ts) and internal (Tb) body temperature loss in hairless pups temporarily within the litter (0 and 3min – pup mass) and separated (6min) from and, during their active (night) and inactive (day) periods; and monitor Ts and Tb body temperature in juveniles. To account for the effect of nesting material in insulation and thermoregulation, we will remove it for 60 min prior to measurements, in half of the cages (assigned randomly), from P15 to P40. To identify the ontogenic onset of endothermy, two tissue samples will be taken before weaning and two afterwards, in spaced time-points (at P4, P15, P23 and P40), namely brain (hippocampus and amygdala), and liver, which are markedly related with thermoregulation control and thermogenesis [16–19], that will be posteriorly sequenced (RNAseq) in order to assess their gene expression.

Type of research

Confirmatory

Hypothesis of your study

Main hypothesis being tested: Endothermic regulation starts around weaning age Secondary hypotheses: 1. Endothermy develops gradually, rather than abruptly 2. Endothermy is a pleiotropic trait, where previously studied metabolic control and temperature-related genes take part. 3. High level of gene expression after weaning indicates the emergence of endothermy

Study design

Experimental unit: litter

Timeline:

P0 - pups birth

from P0 to P40 - Daily TS and TB measurements of the pups (0, 3 and 6 min)

P4 - experimental endpoint - random euthanasia of one pup/litter; dissection and sampling; P15 - experimental endpoint - random euthanasia of one pup/litter; dissection and sampling; P21 - separation of the males and females in two cages by sex; pups weaning

P21-P40 (every 2 days: P22, P24, P28, P30, P32, P34, P36, P38) - random assign of "control" and " experimental" groups to either the male or female cage for the nesting inhibition experiment (30min-1h)

P23 - experimental endpoint - random euthanasia of one pup/litter; dissection and sampling;

P40 - experimental endpoint - random euthanasia of one pup/litter; dissection and sampling;

Method of blinding

The researcher analyzing the data will not be aware of the experimental groups. Allocation concealment for data collection will not be possible, but data extraction will rely on technology (pit tag readers and thermal cameras) which output that cannot be interfered with by the researcher. Performance bias will be addressed by randomization of order by which each litter and each pup within each litter is assessed.

Method of randomization

Software assisted (Excel RAND() function) randomization of treatments to each litter (the order to perform each method/procedure); to each pup of each litter - each pup will have an assigned number that will be randomized; to the cage position in the rack - the cages are also going to be identified with labels and randomized each time.

Additional remarks

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Attachments

Filename	Size (bytes)
study_design.png	134694
study_design_2.png	472569

3. Methods

3. 1. Task A - Pilot study in M. musculus mice breeding:

Description of the method

We carried out a pilot experiment to test our ability to perform the entire experiment from a technical-logistical point of view. Data collected for this purpose will not integrate the study. The pilot informed us on when to set our time points to collect genetic information in the time window in which endothermic thermoregulation is established in developing mice. Based on the preliminary information gathered, we hypothesize that it occurs between the second and third time point (P15 and P23), that is, before and after weaning (P18-P21).

Narcotic/analgesic treatment

Not provided

Drugs/substances

Not provided

Antibodies

Not provided

Cell lines, viruses, DNA or RNA constructs and bacteria

Not provided

3. 2. Task B - Mus musculus mice breeding:

Description of the method

Based on the information from the pilot experiment, we will start the complete experiment with 3 breeding pairs of strain C57BL/6J (from mice descendent from the Jackson laboratory [16] colony) with an average litter size of 6-8 pups/litter [18]. Each breeding pair will produce four litters, generating a total of between 72-96 pups, which should provide sufficient precision, despite the litter being the experimental unit (N=12). For this, three rearing cages (EU Type II cage) should be prepared with maize cob bedding (changed once a week), laboratory food pellets (Teklad Harlan 2014S; Envigo, UK), abundant nesting material (shredded paper) a water bottle (polypropylene bottle, filled with sterilized water) and acrylic and paper tubes to create shelter areas. The ambient temperature is defined between 21-22ºC, humidity between 45-65% and a photoperiod cycle of 12h light/12h dark (with about 100 lux of light intensity in the light period). Male mice will be kept with the mother and litter until weaning age.

