# UNIVERSITÀ DEGLI STUDI DI NAPOLI "FEDERICO II"


**Doctoral Thesis**

# Phenotypic characterization of wild-type animals to bridge a crucial knowledge gap

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*''The simple and familiar hold the secrets of the complex and unknown.''*

Edward B. Burger, The 5 elements of effective thinking

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# Acknowledgments

*"No one who achieves success does so without acknowledging the help of others. The wise and confident acknowledge this help with gratitude."*
Alfred North Whitehead

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I did not sink, because of you all.

# List of Abbreviation

| | |
|---|---|
| AM | Ante meridiem |
| BPS | Balano Preputial Separation |
| CC | Collaborative Crosses |
| CD | Cesarean Derived |
| CNR | Consiglio Nazionale delle Ricerche |
| CRL | Charles River Laboratories |
| DVC | Digital Ventilated Cages |
| EMMA | European Mouse Mutant Archive |
| ES | Embryonic Stem |
| FWHM | Full Width at Half Maximum |
| GM | Genetically Modified |
| GMMS | Gaussian Mixture Models |
| IBBC | Institute of Biochemistry and Cell Biology |
| IMPC | International Mouse Phenotyping Consortium |
| LE | Long Evans |
| GM | Genetically Modified |
| MGD | Mouse Genome Database |
| MGI | Mouse Genome Informatics |
| PCA | Principal Component Analysis |
| PM | Post meridiem |
| QTLs | Quantitative Trait Loci |
| RDI | Regularity Disruption Index |
| RGD | Rat Genome Database |
| WT | Wild Type |

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# Abstract

An accurate understanding of genetic and phenotypic traits of laboratory animal models is crucial to securing validity of scientific results and animal welfare.
The phenotyping pipeline is well established and proactively performed by research institutions, repository and commericial suppliers worldwide most typically on genetically modified animal models, also in the light of regulatory requirements.
Despite the same pipeline is applicable as well to non genetically modified models, the level of in-depth phenotypical characterisation of stock, non mutant rodents strain, is not as systematic as for mutant rodents.
In an effort to widening the characterisation of non-GM rodents phenotype, we focused on two aspects that were either non-characterised or only partially characterised in the pioneering phase of the animal strain.
Particularly, with reference to the laboratory mouse, we undertook a study to disentangle the diurnal activity and feature key aspects of three non-genetically altered mouse strains widely used in research, C57BL/6NCrl (inbred), BALB/cAnNCrl (inbred) and CRL:CD1(ICR) (outbred). With this aim, we conducted a longitudinal analysis of the spontaneous locomotor activity of the mice during a 24-h period for 2 months, in two different periods of the year to reduce the seasonality effect. Mice (males and females) were group-housed in Digital Ventilated Cages (Tecniplast), mimicking standard housing conditions in research settings and avoiding the potential bias provided in terms of locomotor activity by single housing. The recorded locomotor activity was analyzed by relying on different and commonly used circadian metrics (i.e., day and night activity, diurnal activity, responses to lights-on and lights-off phases, acrophase and activity onset and regularity disruption index) to capture key behavioral responses for each strain. Our results clearly demonstrate significant differences in the circadian activity of the three selected strains, when comparing inbred versus outbred as well as inbred strains (C57BL/6NCrl versus BALB/cAnNCrl). Conversely, males and females of the same strain displayed similar motor phenotypes; significant differences were recorded only for C57BL/6NCrl and CRL:CD1(ICR) females, which displayed

higher average locomotor activity from prepuberty to adulthood. All strain-specific differences were further confirmed by an unsupervised machine learning approach. Altogether, our data corroborate the concept that each strain behaves under characteristic patterns, which needs to be taken into consideration in every study design to ensure experimental reproducibility and comply with essential animal welfare principles.

Furthermore, with reference to the laboratory rat, and considering the relevance of exact timing of puberty in preclinical studies, we developed a novel males puberty onset curve, performing a population screening of two outbred strains. Extensive bibliographic resources highlight that in male rats, the age of sexual maturity varies considerably between 40 and 60 days of age. Our screening allowed us to perform a thorough pubertal onset evaluation of Crl:CD(SD) and Crl:LE, relying on the balano-preputial separation test (BPS). Evaluation was carried out on animals under standard barrier conditions, from 4 to 9 weeks of age. In the Crl:CD(SD) population, 90% of males gained the puberty at week 6, and 100% in the following weeks whereas 75% of Crl:LE reached the puberty at week 6, 90% at week 7 and 100% from week 8. Remarkably, in both strains, puberty onset was gained at the average weight of 200 gr suggesting that weight range, not only age range, can be considered a biomarker of puberty onset in these two strains. On the contrary, descended testes cannot be considered an additional factor to identify the full puberty onset either in Crl:CD(SD) and Crl:LE rats.

As a whole, the works reported in this thesis contributed to a better understanding of stock, non genetically modified models. Both studies were succesfully published, confirming the interest of the *in vivo* research community for the phenotypical assessment of commercially available non mutant rodents strains.

# Introduction

## I Genotype *versus* Phenotype

Although these concepts were originally expressed by Mendel, the terms "gene", "genotype" and "phenotype" were introduced by the Danish botanist Wilhelm Johannsen in 1909 (1).
Gene comes from the Greek *genos* (race or offspring) and refers to a unit of heredity. Genotype comes from the Greek *genos* and *tupos* (type) and refers to the genetic constitution of an individual organism (2). In other words, the genotype is defined as the set of genes an organism has. Relevant to note is that genotype can refer to the entire genome of an organism but also to the alleles carried at a particular locus (3). This distinction is particularly relevant when speaking about genetically modified (GM) animals, as in real life environment we are often assessing and discussing about the genotype of GM animals, focusing on mutant alleles, while more rarely the concept of genotype is applied and accurately considered when referring to non GM animals, thus when the concept should be used with reference to the entire genome, or spontaneous mutant alleles, if any.
Phenotype comes from the Greek *phainen* (to show) and *tupos* (type) and refers to the set of observable characteristics of an individual resulting from the interaction of its genotype with the environment; thus, phenotypes reflect the result of the interaction between the inner (genetic) nature of the organism and the outer (environmental) action on the genetic component. (2). The phenotype of an individual is as simple as the physical features of an organism. However, the concept of phenotype includes an enormous range of possible features, embracing potentially any aspect of an organism's morphology, behavior, or physiology as affected by the unique combination of its genotype and its environment (4).

## II The importance of being phenotyped for GM strains

Phenotyping can combine *in vivo* evaluations, including behavioural studies, imaging strategies, and clinical and anatomic assessments to characterize complex phenotypes, including multisystemic phenotypes or syndromes, to develop, standardize and validate animal models.
This approach is systematically and widely applied to GM models, transversally to multiple vertebrates species, ranging, e.g., from zebrafish,

rodents (mice and rats), to rabbits, pigs and nonhumane primates used in research settings.

To this purpose, several initiatives has been built in the last decades, as scientific consortia as well as platform and database available for the scientific community. As an example, The International Mouse Phenotyping Consortium (IMPC) gathers twentyone research institutions in an international effort to identify the function of every protein-coding gene in the mouse genome. The IMPC Consortium collates data from its international member institutes, which collect phenotyping data obtained through standardised phenotyping pipelines guided by their own ethical review panels, licenses, and accrediting bodies that are in line with national and/or geo-political constructs (5).

For GM rodents, particularly mice and rats, international database resources are available open access on the web.

The Mouse Genome Informatics (MGI) is the international database resource for the laboratory mouse, supported by The Jackson Laboratory, providing integrated genetic, genomic, and biological data to facilitate the study of human health and disease (6).

A key project contributing to this resource is the Mouse Genome Database (MGD). MGD includes data on gene characterization, nomenclature, mapping, gene homologies among vertebrates, sequence links, phenotypes, allelic variants and mutants, and strain data. MGD is of particular relevance when looking for phenotypic details in a GM mouse bearing a specific allele as it contains information on mutant alleles, transgenes, strain characteristics, phenotype vocabularies, human disease models, and comparative phenotypes. Integrated access to phenotype and disease model data is accessible via query forms providing genetic, phenotypic, and computational approaches to displaying phenotypic variation sources (single-gene, genetic mutations, QTLs, strains), as well as data on human disease correlation, and mouse models.

Furthermore, phenotypic allele summary and detail reports provide information about the content of phenotype records including observed phenotypes in mouse and genetic background of the mutant (7).

A similar database is existing also for the rat (8). The Rat Genome Database (RGD) was established in 1999 and rapidly became the premier site for genetic, genomic, phenotype, and disease-related data generated from rat research.

A peculiarity of the RGD is that it has expanded to include a large body of structured and standardized data for other species including mouse, human,

chinchilla, bonobo, 13-lined ground squirrel, dog and pig. As MGD, RGD also offers open access tools for querying, analyzing, and visualizing genetic and phenotypical data and resource for researchers worldwide.
A dedicated Phenotypes & Models Portal contains data related to rat strains and phenotypes, as well as essential information for conducting physiological research, identifying disease models, and community forums for gathering feedback from the scientific community. Since 2015, the Rat Genome Database is also running a search engine called PhenoMiner to integrate quantitative phenotype data from the PhysGen Program for Genomic Applications and the National BioResource Project in Japan as well as manual annotations from biomedical literature (9).

## III The importance of being phenotyped for non-GM strains

The relevance of phenotyping is crucial for non-GM models as well. GM mice and rats, particularly, derive from background strains which are bred since decades for research purposes. Genetical integrity data are available for such strains and openly shared by Vendors through model Data Sheets and websites. Nevertheless, phenotypic data of such commercially available models are still partial, with the exception of some specific parameters, such as onset and clinical manifestations of spontaneous pathology (10, 11), lifespan, breeding performances.
Despite the large amount of dataset pertaining to the aforementioned categories, in depth and highly specialized phenotypical characterisation of commercial non-GM strains is still unavailable or, in some cases, available only from outdated and pioneeristic studies.
The choice of a wild type (WT) animal model, being inbred or outbred, used as primary model, or as negative control in an experiment, as well as the choice of the most appropriate background strain to build a specific mutant should be made also taking into account WT strain specific charachteristics. Some are very well known and described. Just as an example, C57BL/6J mice are resistant to audiogenic seizures, have a relatively low bone density, and develop age related hearing loss. They are also susceptible to diet-induced obesity, type 2 diabetes, and atherosclerosis. Macrophages from this strain are resistant to the effects of anthrax lethal toxin. This specific strain is homozygous for Cdh23ahl, the age-related hearing loss 1 mutation, which, on this background, results in progressive hearing loss with onset after 10 months of age (12). This clearly suggests that if the research goal of a specific project focuses on hearing loss, C57BL/6J or a C57BL/6J

background mutant mouse might or might not be the most appropriate choice, based on experimental design and expected outcomes. On the other hand, the mutation Crb1rd8 (associated with retinal distrophy and degeneration, and progressive blindness) has been found to occurr in all sublines of C57BL/6N, but not in any C57BL/6J subline (13). Therefore, if the research goal of a specific project focuses on sight impairment or conversely requires the animals – negative controls included – to have normal sight, C57BL/6N as well as a C57BL/6N background mutant mouse might or might not be the most appropriate choice, again based on experimental design and expected outcomes.

