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Huddling substates in mice facilitate dynamic changes in body temperature and are modulated by *Shank3b* and *Trpm8* mutation

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Social thermoregulation is a means of maintaining homeostatic body temperature. While adult mice are a model organism for studying both social behavior and energy regulation, the relationship between huddling and core body temperature (Tb) is poorly understood. Here, we develop a behavioral paradigm and computational tools to identify active-huddling and quiescent-huddling as distinct thermal substates. We find that huddling is an effective thermoregulatory strategy in female but not male groups. At 23 °C (room temperature), but not 30 °C (near thermoneutrality), huddling facilitates large reductions in Tb and Tb-variance. Notably, active-huddling is associated with bidirectional changes in Tb, depending on its proximity to bouts of quiescent-huddling. Further, group-housed animals lacking the synaptic scaffolding gene *Shank3b* have hyperthermic Tb and spend less time huddling. In contrast, individuals lacking the cold-sensing gene *Trpm8* have hypothermic Tb – a deficit that is rescued by increased huddling time. These results reveal how huddling behavior facilitates acute adjustments of Tb in a state-dependent manner.

Huddling—an active and close aggregation of animals—can serve multiple functions, from thermoregulation to social reward. Thermoregulatory huddling is widespread among animals and is hypothesized to provide an effective means of regulating core body temperature (Tb) and conserving energy. Huddling is cooperative in the sense that individuals bear the costs of donating body heat, but share the benefits[1]. Huddling can alter Tb by increasing the ambient temperature surrounding individuals in close contact, by reducing surface area to volume ratio and therefore heat loss, or by augmenting insulation[2,3]. Notably, most endotherms with the ability to huddle maintain a higher and more stable Tb than their isolated counterparts[2]. However, depending on the species, developmental stage, and environmental conditions, huddling can operate in conjunction with thermoeffector pathways to increase or decrease Tb to maintain a "set point" specific to a particular behavioral state[2,4]. For example, in some species such as adult penguins, huddling causes decreased heat loss to the external environment, allowing for a decrease in metabolic rate and a lower Tb[5]. Similarly, human babies in physical contact with their mother's skin display reduced heat loss and metabolic output[6–8]. In contrast, huddling in rabbit pups results in higher Tb as well as thermoregulatory energy savings that can be channeled into competitive ability[9].

For social animals, the close physical contact experienced during huddling can also reduce stress or be intrinsically rewarding. For example, individuals often display preferences for social contact or for contexts in which they previously experienced social contact[10–12]. Similarly, social contact can buffer the effects of stress[13–15]. In contrast, isolation and removal from physical contact with conspecifics can be aversive[16] and have long lasting consequences[17]. For example, chronically isolated male mice display a reduction of huddle formation, but increased approach behavior, when re-introduced to a group setting[18].

The house mouse, a model organism for studies of energy homeostasis and social behavior alike, displays extensive huddling behavior in the wild[19] and in the laboratory[20,21]. While room temperature (RT, ~21 °C) is thermoneutral (i.e., an ambient temperature where metabolic rate is at a minimum) for humans, it is well below thermoneutrality for mice, largely due to their high surface area to volume ratio[22–24]. This is especially apparent in mouse pups, which are born with immature capacity for thermoregulation[25–27], and sex-specific thermoregulatory huddling

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strategies across the first eight days of postnatal development have been characterized[3,28]. In adult mice, individual housing at RT requires about a third of the energy budget to be devoted to cold-induced thermogenesis[29]. In contrast, group-housed mice at RT display lower thermal conductance (i.e., less heat loss) than individually-housed mice, and this effect is thought to be due to the energy-saving benefits of huddling[30]. In support of this idea, group-housed animals huddle more at lower temperatures[20] and have overall lower metabolic rates than individually-housed mice[21].

Despite the association between group housing and energy savings, the precise details of how huddling affects body temperature over the course of the day in adult mice are poorly understood[31]. For example, mice appear to engage in huddling both when awake and when asleep. While awake and sleep states in isolation are proposed to comprise regulated defenses of upper and lower Tb set points, respectively[4], it is unclear how awake and asleep huddling states map onto these defended set points. Moreover, it is presently unclear how adult thermal states associated with huddling are affected by ambient temperature, sex, and/or genetic factors. One barrier to a more complete understanding of huddling behavior has been the difficulty of automating classification of behavior in a group setting. However, the development of computational tools to classify group-level behaviors will help disentangle the thermoregulatory and social components of huddling. We address these knowledge gaps.

Here, we develop a behavioral paradigm and computational tools to identify huddling-associated thermal states and how they are affected by internal (e.g., sex, age) and external (e.g., housing density, ambient temperature) factors in adult laboratory mice. We also investigated candidate genes expected to influence social interaction and thermosensation. First, *Shank3b* encodes a post-synaptic scaffolding protein, and mutations in this gene are associated with autism spectrum disorder (ASD), Phelan-McDermid syndrome[32], and deficits in social interaction, including in *Shank3b*$^{-/-}$ mouse models[33,34]. Second, the transient receptor potential channels *Trpv1* and *Trpm8* have established roles in thermosensation and thermoregulation. *Trpv1*, also known as the capsaicin receptor gene, is activated by hot stimuli[35], and *Trpm8*, also known as the menthol receptor gene, is activated by cool stimuli[36]. Both *Trpv1*[37] and *Trpm8*[38,39] are involved in thermoregulation. We use animals with mutations in these genes to investigate their association with social thermoregulation.

Altogether, this study quantifies huddling behavior in over 300 48-h recordings at the resolution of seconds. We identify active and quiescent huddling substates that facilitate dynamic changes in body temperature that are more pronounced than the dynamics observed in solo-housed mice. We show that huddling substates in group-housed animals are associated with lower Tbs than those during in solo-housed mice. Intriguingly, active huddling can be associated with either an increase or decrease in core Tb, depending on the context. Finally, we show that the normal patterns of social thermoregulation in mice are fundamentally altered in *Shank3b* and *Trpm8* mutants.

Results

Body temperature is affected by housing density, sex, and ambient temperature

We set out to determine how thermal biology is affected by housing density. To do so, we developed a paradigm in which core body temperature (Tb) and activity of groups of one to three mice are recorded without any human interruption in a home cage recording suite (Fig. 1A). The recordings occur over a 48-hour period with a 12/12 light/dark cycle in two different ambient temperatures (Ta): a standard vivarium temperature (Ta = 23 °C)[40] and a temperature near the lower critical temperature of the thermoneutral zone for adult male and female C57 mice (Ta = 30 °C)[22,24,30,41–43]. Temperature loggers implanted in the abdomen of each mouse recorded Tb once per minute.

First, we found that female Tb was higher when housed at 30 °C than at 23 °C (Fig. 1B), whereas male Tb was not affected by ambient temperature (Fig. 1C). At 23 °C, solo-housed females had significantly lower mean Tb compared to pair- or trio-housed females, but only during the dark cycle (Fig. 1D). In contrast, housing density had no effect on male Tb (Fig. 1E). These results suggest that group size affects female Tb below thermoneutrality, and are consistent with reports that mouse Tb is lower at 23 °C than 30 °C[2,24], and that solo-housed animals have lower Tb compared to group-housed animals at 23 °C[30]. In our system, however, male Tb was unaffected by housing density and ambient temperature. We therefore focused our next set of analyses on female mice.