Narcotic/analgesic treatment

Not provided

Drugs/substances

Not provided

Antibodies

Not provided

Cell lines, viruses, DNA or RNA constructs and bacteria

Not provided

3. 3. Task C - After birth and before weaning period

Description of the method

After the birth of the pups, their health status will be monitored daily (breathing, umbilical cord, milk spot, etc…), as well as that of the mother. The pups will be ear marked as well as labeled with markers in the basis of their tail, for later identification. Every day, twice a day, during their active (dark) and inactive (light) periods, each pup will be collected in random order and will have its body temperature measured with a the subcutaneously injected PIT tags (at P8) and a wireless remote antennae system, under their cage for handoff recordings (TBody) and superficial probe (with a calibrated thermographic camera, TSur). Here, each offspring is quickly transferred, with wooden tweezers, to an arena (with a basis of a medical absorbent pad and surrounded by a grid metal enclosure) , where it will have its temperatures measured . A thermal image will be captured immediately after transferring all the pups, which will capture the image of the mass of the litter at minute zero and at minute 3, and, after P12 and after minute 3, the pups will be separated and at minute 6, another image will be captured for accounting with the individual pup temperature. These images will be achieved with a thermographic camera fixed above the mice arena, where the interval of loss of surface temperature (ºC) will be recorded. Additionally, body temperature will be constantly recorded by the remote antenna system on each pup with an injected PIT tag. Furthermore, the pups and the dam will be weighted weekly, for health control. On postnatal days 0, 4, 15 and 23, which are

the experimental time points, after this previously stated daily procedure, one pup from each litter will be sacrificed by decapitation to collect organ samples, namely from two regions of the brain (hippocampus and tonsil) and liver, which will be immediately preserved in PCR tubes filled with RNAlater, and subsequently placed in a freezer at -80ºC. For this procedure, we accounted for a total of about 48 pups sacrificed in the 4 experimental cycles, 12 for each of the 4 time points. Around P18-P21 all remaining pups will be weaned and we will transition to solid food (Teklad Harlan 2014S; Envigo, UK and sunflower seeds), at which point the mothers will be transferred back to a new breeding cage to enter in a new experimental cycle, in the case of laboratory mice

Narcotic/analgesic treatment

Not provided

Drugs/substances

Not provided

Antibodies

Not provided

Cell lines, viruses, DNA or RNA constructs and bacteria

Not provided

3. 4. Task D - After weaning period until P40

Description of the method

After the third time point on the 23rd postnatal day, we will continue to measure and compare Tbody and Tsup of juvenile mice, twice a day: during the most (dark) and least active (light) period. Here, each offspring is quickly transferred, with wooden tweezers, to an arena (with a basis of a medical absorbent pad and surrounded by a grid metal enclosure), where it will have its temperatures measured. A thermal image will be captured immediately after transferring all the pups, which will capture the image of the mass of the litter at minute zero and at minute 3, and, and at minute 6, the pups are separated from eachother and another image will be captured for accounting with the individual pup temperature. These images will be achieved with a thermographic camera fixed above the mice arena, where the interval of loss of surface temperature (ºC) will be recorded. Additionally, body temperature will be constantly recorded by the remote antenna system on each pup with an injected PIT tag. Furthermore, the pups and the dam will be weighted weekly, for health control. As at this stage the animals will already have some fur, therefore, after weaning (P21) the pups will be separated according to their sex into two new cages. These cages, from P22- P40, every two days will be randomly assigned to either group 1 – control where the mice are just handled to not produce bias; or group 2 – experimental group, where the mice are handled and all the nest material is removed from the cage for 30min;in both cases, this entire process will be recorded with the thermal camera, and automatically with the remote PIT tag system to see the temperature variation (minute 0 and minute 30) and establish a comparative analysis. Furthermore, at P40, which is the last experimental time point, after this daily procedure, a juvenile mouse of each litter will be sacrificed for collecting organ samples, namely from two regions of the brain (hippocampus and amygdala) and the liver, which will be immediately preserved in PCR tubes filled with RNAlater, and subsequently placed in a freezer at -80ºC. The experiment continues until all three batches are completed. In the third experimental cycle, all surplus mice, as well as the initial parents, will be rejected at the end of that cycle.

Narcotic/analgesic treatment

Not provided

Drugs/substances

Not provided

Antibodies

Not provided

Cell lines, viruses, DNA or RNA constructs and bacteria

Not provided

3. 5. Task E - Sampling, RNA sequencing and data analysis

Description of the method

In each of the four moments, 1 mouse per litter will be sacrificed, totaling about 48 mice in the four experimental cycles. As mentioned earlier, these mice will be euthanized and immediately, by cervical dislocation, dissected and sampled from total brain and liver. Samples will be preserved in RNAlater. In addition, total RNA will be extracted from liver, amygdala and hippocampus tissues to detect expression changes underlying behavioural (brain) and physiological (liver) differences and to further establish a transcriptome pool. The RNA will be transformed into libraries for sequencing through paired-end reads with 2x100bp (Illumina, HiSeq 2500). The RNA-Seq readings will be mapped to reference rodent genomes (*Mus musculus*). The R-packages (DESeq2) will be used to detect differentially expressed genes. To detect subtle changes, we will use established R scripts to identify co-expression networks. Finally, we will perform GO enrichment on co-expression network modules to detect clusters of functions/pathways that responded to thermoregulatory expression and further interpret their gene-gene networks with the Wto and codina R packages.