Similarly in rats, differences between strains in relation to the dopaminergic system are also well described (14). As an example, Lewis rats show a blunted HPA axis response to a variety of stressors, while Fischer 344 rats, on the other hand, display a strong HPA axis response.

A furher acknowledgmet of the relevance of genetic potential retained into non-GM animals can be seen in the so-called Collaborative Crosses (CC) project. This project, initiated as a worldwide effort between the end of the 20th Century and the very beginning of the 21st, aimed to generating, genotyping and phenotyping approximately a thousand of recombinant inbred mouse lines. These lines are the products of an eight-way funnel breeding design involving eight genetically diverse mice founder strains provided by The Jackson Laboratory: A/J, C57BL/6J, 129S1/SvImJ, NOD/ShiLtJ, NZO/HiLtJ, CAST/EiJ, PWK/PhJ, and WSB/EiJ (15). The autosomal genomes of each line have equal contributions from each founder strain, and the recombinations that accumulate during the breeding process is independent between lines. These strains, deriving from well genetically charachterised stock inbred strains, show a long-term genetic stability, and allow to overcome the limitations of existing mouse genetic resources for analysis of phenotypes caused by combinatorial allele effects. The CC in fact (16) offer large genotypic and phenotypic diversity with typical stable genomes from inbred strains, modelling the complexity of the human genome and supporting analyses of common human diseases with complex etiologies originating through interactions between allele combinations and the environment (17). All these genetic and phenotypic resources are deriving from non-GM, inbred strains, with the further huge andvantage, also from the animal welfare perspective, that genotyping only needs to be performed on the panel, not on each individual mouse (18).

## IV The challenge of Substrains

Thousands of articles are published each year, with reference to GM mice, yet genetic background issues are rarely considered. Awareness and subsequent assessment of such issues are not, but should become, routine norms of murine experimentation (19). A comprehensive approach to the problem should take into account the role played by substrains variability. It is widely acknowledged that phenotypes of mutant mice are strongly influenced by genetic background, including flanking alleles, as well as the targeted genes, thus appropriate control for genetic background is essential for adequate experimental design, including the choice of the model, and the proper interpretation of data (20).

Well known examples of substrains associated confounding factors include both considerations affecting embryonic stem (ES) cells production as well as later stage *in vivo* drawbacks.
The 129 mouse is the most widely used strain in gene targeting experiments. It was showed (21) that 129/SvJ is significantly different from the other 129 substrains and is more accurately classified as a recombinant congenic strain (129cX/Sv), being derived from 129/Sv and an unknown strain. Mixed genetic backgrounds could confound or impair gene targeting experiments by reducing homologous recombination efficiency when constructs and ES cells are not obtained from the same 129 substrain. Additionally, different genetic backgrounds may lead to different phenotypes of genes targeted in different 129-derived ES cell lines (21).
Moreover, the extensive genetic variability among 129 substrains derived from both intentional and unintentional outcrossing, leaded to extensive variability of embryonic stem cells derived from them, allowing consideration of its negative impact on targeting technology, including: homologous recombination frequencies, preparation of inbred animals, and availability of appropriate controls (22).
Since its development in the 1930s, the C57BL/6 strain diverged into two major groups in the 1950s, C57BL/6J and C57BL/6N, and more and more substrains have been established from them worldwide in the next decades. Currently available C57BL/6 substrains, derived from the C57BL/6 founder line, are more and more systematically reported to differ in several phenotypes (23).
As with 129, great care must be taken when working with mice engineered by using C57BL6 embryonic stem cell lines because control groups,

backcrosses, and intercrosses could inadvertently introduce phenotypically significant polymorphic alleles or environmental confounds (24) – including vendor or other sources of the substrain. On the other hand, as with Collaborative Crosses, deliberate crosses between B6 substrains may provide an opportunity to map polymorphic loci that contribute to variability in a trait on largely homogenous backgrounds, which has the potential to improve mapping resolution and aid in the selection of candidate genes (24). Significant phenotypic differences arising from C57BL6 substrain, both spontaneously or under experimental conditions, impact among others behaviour and neurology, cardiovasculas system, homeostasis and metabolism, integument, immune system, and neoplasm susceptibility (25). Similar findings have been documented in rats as well, particularly in the fields of neurobiology and behaviour. By way of example, with substrains obtained from different vendors, differences in autism-related behavioral phenotypes are described in Wistar-Kyoto rats (26), while different sensorimotor gating-disruptive effects of dopamine agonists, and neuropathic pain behaviors are reported in Sprague Dawley rats (27, 28).

## V The path forward

It is crucial to remember that phenotype is the combination of genetic component and environmental impact (29), thus constant and active assessment of non-GM and GM strains should be part of each Breeder or research Institution's animal care and use program, when dealing with engineered models.

In the light of the relevance of non-GM models phenotypic potential, and to contribute to the widening of the characterisation of non-GM rodents phenotype, we focused on two aspects that were either non-charachterised or only partially characterised in the pioneering phase of the animal strain.

Particularly we focused our attention on spontaneous, undisturbed motor activity in three commercially available mice strains: C57BL/6NCrl (inbred), BALB/cAnNCrl (inbred) and CRL:CD1(ICR) (outbred).

We also focused our attention on the phenotypic charachterisation of puberty onset in male rats, particularly using the Balano preputial separation (BPS) test, on Crl:CD(SD) and Crl:LE, both outbred.

Interestingly, data on spontaneous motor activity in the aforementioned murine strains were totally new, thus we first charachterise this kind of activity, and behavioural phenotype, for the first time, covering lifespan ranging from weaning to sexual maturity and adulthood, in both sexes.

# Introduction

Data obtained from rats provided an update on Crl:CD(SD) consistency with individuals from the same colony under genetic integrity standard, serving under some perspective as a quality control for the strain consistency. Information on Crl:LE provided the first pubertal curve for this specific substrain, and first data from the pioneeristic study of Long and Evans, from the beginning on 20th Century.

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# Chapter 1
# Bridging the gap in the mouse (*Mus musculus*)

Phenotyping spontaneous locomotor activity in inbred and outbred mouse strains by using Digital Ventilated Cages

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## 1.1. Introduction

An accurate knowledge of the animal model is critical to ensure appropriate experimental design and subsequent reproducibility, reduce animal waste and comply with essential animal welfare principles. In the process of thoroughly comprehending and choosing a reliable animal model, phenotyping circadian rhythms and motor activity can provide information of paramount importance for the correct setting of - among other types of studies - behavioral, metabolic, neuroscience and cancer studies (1,2,3,4). Circadian rhythmicity controls a wide variety of physiological events, including body temperature, activity, sleep, metabolism, heart rate, blood pressure and hormone and neurotransmitter secretion (5). With the development and validation of multiple non-invasive recording instruments and variables of interest for different species, increasing numbers of research papers, ranging from assessment of focal behaviors mostly under experimental conditions (i.e., out of cage) to in-cage recording have been released, showing the scientific relevance of broadening the understanding of spontaneous behavior of undisturbed animals.

Despite the availability of several studies, to date, most focus on systematically reviewing C57BL/6J, C57BL/6J-related or genetically altered murine strains (2,6,7). Little systematic data about characterization of spontaneous in-cage motor activity in inbred and outbred mouse stock strains are currently available from different vendors. Lack of such information can lead to inappropriate model choice, steering researchers to wrong experimental designs and confounding factors in experimental data analysis. On the other hand, a clear, unbiased characterization of spontaneous in-cage behaviors could improve comparability and reproducibility of models and data obtained apparently from similar strains but differently originated. Extensive literature documents strain-specific differences in circadian rhythms as well as remarkable differences in diurnal activity patterns (8) among inbred and hybrid strains (9). Natural genetic polymorphisms manifested by inbred strains also indicate that background affects circadian rhythmicity (9). The choice of mouse strain is thus the most important consideration for mouse circadian rhythm screen and ultimately dictates the ability to identify mutants. The implementation of large-scale phenotyping datasets may positively affect reduction measures, according to the 3Rs principles and policy (10), and accelerate global animal research.

On the basis of systematic observations made through extensive experience in the breeding of both outbred and inbred mouse strains, we decided to verify the existence of and eventually record relevant differences in circadian rhythms and spontaneous locomotor activity among different stock mouse strains. We focused on three non-genetically altered mouse strains widely used in research: C57BL/6NCrl (inbred), BALB/cAnNCrl (inbred) and CRL:CD1(ICR) (outbred). The choice of these strains was based on the following facts. First, although C57BL/6NCrl is commonly used for research purposes, it is less characterized than the substrain C57BL/6J. Remarkably, the two substrains, having clear phenotypic differences in various aspects, cannot be used interchangeably (11). Second, because of the low genetic variability and phenotypic instability compared to the other substrains (12), BALB/cAnNCrl is frequently used in longitudinal neurobehavioral analyses. Third, among outbred strains, CRL:CD1(ICR) is the most commonly used in laboratories worldwide.

We screened our mice by using an automated recording home-cage device, the Digital Ventilated Cage (DVC by Tecniplast) to obtain an unbiased understanding of in-cage spontaneous mouse behavior and to track locomotor activity in the two sexes during a 24-h period. The DVC system, which relies on the detection of animal activity via the generation of tiny electromagnetic fields, has been proven to be safe for animals (13) and does not affect their behavior or welfare (14). A previous study comparing C57BL/6NCrl and BALB/cAnNCrl mice housed in the DVC system has reported differences in measures such as bodyweight, water utilization and position within the cage, as well as a common test of anxiety-related behavior and cognition (14).

Here, we introduce new and different circadian metrics to analyze data obtained only from in-cage recording. We compared the 24-h spontaneous locomotor activity of the mice and extrapolated key aspects of the day and night activity patterns for each strain. All analyzed metrics clearly show significant differences in the circadian activity of the three selected strains, not only identifying differences when considering inbred versus outbred strains, but—consistent with available literature (2) —characterizing strain-specific spontaneous locomotor patterns during the 24-h period, proving once more that different strains have peculiar diurnal motor phenotypes. The strain-specific differences are further confirmed by an unsupervised machine learning approach.