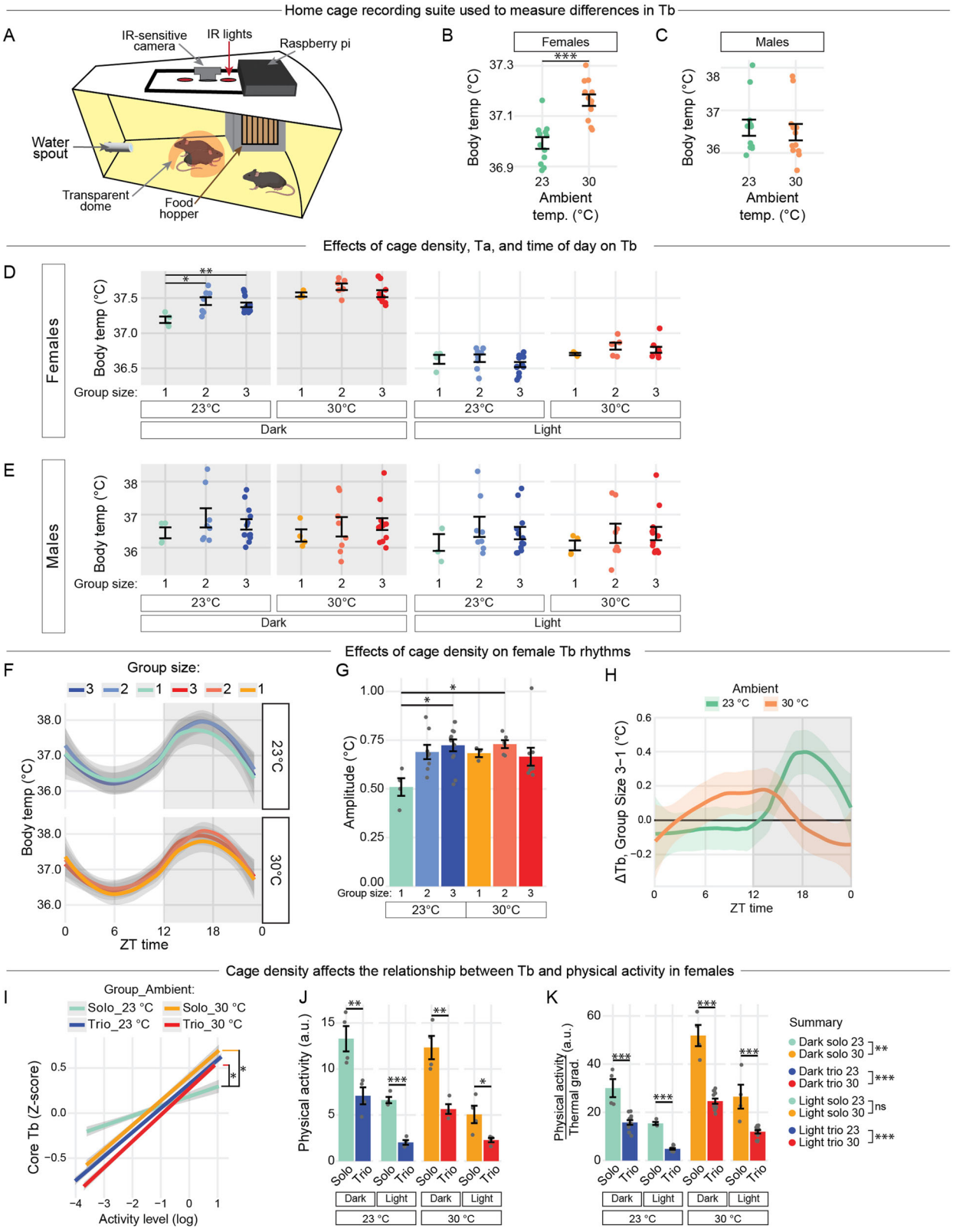
To determine the effect of housing density on diurnal rhythms of female Tb, we performed cosinor analysis (Fig. 1F; SOM methods). Analysis of Tb over time revealed that 23 °C solo-housed animals had decreased Tb amplitude (Fig. 1G), likely due to an inability to achieve peak Tb during the dark cycle. In males, no effects of group size or ambient temperature were observed. To account for inter-individual variation in Tb, we next calculated the difference in diurnal Tb when females were trio-housed vs. solo-housed, on a per-individual basis. At 23 °C, females were, on average, cooler during the inactive period and warmer during the active period when trio-housed vs. solo-housed. However, at 30 °C, this patten was abolished and partially reversed: females were warmer during the inactive period when trio-housed vs. solo-housed (Fig. 1H). These results suggest that housing density affects female Tb rhythms in an ambient temperature-dependent fashion, with the span of Tb reduced during solo housing. Our findings corroborate previous reports that solo-housed animals have an overall lower Tb due to increased heat loss[2,30].

Because physical activity and Tb are related, and because physical activity may differ between social contexts, we next investigated the relationship between Tb and physical activity according to both housing density and ambient temperature using regression analysis. We measured physical activity in female mice using frame-to-frame pixel changes in the 48-h video recording. The slope between activity level and Tb in 23 °C solo-housed females was significantly weaker than both 23 °C trio-housed mice and mice housed at 30 °C, regardless of housing density (Fig. 1I). Thus, solo-housed females have a dampened relationship between physical activity and Tb, likely due to excessive heat loss; moreover trio-housed animals maintain an equivalent thermal profile at 23 and 30 °C.

We next determined how cumulative activity levels were affected by housing density and ambient temperature. Solo-housed animals had significantly higher activity levels than trio-housed animals across both ambient temperatures and light/dark conditions (Fig. 1J). These results suggest that the lower Tb in 23 °C solo-housed females (Fig. 1D) cannot be explained by a decrease in physical activity. To further examine the relationship between physical activity, ambient temperature, and Tb, we analyzed an $\frac{Activity}{Thermal\ gradient}$ quotient, where thermal gradient is defined as $[T_b - T_{ambient}]$. Because, under most conditions, physical activity is tightly correlated with total energy expenditure[29,43], this metric is related to thermal conductance (i.e., $\frac{Total\ energy\ expenditure}{Thermal\ gradient}$). Here, we found that solo-housed animals had a greater $\frac{Activity}{Thermal\ gradient}$ quotient than their trio-housed counterparts, a result driven by both lower Tb and higher activity (Fig. 1K). Thus, solo-housed animals may have a reduced ability to conserve heat during inactive periods of the day, particularly at 23 °C. Together, these results suggest that group size and ambient temperature play important roles in determining the thermal profiles of laboratory mice.

Huddling behavior is affected by sex, light/dark cycle, and ambient temperature

We next addressed how huddling behavior might contribute to our observation that housing density alters the thermal biology of mice. To do so, we used the home cage recording suite to monitor aspects of behavior in groups of three mice for 48 h (Fig. 1A). For group-housed mice, we defined three behavioral states: (1) locomotion (LM; group members all display high levels of physical activity), (2) active huddle (AH; all group members are huddling while displaying some physical activity), and (3) quiescent huddle (QH; all group members are huddling, but not displaying physical activity). Active and quiescent huddling substates were defined by direct physical



contact between group members. Together, behavioral states were determined by a combination of location, physical contact, and activity level (Fig. 2A, B). The IR-transparent dome hut placed in the cage helps consolidate huddling to a single location, improving analysis performance without impact on the quantity of huddling (Fig. 2C). This surveillance-style recording suite, in conjunction with an automated analysis pipeline, quantifies huddling substates with approximately 90% accuracy compared to manual scoring (Fig. 2D, E). Thus, the home cage recording suite is a novel system for automated analysis of huddling substates over 48-hr time periods.

