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The critical role of vestibular graviception during cognitivo-motor development

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List of non-standard abbreviations

Het: Head Tilted PND: Postnatal days

USVs: Ultrasonic vocalizations

Declarations of interest:

None

Abstract

Earth's gravity acts both as a mechanical stimulus on the body and as a sensory stimulus to the vestibular organ, which is transmitted into the brain. The vestibular system has been recently highlighted as the cornerstone of the multisensory cortex, of the dorsal hippocampus related to spatial cognition. Consequently, we have hypothesized that the vestibular sensory perception of gravity by the otoliths might also play a crucial role during the first stages of development in both sensorimotor and cognitive functions and the construction and perception of the 'self' and related functions of orientation and navigation. We have investigated an original mouse model (Head Tilted mice, B6Ei.GL-*Nox3*^{het}/J) suffering from a selective congenital absence of vestibular otolithic gravisensors. We report that mouse pups suffered from a delay in the acquisition of sensorimotor reflexes, spatial olfactory guidance, path integration, and ultrasonic communication, while maternal care remained normal. We demonstrate that development has a critical period dependent on the vestibular otolithic sensory perception of gravity, probably temporally between the somesthetic and visual critical periods. The symptoms expressed by the congenital otolithic-deficient mice are similar to validated mouse models of autism and highlight the significance of vestibular graviception in the pathophysiology of development.

Keywords: otolith, early development, B6Ei.GL-*Nox3*^{het}/J, gravity, vestibular system, autism

Highlights

- Earth's gravity influences early cognitive-motor development through vestibular sensory graviception.
- Development has a critical period dependent on the vestibular sensory perception of gravity, probably temporally between the somesthetic and visual critical periods.
- Vestibular sensory perception of gravity might play a crucial role during the first stages of development in both sensorimotor and cognitive functions and the construction and perception of the 'self' and related functions of orientation and navigation.
- Congenital otolithic-deficient mice exhibit similar symptoms as validated mouse models of autism and highlight the significance of vestibular otolithic graviception in the pathophysiology of development.

1. Introduction

The vestibular organs located within the inner ear have been explored during the last decades as a sensory system essential for maintaining gaze and postural stabilization based on encoding, in three dimensions, the body's position and motion with respect to earth gravity [1]. Numerous investigations have demonstrated their crucial role at the brainstem level and have led to the recognition of the vestibular organs as ensuring the basic function of balance [1]. However, the vestibular system is an ancestral sensory organ originating from the protochordates more than 500 million years ago [2], which then links the brain to gravitational force well beyond the brainstem reflex networks. Therefore, this indicates that Earth's gravity comprises two main components acting on any living species, namely, a mechanical loading factor which affects the physiology, and as a form of gravitational sensory stimulation which affects the whole brain. Remarkably, the extent of vestibular function was discovered during the last decade, in particular, its crucial involvement in many high-level brain functions: (i) spatial cognition (including self-perception, sense of direction, verticality perception, internal representation of gravity, orientation, navigation and spatial memory, and mental calculation) [3–10]; (ii) social cognition [11]; and (iii) emotional regulation [12,13]. Recent studies have highlighted numerous direct and indirect cortical projections of vestibular inputs to numerous brain areas [14] including the opercular insular/retro-insular cortex and surrounding regions [3,5,15–17]. These regions encode an absolute geocentric reference of living species, up to humans, devoted to the crucial role of gravity-related verticality perception [18]. This idiothetic referential frame is supplemented by the overlap of egocentric and allocentric frames provided by the somatosensory and visual systems, respectively, and altogether represent the multisensory integration processes [3,19]. All of these highly complex cortical functions, based at least partly on the vestibular perception of Earth's gravity, allow us to appreciate their importance for the individual in the construction and

perception of the ‘self’, in its interaction with the surrounding world and with others. And yet, adult patients suffering from vestibular deficits as well as patients with stroke and epilepsy occurring within the parieto-temporal junction and surrounded regions, report a large number of cognitive (e.g., [20,21]) and neuro-psychiatric symptoms [12]. The vestibular projections
30 of vestibular gravitational information towards areas of consciousness, can result in feelings of depersonalization, projection out of the body, and loss of spatial landmark [12]. As mentioned above, all these motor and mental disorders were investigated at adulthood and resulted in a deterioration of a functional system. One can easily envisage that early alteration of the vestibular system (during pre- or post-natal development) could deeply affect the development
35 of individuals, particularly considering its prenatal maturation [2,22].

The vestibular organ is mature at 4 months of pregnancy in humans and functional from birth [2,22]. Some authors have suggested indeed that locomotor and postural development are influenced by the effect of gravity [23–25], which is reinforced by studies conducted in hypergravity environments [26]. However, the respective roles of the mechanical and the
40 sensory components of gravity on the species studied remain poorly defined [27]. The idea that vestibular information has an influence on sensory-motor development was recently raised by Wiener et al. [28] from a few children’ data with complete and bilateral vestibular loss, who showed a delay in motor milestones [28], and poorly dapted strategies [29]. They could also have some difficulties in high-level cognitive functions, peer interactions and communication,
45 as reported in some case forms of Autism Spectrum Disorders (ASD) [30]. This has become all the more meaningful since we know that the vestibular system participates in self-perception in the adult, in our body schema construction and our relationship with the environment and others, as mentioned above.

Recent studies have provided direct evidence that the otoliths, which sense linear
50 acceleration, including linear gravitational acceleration, may contribute to spatial memory.

First, it has been reported that otolith-deficient mice exhibit deficits in spatial memory tasks [31–35]. Second, otolith deficiency has been reported to result in a degradation of both thalamic head direction cell and hippocampal place cell responses [36,37]. Third, in epidemiological studies, cVEMP and oVEMP function, which provide an index of saccular and utricular function, respectively, have been demonstrated to be predictors of cognitive dysfunction as a result of aging (see [38] for a review). Electrical stimulation of the otolith receptors alone in guinea pigs and rats has also been reported to evoke widespread responses throughout the hippocampus, which is well known to be critical for spatial memory [39,40]. The otoliths are not the only part of the vestibular system which can sense gravity of course, since the vertical canals can contribute to this; however, they are a major contributor to the vestibular sense of gravity (e.g., [41]).

Therefore, to further explore the effects of vestibular graviception on cognitive function and in global self-construction, and to examine the existence of a gravity-related vestibular sensory window during early development, we have evaluated the behavioural consequences of a selective otolith-deficient mouse model of Head Tilted mice (*Het*) [42], i.e. devoid of vestibular graviception attributable to the otoliths, and we have looked for a potential maternal care effect, and visuo-vestibular and somesthetic-vestibular interactions. Our hypothesis was that mice devoid of otoliths would develop abnormally in terms of sensorimotor and spatial memory function.