## **1.2** Matherials and Methods

### 1.2.1 Mice

The three strains here analyzed, C57BL/6NCrl, BALB/cAnNCrl and CRL:CD1(ICR), were obtained from Charles River Laboratories. The mice were bred under barriered specific pathogen free–condition facilities at the Charles River Laboratory facility in Calco, Italy according to internal breeding standard operating procedures, which include a genetic stability program and specific pathogen free conditions. At 3 weeks of age, after weaning, the mice were moved to the CNR-IBBC/EMMA-Infrafrontier-IMPC Core Structure (Monterotondo, Rome, Italy)—Consiglio Nazionale delle Ricerche (Rome, Italy) and housed in DVC racks for the whole duration of the study. After acclimatization, mice of each strain were housed in groups of three individuals per cage, fed ad libitum with standard diet (4RF21; Mucedola), under standard controlled environmental parameters (temperature = 21 ± 2 °C; relative humidity = 55% ± 15%), and mice were kept in a 12-h light/12-h dark cycle (7 AM—7 PM: lights on) with 12–15 air changes per hour and a 12:12 light cycle. Light intensity at room level was 230 lux, while cages were exposed to slight differences according to their position within the rack. Variations of light intensity at cage level were recorded, with lux levels ranging from 29 to 12 lux. Certified dust-free wood bedding (Scobis one; Mucedola) was provided in the cages. Mice were provided chlorinated, filtered water ad libitum. 2-week-interval cage changes were adopted with unaltered standard procedure and timing (Mondays at 10 AM). Differently from other studies (7), cage density was standardized to three mice per cage, with the intent to mimic possible standard housing conditions in research settings, avoiding the potential bias provided in terms of locomotor activity by single housing (i.e., absence of interaction with cage mates and altered (increased) time to integrate into the nest, leading to a prolonged activity time (41).

Experimental groups were divided in two separate cohorts of mice in two different periods of the year (springtime and late summer/early autumn) to reduce the seasonality bias, as follows: C57BL/6NCrl mice, n = 18 males (6 cages); n = 18 females (6 cages); BALB/cAnNCrl mice, n = 18 males (6 cages); n = 18 females (6 cages); CRL:CD1(ICR) mice, n = 18 males (6 cages); n = 18 females (6 cages). Each cohort was thus composed of 54 individuals (27 females plus 27 males equally divided per strain).

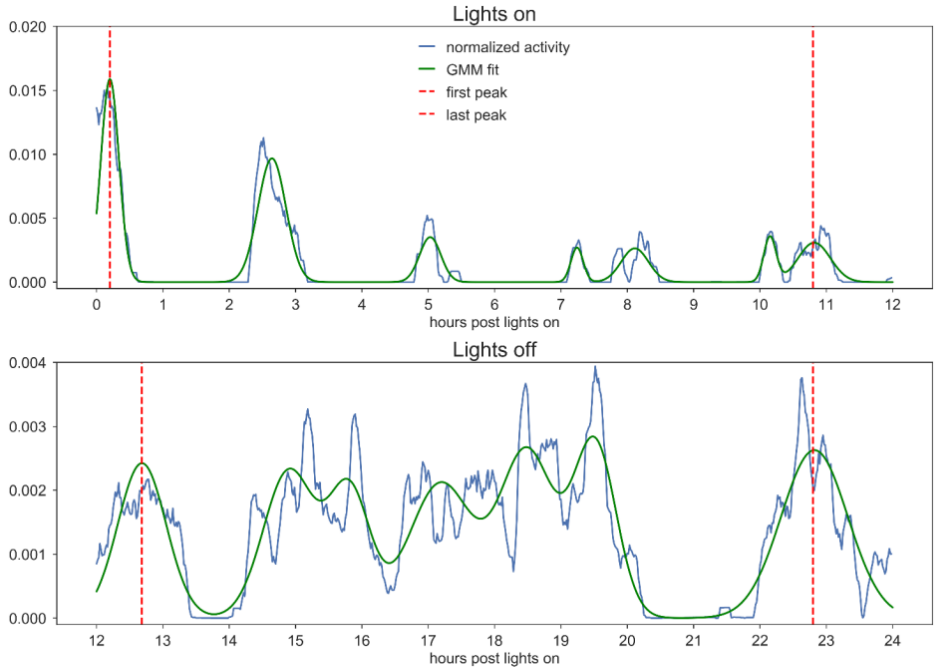
### 1.2.2 Home-cage activity monitoring: DVC system and activity metrics

All cages were kept in a DVC rack, a home monitoring system that automatically measures animal activity 24/7(19). An electronic capacitance sensing board is positioned below each cage and consists of 12 contactless electrodes that record the animal's presence in each electrode surrounding. We used the 'activation density' metric to capture mouse activity in the cage (19), aggregated in 1-min bins. We then analyzed lights-on activity (the average of all the 1-min bins within 12 h of daytime) and lights-off activity (the average of all the 1-min bins within 12 h of nighttime). On the basis of previous reports (42), we calculated 'diurnality', which is the (daily) fraction of activity performed during the lights-on phase with respect to the total activity performed in the whole day, measured as the sum of lights-on and lights-off activity. We also calculated the regularity disruption index (RDI) during both lights-on and lights-off phases (6). This metric captures the level of irregularity of the activity pattern: a time series in which all minutes have similar activity levels gives a low RDI, whereas a high RDI indicates that minutes of activity are very different from each other.

### 1.2.3 Responses to procedures estimated with Gaussian mixture models (GMMs)

To determine the location of activity peaks during the 24 h, especially those related to responses to lights-on and lights-off phases, we used GMMs. We used the library scikit-learn (version 0.19.1) for Python (43) to fit each 720-min time series (12 h of the lights-on or lights-off phase) as a mixture of several normal-density components so that we could calculate their means, weights and standard deviations. We used a fixed number M of components for all the time series to homogeneously compare results between cages and groups, and we set M = 7 after empirically observing the model fitting and mean absolute error for different M's. Among these seven, we used the means of first and last components during both daytime and nighttime as estimates of the time of responses to lights-on and lights-off phases (Fig. 1).

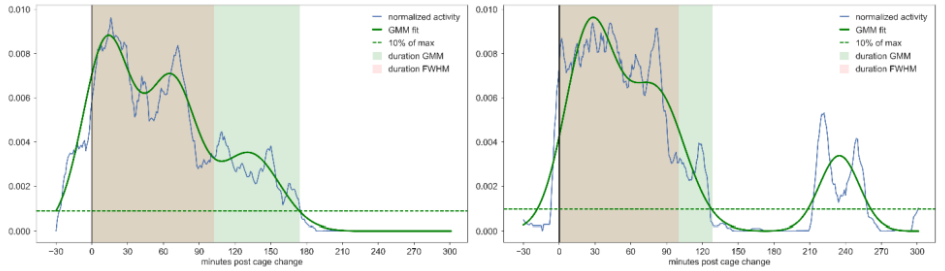
**Figure 1: GMM fit during lights on and lights off period.**




Exemplifying activity time series during lights on (A) and lights off (B), fitted with a GMM with M = 7 gaussian components. Red dashed lines indicate the time of the first and last components. Activity is here normalized to the sum of all the activity during the whole 24 hours.

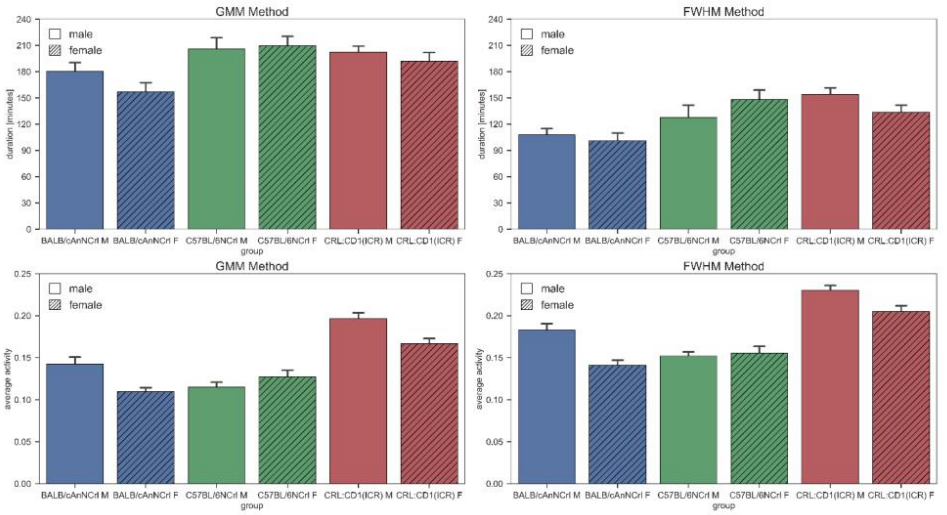
We used a similar approach to calculate the duration of the response to the cage change. We selected the 5-h time series after each cage change and fit a GMM with M = 3 components. We determined the duration as the interval between the time of cage change and the time at which the fitted curve goes below 10% of its peak. We also made a comparison between the results with GMMs and those with the full-width half-maximum method already described (19) (Figs. 2 and 3).

**Figure 2: Duration of response to cage change estimated with GMM and FWHM.**




The figure shows two examples of the activity time series within the 5 hours after cage change. The GMM fit (with M = 3 components) was able to capture the clear response in A and to separate the response to cage change and the later burst of activity in B.

**Figure 3: Response to cage change with GMM and FWHM.**



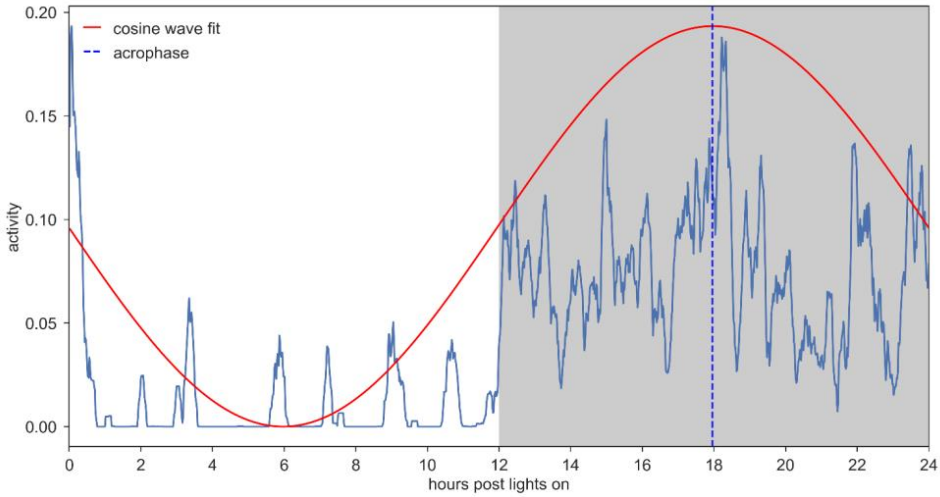

The results obtained with the use of GMMs are reported in A-C and compared to the ones obtained using the FWHM methodology proposed in Pernold et al (51).