Using this paradigm, we quantified how huddling behavior changes due to different internal (e.g., sex, age) and external (e.g., light/dark cycle,

Fig. 1 | Body temperature is affected by sex, housing density, ambient temperature, and activity. A Schematic showing home-cage recording suite (HCRS) setup. **B**, **C** Mean Tb according to ambient temperature for females (**B**) and males (**C**). Data include all group sizes. $N = 24$ animals (12 male, 12 female). **D**, **E** Mean Tb according to group size, ambient temperature, and light/dark cycle for females (**D**) and males (**E**). **F** Mean Tb for each group size in each ambient temperature across zeitgeber time (ZT). Gray outline represents SEM. **G** Tb amplitude (deviation from the cosinor fitted mean) across ZT according to ambient temperature and group size. **H** Delta Tb is difference between group sizes 3 and 1 across ZT. Black line represents no difference in Tb between group sizes. **I** Linear regression model plotting activity level with normalized Tb for solo- and trio-housed in each ambient temperature. Gray outline represents SEM. $R^2 = 0.1149$. **J** Cumulative physical activity according to group size, ambient temperature, and light/dark cycle. K. $\frac{Activity}{Thermal\ gradient}$ quotient according to group size, ambient temp, and light/dark cycle. Thermal gradient = $[T_b - T_{ambient}]$. "Summary" compares means according to ambient temperature. For **D**–**K**, $N = 12$ mice (12 trio-housed split into 4 solo- and 8 pair-housed). **F**–**K** uses only female data. Data are mean ± SEM. Pairwise comparisons are from Tukey post-hoc test of linear mixed-effects model (**B**–**E**, **I**, **K**) or linear model (**G**, **J**); $P < 0.05$: *, $P < 0.01$: **, $P < 0.001$: ***. Full statistical analysis is in Table S1.

ambient temperature) factors in trios of mice. For males and females from five to 10 weeks of age at 23 °C, the cumulative time spent active huddling was approximately five hrs (300 min), and cumulative time spent quiescent huddling was approximately 7.5 h (450 min). While males and females spent an equivalent time active huddling, males spent more cumulative time quiescent huddling (Fig. 2F). From five to 10 weeks of age, males gradually reduced the amount of time spent active huddling, while time spent quiescent huddling was stable across this period. In contrast, females spent an equivalent and stable amount of time active and quiescent huddling across this period (Fig. 2G). In the huddling ethogram, time spent active and quiescent huddling appears to be time-of-day dependent (Fig. 2D). We therefore quantified the effect of the light/dark cycle on huddling. Although readily detectable throughout the 24-hr period, active and quiescent huddling substates were more common during the light cycle compared to the dark cycle in males and females (Fig. 2H). Thus, huddling behaviors are dependent on age, sex, and time of day.

The physical contact experienced during huddling can be considered either a thermoregulatory behavior[1,2,9] or a rewarding social behavior[12,44–47]. The adult mouse thermoneutral zone (approximately 29–33 °C[22,24,30,41–43]) is well above the standard ambient temperature of animal vivaria[40], and the drive to huddle for thermoregulatory benefit may be particularly strong at 23 °C. To determine whether mice are motivated to huddle for social reward in the absence of thermoregulatory need, we quantified cumulative huddling behavior in five- to 10-week-old mice at 30 °C. Notably, huddling was nearly abolished in males and significantly reduced in females across the 48-h period (Fig. 2I, J). Nevertheless, at 30 °C, there were still periods when animals were quiescent but not making physical contact (i.e., "quiescent without huddling") (Fig. 2I), a behavior rarely observed at 23 °C (e.g., Fig. 2D), suggesting animals prefer to sleep alone at 30 °C. While both males and females showed decreased time spent huddling at 30 °C, females displayed significantly more time in AH and QH than males (Fig. 2J), suggesting a combination of social and thermoregulatory components of huddling in females. Together, these data indicate that huddling behavior is dependent on ambient temperature and, at standard room temperature, serves a primarily thermoregulatory function.

Huddling among females facilitates an energy saving thermal profile at room temperature

Next, to directly link thermal and behavioral states in solo and group-housed animals, we monitored Tb and behavior over 48-h periods using the home cage recording suite. Using housing rotations, we examined the same individuals either separated, with one sibling, or with two siblings during 48-h recordings (SOM Methods). This design allowed us to control for between-individual variation while preventing long-term effects of isolation such as cold adaption[48] and antisocial behavior[49]. For solo-housed animals, we designated three different behavioral states corresponding to the behavioral states of group-housed animals: (1) locomotion (Lm; high levels of physical activity), (2) grooming (Gr; low levels of physical activity), and (3) quiescence (Qu; no physical activity) (Fig. 3A and S1A). Here, we aggregated data across the light/dark cycle to maximize the number of behavioral bouts in our linear mixed-effect modeling of Tb (SOM methods).

We compared mean Tb for each behavioral state in solo-, pair-, and trio-housed mice at ambient temperatures of both 23 and 30 °C. In females, we observed a significant decrease in Tb from the highest to lowest activity behavioral state across all group sizes and ambient temperatures (Fig. 3B, D, S1B–D). These findings are consistent with other reports showing Tb is positively correlated with activity level[24,29,30,50]. However, patterns between solo and group-housed females were different. In solo females, quiescence was significantly lower than grooming and locomotion (Fig. 3B, S1B). In contrast, in group-housed females, both active and quiescent huddling were lower than locomotion (Fig. 3C, D). Thus, both huddling substates are associated with reduced Tb, even in ambient temperatures with minimal thermal stress (Fig. S1C, D).

To quantify the magnitude of change in Tb (ΔTb), we compared mean ΔTb between behavioral states in solo-housed animals (i.e., locomotion to grooming Lm→Gr, locomotion to quiescence Lm→Qu, and grooming to quiescence Gr→Qu), and equivalent behavioral state transitions in group-housed animals (i.e., locomotion to active huddle LM→AH, locomotion to quiescence huddle LM→QH, and active huddle to quiescence huddle AH→QH). We first examined ΔTb at 23 °C (Fig. 3E–G). For solo-housed animals, there was a negative ΔTb between quiescence and both grooming and locomotion (Fig. 3F, G, light green points), confirming previous reports that sleep is associated with lower Tb[51–53]. In contrast, for group-housed animals, there was a strong negative ΔTb between locomotion and both active and quiescent huddling (Fig. 3E, F, light and dark blue points), and these huddling-associated decreases in Tb were significantly larger than the decreases in Tb from locomotion to grooming and quiescence in solo-housed animals (Fig. 3E, F). These results suggest that, below the thermoneutral zone (i.e., 23°C), active and quiescent huddling in females facilitate reductions in Tb that are stronger than those during comparable behavioral transitions in solo females, consistent with an energy saving model of social thermoregulation.