Narcotic/analgesic treatment

Not provided

Drugs/substances

Not provided

Antibodies

Not provided

Cell lines, viruses, DNA or RNA constructs and bacteria

Not provided

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4. Statistics

4. 1. differential expresion analysis - DESEQ2

Assigned method(s)

Task E - Sampling, RNA sequencing and data analysis

Main endpoints

Differential gene expression between each experimental timepoint.

Secondary endpoints

Differential gene expression between each group (control and experimental) between the third and fourth experimental timepoints (nest restriction experiment).

Sample size calculation

We will use 3 breeding pairs of mice of the C57BL/6J strain, with an average litter size of 6-8 pups/litter. Each breeding pair will produce four litters, generating a total of between 72-96 offspring. The litter will be the experimental unit (N=12), as there is a marked relationship of the contribution of each littermate to the temperature of the others. While this would only allow detecting considerably large effect sizes, our pilot study suggests such effects are expected, not only because of low variability in regards pup body temperature but also very large temperature variations as a result of the treatments, until endothermy is established. Moreover, data is pooled from all pups of each litter, with considerable gains in precision of the estimate. Moreover, using a randomized block design will also contribute to reducing experimental “noise”, which will be furthermore reduced by using an inbred strain. For gene expression comparison, the observational unit will be pup from each time-point (4 time-point, two groups of 6 pups per group (control/experimental), i.e. 48 observational units). Breeding pair from which each litter is bred will be a random factor (block).

Primary statistical analysis

PCA and variance-stabilizing transformation (vst) to see: sample distribution and noise of metadata factors.

Exclusion criteria

All animals presenting congenital malformations, injuries (e.g. from fighting) or any unexpected health and welfare issues that cannot be mitigated will be excluded.

4. 2. GO enrichement analysis

Assigned method(s)

Task E - Sampling, RNA sequencing and data analysis

Main endpoints

GO annotation terms of Biological Process (BP) and Molecular Functions (MF) of previously found Differential gene expression between each experimental timepoint.

Secondary endpoints

GO annotation terms of Biological Process (BP) and Molecular Functions (MF) of previously found Differential gene expression between each group (control and experimental) between the third and fourth experimental timepoints (nest restriction experiment).

Sample size calculation

see above

Primary statistical analysis

Over representation analysis (ORA) (Boyle et al. 2004) to determine whether known biological functions or processes are over-represented (= enriched) the previously found differential expressed genes, where the p-value is calculated by hypergeometric distribution. p=1−k−1∑i=0(Mi)(N−Mn−i)(Nn) In this equation, N is the total number of genes in the background distribution, M is the number of genes within that distribution that are annotated (either directly or indirectly) to the gene set of interest, n is the size of the list of genes of interest and k is the number of genes within that list which are annotated to the gene set. The background distribution by default is all the genes that have annotation. P-values should be adjusted for multiple comparison.

Exclusion criteria

All animals presenting congenital malformations, injuries (e.g. from fighting) or any unexpected health and welfare issues that cannot be mitigated will be excluded.

4. 3. Gene-gene Network analysis - Wto and Codina

Assigned method(s)

Task E - Sampling, RNA sequencing and data analysis

Main endpoints

Comparing the type of expression of our gene termoregulatory networks (wTO) and classifying the types of nodes and links establishing a comparison between the networks of the several experimental timepoints and between the control and experimental group of the nest restriction part (P22-P40).

Secondary endpoints

Not provided

Sample size calculation

see above

Primary statistical analysis

WTO calculates the weighted topological overlap (wTO), considering the up and down regulated (+/-) differently expressed genes and p-value calculation (raw and adjusted) for each pairwise gene score, using a Pearson correlation. CoDiNA detects and classifies the links and nodes in either shared, specific or different among the control and experimental networks, normalizing the data between these different categories.

Exclusion criteria

All animals presenting congenital malformations, injuries (e.g. from fighting) or any unexpected health and welfare issues that cannot be mitigated will be excluded.