2. Methods

2.1. General procedures:

Experiments were carried out in accordance with the European Communities Council
75 Directive 86/6609/EEC as well as French legislation. Mice were housed under constant
temperature (21 ± 1 °C) and humidity ($55\pm 10\%$) conditions. Mice were kept under a 12:12 h
normal light: dark cycle (lights on at 8:00 AM, progressively increasing to 400 Lux) with food
and water available *ad libitum*. General parameters like weight and welfare were regularly
evaluated. The B6Ei.GL-Nox3het/J mice present a mutation on chromosome 17 inhibiting
80 NADPH oxidase 3 gene expression [43] and this leads, in the recessive condition (Het -/-), to
a specific and complete lack of saccular and utricular otoliths within the inner ear [42],
making the mice unable to detect gravity with those organs. Two couples of homozygote mice
B6Ei.GL- Nox3het/J (Jackson Laboratory) were cross-bred and the genotype of each animal of
the colony was checked by polymerase chain reaction (PCR). All tests were individually
85 and randomly performed in 27 *Het -/-* mice and 36 *Het +/-* mice serving as control in blind
conditions (CURB, Caen, France, animal ethical agreement received by the CENOXEMA
0412-01), the phenotype and genotype being determined by PCR investigation after the
evaluations. *Het-/-* mice were investigated from birth and compared to heterozygote normal
littermates (*het-/+*) serving as controls (n = 27 *Het-/-* versus 36 *Het-/+*). All mice were
90 tested in all of the tests described hereafter. The sensorimotor development of newborn pups
was tested from postnatal days (PND) 2 to 10 with a panel of behavioural tasks (righting
reflex, cliff avoidance, negative geotaxis, whiskers' stimulation response and grasp reflex
tests) selected for their ability to measure classic reflex acquisition [44]. Because the *Het*
strain (type +/-) presented similar developmental milestones to mouse strains free of genetic
95 manipulation (*C57BL6*), we preferred to compare *Het-/-* (recessive NADPH oxidase 3
inhibition) with *Het-/+* serving as control mice, avoiding development-related strain effects

and selectively focusing on the role of the sensory otolith component. The time frame of the scheduled tests was similar for each animal and chosen in order to minimize the influence of the experimenter's manipulations and possible test interference. If no differences were detected in comparisons of male and female mice in each test, data were collapsed across sex. The technical details of each of the test conditions (age, method, size of groups) are detailed below.

2.2. *Sensorimotor developmental milestones tests*

Young mice were individually monitored daily during the pre-weaning period for the appearance of developmental landmarks. Each day of the testing sessions, the pups were weighed (to the nearest 0.01g), the global development was observed (head support, pelvis and shoulder support, onset of motion, limb coordination...), but not quantified, in order to detect and exclude animals with potential malformations. The age for the eyes' opening was noted for each pup. The pups were tested from 2 to 10 postnatal days (PND) as detailed below and weight gain was considered as a sign of growth of the pups and indirectly as an indication of appropriate maternal feeding. The development of new-born mice was quantified from a panel of five behavioural tasks (righting reflex test, cliff drop aversion test, negative geotaxis test, whisker stimulation response evaluation, forelimb grasping reflex test) based on the work of Fox [44] and chosen to evaluate the motor and cognitive development in rodents, especially focused on the cerebellum, sensory systems and vestibular system maturation. These tests measure the typical motor development of the pup involving body righting mechanisms and balance, notably through the perception of verticality and body perception, body orientation, and individual orientation as a result of vestibular information integration.

The righting reflex is a primitive reflex of the pups which allows them to restore a normal orientation of the body i.e., the four limbs on the ground, when a pup is on its back.

This reflex was evaluated by placing the young mouse on its back and by measuring the time required by the animal to turn over to return to a normal position with all four limbs on the ground. This task evaluates upright position and verticality perception from vestibular information.

The negative geotaxis test is based on the natural aversion of mice to have their head and forelimbs in a ventral position compared to the pelvis and hindlimbs and the observation of a righting reflex to restore them to a safe position. In this test, the mouse was placed on a sloping surface/inclined plane at 45° in an upright position with its head tilted down and the time required by the animal to turn around, crawl up the slope and come back to an up-right head position, was noted.

The cliff drop aversion test represents a reflex to avoid a dangerous situation including an empty space and falling. To measure this reflex, the pup was placed on the edge of a cliff (i.e., a turned-over cage) with the tip of its forelimbs and nose over this edge and the time required by the animal to turn and crawl away from the cliff drop in order to escape the void, was measured.

The whiskers' placing response test consists of bilaterally stimulating the pup's face, by placing a solid object (a paintbrush was chosen here) in contact with vibrissae and measuring the reaction of the mouse, i.e., a raising of the head and an extension of the forelimbs in order to grasp the object. The duration to succeed in each test was measured (60s max, success =1, failure = 0).

The forelimb stick grasping reflex consists of bilaterally stimulating the pup's forelimbs, by approaching and putting a solid object (a toothpick was chosen here) in contact with the palmar face of the forelimbs and measuring the reaction of the mouse, i.e., the ability of pups to grasp the object. The duration to succeed in each test was measured (60s max, success =1, failure = 0).

2.3. *Ultrasonic vocalization (USV)*

During the neonatal stage, the ability of pups to respond to maternal separation and
150 isolation by the temporary emission of ultrasonic vocalizations (USVs), constitutes a survival
instinct and an indicator of normal development [45]. The recording and quantification of USV
emitted by pups following maternal separation and cold stress is useful to investigate
neurodevelopmental disorders in rodents [46]. Here the earlier USVs of *Het* pups (n = 10 litters,
n = 63 pups) were recorded at baseline using SONOTRACK 2.4.0 (Metris B.V., Netherlands)
155 at PNDs 2, 4, 6, 8 and 10 during a 5-min session of dam-littermate separation. On each day of
testing, each pup was removed from the home cage and placed into an empty plastic container
(length 25 cm, width 8 cm, height 10 cm) located inside a sound-attenuating and temperature-
controlled box, and assessed for USVs during a 5-min session. At the end of the recording
session, each pup was replaced in the home cage with dam and littermates. The average success
160 to generate USV emissions, the average of the number of calls per min, the average duration of
calls (ms) and the average frequency of the calls (kHz), were measured and analysed.

2.4. *Home retrieval test (PND 9)*

This test was adapted from the Homing test reported by Scattoni et al. [47] and is based
165 on the natural tendency of blind pups to return to the safety of their nest due to olfactory cues,
i.e., when motor development allows short distance travelling. On PND 9, individual pups were
transferred to a Plexiglas cage (36 cm x 22.5 cm, walls 10 cm high). Wood shavings removed
from the home cage were evenly spread on one third of the surface of the cage and cotton wool
from the nest was dispersed on this dirty litter (12 cm x 22.5 cm, nest area). The rest of the cage
170 was covered with clean bedding (two thirds of the cage, 24 cm x 22.5 cm, clean neutral area).
The pup was placed in the middle of the surface of the Plexiglas cage, i.e., in the clean neutral

area at 18 cm from the cage edge, facing the edge for 3 min. Homing performance was scored according to 4 criteria: (a) latency to reach the area containing nest litter; (b) time spent in the area containing nesting litter; (c) percentage of success reaching the nest area; and (d) the average latency to start to move. Furthermore, the locomotor activity of the pups was assessed by qualitative observation.