We next compared ΔTb between behavioral states at 30 °C, where huddling is far less common (Fig. 2J). For group-housed females, active huddling was associated with a negative ΔTb compared to locomotion, and this decrease was greater than the decrease in Tb during grooming compared to locomotion in solo-housed females (Fig. S1E). In contrast to the 23 °C data, at 30 °C the ΔTb from locomotion to quiescence huddling was equivalent to the ΔTb from locomotion to quiescence in solo animals (Fig. S1F). Finally, for solo-housed animals, the ΔTb from grooming to quiescence resulted in a decrease in Tb that was significantly greater than the ΔTb from active huddling to quiescent huddling (Fig. S1G). These results suggest that, at 23 °C and 30 °C, both active and quiescent huddling can facilitate drastic decreases in Tb.

Huddling substates could facilitate transitions to a Tb set-point for rest (regardless of housing density), or to a set-point unique to huddling. To address this, we examined the absolute Tb associated with different behaviors. At 23 °C, locomotion-associated Tb was higher in group-housed compared to solo-housed females (Fig. S1H). In contrast, active huddling was associated with a lower Tb than solo grooming, and quiescent huddling in trios was associated with a lower Tb than solo quiescence (Fig. S1I, J). These trends were largely lost at 30 °C (Fig. S1K–M). Thus, under conditions of cold-induced thermogenesis, although group-housed animals become warmer than solo animals during locomotion, huddling facilitates lower rest-associated Tbs. Because group-housed animals, compared to solo-housed animals, have higher Tb during the active phase (Fig. 1D and[30]), lower physical activity (Fig. 1J and[30]), and lower $\frac{Activity}{Thermal\ gradient}$ quotient (Fig. 1K), these data

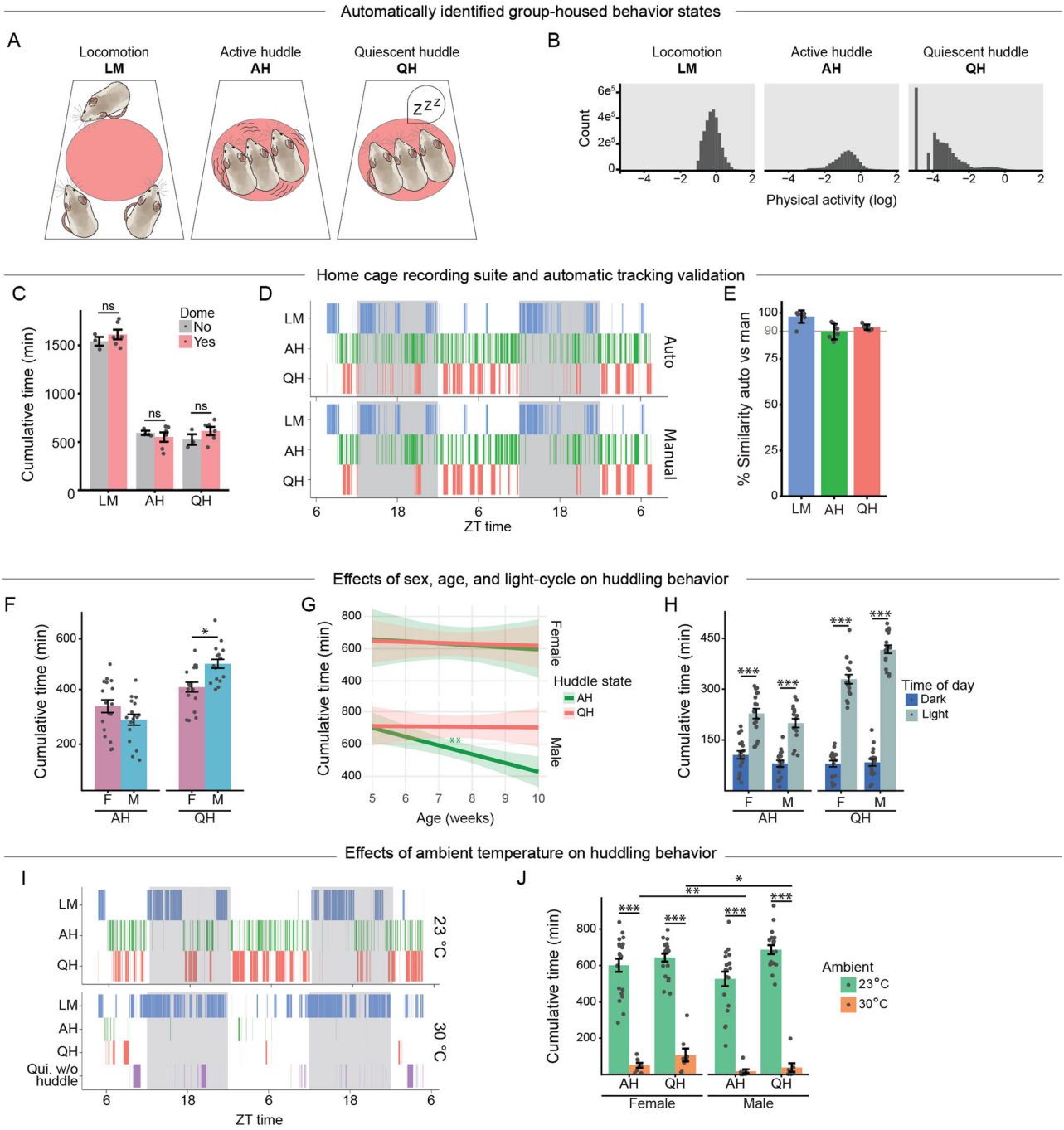


Fig. 2 | Huddling behavior is affected by sex, light/dark cycle, and ambient temperature. A, **B** Illustrations representing huddling behavior substates (**A**) and corresponding activity histograms (**B**). **C** Mean cumulative time (min) spent in each huddling state with and without dome. $N = 10$ groups (4 no, 6 yes). **D** Example ethogram showing automatically-scored compared to manually-scored data. **E** Percent similarity between auto and manual tracking, measured at 5 frames per-second. Gray line represents target 90% accuracy. N = 8 groups. **F**–**H** Mean cumulative time (min) spent in active and quiescent huddle states, according to sex (**F**), age (**G**), and time of day (**H**). $N = 35$ groups (18 female, 17 male). **I** Example ethograms showing huddling behaviors in 23 °C (top) and 30 °C (bottom). "Qui. w/o huddle" represents a fourth behavioral state where all animals are quiescent, but without huddling. **J** Mean cumulative time (min) spent in each huddling state according to sex and ambient temperature $N = 35$ groups in 23 °C (18 female, 17 male), 16 groups in 30 °C (8 female, 8 male). Data are mean ± SEM. "Group" refers to a trio of mice. Pairwise comparisons are from Tukey post-hoc test of linear mixed-effects model (**F**–**H**, **J**) or linear model (**C**, **J** summary); $P < 0.05$: *, $P < 0.01$: **, $P < 0.001$: ***. Full statistical analysis is in Table S1.

suggest that both active and quiescent huddling facilitate long-lasting energy-savings.