### 1.2.4 Analysis of circadian rhythmicity

Typical measures used in the analysis of circadian rhythmicity are 'acrophase' and 'activity onset'(44). Acrophase is the time at which the peak of the circadian rhythm occurs, and thus it is an estimate of the centrality and concentration of the activity during a 24-h period (45). Activity onset is the time that animals start being active, and in the case of rodents, it typically refers to the time around the lights-off phase. Conventional approaches to numerically determine these metrics are generally based on a clear separation between day (extremely low or zero activity) and night (very high activity). This is common when using running wheels, whereas with spontaneous locomotion, the separation is not always so clear, and conventional approaches possibly need to be modified (46).

We applied cosinor analysis (47) to fit a cosine wave with known period (t = 24 h) to each daily activity time series (1,440 min, i.e., 24 h). The acrophase is determined as the time at which the fitted curve reaches its maximum value (Fig. 4).

**Figure 4: Cosinor analysis.**

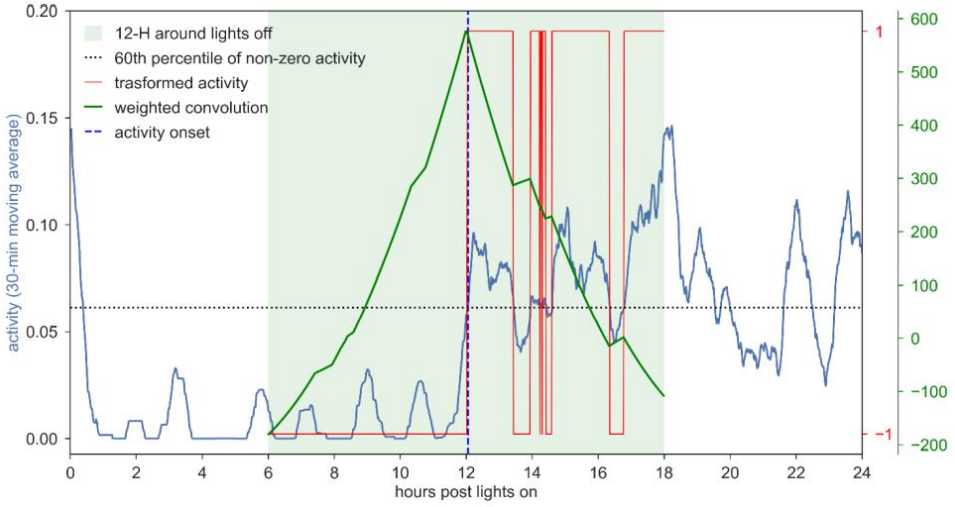



An exemplifying 24-hours minute-based activity time series (1440 minutes), fitted with a cosine wave with period T = 24 hours. The peak of the wave determines the acrophase (blue line).

We estimated activity onset time by the template-matching algorithm used by the ClockLab analysis package (Actimetrics Inc.), which we empirically

adapted to spontaneous activity data, for which the separation between day and night is not always so sharp. We considered only data lying in an interval of 12 h centered on lights-off time, smoothed with a 30-min moving average. Each time series (12 h) was transformed to an array of 1's and −1's depending on whether each minute exceeded or fell below the 60th percentile of all non-zero activity data. We then computed the convolution (a mathematical operator that returns the product between one fixed sequence and another sequence that slides) between the transformed time series and a template of N hours of −1's followed by M hours of 1's (M = 6, N = 6; i.e., 720 min of –1's and 720 minutes of 1's). Finally, we weighted the convolution for the number of samples of the time series overlapping it and determined the onset time with the location of the maximum of this weighted convolution (Fig. 5).

**Figure 5: Activity onset. An exemplifying 24-hours minute-based activity time series (1440 minutes).**




The green span is the 12-hours interval within which we applied the template matching algorithm. The red line is the transformed time series, depending on whether the activity exceeds or fall below the 60th percentile of non-zero activity data (dotted black line). The green line is the convolution with a template of N hours of -1's followed by M hours of 1's (M = 6, N = 6, i.e. 720 minutes of –1's and 720 minutes of 1's), weighted for the number of samples of the time series overlapping it. Finally the blue dashed line

indicates the peak of the weighted convolution and thus the estimated activity onset (in this example, 723 minutes post lights on, i.e. 3 minutes after lights off).

### 1.2.5 Cluster analysis

Machine learning can be a novel approach to model complex data in animal behavior studies (22). Cluster analysis is one of the most common unsupervised learning techniques, aiming to find groups composed of units similar to each other and different from the units of other groups. Here, we decided to apply a K-means algorithm to cluster the daily data and see if strains do separate in an unsupervised and data-driven approach. All the previously described metrics (lights-on and lights-off activity, diurnality, lights-on and lights-off phases, RDI, acrophase, activity onset and all metrics relative to responses to lights-on and lights-off conditions) were used as input for the clustering algorithm. We applied principal component analysis (PCA) to reduce dimensionality and then applied K-means with K = 3 clusters, with the aim of separating strains and not sex (which was not always a significant factor in our analyses). Each day of each cage was therefore classified in a specific cluster.

### 1.2.6 Statistical tests

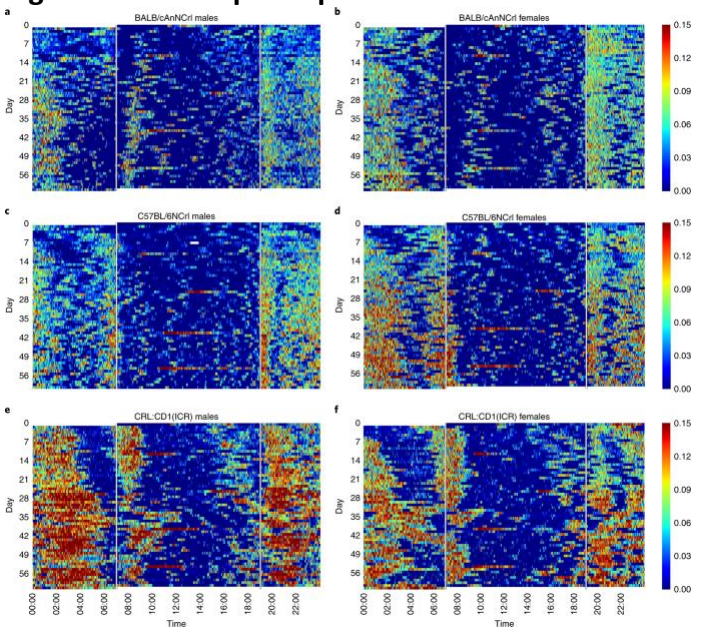
Because the same individuals were assessed over time and for a long period (60 d), we used general linear mixed models to quantitatively evaluate differences between strains, sexes and time and light conditions. We used lmerTest R software package to model data and test for fixed effects (48). We resorted to a top-down approach and successive likelihood ratio tests to define the model best explaining the data (49).

We used Python to process and visualize data and R (version 3.4.3) to run all statistics, with significance level $\alpha = 0.05$. We excluded days of cage changing from the analysis, as well as days with missing values or with some technical issues. As a consequence of group housing, the statistical unit is the cage (50): DVC measures the overall aggregated value of activity of the mice for each cage, with a reduction of statistical power that is not necessary scaled down exactly with the aggregation factor, because of probable intra-cage correlation (24).

## **1.3** Results

The DVC system allowed us to monitor the activity of three commonly used mouse strains (C57BL/6NCrl, BALB/cAnNCrl and CRL:CD1(ICR)) from 4 to 12 weeks of age, covering the period between weaning, sexual maturity and early adulthood. Heat maps of representative cages of male and female C57BL/6NCrl mice, used as a reference strain, BALB/cAnNCrl mice and CRL:CD1(ICR) mice show how the activity was distributed across the 24 h of the experiment (Fig. 6). As expected, the overall highest recorded activity was concentrated during the night, although clear differences were observed between the three strains. To better disentangle the circadian phenotype of the three strains, we used the metrics decribed below.

**Figure 6: Heatmaps of spontaneous locomotor activity.**



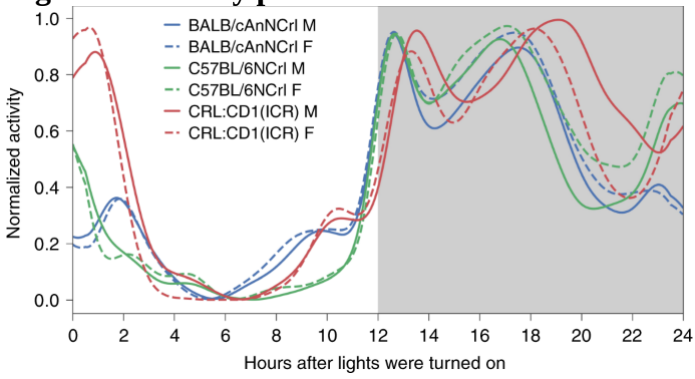

**a**–**f**, Each panel shows 24-h activity during the experiment in an exemplifying cage of BALB/cAnNCrl males (**a**), BALB/cAnNCrl females (**b**), C57BL/6NCrl males (**c**), C57BL/6NCrl females (**d**), CRL:CD1(ICR) males (**e**) and CRL:CD1(ICR) females (**f**), with $n$ = 3 mice per cage.

### 1.3.1 24-h locomotor activity pattern

We first qualitatively analyzed the average pattern of recorded locomotor activity for males and females of each strain, C57BL/6NCrl, BALB/cAnNCrl and CRL:CD1(ICR), for 24 h and 7 days a week (24/7) for

the two entire experimental periods. The activity of the three strains was not entirely confined to the dark phase, but cyclical patterns of increased and decreased activity over the light and dark phase were detected (Fig. 7). The locomotor activity of C57BL/6NCrl and CRL:CD1(ICR) mice began before dawn, and it lasted ~1 h. In contrast, BALB/cAnNCrl mice activated 2 h after lights were turned on. A clear pre–dark phase anticipatory activity (2 h before lights were turned off) was observed in the pattern of CRL:CD1(ICR) and BALB/cAnNCrl mice and progressively increased during the transition phase between light and dark. The peak of activity was recorded during the dark phase for the three strains, with a strain-specific pattern: C57BL/6NCrl mice showed remarkable peaks throughout the night, with extended activity for up to 1 h after lights were turned on; CRL:CD1(ICR) mice also displayed peaks of activity during the whole dark phase, but there was a gradual increase in activity at the start of the lights-off phase, reaching a peak 2 h later, and then alternated decreased and increased activity for up to 2 h after lights were turned on. In contrast, the recorded activity of BALB/cAnNCrl mice displayed bouts of intermediate activity beginning with clear anticipatory activity before the lights-off phase and continuing during the dark phase. BALB/cAnNCrl mice also showed a clear reduction in activity toward the end of the dark phase and had an additional short bout of activity 2 h after lights were turned on. No clear difference was observed between males' and females' cages, except for CRL:CD1(ICR) males revealing a more intense activity during the night compared to females.