2.5. *Maternal care and parental behaviour*

Maternal behaviour requires continuous observation of the dam during the first post-natal period. The behaviour of the dam before each test and during the progress of the experiments was monitored as well, but not quantified, in order to detect potential defects inducing biases in the experiments. Pups are extremely vulnerable to predator attack and cold temperature and one of the many roles of the dam is to ensure that pups are in a safe condition, i.e., inside the nest. The retrieval assay generates an instinctive maternal behaviour and provides a measure of the ability of the dam to find pups moved outside the nest. Two tests were performed here in order to evaluate maternal basic instincts: the all pup retrieval test (2 sessions) and the one pup retrieval test (5 sessions) in both groups of dams (n = 5 dam *Het* $-/-$ versus 5 dam *Het* $-/+$).

The all pup retrieval assay (PND 4 & PND 6) was performed at PND 4 and PND 6 in female mice and the littermate's home cage during a 10-min period. To perform the test, the dam was briefly separated from its pups for 30 s in order to generate stress and removed from its home cage while the pups were removed from the nest and placed together in the opposite corner of their cage, always at the same distance from the nest. The dam was then reintroduced into her cage facing the wall and in the empty nest. The behaviour of the dam was observed during a 10-min period and the latency to retrieve the first pup and to take it back to the nest was measured.

The one pup retrieval assay (PND 2 to 10) was performed each day of the testing sessions in female mice and the littermate's home cage during a 3-min period of induced stress. To perform the test, the dam was left in the home cage with her pups while the
200 experimenter removed one pup, randomly chosen, to the nest and placed it in the opposite corner of the cage, always at the same distance from the nest. The latency to retrieve the isolated pup and to take it back to the nest was measured. The dam had 3 min to perform the test and if the dam failed to place the pup inside the nest after the 3 min session test, the pup was placed inside the nest by the experimenter and the test was considered as a failure.

205

2.6. Statistical analysis

All statistical analyses were carried out using the software R 3.3.1 [48]. First, continuous, numeric responses were analysed using linear mixed models [49] and the R package nlme [50]. Second, binary response variables were analysed using generalized linear
210 mixed effects models [51] and the R package lme4 [52]. Third, ordinal responses or numerical responses with only few possible values were analysed using cumulative link mixed models [52] and the R package ordinal [53]. In the case of non-normally distributed data, Mann-Whitney U tests were used, except for longitudinal data where a non-parametric analysis was implemented in the R package nparLD [54]. Frequency data were analysed using a Chi-squared
215 test [54]. The level of statistical significance for all analysis was set at $p \leq 0.05$.

3. Results

3.1. General developmental observations

220 The observation of the age of eye opening in pup mice revealed no significant genotype effect (Fig. 1, p -value > 0.05 , ns) while the age of eye opening was about 14 days in both *Het*^{-/-} and *Het*^{-/+} groups i.e., within the time window described in the literature for C57BL6 mice.

No differences were detected between males and females; therefore, data were collapsed across sex (p -value > 0.05).

225

3.2. Sensory and motor skills development assessment

Weight measurements in the pup mice ($n=27$ *Het*^{-/-} vs $n=36$ *Het*^{-/+}) revealed no group effect: the body weight (Fig. 2A) was similar between *Het*^{-/-} and *Het*^{-/+} control pups (Mann-Whitney test, p -value > 0.05 , ns), showing evidence of good physiological growth provided by appropriate maternal feeding. The performance of mice for the grasping reflex was also similar
230 in both *Het*^{-/-} mice and controls (data not shown, 100% success for all animals).

The progression of the global developmental score (calculated from all rated tasks: righting reflex, cliff drop aversion test, negative geotaxis test and whisker stimulation response), requiring vestibulo-somesthetic and cerebellar integration, was lower in otolith-deficient mice (*Het*^{-/-}) compared to control (*Het*^{-/+}) mice (Fig. 2B); *Het*^{-/+} mice showed a positive age trend (0.242 ± 0.023 , p -value ≤ 0.001), whereas the age trend in *Het*^{-/-} was significantly weaker (0.099 ± 0.0307 , p -value ≤ 0.001) from PND2 to PND10, with a difference between PND6 and PND10 (PND6-PND8 p -value ≤ 0.001 ; PND10 p -value ≤ 0.01).
235

240 The developmental tests requiring perception of body orientation in space (righting reflex, cliff drop aversion and negative geotaxis test, Fig. 2C, 2D and 2E, respectively) revealed a poorer performance in *Het*^{-/-} compared to *Het*^{-/+} mice (righting reflex and cliff drop

aversion test, p -value ≤ 0.001 ; negative geotaxis test, p -value ≤ 0.01). In the righting reflex (Fig. 2C), both groups improved day after day (*Het*^{-/+}, 0.597 ± 0.081 , p -value ≤ 0.001 , *Het*^{-/-}, 1.402 ± 0.422 , p -value ≤ 0.001), with a significant genotype effect (p -value ≤ 0.01). A pseudo post-hoc analysis carried out at each age via a Chi-squared test revealed a significant difference from PND4 to PND6 (unadjusted p -value ≤ 0.05). Analysis of the cliff drop aversion test (Fig. 2D) revealed poorer performance in the *Het*^{-/-} group compared to *Het*^{-/+}: *Het*^{-/+} mice showed a positive age trend (0.4043 ± 0.133 , p -value ≤ 0.01), while the age trend in *Het*^{-/-} mice showed a significantly negative progression (-0.1573 ± 0.150 , p -value ≤ 0.001). The pseudo post-hoc analysis carried out at each age via a Chi-square test revealed a significant difference from PND6 to PND10 (PND6, unadjusted p -value ≤ 0.05 ; PND8-PND10 unadjusted p -value ≤ 0.01 vs PND8-PND10 adjusted p -value ≤ 0.05).

The negative geotaxis test analysis (Fig.2E) revealed poorer performance in the *Het*^{-/-} compared to the *Het*^{-/+} group: The *Het*^{-/+} group showed a positive age trend (0.834 ± 0.133 , p -value ≤ 0.001) while the age trend in *Het*^{-/-} mice showed a significantly weaker progression (0.386 ± 0.156 , p -value ≤ 0.01). A pseudo post-hoc analysis carried out at each age via a Chi-square test revealed that the difference was observed from PND6 to PND10 (adjusted p -value ≤ 0.001). Interestingly, *Het*^{-/-} mice presented a greater response to whisker stimulation during the early stages of development compared to controls (p -value ≤ 0.05 , Fig. 2F), particularly at an early stage at PND2 (pseudo post-hoc, unadjusted p -value ≤ 0.05) and at PND6, but this difference was only marginally supported by statistical analyses at age PND6 (p -value ≤ 0.05).