In addition to lowering Tb, reducing short-term Tb variance may be another way to conserve energy[54]. This notion is based on the principle that, all else being equal (e.g., for an animal of a given body mass and heat capacity, within a particular physiological state, and maintaining a particular defended Tb set point), a mean Tb with high variability will require more work than the same Tb with low variability. We therefore evaluated mean Tb standard deviation (i.e., Tb SD) between solo and group housed females at comparable behavioral states. At 23 °C, whereas Tb SD during locomotion was equivalent in solo- and group-housed females (Fig. 3H), Tb SD during active huddling and quiescent huddling were lower than grooming

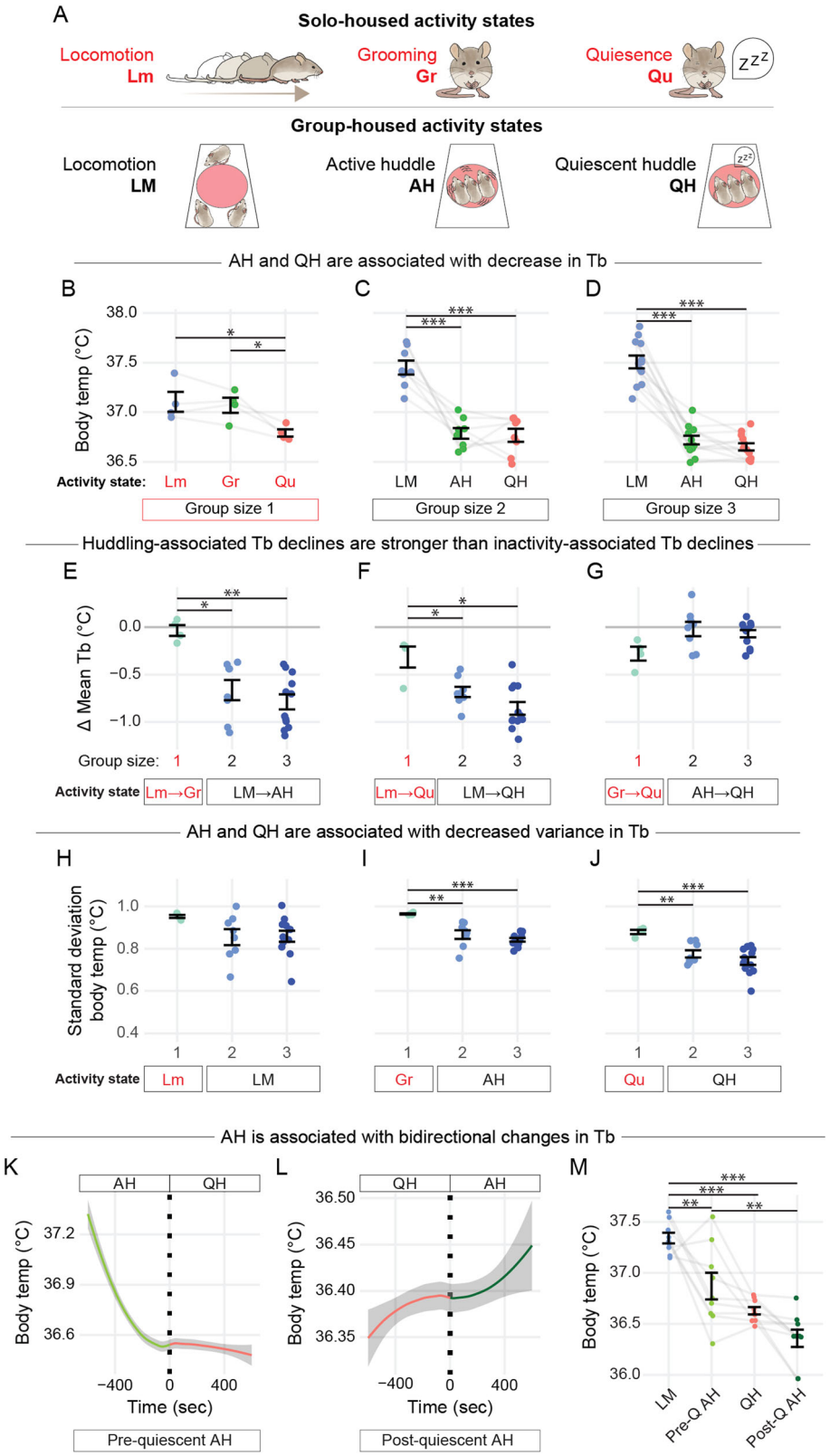


Fig. 3 | Huddling among females facilitates an energy saving thermal profile at room temperature. A Illustrations showing solo-housed mice activity states and corresponding group-housed activity states. **B**–**D** Mean Tb during each activity state for solo-housed (**B**), pair-housed (**C**), and trio-housed. (**D**) groups. Red box and activity state labels represent solo-housed activity states. **E**–**G** Magnitude of Tb change as measured by difference (Δ) in Tb between group sizes for each transition in behavior: Lm to Gr and LM to AH (**E**), Lm to Qu and LM to QH (**F**), and Gr to Qu and AH to QH (**G**). Gray line represents no difference in Tb between states. Red boxes and state labels represent solo-housed animals. **H**–**J** Variance (SD) of Tb comparing between group sizes, for locomotion (**H**), grooming and active huddling (**I**), and quiescence and quiescent huddling (**J**). Red boxes and state labels represent solo-housed animals. **K**, **L** Mean Tb during transitions from AH to QH and from QH to AH. Data are aligned to QH start (time 0 in **K**) and QH end (time 0 in **L**). Plots display 10-minute intervals of AH followed by QH (**K**) and QH followed by AH (**L**). Dotted line represents the time of transition. M. Quantification of **K**, **L**, summarizing mean Tb according to huddling substate. AH is split into bouts that are pre- and post-quiescent huddle bouts, respectively (i.e., Pre-Q AH and Post-Q AH). $N = 9$ trio-housed mice. Data are mean ± SEM. For **B**–**J**, $N = 12$ mice (12 trio-housed split into 4 solo and 8 pair, SOM Methods). Pairwise comparisons are from Tukey post-hoc test of linear mixed-effects model (**B**–**J**, **M**); $P < 0.05$: *, $P < 0.01$: **, $P < 0.001$: ***. Full statistical analysis is in Table S1.

or quiescence in solo-housed animals, respectively (Fig. 3I, J). At 30 °C these trends were diminished, although Tb SD during quiescent huddling was still lower than solo quiescence (Fig. S1A–D). These results suggest that huddling affords stabilization of Tb that is otherwise not available to solo-housed animals, and this stability is more apparent at 23 °C than 30 °C, consistent with the notion that huddling is an energy saving behavioral strategy.

Huddling has weaker effects on male thermal profiles

We next investigated social thermoregulation in males using the same approach (Fig. S3). While female Tb decreased from the highest to lowest activity state across all group sizes and ambient temperatures (Fig. 3B–D, S1B–D), these trends were weaker in males (S3B, C). This was especially evident when looking at the magnitude of change (ΔTb). Group-housed

females displayed stronger decreases in Tb from locomotion to huddling states than solo-housed females did from locomotion to inactive states (Fig. 3E, F, S1E, F). This pattern was not observed in males: decreases in Tb from active to huddling states in groups were equivalent to decreases from active to inactive states in solo-housed males (Fig. S3D, E left and middle panels). Next, we discovered that, compared to solo-housed females, group-housed females displayed higher Tb during locomotion, but lower Tb during quiescent states, (Fig. S1H–J), and this pattern was not observed in males: there were no differences in Tb comparing solo- and group-housed males for any behavior or ambient temperature (Fig. S1F, G). Finally, at 23 °C, female Tb SD tends to decrease from locomotion states to quiescence, regardless of group size, whereas this effect is diminished at 30 °C (Fig. S2B–D). However, this pattern is eliminated in males, where they show no effect of behavior on Tb SD in 23 °C, and only a decrease in Tb SD for trios at 30 °C comparing high to low activity states (Fig. S3H, I). Thus, although male groups display both active and quiescent huddling, these behaviors do not confer significant body temperature changes as seen in female groups.