**Figure 7: Activity pattern over 24 h of the three analyzed strains.**



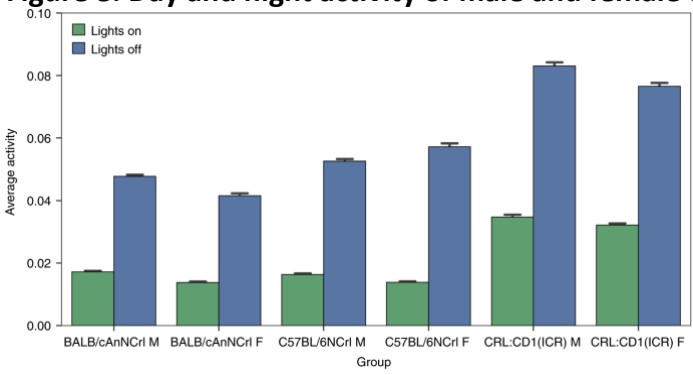

The figure shows the average distribution of activity over the 24 h of a day (lights-on and lights-off periods). Each daily activity time series was

normalized to its peak activity (=1.0), to compare groups by their relative 24-h pattern and not by the absolute level of activity. F, females; M, males.

### 1.3.2 Day and night activity

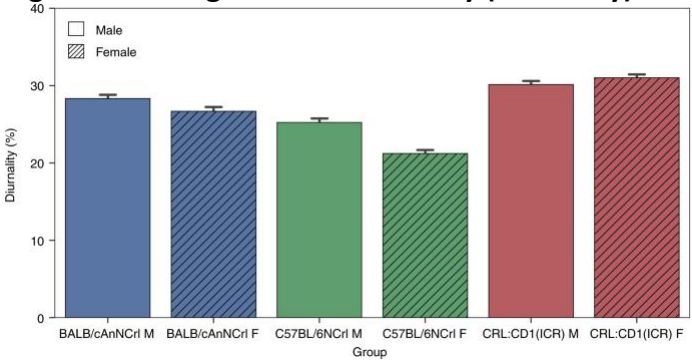
We then characterized more in depth the day and night level of activity for each strain. With this aim, we measured the average activity of each cage during 12 h of light and dark (Fig. 8). We used linear mixed models to question which of the following effects, including strain, sex, time and light, quantitatively correlates with the observed differences in the day and night activity levels. Because the mice are nocturnal, the average activity of the three strains in both males and females was much higher during the night than during light hours ($P_{light} < 0.001$). Indeed, the impact of light on average activity displayed a positive slope in all cages over time, with a shift depending on light and time-light interactions ($P_{time\text{-}light} < 0.001$). Although we did not observe significant differences in activity levels between BALB/cAnNCrl and C57BL/6NCrl mice ($P_{BALB/cAnNCrl} > 0.05$), CRL:CD1(ICR) mice displayed significantly more intense average activity during day and night compared to C57BL/6NCrl mice ($P_{CRL:CD1(ICR)} < 0.001$). The sex factor was not significant and was thus excluded from the model. Finally, we observed an increasing trend of activity over time ($P_{time} < 0.001$), with an estimated positive slope of $3.31 \times 10^{-4}$. We then compared the average activity during the second, fifth and eighth weeks of the experiment (i.e., 5, 8 and 12 weeks of age), probably corresponding to the pre-pubertal, post-pubertal and adulthood phases, respectively (15). Our results confirmed that the activity significantly changed over these biological cornerstones ($P_{weeks} < 0.001$).

**Figure 8: Day and night activity of male and female cages of each strain.**



Average (± s.e.m.) activity during lights-on and lights-off phases across multiple days and cages of each group.
We observed that, overall, BALB/cAnNCrl and CRL:CD1(ICR) mice displayed a higher daylight activity than C57BL/6NCrl mice (Fig. 9). Specifically, CRL:CD1(ICR) mice had a significantly higher diurnality ($P_{CRL:CD1(ICR)} < 0.001$) than BALB/cAnNCrl mice ($P_{BALB/cAnNCrl} < 0.05$), independently of sex differences.

**Figure 9: Average of diurnal activity (diurnality).**



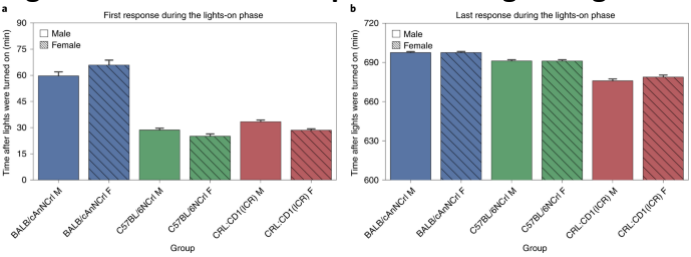

The graph shows the percentage of recorded spontaneous locomotor activity during the lights-on phase with respect to the total activity recorded for 24 h. The percentage was measured in male and female cages of each strain, across multiple days and cages of each group.

### 1.3.3 Responses to lights being on and lights being off

Given that light deeply correlates with activity over 24 h, we decided to better analyze the locomotor activity in relation to light by identifying four critical moments over 24 h: (i) the first response during the lights-on phase, (ii) the last response during the lights-on phase, (iii) the first response during the lights-off phase and (iv) the last response during the lights-off phase. The first response during the lights-on phase (Fig. 10a) occurred in a short time after lights were turned on for both C57BL/6NCrl and CRL:CD1(ICR) mice, whereas it was substantially delayed for BALB/cAnNCrl mice (PBALB/cAnNCrl < 0.001). In contrast, the last response during the lights-on phase (Fig. 10b) appeared earlier for CRL:CD1(ICR) mice ($P_{CRL:CD1(ICR)} < 0.001$) and slightly later for BALB/cAnNCrl mice ($P_{BALB/cAnNCrl} < 0.01$)

compared to the reference strain. The effect of sex was excluded from both models, because no significant difference was observed.

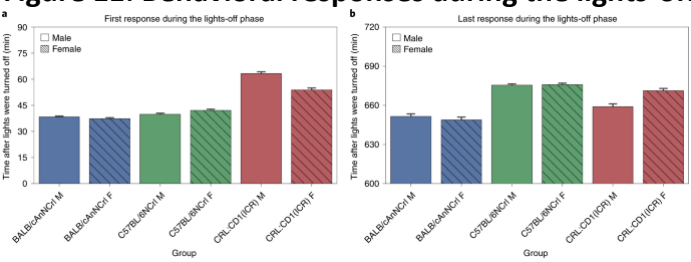
**Figure 10: Behavioral responses during the lights-on phase.**




**a**, The graph shows the average (± s.e.m.) time of the first peak of activity during the lights-on phase across multiple days and cages of each group. **b**, Average (± s.e.m.) time of the last peak during the lights-on phase. The time is expressed as minutes after lights were turned on.

The first response to the lights-off phase also suggested a different behavior between strains: whereas BALB/cAnNCrl mice ($P_{BALB/cAnNCrl} > 0.05$) displayed an early response to lights being turned off, similarly to the reference strain, CRL:CD1(ICR) mice showed a clear delayed response to lights being turned off ($P_{CRL:CD1(ICR)} < 0.001$). This was particularly evident in males' cages ($P_{CRL:CD1(ICR):male} < 0.001$) (Fig. 11a). Conversely, in correspondence with the end of the dark phase, we observed a clear significant anticipation of the last peak of activity of BALB/cAnNCrl and CRL:CD1(ICR) mice, in either males or females, compared to C57BL/6NCrl mice ($P_{BALB/cAnNCrl} < 0.001$; $P_{CRL:CD1(ICR)} < 0.05$) (Fig. 11b).

**Figure 11: Behavioral responses during the lights-off phase.**

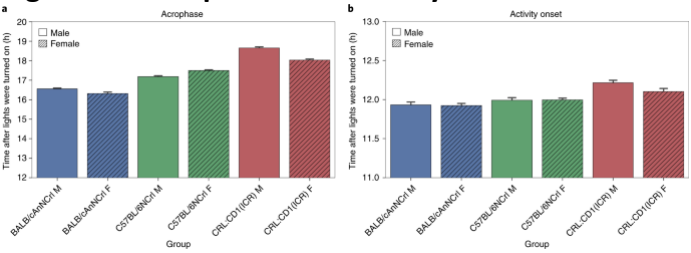


**a**, The graph shows the average (± s.e.m.) time of the first peak of recorded locomotor activity during the lights-off phase across multiple days and cages of each group. **b**, Average (± s.e.m.) time of the last peak of recorded locomotor activity during the lights-off phase. The time is expressed as minutes after lights were turned off.

### 1.3.4 Acrophase and activity onset

The acrophase and activity onset were evaluated in both males' and females' cages of the three strains to characterize the locomotor circadian rhythm. The acrophase was anticipated for BALB/cAnNCrl mice ($P_{BALB/cAnNCr} < 0.001$) compared to the reference strain (Fig. 12a) and clearly delayed in cages of CRL:CD1(ICR) mice ($P_{CRL:CD1(ICR)} < 0.001$). Slight but significant differences were seen when measuring the activity onset (Fig. 12b): whereas the beginning of activity of C57BL/6NCrl mice probably corresponded to the transition from the lights-on phase to the lights-off phase, it was slightly anticipated in BALB/cAnNCrl mouse cages ($P_{BALB/cAnNCrl} < 0.01$) and clearly delayed in CRL:CD1(ICR) mice ($P_{CRL:CD1(ICR)} < 0.001$).

**Figure 12: Acrophase and activity onset.**



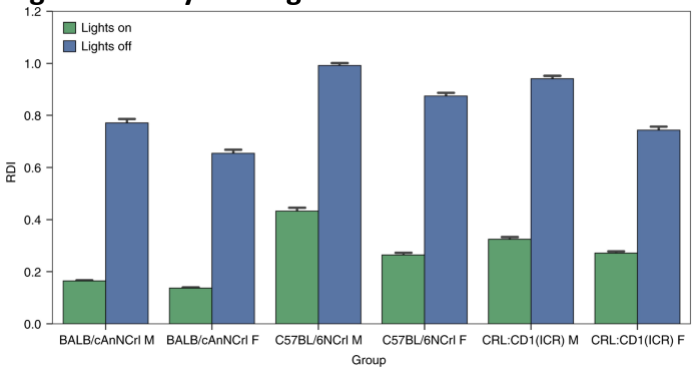

**a**, The graph shows the average (± s.e.m.) of acrophase across multiple days and cages of each group, expressed as hours after the lights-on time. **b**, Average (± s.e.m.) of activity onset across multiple days and cages of each group, expressed as hours after the lights-on time.

### 1.3.5 Regularity disruption index (RDI)

Finally, we calculated the RDI for females and males of each strain, to capture possible irregular mouse activity patterns during lights-on and lights-off phases over the entire experimental period. We observed that during the lights-on phase, C57BL/6NCrl and CRL:CD1(ICR) mice

frequently changed their status, compared to BALB/cAnNCrl mice ($P_{BALB/cAnNCrl} < 0.001$) (Fig. 13), which displayed the most stable locomotor behavior during the lights-on phase. As expected, RDI was much higher during the lights-off phase in all strains ($P_{light} < 0.001$). Remarkably, RDI was slightly higher in males of all strains than in females ($P_{female} < 0.05$).