265 3.3. *Spatial orientation with olfactory guidance*

Spatial orientation with olfactory guidance in pups, tested by observing the strong natural tendency of the immature and blind pup to find its way back to the nest, was impaired

in *Het*^{-/-}-pups. *Het*^{-/-} pups took significantly longer to reach the nest area (“Latency to reach the nest”, Mann-Whitney test, $p \leq 0.01$, Fig. 3A) and spent less time in the nest area than controls (Mann-Whitney test, p -value ≤ 0.01 , Fig. 3B) at PND9. The otolith-deficient *Het*^{-/-} pups showed a lower percentage of success reaching the nest area compared to *Het*^{-/+} mice (Chi²-tests for equality of proportions, p -value ≤ 0.01 , Fig. 3C), while the latency period before the first movement reaction in pups remained similar in both groups (Mann-Whitney test, p -value > 0.05 , ns, Fig.3D). Two aberrant values were excluded from analysis in the Home retrieval test performed at PND9 (n=25 *Het*^{-/-} versus 36 *Het*^{-/+}).

3.4. Ultrasonic vocalizations (USV)

The ultrasonic vocalizations were altered in *Het*^{-/-} mice. The change in probability of success in USV emission in *Het*^{-/-} pups was different compared to controls: while *Het*^{-/+} mice showed a negative age trend (-0.269 ± 0.112 , p -value ≤ 0.05), the age trend in *Het*^{-/-} mice showed a significantly different positive progression (0.28 ± 0.144 , p -value ≤ 0.001 , Fig. 4A). This led to lower values for the average success for the USV emissions of *Het*^{-/-} mice at PND2 (p -value ≤ 0.05) and higher values at PND10 (p -value ≤ 0.01) compared to controls. However, the number of vocalizations emitted per min, restricted to animals emitting USVs, was impaired in *Het*^{-/-} mice (p -value ≤ 0.05 , Fig. 4B), while both the average duration of calls and the average frequency of calls showed a statistically significant age effect (p -value ≤ 0.001 ; Fig. 4C) in both groups, but no significant genotype effect (p -value > 0.05 , ns, Fig. 5d) from PND2 to 10.

3.5. Maternal care and parental behaviour

The estimation of care for *Het*^{-/-} and control dams towards their pups was similar (n=5 dam *Het*^{-/-} versus n=5 dam *Het*^{-/+}). The first test (one pup retrieval test) analysis revealed no

significant genotype effect at each day when the test was performed, of the ability of dams to find and to put the isolated pup in the nest with the littermates (p-value ≤ 0.05 ; Fig. 5A and 5B).

295 The results obtained in the second test (all pups retrieval test) analysis revealed no genotype effect of the ability of dams to find and to put the isolated pup in the nest with the littermates at PND6 and 8 (p-value > 0.05 ; Fig. 6A and 6B).

4. Discussion

300 The absence of difference in body weight kinetics between *Het*^{-/-} and *Het*^{-/+} control pups revealed parallel evidence of good physiological growth provided by appropriate maternal feeding. The lowest level of archaic sensorimotor reflex, devoid of vestibular influence (the primitive grasping reflex), was similar in both *Het*^{-/-} mice and controls, suggesting that a congenital lack of otoliths did not influence basic sensorimotor loop implementation during
305 gestation. In our study, the progression of the global developmental score requiring vestibulo-somesthetic and cerebellar integration was decreased in otolith-deficient mice (*Het*^{-/-}) compared to control (*Het*^{-/+}) mice. *Het*^{-/+} mice showed a positive age trend whereas the age trend in *Het*^{-/-} mice was significantly weaker during the early post-natal developmental period from PND2 to PND10. The maximum difference between PND6 and PND10 confirmed the
310 existence of a sensitive developmental period for gravity information. Some studies with animal models of a modified gravity environment corroborate such an hypothesis (see [55] for a review). However, these latter studies where young rats were transiently exposed to altered gravity conditions (hyper- or microgravity) revealed temporary impairment and quick recovery when they were replaced in 1g normal conditions (see [55] for a review). The developmental
315 tests requiring perception of body orientation in space (righting reflex, cliff drop aversion and negative geotaxis test) revealed poorer performance in *Het*^{-/-} compared to *Het*^{-/+} mice. The results of the *Het*^{-/-} mice, offering a sustainable inhibition of the vestibular perception of

gravity by the otoliths while preserving its direct mechanical stimulus, suggest that a large part of the delay in sensorimotor development is related to the vestibular gravisensors, as previously reported in studies performed in microgravity or hypergravity conditions (see [55] for a review). Obviously, the vertical canals also make a contribution to the perception of gravity (e.g., [41]); however, our results suggest this is not sufficient to compensate for the loss of the otolithic component of graviception.

We are confident that the behavioural effects observed in this study are a consequence of *development* without functional otoliths rather than simply reflecting the effects of the loss of otoliths in general. First, the heterozygous *tilted* mice did not exhibit the behavioural abnormalities of the homozygous *tilted* mice during the equivalent developmental period. Although selective otolith lesions have never been achieved in experimental behavioural studies in adult animals due to the surgical difficulty of lesioning them alone, without affecting the semi-circular canals, a large literature has demonstrated that complete vestibular lesions result in spatial memory deficits in adult rats but without the other sensorimotor changes observed here (see [10] for a review. It is very likely that the lack of otolith function in the *tilted* mice was responsible for the developmental sensorimotor function and spatial memory deficits observed in this study. However, we cannot selectively lesion the otoliths in either adult mice nor young and new-born mice in order to compare them with *tilted* mice due to the surgical limitations or limitations of chemical injections; and even if we could do this the comparison would not be valid because the *tilted* mice do not have surgical lesions of the otoliths. Essentially, it is impossible to compare the genetic cause of the loss of the otoliths in *tilted* mice to any adult, unless the mice survived into adulthood; however, this would not control for the developmental effects of the genetic defect.

Spatial orientation with olfactory guidance in pups, was impaired in *Het-/-* pups at PND 9, suggesting a vestibular otolithic contribution to spatial orientation. This also suggests that

the otolithic inputs are involved early in motion planning to reach a goal. Interestingly, we previously reported lasting spatial navigation and spatial memory impairments in *Het*^{-/-} mice and mice with otolith deficiency in adulthood [31,35]. Therefore, one can speculate that the spatial disorientation observed at PND 9 is not compensated by either the visual (allocentric frame) system or the somesthetic system (egocentric frame) at the adult stage, reinforcing the hypothesis that developmental sensory disorders influence long-term spatial cognitive abilities. We may suggest that at the adult stage, alteration might also be due to disorders in building cognitive maps.

The existence of sensory-related critical periods throughout development, i.e. time windows of higher vulnerability to environmental changes, has previously been demonstrated with visual system ontogenesis [56]. Early visual deprivation influences the types of spatial representation and strategies of navigation (response strategy versus spatial strategy) [57]. Here, we showed that development in mice has an early sensory component dependent on the vestibular perception of Earth's gravity, probably combined with its mechanical component.