Female active huddling facilitates bidirectional body temperature changes before and after quiescent huddling

Group-housed animals active huddle more at 23 °C compared to 30 °C (Fig. 2J), and the temporal patterning of active huddling appears correlated with quiescent huddling in the 48-h ethogram (Fig. 2D, I). These observations suggest that active huddling may be a motivated behavior to facilitate Tb changes leading into and out of the energy-saving quiescent huddling state. To understand how Tb is affected by transitions between active and quiescent huddling, we used a computational strategy to characterize active huddling at the borders of quiescent huddling. We organized bouts of huddling into continuous "epochs" that were sustained for at least 900 frames, or three minutes. Next, we characterized active huddling epochs as either preceding a quiescent huddle (i.e., pre-QH active huddle) or following a quiescent huddle (i.e., post-QH active huddle), depending on whether they occurred within 10 min before the start, or 10 min after the end, of a quiescent huddle epoch (SOM methods). For these experiments, we focused on trios at 23 °C, which exhibited strong active huddling associated decreases in Tb (Fig. 3D). Analysis of these huddling substates showed that during pre-QH active huddle epochs, Tb drops until approximately four minutes before the onset of QH and then stabilizes as the QH epoch begins (Fig. 3K). The opposite effect is seen in post-QH active huddle epochs, where Tb is stable coming out of the QH epoch but starts increasing about six minutes after the end of QH (Fig. 3L). These results suggest that active huddling operates in conjunction with physiological processes (e.g., vasomotor pathways or brown fat thermogenesis) to facilitate bidirectional changes in Tb, depending on whether it occurs before or after quiescent huddling.

We then examined the mean Tb of each of these huddling substates. Consistent with our observation that Tb declines during huddling (Fig. 3D), we found that pre-QH active huddle, post-QH active huddle, and QH were all lower than the locomotion state (Fig. 3M). Moreover, mean Tb for active huddling was warmer during pre-QH compared to post-QH. An important consideration in the interpretation of these results is thermal inertia, where the actual Tb lags behind acute changes in the Tb setpoint (on the order of minutes for small rodents[55]). In light of this information, our results suggest that pre-QH active huddling is associated with a cooling transition that goes from a high to low Tb, while post-QH active huddling is associated with a warming transition that goes from a low to high Tb. Because post-QH active huddling is the lowest observable Tb in our system (i.e., ~36.4 °C, Fig. 3M), these data indicate that pre-QH active huddling may be a strategy to facilitate an energy saving state (and consequently heat loss) to reach the low defended Tb set-point of rest; similarly, at the low-point of this energy-saving state, post-QH active huddling may then be used to elevate Tb in preparation for the higher defended Tb set point of the active state[4].

Shank3b mutation is associated with decreased huddling and increased Tb

We next addressed how a genetic factor with an established role in prosocial interaction affects social thermoregulation at 23 °C. The gene *Shank3b* encodes a post-synaptic scaffolding protein[56,57], and, in humans, mutations in the gene are associated with autism spectrum disorder and Phelan-McDermid syndrome[58–60]. *Shank3b*$^{-/-}$ mice show repetitive grooming behaviors and deficits in social interactions, particularly in the three-chamber sociability assay[34]. Here, we compared cumulative huddling time in *Shank3b*$^{-/-}$ mutants[34] and wildtype (WT) animals. Time spent active huddling was not affected by genotype. In contrast, mutant females spent less time quiescent huddling than WT females and, while males trended in the same direction, the difference was non-significant (Fig. 4A). These results suggest the antisocial effects described in *Shank3b*$^{-/-}$ animals may generate a deficit in quiescent huddling, especially among females. We therefore focused all subsequent experiments on female mice.

We next analyzed the effect of *Shank3b* mutation on body temperature. Trio-housed mutants showed a significantly higher Tb during both light and dark cycles; solo-housed mutants showed a similar pattern, but the differences were non-significant (Fig. 4B). Thus, group-housed *Shank3b*$^{-/-}$ animals spend less time quiescent huddling, and, unexpectedly, have a hyperthermic Tb compared to WT controls.

We then examined diurnal rhythms of Tb according to genotype. Mutant animals had on average higher mean Tb across all times of day (Fig. 4C). We further quantified these results by performing cosinor analyses and found that *Shank3b*$^{-/-}$ animals had a lower Tb amplitude than their WT counterparts (Fig. 4D). Mutation did not affect phase angle, indicating that circadian Tb rhythm is dampened but not shifted (Fig. 4E). We next addressed whether high Tb in mutants was due to changes in physical activity. Surprisingly, solo-housed mutant animals had significantly less physical activity than solo-housed WT animals, while no differences in cumulative physical activity were measured in trio-housed animals (Fig. 4F). Together, these results suggest that *Shank3b* mutation affects circadian Tb rhythms by shifting them upwards and compressing them and that these Tb increases are not due to increased physical activity.

We next investigated the relationship between behavioral states and Tb in *Shank3b*$^{-/-}$ animals. Like WT (Fig. 4G and Fig. 3B–D), mutant solo- and trio-housed animals showed Tb declines during grooming/quiescence and during active and quiescent huddling, respectively, although these declines were generally more significant than those observed in WT mice (Fig. 4G). Despite steeper Tb declines during huddling substates, group-housed *Shank3b*$^{-/-}$ animals maintained higher Tb than wildtypes during all behavioral states, whereas solo-housed mutants showed no difference in Tb compared to WT in any behavioral states (Fig. 3G Between-strain comparison). We next addressed the effect of *Shank3b* mutation on variance in Tb (Tb SD). Solo-housed animals displayed no effect of *Shank3b* on Tb SD for any behavioral state, whereas group-housed *Shank3b*$^{-/-}$ animals showed a much lower SD Tb than wildtypes (Fig. 4H), suggesting that Tb may be at a maximum.

Taken together, these results suggest that group housing induces hyperthermia in *Shank3b*$^{-/-}$ females compared to WT. Although huddling can stabilize and reduce Tb in mutants, they do less of it, and it is insufficient to restore a normal Tb.