**Figure 13: Day and night RDI.**



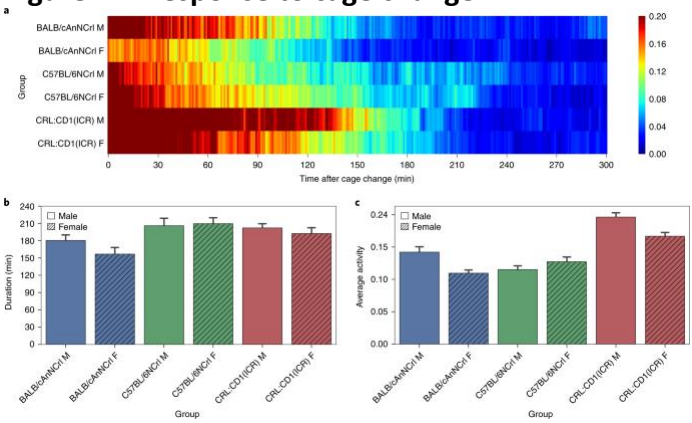

The graph shows the average (± s.e.m.) of RDI during lights-on and lights-off phases across multiple days and cages of each group.

### 1.3.6 Behavioral response to the cage change

We then decided to analyze and measure the locomotor activity within a range of 5 h after the cage change (Fig. 14a), to evaluate the response to such a stressful moment in the husbandry and management of mice (16,17). The cage-change procedure was performed by trained animal care technicians under standardized practices: every 2 weeks, during the light phase of the light/dark cycle, from the dirty cage to the clean one and shortly restraining and moving the mice by tail grasping and suspension. We focused on two measurements: duration, as the average (± s.e.m.) estimate of the duration of the response to cage change, and average activity, as the average (± s.e.m.) activity recorded within the estimated response duration (Fig. 14b and c). BALB/cAnNCrl mice ($P_{BALB/cAnNCrl} < 0.05$) showed a significantly shorter response in terms of duration than did C57BL/6NCrl and CRL:CD1(ICR) mice (Fig. 14b). C57BL/6NCrl mice showed a longer duration of locomotor response to cage change, with slightly higher values in females than males, in contrast to BALB/cAnNCrl and CRL:CD1(ICR) mice (Fig. 14b). Slightly significant sex differences were observed in

average activity, which increased in males—with the only exception being C57BL/6NCrl mice (Fig. 14c)—suggesting a potential correlation with strain and sex-related exploratory and marking behavior (18). With the only exception of a clearly longer duration of locomotor response to cage change showed by C57BL/6NCrl mice (both males and females), other data on behavioral response to cage change should be investigated further to evaluate potential correlations between strain, handling and restraining techniques and sex-related exploratory and marking behavior. Further analyses are now ongoing, implementing new metrics to better understand the reaction to cage change under the behavioural perspective, to identify any spatial preference in the cage right after the cage change, as well as right before the next cage change – meaning the moments of minor and major habituation to the home cage environment. The goal of further analysis of these kind will be to move the assessment of the motor activity from a quantitative to a more qualitative interpretation.

**Figure 14: Response to cage change.**




**a**, The heatmap shows the minute-based activity recorded within 5 h after cage change, averaged across all days of cage change and all cages of the corresponding group (as rows). **b**, The graph shows the average (± s.e.m.) estimate of the duration of the response to cage change. **c**, The graph shows the average (± s.e.m.) activity recorded within the estimated response duration.

### 1.3.7 K-means clustering

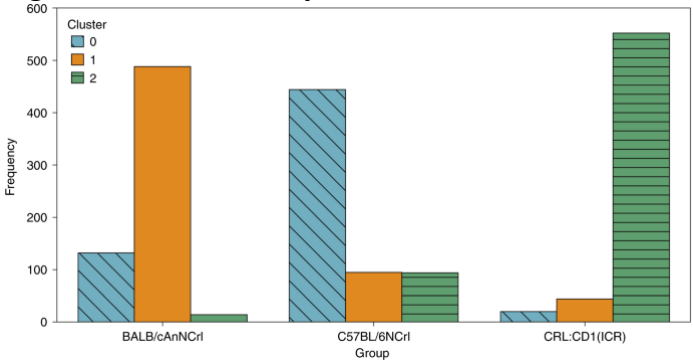
All analyzed metrics clearly highlighted differences in the circadian activity of the three selected strains (Table 1). To further confirm our results, we undertook the K-means clustering method and included each previously analyzed metric. To reduce the dimensionality, we first applied principal component analysis (PCA) and successive K-means clustering, aiming at separating only strains, and not sex. We were able to record ~600 measurements per strain (one measurement is one cage per day), obtained as 12 cages for 50 d per strain. We set K-means with three clusters. We observed that each cluster contains measurements mostly from a single strain, meaning that cages of the same strain are more similar to each other than to other strains. As represented in Fig. 15, BALB/cAnNCrl was assigned to cluster 1, C57BL/6NCrl to cluster 0 and CRL:CD1(ICR) to cluster 2. We further confirmed these results for each strain by calculating how many times each cage could be classified in the specific cluster over the two experimental periods. Our results show that each cage was classified according to the corresponding cluster of its strain, except for one C57BL/6NCrl cage that was classified in the BALB/cAnNCrl corresponding cluster (Table 2).

**Table 1 Key patterns of spontaneous locomotor activity recorded for each cage housing BALB/cAnNCrl, C57BL/6NCrl and CRL:CD1(ICR) mice of both sexes**

| Strain | | Day and night activity | Diurnality | Response to the lights-on phase | Response to the lights-off phase | Activity from pre-puberty until adulthood | Acrophase | Activity onset | RDI |
|---|---|---|---|---|---|---|---|---|---|
| BALB/cAnNCrl | Males | ++ | ++ | Delayed | Early | + | Early | Early | ++ |
| | Females | ++ | ++ | Delayed | Early | + | Early | Early | + |
| C57BL/6NCrl | Males | ++ | + | Early | Early | ++ | Delayed | Concomitant | +++ |
| | Females | ++ | + | Early | Early | +++ | Delayed | Concomitant | ++ |
| CRL:CD1(ICR) | males | +++ | +++ | Early | Delayed | ++ | Delayed | Delayed | +++ |
| | females | ++ | +++ | Early | Delayed | +++ | Delayed | Delayed | ++ |

+++, ++, + indicate intense, medium and low average of locomotion, respectively.

**Figure 15: Cluster analysis.**




Each measurement (day per cage) was classified in a specific cluster by the K-means algorithm that took as input all the previously analyzed metrics (after a dimensionality reduction with PCA). The graph clearly shows three predominant clusters, each corresponding to a strain (cluster 0 to C57BL/6NCrl, cluster 1 to BALB/cANCrl, and cluster 2 to CRL:CD1(ICR)). Notably, CRL:CD1(ICR) shows the highest percentage of measurements classified in a single cluster.

**Table 2 Relative frequencies of classification of cages in each cluster**

| Group | Cage | cl 0 (%) | cl 1 (%) | cl 2 (%) | Group | Cage | cl 0 (%) | cl 1 (%) | cl 2 (%) | Group | Cage | cl 0 (%) | cl 1 (%) | cl 2 (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| BALB/cAnNCrl M | C_01 | 18.5 | 77.8 | 3.7 | C57BL/6NCrl M | C_04 | 70.4 | 13.0 | 16.7 | CRL:CD1(ICR) M | C_11 | 9.3 | 5.6 | 85.2 |
| BALB/cAnNCrl M | C_03 | 9.3 | 83.3 | 7.4 | C57BL/6NCrl M | C_06 | 53.7 | 3.7 | 42.6 | CRL:CD1(ICR) M | C_13 | 1.9 | 0.0 | 98.2 |
| BALB/cAnNCrl M | C_17 | 9.3 | 87.0 | 3.7 | C57BL/6NCrl M | C_08 | 18.9 | 45.3 | 35.9 | CRL:CD1(ICR) M | C_16 | 5.6 | 9.3 | 85.2 |
| BALB/cAnNCrl M | C_20 | 46.2 | 53.9 | 0.0 | C57BL/6NCrl M | C_19 | 90.4 | 9.6 | 0.0 | CRL:CD1(ICR) M | C_21 | 0.0 | 0.0 | 100 |
| BALB/cAnNCrl M | C_23 | 26.9 | 73.1 | 0.0 | C57BL/6NCrl M | C_22 | 100 | 0.0 | 0.0 | CRL:CD1(ICR) M | C_24 | 0.0 | 0.0 | 100 |
| BALB/cAnNCrl M | C_26 | 36.5 | 61.5 | 1.9 | C57BL/6NCrl M | C_25 | 92.0 | 4.0 | 4.0 | CRL:CD1(ICR) M | C_27 | 0.0 | 0.0 | 100 |
| BALB/cAnNCrl F | C_10 | 17.3 | 76.9 | 5.8 | C57BL/6NCrl F | C_02 | 50.0 | 40.7 | 9.3 | CRL:CD1(ICR) F | C_05 | 1.9 | 18.5 | 79.6 |
| BALB/cAnNCrl F | C_12 | 11.1 | 87.0 | 1.9 | C57BL/6NCrl F | C_15 | 44.4 | 13.0 | 42.6 | CRL:CD1(ICR) F | C_07 | 0.0 | 6.4 | 93.6 |
| BALB/cAnNCrl F | C_14 | 5.6 | 92.6 | 1.9 | C57BL/6NCrl F | C_18 | 61.1 | 37.0 | 1.9 | CRL:CD1(ICR) F | C_09 | 11.5 | 15.4 | 73.1 |

| Group | Cage | cl 0 (%) | cl 1 (%) | cl 2 (%) | Group | Cage | cl 0 (%) | cl 1 (%) | cl 2 (%) | Group | Cage | cl 0 (%) | cl 1 (%) | cl 2 (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| BALB/cAnNCrl F | C_29 | 9.6 | 90.4 | 0.0 | C57BL/6NCrl F | C_28 | 78.9 | 7.7 | 13.5 | CRL:CD1(ICR) F | C_30 | 0.0 | 6.3 | 93.8 |
| BALB/cAnNCrl F | C_32 | 28.9 | 71.2 | 0.0 | C57BL/6NCrl F | C_31 | 96.2 | 3.9 | 0.0 | CRL:CD1(ICR) F | C_33 | 1.9 | 1.9 | 96.2 |
| BALB/cAnNCrl F | C_35 | 32.7 | 67.3 | 0.0 | C57BL/6NCrl F | C_34 | 90.4 | 0.0 | 9.6 | CRL:CD1(ICR) F | C_36 | 5.8 | 21.2 | 73.1 |

The table shows how many times (as percentages) each cage was classified in the three clusters (cl 0, cl 1 and cl 2). On most of the days, each cage was classified to the corresponding cluster of its strain, except for cage C_08 of C57BL/6NCrl M group. CRL:CD1(ICR) cages show the overall highest percentages of being classified in their specific cluster (cl. 2).