Similarly, vestibulo-deficient infants suffer from motor impairments [28] characterized by delayed walking, higher risk of falling, and long-term postural instability), varying with age and walking-dependency. Additionally, spatial cognitive processes could be affected, with difficulty in detecting one's own movement in space and in estimating distance of motion, probably through path integration impairment [28]. However, this deficiency remains to be investigated during childhood. Our present observations in mice highlight the possible gravisensory role of vestibular pathology during human development.

The ultrasonic communication to respond to maternal separation was altered in *Het*^{-/-} mice, since the number of emissions was decreased or increased. Unlike control mice, maternal separation and a cold stimulus did not always cause USV emissions at PND2 in *Het*^{-/-} mice, but their higher rate of response at PND10 revealed a temporal shift in the optimal response

rate in this test, highlighting a potential delay in the establishment of mother-pup communication. The delay in USV emissions in *Het*^{-/-} mice compared to controls may be the
370 result of: (i) a mechanical inability to produce USVs at an early age; (ii) a different level of sensitivity to thermal variations; (iii) a different stress response and management due to maternal separation; or (iv) an underlying cause of a social communication deficit and emotional reactivity. *Het*^{-/-} pups seemed to exhibit a delay in the emissions, with no changes in the profile of USVs. Although the exact role of the USVs temporarily emitted by isolated pups is still
375 debated (social communication, instinctive behaviour of pup nest homing, or physiological response to homeothermic stress) [58,59], the modulation of isolated emissions due to genetic manipulation and/or environmental conditions is an index of delayed development [45,60]. Moreover, maternal care, known to play a critical role in the development of pups, cannot be involved in USV impairments in the present study since the behaviour of *Het*^{-/-} and control
380 dams towards their pups was similar throughout two control tests.

All of the developmental disorders reported here in *Het*^{-/-} mice have been documented in validated mouse models of autism spectrum disorders (ASD), i.e. sensorimotor development, spatial orientation, and social communication impairments [60–62]. Interestingly, *Het*^{-/-} mice presented a greater response to whisker stimulation during the early stages of development,
385 demonstrating a hypersensitivity to somesthetic stimulation, which is one of the key symptoms in the early diagnosis of ASD in children with social disability [63–65]. Children with ASD or attention-hyperactivity disorders present body perception impairments, alteration of spatial orientation (particularly body orientation and body scheme construction), a delay in motor development, and impaired behavioural motor responses sometimes supplemented by
390 emotional and social disturbances [66–70], including some clinical signs that are not consistent with gravity-related vestibular symptoms. Taken together, these similarities suggest that the gravity-related sensory vestibular component might play a substantial role in the

pathophysiology of ASD during an early stage of development. This idea is not new [71,72]; however, the lack of systematic data on vestibular function in patients with ASD make it impossible to draw firm conclusions at this time.

5. Conclusion

Our work highlights the notion that development has a critical period which is dependent on the vestibular sensory graviception in mice. The brain-gravity interaction on Earth has thus two crucial components: one mechanical and the other sensory. The symptoms expressed by the congenital otolithic-deficient mice are similar to validated mouse models of autism and highlight the significance of vestibular graviception in the pathophysiology of development. On the other side, our data reinforce the role and the relevance of the protocols of psychomotricity involving vestibular stimulation. Cognitive-motor consequences in vestibulo-deficient infants remain to be investigated with the perspective of a better screening, diagnosis and treatment.

Authors Contribution

Le Gall, Hilber, Besnard have designed the research, analysed data and wrote the paper, Chesneau and Bulla have performed the statistical analysis, Le Gall, Toulouse, Machado and Philoxène have performed research, Smith has performed the statistical analysis and wrote the paper.

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Legends

Figure 1. Sensorimotor development evaluation and weight gain of pup mice from PND2 to PND10 ($n=27$ *Het*^{-/-} versus 36 *Het*^{-/+}). No differences were detected between male and female, therefore data were collapsed across sex (p -value > 0.05). **a**, The weight gain of pups from PND2 to PND10 served as controls for physiological development. The follow-up of weight in pup mice revealed no group effect: the weight gain was similar in both groups arguing, in parallel, for good growth and appropriate maternal feeding (Mann-Whitney test, p -value > 0.05 , ns, data \pm sem). **b**, The global score of development (calculated from all rated tasks) showed a lower level of sensorimotor development in otolith-deficient mice (*Het*^{-/-}) compared to control *Het*^{-/+} mice: *Het*^{-/+} showed a positive age trend (0.242 ± 0.0233 , p -value ≤ 0.001 , ***), whereas the age trend of *Het*^{-/-} showed a significantly weaker progression (0.099 ± 0.0307 , p -value ≤ 0.001 , ***) from PND2 to PND10 with a maximum difference between PND6 and PND10 (PND6-PND8, p -value ≤ 0.001 , ***, PND10, p -value ≤ 0.01 , **). The *Het*^{-/+} group present similar developmental milestones and global scores compared to mice strains free of genetic manipulation (C57BL6), so we preferred to compare mice of the same strain, i.e., *Het*^{-/-} and *Het*^{-/+} controls. Results revealed that the sensorimotor development in *Het*^{-/-} mice is delayed and poorer compared to *Het*^{-/+} mice and normal development described in the literature. **c**, In the righting reflex, both groups improved day after day (*Het*^{-/+}, 0.597 ± 0.081 , p -value ≤ 0.001 , ***, *Het*^{-/-}, 1.402 ± 0.422 , p -value ≤ 0.001 , ***), but a genotype effect was observed (p -value ≤ 0.001 , ***). A pseudo post-hoc analysis carried out at each age via a Chi-squared test revealed that the difference observed was significant from PND4 to PND6 (unadjusted p -value ≤ 0.05 , *). **d**, Analysis of the cliff drop aversion test revealed poorer performance in the *Het*^{-/-} group compared to *Het*^{-/+}: *Het*^{-/+} mice show a positive age trend (0.4043 ± 0.133 , p -value ≤ 0.01 , **), while the age trend in

Het^{-/-} mice showed a significantly negative progression (-0.1573 ± 0.150 , p -value ≤ 0.001 , ***). An additional group effect was observed (p -value ≤ 0.05 , *). A pseudo post-hoc analysis carried out at each age via a Chi-square test revealed that the difference observed was significant from PND6 to PND10 (PND6, unadjusted p -value ≤ 0.05 , *; PND8-PND10 unadjusted p -value ≤ 0.01 , ** vs PND8-PND10 adjusted p -value ≤ 0.05 , *). **e**, The negative geotaxis test analysis showed a poorer performance in the *Het*^{-/-} compared to the *Het*^{-/+} group: The *Het*^{-/+} group showed a positive age trend (0.834 ± 0.133 , p -value ≤ 0.001 , ***) while the age trend in *Het*^{-/-} showed a significantly weaker progression (0.386 ± 0.156 , p -value ≤ 0.01 , **). A pseudo post-hoc analysis carried out at each age via a Chi-squared test revealed that the difference observed was significant from PND6 to PND10 (adjusted p -value ≤ 0.001 , ***). **f**, Conversely, *Het*^{-/-} mice results for the response to whisker stimulation were higher overall compared to control mice (genotype effect, p -value ≤ 0.05 , *), particularly at an early stage at PND2 (pseudo post-hoc, unadjusted p -value ≤ 0.05 , *) and at PND6, but this difference was not validated by statistical analyses at age PND6 (p -value = 0.054).