4. 4. Thermal evaluation

Assigned method(s)

Task C - After birth and before weaning period
Task D - After weaning period until P40

Main endpoints

Surface temperature variation of the hurdle of pups (pooled) Surface temperature of individual pups (pooled)

Secondary endpoints

Subcutaneous temperature variation of the individual pups (pooled)

Sample size calculation

see above

Primary statistical analysis

A repeated measures two-way ANOVA will be performed, with sex and nesting set-up (nest vs. no-nest) as between-subjects fixed factor, and measures at three time-points for within-subjects analysis. Breeding pair from which each litter is bred will be a random factor (block).

Exclusion criteria

All animals presenting congenital malformations, injuries (e.g. from fighting) or any unexpected health and welfare issues that cannot be mitigated will be excluded.

5. Animals

5. 1. Mice (Mus musculus)

Animal strain/breed

C57BL/6J

Genetically modified

No

Sex

Female
Male

Further characteristics of the animals (e.g. age, body weight, size)

pups from P0 to P40, resulting from 4 consecutive breedings with 3 C57BL/6J mice couples

Housing conditions

One rearing cage per mice couple (3, EU Type III cage) should be prepared with maize cob bedding (changed once a week), laboratory food pellets (Teklad Harlan 2014S; Envigo, UK), abundant nesting material (shredded paper) a water bottle (polypropylene bottle, filled with sterilized water) and acrylic tubes to create shelter areas. The ambient temperature is defined between 21-22ºC, humidity between 45-65% and a photoperiod cycle of 12h light/12h dark (with about 100 lux of light intensity in the light period). Male mice will be kept with the mother and litter until weaning age.

Afer weaning (P21), the resulting pups, from each litter will be separated by sex into two cages (total 6, U Type II cage) and these should be prepared with maize cob bedding (changed once a week), laboratory food pellets (Teklad Harlan 2014S; Envigo, UK), abundant nesting material (shredded paper) a water bottle (polypropylene bottle, filled with sterilized water) and acrylic tubes to create shelter areas. The ambient temperature is defined between 21-22ºC, humidity between 45-65% and a photoperiod cycle of 12h light/12h dark (with about 100 lux of light intensity in the light period).

Roll paper tubes will also be added throughout the experiment to increase the enrichment.

Refinement

1 - For Mus musculus we chose an “inbred” strain, C57BL/6J, which will be acquired from Jackson laboratories. It is a common mouse strain bred specifically for laboratory studies and has no relevant mutations for any phenotypic abnormalities during its development, being considerably prolific. Each couple will be accommodated within the i3S animal facilities, in a type II cage (EU type II cage) that will be prepared with corncob bedding (changed once a week), laboratory food pellets (Teklad Harlan 2014S; Envigo , UK), abundant nesting material (shredded paper), a water bottle (polypropylene bottle, filled with sterilized water) and acrylic tubes to provide shelter. The ambient temperature is set between 21-22ºC, humidity between 45-65% and a photoperiod cycle of 12h light/12h dark (with around 100 lux light intensity in the light period). Male mice will be kept with their mother and litter until weaning age.

Critical limits: If by any means the experimental interventions cause high pain or suffering that cannot be mitigated, the animals will be euthanized. These effects are not expected to occur, but given that there are interventions with some level of invasiveness, such as rectal temperature measurement or tattooing/ear marking, there is always a small chance of problems resulting from them, which will nevertheless be avoided as much as possible.

Regarding the care to be provided to animals:

i. Animals may manifest stress and anxiety in response to changes in their environment or routine (such as cage changes or cleaning), as well as physical handling or social disruption by researchers/ caretakers. In the case of the laboratory environment, we will only perform measurements on the offspring, some of which are non-invasive, using the thermography method (TS) and we will also take internal temperature measurements (TB) with adapted rectal probes (B-RET-3) , where the procedure lasts less than 30s per animal. There is the issue of handling the pups to obtain temperature records, which will be done quickly, handling the pup/adult by the cervical fold.

ii. If for any reason the animals lose any body condition (not expected), the monitoring frequency will be increased. To minimize these effects during the experimental period, all animals will be weighed daily and evaluated using score sheets.

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iii- Special attention will be paid to behavior, in order to identify changes indicative of possible added stress, namely we will keep the litters together with their mother until weaning, who will have access to nesting material, cardboard tubes and paper refuges, such as shelter.

iv. Even if no illnesses or injuries are expected to result from experimental procedures, attention will be paid to the possibility of traumatic injuries or infections, which will be prevented as much as possible. If these occur, appropriate treatment will be applied to the animal, in accordance with veterinary recommendations. If necessary, even if not expected, it will be euthanized, thus applying a humane critical limit.

6. Updates

2023-11-10

We decided best to finish the experiment at P35, since our hypothesis aims to the weaning period. Thus the final sampling timepoint would change to P35.

Legally our veterinarian department only allowed to insert the PIT tags on the pups at P10