Increased huddling in *Trpm8*$^{-/-}$ mutants rescues hypothermic body temperature

We next addressed how two genetic factors with established roles in thermosensation affect social thermoregulation at 23 °C. The cold-sensing menthol receptor *Trpm8* is activated at temperatures of approximately 26 °C, with increasing activation as temperatures decrease to 8 °C[61,62]. *Trmp8*$^{-/-}$ mutants have disrupted thermosensation and thermoregulation and have lower Tb[38,39,63]. The warm-sensing capsaicin receptor *Trpv1* is activated at temperatures >43 °C, a threshold similar to where heat evokes pain[62]. We compared huddling time in *Trpm8*$^{-/-}$[63] and *Trpv1*$^{-/-}$[64] mutant and homozygous WT animals. There was no effect of mutation on active

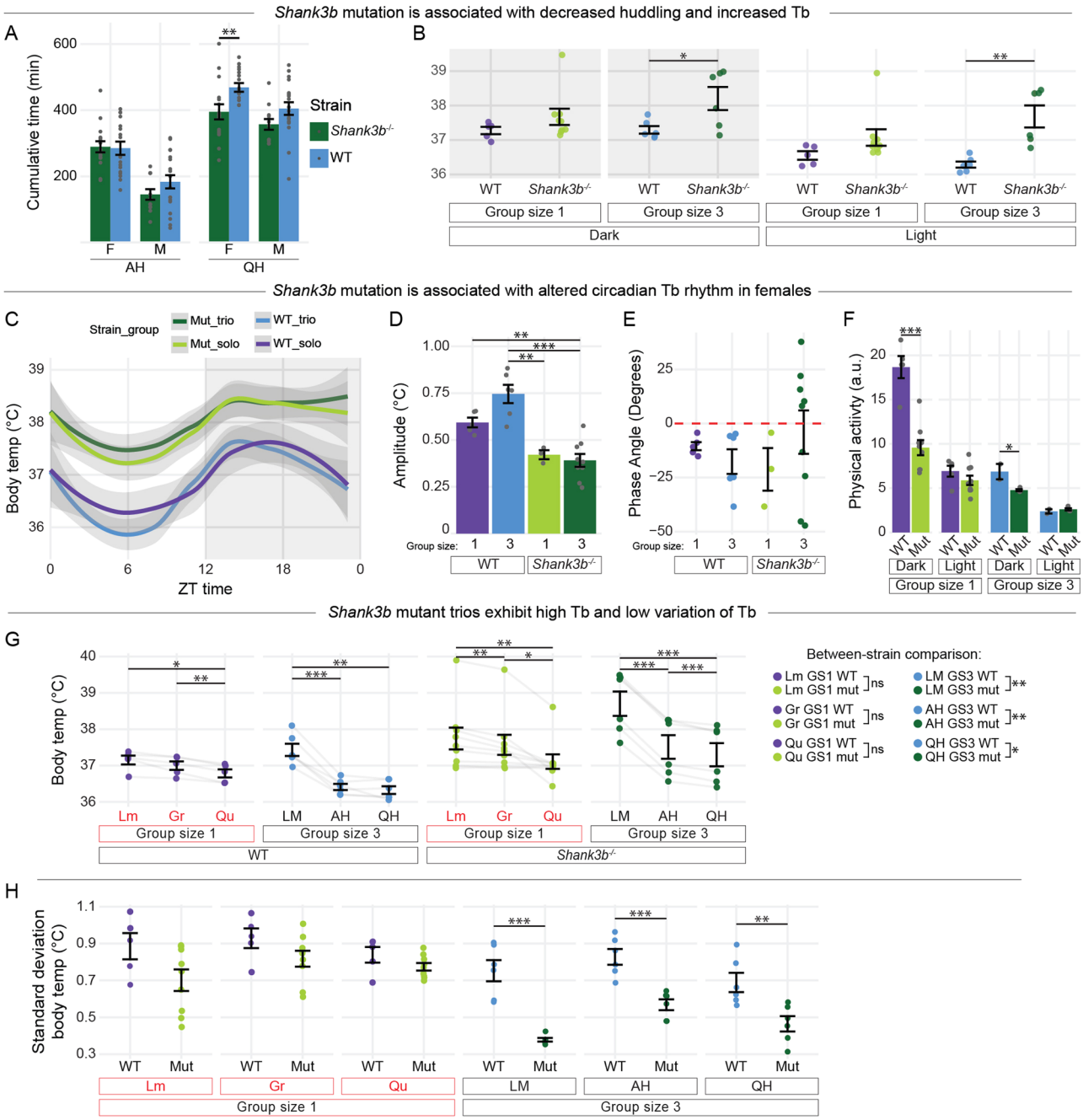


Fig. 4 | *Shank3b*-/- mutation is associated with decreased huddling and increased Tb in females. A Mean cumulative time spent active huddling (AH) or quiescent huddling (QH) according to sex and genotype. N = 24 WT groups (9 male, 15 female), 34 *Shank3b*-/- groups (18 male, 16 female). **B** Mean Tb comparing genotypes across group size and light/dark cycle. **C** Mean Tb of each genotype and group size across ZT. Mutants had significantly higher Tb than WT for both group sizes ($P < 0.001$). **D**, **E** Cosinor analysis of Tb across ZT shows amplitude (**D**) and phase angle (**E**) of each group. **F** Cumulative physical activity according to genotype, group size, and light/dark cycle. $N = 7$ WT groups (5 solo-, 2 trio-housed), 13 *Shank3b*-/- groups (9 solo-, 4 trio-housed). **G** Mean Tb comparing behavioral states across genotype and group size. "Between-strain comparison" shows effect of genotype within the same state and group size. **H** Variance (SD) of Tb comparing genotype differences within each state and group size. Data are mean ± SEM. For **B**–**E** & **G**, **H** $N = 15$ *Shank3b*-/- animals (9 solo-, 6 trio-housed), 11 WT animals (5 solo-, 6 trio-housed). Pairwise comparisons are from Tukey post-hoc test of linear mixed-effects model (**A**, **B**, **G**, **H**) or linear model (**D**–**F**, **G** summary); $P < 0.05$: *, $P < 0.01$: **, $P < 0.001$: ***. The Full statistical analysis is in Table S1.

huddling. In contrast, female and male *Trpm8*$^{-/-}$ mutants showed a significant increase in quiescent huddling (Fig. 5A). These results suggest that the absence of *Trpm8*, but not *Trpv1*, results in altered huddling behavior at 23°C. Subsequent experiments therefore investigated *Trpm8*$^{-/-}$ mutation in females, which display stronger thermoregulatory effects of huddling (Fig. 3).

We analyzed the effect of *Trpm8* mutation in solo- and trio-housed conditions. Trio-housed *Trpm8*$^{-/-}$ and WT females showed equivalent Tb. In contrast, solo-housed *Trpm8*$^{-/-}$ animals displayed a significant decrease in Tb during the light phase of the day (Fig. 5B). We then analyzed the effect of *Trpm8* mutation on diurnal rhythms of Tb and found that solo-housed *Trpm8*$^{-/-}$ animals were notably different from WT (Fig. 5C). Cosinor analysis revealed that solo-housed *Trpm8*$^{-/-}$ animals had a higher Tb amplitude compared to WT (Fig. 5D), indicating an increase in diurnal variation, and significantly lower phase angle than all other groups (Fig. 5E), due to a left shift in their circadian Tb rhythm. These results suggest that