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Figure 2. The Home Retrieval test performed at PND9. No differences were detected between males and females; therefore data were collapsed across sex (p -value > 0.05 , $n=25$ *Het*^{-/-} versus 36 *Het*^{-/+}). Two aberrant values were excluded in this test. **a**, The *Het*^{-/-} group needed significantly more time to reach the nest area compared to the *Het*^{-/+} control group (“Latency to reach the nest”, Mann-Whitney test, $p \leq 0.01$, **, data +/- sem). **b**, The time spent in the nest area was significantly lower in the *Het*^{-/-} group compared to the *Het*^{-/+} control group (Mann-Whitney test, $p \leq 0.01$, **, data +/- sem). **c**, The otolith-deficient *Het*^{-/-} pups showed a lower percentage of success reaching the nest area compared to *Het*^{-/+} mice (Chi²-tests for equality of proportions, $p \leq 0.01$, **, data +/- sem). **d**, Conversely, the latency before the first

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655 movement reaction of pups (Mann-Whitney test, $p > 0.05$, ns, data +/- sem) was similar in both groups (*Het*^{-/-}, *Het*^{+/-}).

Figure 3. Ultrasonic vocalizations (USVs) in pup mice on PND2, PND4, PND6, PND8, and PND10. No differences were detected between males and females, therefore data were collapsed across sex (p -value ≤ 0.05 , $n=27$ *Het*^{-/-} vs 36 *Het*^{+/-}). **a.** The percentage of animals emitting USVs and thus the probability of success in the test in *Het*^{-/-} pups was lower at PND2 (p -value ≤ 0.05) and higher at PND10 (p -value ≤ 0.01) compared to controls: while *Het*^{+/-} showed a negative age trend (-0.269 ± 0.112 , p -value ≤ 0.05), the age trend in *Het*^{-/-} showed a significantly different positive progression (0.28 ± 0.144 , p -value ≤ 0.001). **b.** The number of vocalizations emitted per min restricted to animals emitting USVs, were impaired in *Het*^{-/-} mice (p -value ≤ 0.05). **c.** The average duration of calls (ms) showed a statistically significant age effect (p -value ≤ 0.001) in both groups, with no genotype effect (p -value > 0.05 , ns) at PND2, PND4, PND6, PND8, and PND10 during a five-min session after a short maternal separation. **d.** The average frequency of calls (KHz) showed a statistically significant age effect (p -value ≤ 0.001) in both groups, with no genotype effect (p -value > 0.05 , ns) at PND2, PND4, PND6, PND8, and PND10 during a five-min session.

Figure 4. The One Pup Retrieval test performed at PND2, PND4, PND6, PND8, and PND10. 10 females were tested ($n = 5$ dam *Het*^{-/-} versus 5 dam *Het*^{+/-}). **a.** The latency to retrieve the separated pup was statistically similar in *Het*^{-/-} and *Het*^{+/-} groups between PND2 and PND10 (Nonparametric Analysis of Longitudinal Data, p -value > 0.05 , ns, data +/- sem). **b.** No significant group effect on latency to put the retrieved pup into the home nest was observed in the test (Nonparametric Analysis of Longitudinal Data, p -value > 0.05 , ns, data +/- sem).

680 **Figure 5. The All Pups Retrieval test performed at PND4 and PND6.** 10 females were tested
($n = 5$ dam *Het*^{-/-} vs 5 dam *Het*^{-/+}). **a**, The latency to retrieving the separated pups was
statistically similar in *Het*^{-/-} and *Het*^{-/+} groups at PND4 and PND6 (Nonparametric Analysis
of Longitudinal Data, p -value > 0.05 , ns, data \pm sem). **b**, No significant group effect for latency
to put the retrieved pups into the home nest was observed at PND4 and PND6 in this test
685 (Nonparametric Analysis of Longitudinal Data, p -value > 0.05 , ns, data \pm sem).