## 1.4 Discussion

The increasing number of available mouse strains and their genetically diverse background call for a need to identify strain-specific features to better guide the appropriate choice of models. This is even more relevant when conducting experiments to compare negative controls with the transgene, when modeling certain neurological conditions, as well as in the case of metabolic and cancer diseases, neurodegenerative and aging studies, among others. In parallel with the need for accurate phenotypic characterization, the scientific community is putting great effort into developing and validating continuous automated and non-intrusive home-cage analysis systems as unbiased approaches for behavioral evaluation (19,20,21,22) with the advantage of reducing the effect of human handling and therefore improving animal welfare according to the 3Rs principles, without affecting experimental outcomes. Moreover, such technologies, allowing longitudinal observations, contribute to reducing the number of animals used per experiment or study, by enabling researchers to obtain either comparable levels of information from fewer animals or more information from the same number of animals, thereby avoiding further animal use.

The aim of this study was to characterize in depth and compare the spontaneous circadian rhythms of three commonly and widely used mouse strains, C57BL/6NCrl (inbred), BALB/cAnNCRL (inbred) and

CRL:CD1(ICR) (outbred) in biomedical research. A longitudinal analysis of the circadian activity was conducted 24/7 in group-housed mice in the DVC system for 2 months in two cohorts in late summer and early spring, to avoid seasonal effects. To our knowledge, this is the first attempt to capture the diurnal phenotypic differences of the three selected strains, achieved by introducing new circadian metrics and confirming the results with a machine learning approach, which is a useful addition to the animal behaviorist's analytical toolkit (23).

As nocturnal animals, mice are active mainly during the dark phase, when the endogenous circadian clock dictates the behavior of the animal (5). We observed that in all cages, the spontaneous locomotor activity revealed a clear rhythmicity, with the peak during the dark phase and the lowest activity during light hours (20,22,24). C57BL/6NCrl and CRL:CD1(ICR) mice displayed an increased activity before the end of the dark phase, which lasted also during the first 1–2 h of the lights-on phase, confirming that circadian rhythms are internally generated patterns of activity (25) and function as an innate clock and that their development is genetically programmed independently of the environment (26). The circadian phenotype of C57BL/6NCrl, herein used as a reference strain, matched with the description of C57BL/6J previously documented (19,20). This represents a non-obvious observation, because several gene differences, some of which may regulate circadian clock function, including *Adcy5* (which influences locomotor activity levels), *Pmch* (which mediates sleep and arousal) and *Crb1* (which controls retina photoreceptor structure), have different regulation in the two substrains (27). Furthermore, different behavioral and physiological responses to circadian disruption and wheel-running access have been demonstrated in male C57BL/6NCrl and C57BL/6J mice (28). However, against C57BL/6J, we were able to compare only the day and night activity pattern and the effect of cage change. Future experiments with the DVC system are necessary to dissect possible behavioral differences in the circadian activity of the two substrains.

C57BL/6NCrl and BALB/cAnNCrl mice showed both similarities (day and night activity levels, the first response to the lights-off phase and the last response to the lights-on phase) and differences (the first response to the lights-on phase, the last response to the lights-off phase, acrophase, RDI and the response to cage change) in their spontaneous locomotor activity, which

supports previous studies comparing phenotypic characteristics of C57BL/6NCrl mice with BALB/cAnNCrl mice in different behavioral experimental settings (29,30). Compared to C57BL/6NCrl mice, we observed that BALB/cAnNCRL mice showed a substantially delayed response to the lights-on phase and an anticipated peak toward the end of the night. Another evident difference was observed in the RDI, a digital biomarker used for phenotyping the onset and the evolution of neuromuscular diseases in murine models (6). Notably, the recorded activity of BALB/cAnNCrl mice did not reveal significant irregularity and/or disturbances in the rest/sleep behavior during light hours compared to C57BL/6NCrl mice. The more stable locomotor activity in BALB/cAnNCrl mice, in either males or females, compared to the other two strains is in line with previous reports (31) and could be ascribed to the low sociability and conspecific interaction of this strain (12,32).

CRL:CD1(ICR) mice exhibited the most clearly differentiated patterns in all metrics compared to the two inbred strains and had the highest average 24/7 activity recorded. Remarkably, only in this strain we observed sex differences, with males more active than females, although not statistically significant in all measurements. Our findings thus extend previously observed sex differences reported for this strain (33).

We also evaluated activity at three different time points, targeting cornerstones of mouse development from prepuberty to adulthood (15). Previous studies involving wheel-running activity showed that daily activity reaches a peak and plateaus at 9–10 weeks of age in mice (34). According to our data, free movement activity intensity significantly changes over the selected time points, confirming an increase in spontaneous activity and showing strain differences with a distribution pattern from CRL:CD1(ICR), the highest, to BALB/cAnNCrl, the lowest. Remarkably, BALB/cAnNCrl males and females and C57BL/6NCrl males showed a homogeneous activity pattern over the three time points, whereas C57BL/6NCrl and CRL:CD1(ICR) females showed a clear, progressive increased activity pattern. With wheel running, sex proved to be a significant factor in daily activity, with females showing higher intensity than male mice (34). Conversely, our data show on average a higher activity intensity in male BALB/cAnNCrl and CRL:CD1(ICR) mice, and only C57BL/6NCrl females displayed higher activity intensity, suggesting that evaluation of

spontaneous activity in cage locomotion provides a different perspective on activity intensity because it is a permanent and long-term parameter avoiding artefacts (35) and habituation bias (36).

Very interestingly, we confirmed our phenotypic analyses by an unsupervised machine learning approach. Each strain corresponded to a cluster, and notably the repeated and longitudinal measurements of all circadian metrics confirmed that data referring to cages housing each strain were included in the corresponding cluster, except for one C57BL/6NCrl cage that was classified in the BALB/cAnNCrl corresponding cluster, corroborating the diversity of circadian phenotype of the three strains. Interestingly, all CRL:CD1(ICR) cages have been classified in the same cluster with very high rates, compared to the inbred strains. These observations further confirm a higher similarity in the diurnal locomotor activity between the two inbred strains and the phenotypic variability of outbred strains (37).

Finally, thanks to the automated home-cage 24/7 monitoring system, which allows researchers to longitudinally monitor individual group-housed cages without adverse behavioral and physiological effects, we were able to portray key features of C57BL/6NCrl, BALB/cAnNCrl and CRL:CD1(ICR) mice, relying on their spontaneous locomotor activity, with CRL:CD1(ICR) mice more active and dynamic, C57BL/6NCrl mice more susceptible to environmental stimuli and BALB/c mice the least active strain. Overall, the systematic in-cage data recording potentially creates a large-scale and open behavioral database with a specific focus on spontaneous, unbiased locomotor activity patterns. The availability of such data from both non-genetically and genetically modified mice will allow precise comparison between strains and mutations (38), leading to more accurate understanding of deviations from baselines, pondered welfare assessment and phenotyping of genetically modified animals (39), with a further positive impact on implementation of refinements, including endpoints, increasing reproducibility and awareness in selecting appropriate models. We are confident that these phenotypic features will be helpful when selecting an appropriate model, independently also of the genetic variability of strains (inbred versus outbred), contributing thus to the effort to overcome the classical dichotomy of inbred versus outbred strains (40).

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# Chapter 2:
# Bridging the gap in the rat (*Rattus norvegicus*)

## Puberty onset curve in CD (Sprague Dawley) and Long Evans outbred male rats

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## 2.1 Introduction

Defining the precise dating of puberty is crucial for preclinical studies due to the complex neuroendocrine controlling networks action. Onset of puberty has proven to be a reference target in neurobiological studies (1), repro-toxicology essays (2, 3), endocrinology (4, 5), nutrition and development research (6) as well as a threshold to appropriately plan pre/post pubertal surgical interventions on reproductive system (vasectomy and orchiectomy). In rodent males, among the most common non-invasive methods to evaluate pubertal onset is the preputial or balano-preputial separation test (BPS) (7). By convention, such threshold is targeted on age. In this report we provide puberty onset curve for two widely used outbred rat strains, Crl:CD(SD) and Crl:LE, analyzing preputial separation in the light of two parameters: age and weight. Crl:CD(SD) is an outbred strain originated in 1925 by Robert W. Dawley from a hybrid hooded male and a female Wistar rat, and is widely used as general multipurpose model, ranging from safety and efficacy testing, ageing, nutrition, diet-induced obesity, oncology and surgical model preparation. Crl:LE is an outbred strain originated by Drs. Long and Evans in 1915 by crossing several Wistar Institute white females with a wild gray male, and is widely used as general multipurpose model, specifically in behavioral research and diet-induced obesity studies.

## 2.2 Materials and Methods

### 2.2.1 Rats

Data were collected from production colonies housed at Charles River Laboratories Italy by veterinary staff and trained animal care technicians. Animal breeding and management were performed in accordance with Corporate guidelines and AAALAC International requirements and in full compliance with Italian Legislative Decree 26/2014. Animals were group housed in open cages, in barrier room under SPF conditions and controlled environmental parameters, with T range of 22°±2°, RH set point at 50% ±20% and light/dark cycle of 12:12 hours. Rats received ad libitum a commercial standard rodent diet (SDS VRF1). Crl:CD(SD) colony is bred under International genetic stability standard.
BPS test was performed on male rats from 4 to 9 weeks of age:

- 45 animals/week for Crl:CD(SD) population (total 270);
- 20 animals/week for Crl:LE rats population (total 120).

The sample size was calculated with an a priori power analysis ($\alpha$= 0.05; 1-$\beta$= 0.80) taking into consideration the numerosity of males of the two production colonies (CRL:CD (SD) and CRL:LE) available for each time point analyzed.

The assessment was performed as a nonblinded population screening involving animals consolidated from multiple litters, in two different sessions and simultaneously covering different ages, meaning that the assessment was not repeated on the same animals at progressive time points. Animals were also screened for clinical and phenotypical integrity; one animal within the 4 weeks old CRL:LE cohort was discarded due to congenital hindlimb malformation.

The correspondence between weeks and days of age used by the breeder to consolidate animals is summarized in Table I. Statistical analyses were performed by using GraphPad Prism 9. Wilcoxon signed-rank test and Pearson correlation were applied for assessing how preputial separation correlated with age, weight, and descended testes in the two strains.

### 2.2.2 Balano-preputial separation test

The assessment was performed accordingly to Korenbrot et al (7) and Lewis et al (8) by restraining the animals in supine position, and manually retracting the prepuce applying a gentle pressure to the prepuce sides, downward. The anatomical structure identified were – from top to bottom – the urinary orifice, the glans and prepuce.