FIGURE 1

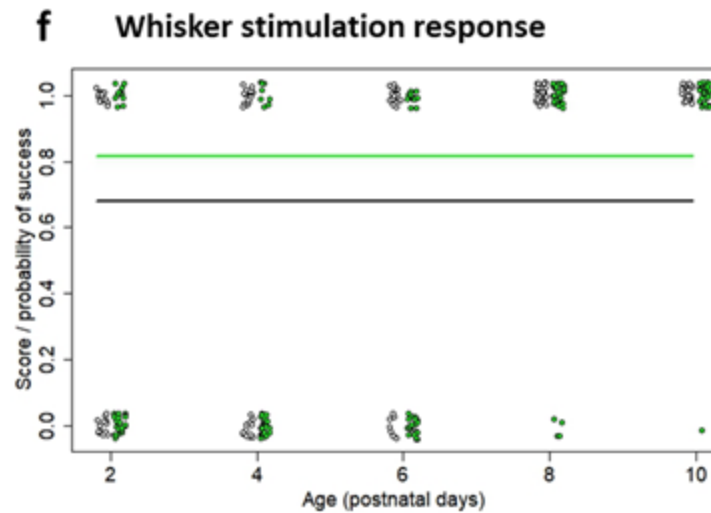
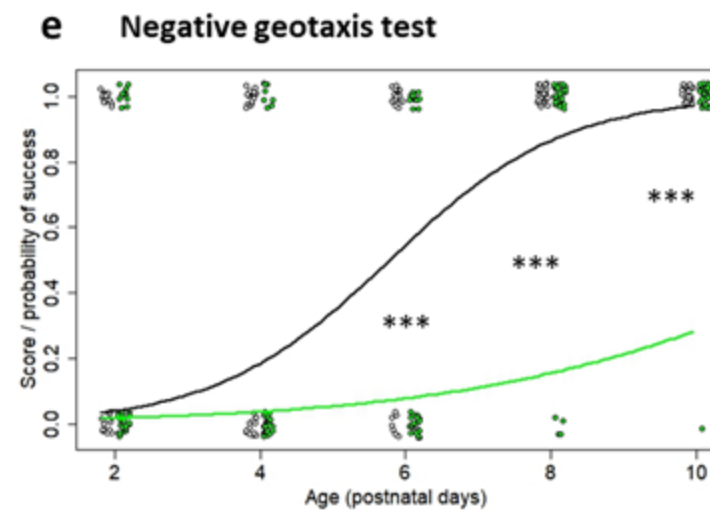
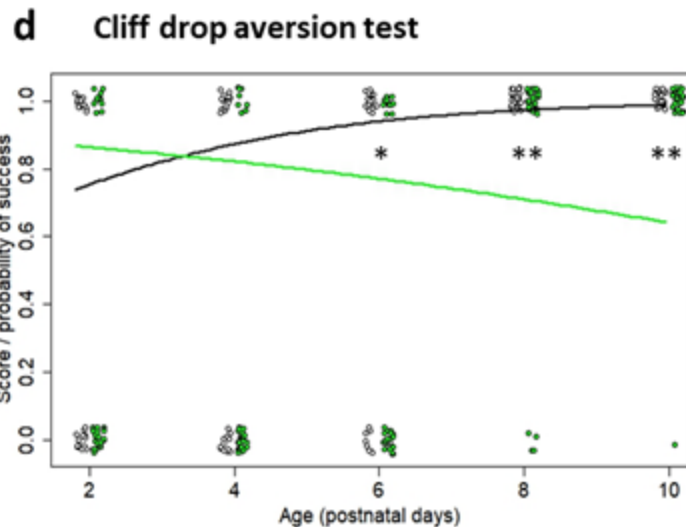
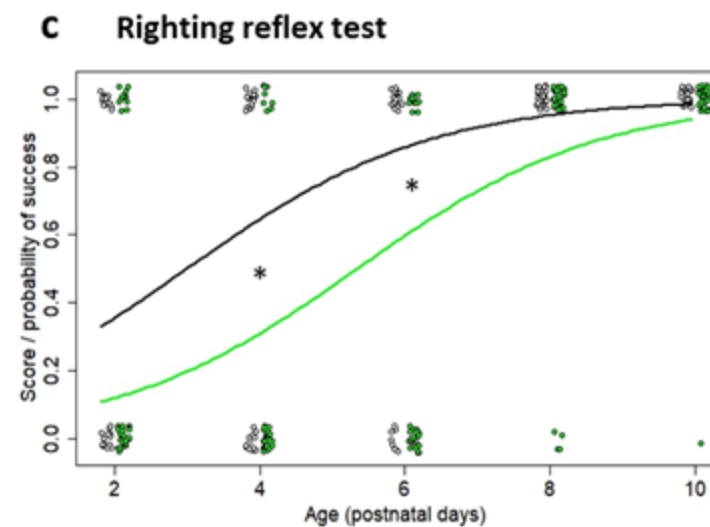
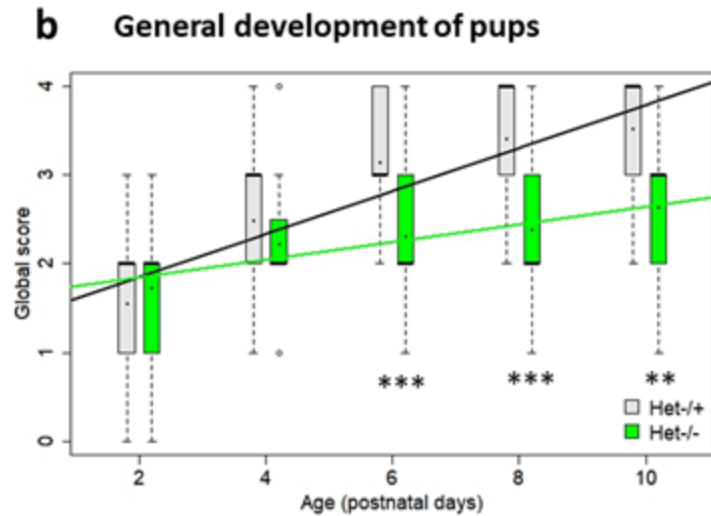
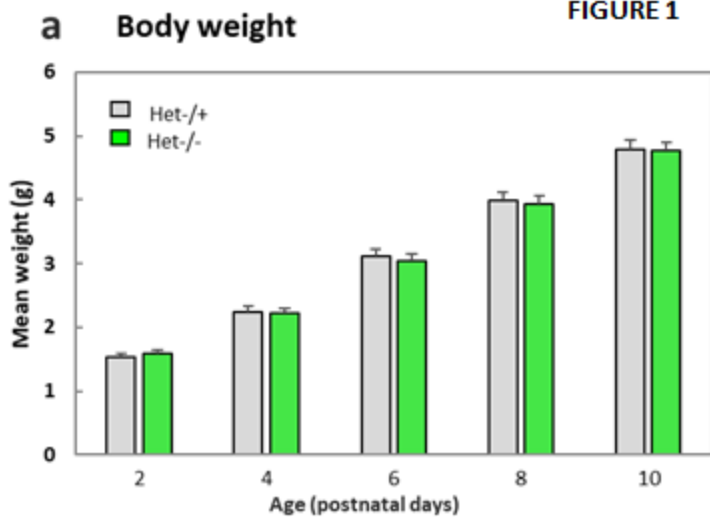


FIGURE 2

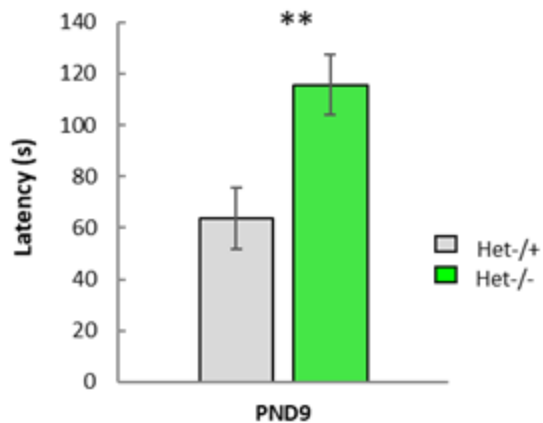
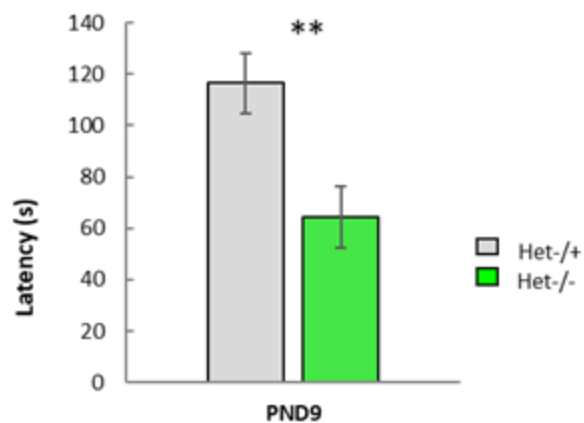
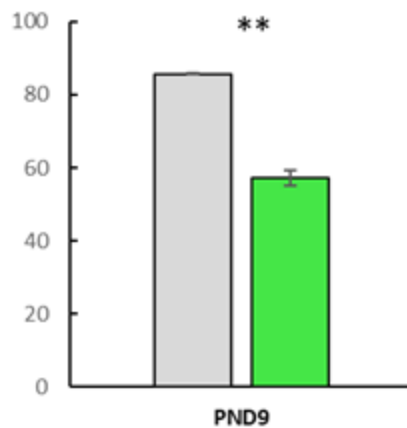
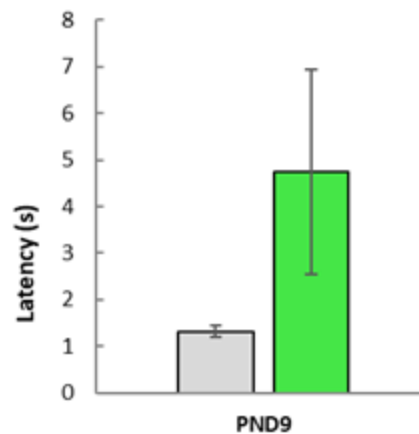
a Latency to reach the nest area**b** Time spent in the nest area**c** % of success to reach the nest area**d** Latency to start the move

FIGURE 3

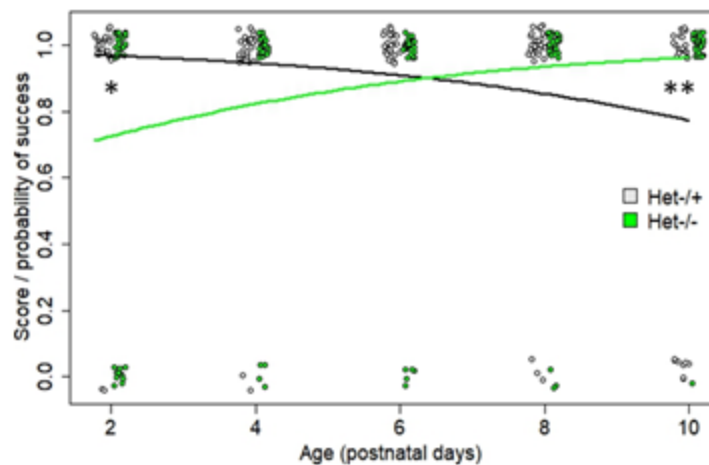
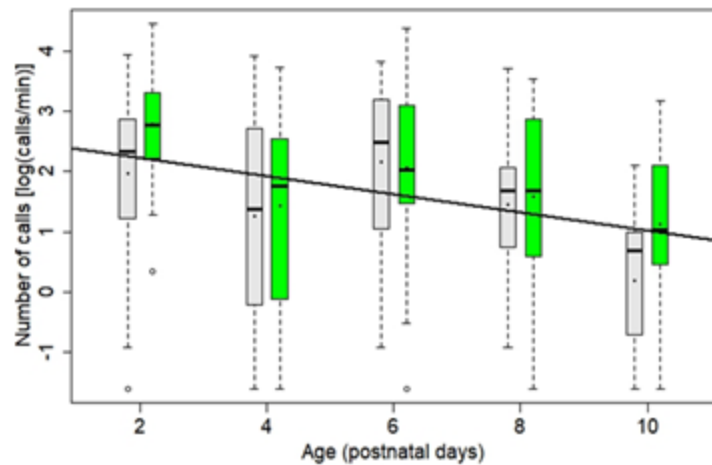
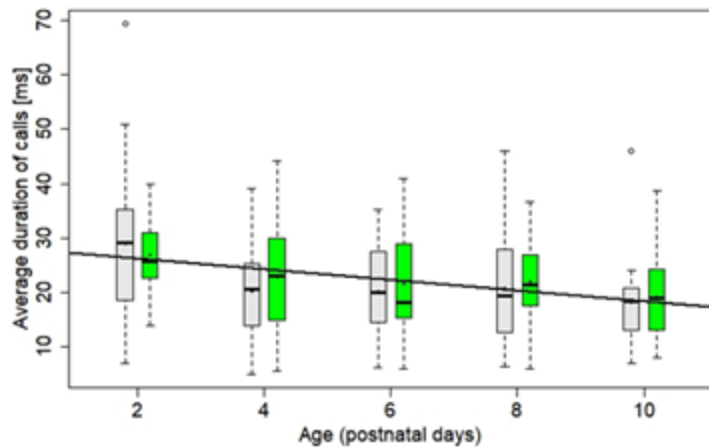
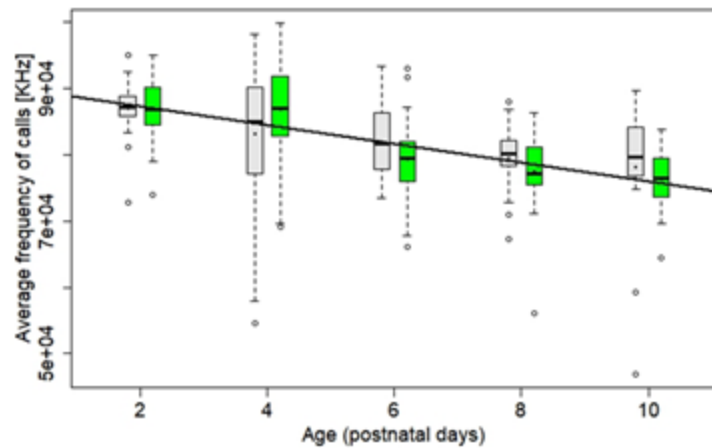
a Average of success to USVs emissions**b** Number of calls/min**c** Average duration of calls (ms)**d** Average frequency of calls (KHz)

Figure 4

The One Pup Retrieval test

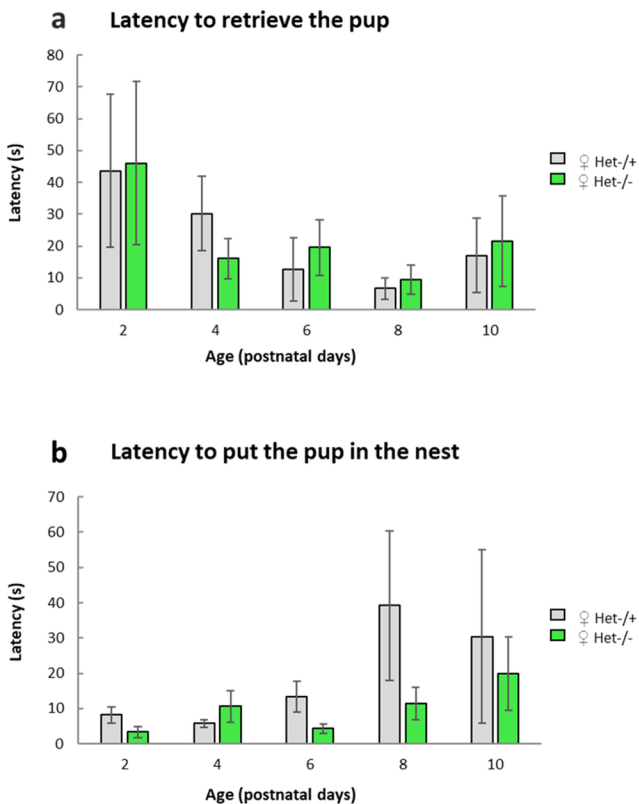


Figure 5

The All Pups Retrieval test