Immature rats, during unpubertal phase, show a flattened surface at the top of the penis, with urinary orifice visible at the centre of the structure and the prepuce fully covering, the glans (Fig.1A-A). Trying to retract the prepuce may enlarge the urinary orifice, without significantly exposing the glans. In prepubertal rats, the same retraction can partially exposing the glans, without allowing a full detachment of the prepuce from the structure underneath (Fig.1A-B). BPS test is considered positive only upon full retraction of prepuce, to expose the entire glans surface (Fig.1A-C).

## 2.3 Results

Animals of both strains were negative to the BPS, schematically represented in Fig. 1A, at week 4 and 5: in the Crl:CD(SD) population, 90% of males gained the puberty at week 6, and 100% in the following weeks (Fig. 1B); in CRL:LE the puberty onset appeared more gradually, with 75% of males positive to test at week 6, 90% at week 7 and 100% from week 8 (Fig. 1B) (Table I). Males at 9 weeks old were checked to validate the data recorded in 8 weeks old population. We observed slope in the weight of both strains ($p>0.005$), with a discrepancy of 203 (Crl:CD (SD) vs 199 (CRL:LE), while no statistically significant differences were assessed with regards to the age of preputial separation ($p<0.005$) (discrepancy 3 vs 825). In both strains, a positive correlation between preputial separation and age (r(Crl:CD(SD)BPS:age)=0.89; r(Crl:LE BPS:age)=0.94) as well as between preputial separation and weight (r(Crl:CD(SD)BPS:weight)=0.84; r(Crl:LE BPS:weight)=0.90) was calculated. Remarkably, descended testes were 100% visible in Crl:CD(SD) from week 4 onward, while 75% in Crl:LE from week 4 (Fig. 1C).

| Week | Day – Min | Day – Max | % BPS Crl:CD(SD) | % BPS Crl:LE | % Descended testes Crl:CD(SD) | % Descended testes Crl:LE |
|---|---|---|---|---|---|---|
| 4 | 28 | 34 | 0% | 0% | 100% | 75% |
| 5 | 35 | 41 | 0% | 0% | 100% | 100% |
| 6 | 42 | 48 | 90% | 75% | 100% | 100% |
| 7 | 49 | 55 | 97,5% | 90% | 100% | 100% |
| 8 | 56 | 62 | 100% | 100% | 100% | 100% |
| 9 | 63 | 69 | 100% | 100% | 100% | 100% |

Table I: Summary of BPS screening in the two outbred strains populations.

## 2.4 Discussion

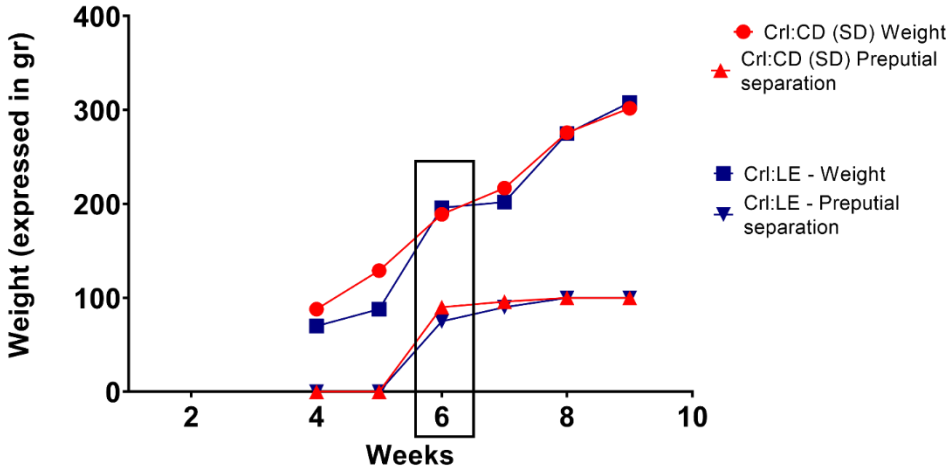
BPS is an androgen-dependent event necessary for complete copulatory behaviour and is therefore a commonly used index of pubertal development (7). Sexual maturity does not mark the beginning of adulthood, rather denotes the beginning of adolescence as in humans. Rats progress through a

period of adolescence characterized by behaviors such as increased risk-taking and social play, which extend well beyond the pubertal period through the transition to adulthood (9). Therefore, defining the puberty is a fundamental tool when using rats as translational models. The classical studies of Kennedy and Mitra (10) established that the weight is well associated with the timing of sexual maturation and with the concept that the body weight of an animal can be considered an approximate marker of its age. Here, we report that almost 100% of sexual maturity is reached by animals with a weight above 200gr in both strains, at an average age of 6 weeks in the case of Crl:CD(SD) and 7 weeks in Crl:LE. The work from CRL US colleagues (8) highlighted a few crucial aspects of the BPS test. Particularly a) the greater accuracy of the assessment when performed on more than 1pup/sex/litter and b) the risk for inter laboratory differences in results when a non-standardised method for the assessment of BPS is applied (or a locally standardized, but not generally acknowledged one).
We applied the same BPS test principles described in the paper – as per CRL common practice – and our data for Crl:CD(SD) are comparable to the ones presented in 2001 even though a granular comparison is not possible, as their assessment was made on a daily basis, our assessment on a week range basis. Consistently with their work, we observed no positivity for BPS under post-natal day 39 and that BPS positivity window widens when multiple animals per litter are assessed compared to single animal/sex/litter assessment range. The timing observed in Crl:LE is in line with observation of an average age of 50 days after birth (P50) made by Long and Evans in their pioneering work. Remarkably, we have also analyzed animals weights, positively consistent with official models growth curves, and observed a higher growth variability in Crl:LE compared to Crl:CD(SD) and reinforcing the fact that weight is a relevant biomarker in defining puberty onset.
We also evaluated descended testes, an indicator of incipient puberty, being this phase predominantly under the androgens control (11). Interestingly, we observed that testicle descent is more gradual in Crl:LE, with a progression from 75% (4 weeks old) and 100% at week 5. While in CD the passage is precocious with 100% at 4 weeks old. As testicles descent is visible in 100% of the male population of both strains long beforehand than BPS - week 4 versus week 8 in Crl:CD(SD) and week 5 versus week 8 in Crl:LE rats, respectively - this factor should not be considered as valuable biomarker of puberty onset. Overall, these data confirm that the timing of full sexual

maturity varies between strains and suggest that weight, not only age, should be considered a biomarker of puberty onset in these two outbred strains.

(A)


(B)



(C)

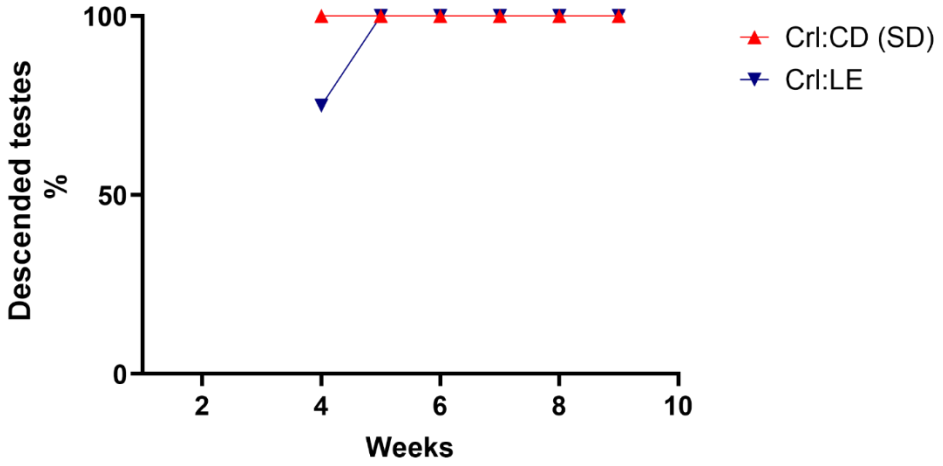



**Fig. 1:** (A) Schematic drawing of BPS (rearranged by Lewis et al. (8) and Yamasaki et al. (12) representing A unpuberal, B prepuberal and C puberal rat with a different color code identifying the anatomical structures:
Lilac: external urethral orifice
Light pink: glans
Dark pink: prepuce
(B) Comparison of growth curves and puberty onset (based on BPS) between the two rat strains by applying Wilcoxon signed rank, demonstrating that the minimum weight for positive BPS test is above 200gr in both strains, corresponding to the week 6 of age. Significant differences ($p>0.05$) were observed when comparing the weight at different weeks of age in both strains.
(C) Average of descended testes in Crl:CD(SD) and Crl:LE and the correlation with age (weeks).

# Bibliography of Chapter 2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# Conclusions

The importance of extensively characterising under the phenotypical perspective stock, non-GM laboratory rodents is crucial to securing cornerstone of current high-quality *in vivo* research such as model validity, data reproducibility, and welfare assessment.
On top of this remarkable advantages, which alone should advocate for promoting and progressing phenotyping pipelines of such models, there are two further considerations that are worth to be highlighted.

The first one pertains to the phenotypical assessment of spontaneous motor activity in inbred and outbred mice. This specific assessment was possible thank to a relatively novel, and still non widely diffused, cage type. Digitally ventilated cages provide the ideal environment to allow for continuous (H 24), long-termed, undisturbed recording of spontaneous, home-cage activities. This way of assessing animals in their cage provides, especially under the behavioural perspective, a clear picture of the real, spontaneous mouse behaviour, in an animal facility setting, with no bias associated with external interference. Furthermore, in the light of the 3Rs principle, cage digitalisation can open new, revolutionary perspective to implement each of the three Rs. Being able to contantly monitor the cage environment and the status of the animals inside can significantly improve refinement, by early detecting any deviation from baseline values in the mouse activity, contributing - and in this sense baseline data from stock, background strains can support – to identify early endpoint and reduce the burden. What is even more interesting though, is the possibility to implement reduction and potentially replacement. The large amount of data recorded remains undefinitely available for the user, allowing to reassess the same data retrospectively, in the light of potential later questions arising. Questions might come from peer reviewers, as well as from research group internal discussion. In both cases, new metrics could allow to extrapolate new scientific data without repeating any experiment, promoting reduction, and potentially replacement. This data Recycling approach – a further R - can, in principle, allow to save animals, money and time.

A second and final consideration arises from the phenotyping of BPS in male rats. Such detailed assessment was not available anywhere for Long

Evans rats, thus we provided the first published puberty onset curve for this strain, updating original pioneeristic data from Dr Long and Dr Evans.
CD rats were, conversely, more charachterised with datasets available from different breeding and experimental sites. Interestingly, we confirmed very similar results to the ones obtained by colleagues working on the same strain. This new assessment can be positively seen as a quality control screening, showing a remarkable integrity of this outbred strains, proving how genetic stability programs worldwide, along with colony maintenance under standardised environmental conditions, can secure the availability of homogeneous models, modulating the interaction of genotype and environment – wich includes environmental paramethers as well as food, bedding, microbiological status, etc. – standardising phenotypic outcomes.