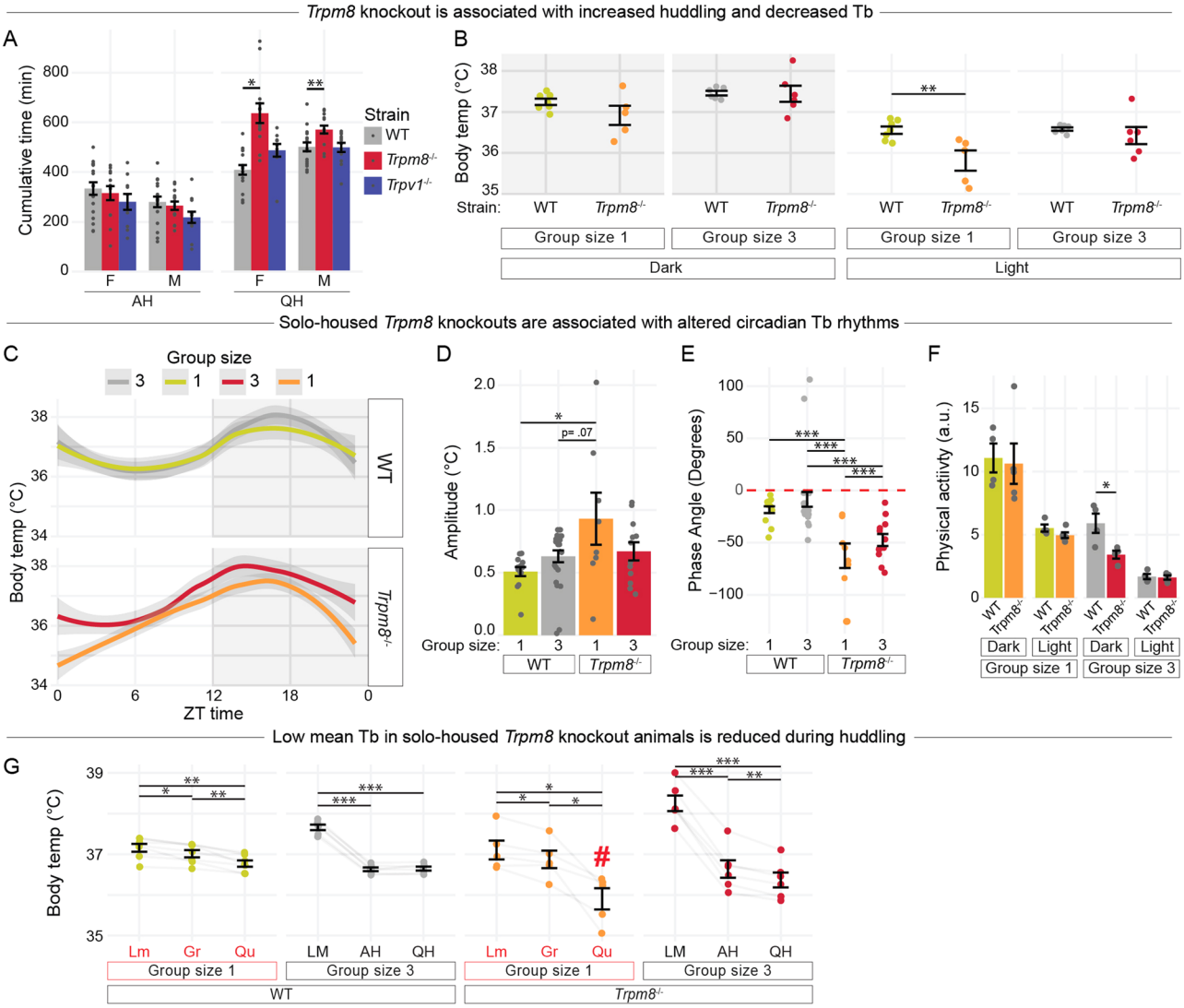


Fig. 5 | Increased huddling in *Trpm8*-/- mutants rescues hypothermic body temperature in females. A Mean cumulative time spent active huddling (AH) or quiescent huddling (QH) according to sex and genotype. $N = 30$ WT groups (15 male, 15 female), 26 *Trpm8*-/- groups (13 male, 13 female) and 20 *Trpv1*-/- groups (10 male, 10 female). **B** Mean Tb comparing genotypes across group size and light/dark cycle. **C** Mean Tb of each genotype and group size across ZT. **D**, **E** Cosinor analysis of Tb across ZT shows amplitude (**D**) and phase angle (**E**) of each group. **F** Cumulative physical activity according to genotype, group size, and light/dark cycle. $N = 7$ WT groups (5 solo-, 2 trio-housed), 13 *Trpm8*-/- groups (9 solo-, 4 trio-housed). **G** Mean Tb comparing behavioral states according to genotype and group size. Red "#" indicates lower body temperature than all other conditions (all P values < 0.05). Data are mean ± SEM. For **B**–**E** & **G**, $N = 11$ *Trpm8*-/- animals (5 solo-, 6 trio-housed), 13 WT animals (7 solo-, 6 trio-housed). Pairwise comparisons are from Tukey post-hoc test of linear mixed-effects model (**A**, **B**, **G**) or linear model (**D**–**F**); $P < 0.05$: *, $P < 0.01$: **, $P < 0.001$: ***. Full statistical analysis is in table S1.

solo-housed *Trpm8*$^{-/-}$ females display both hypothermia and abnormal Tb rhythms compared to trio-housed *Trpm8*$^{-/-}$ and WT females.

We next investigated the relationship between behavioral states and Tb in *Trpm8*$^{-/-}$ animals. Compared to WT, trio-housed *Trpm8*$^{-/-}$ animals exhibited decreased physical activity during the dark. In solo-housed animals, there was no effect of mutation on physical activity, indicating that reduced activity is not responsible for lower Tb in *Trpm8*$^{-/-}$ mutants (Fig. 5F). Next, solo-housed WT females showed a steady decrease in Tb from the highest to lowest activity state (Fig. 5G). However, solo-housed *Trpm8*$^{-/-}$ females displayed an abnormally low Tb during quiescence that was lower than all other conditions examined (Fig. 5G "#", all p-values < 0.05). This was especially evident when looking at individual traces of Tb for solo-housed animals, where there were sudden drops in Tb to nearly 28 °C (Fig. S4A, B), resembling bouts of torpor. These drastic drops in Tb were eliminated when the same animals were group-housed (Fig. S4C). Trio-housed WT and *Trpm8*$^{-/-}$ females also showed similar trends of decreasing Tb at lower activity states (i.e., active and quiescent huddling). These results suggest that, compared to WT, *Trpm8*$^{-/-}$ solo-housed females display abnormally low Tb during quiescence, but normal Tb during huddling when group housed.

We next addressed the effect of *Trpm8* mutation on variance in Tb (Tb SD). For solo-housed animals, wildtypes displayed a reduction in Tb SD during quiescence compared to grooming. In contrast, Tb SD in *Trpm8*$^{-/-}$ solo-housed animals trended upwards during quiescence compared to grooming and locomotion (Fig. S4D), a pattern not observed in other experiments. For trio-housed animals, *Trpm8*$^{-/-}$ again displayed an unusual trend of increasing Tb SD with lower levels of activity, although Tb SD during quiescent huddling was lower than that of active huddling (Fig. S4D).

Taken together, these results suggest that solo-housed *Trpm8*$^{-/-}$ animals have a deficit in maintaining stable Tb and Tb rhythms, especially during quiescence. Because group-housed *Trpm8*$^{-/-}$ animals exhibit increased huddling time and more normal Tbs, this deficit may be rescued by huddling.

Discussion

Adult wild and laboratory rodents use huddling to thermoregulate[1,2,19,20], but also as a form of social interaction[12,14,65–67]. Because laboratory mice are a model organism for the study of energy regulation and social behavior, there is a need to understand the precise details of how huddling relates to body temperature (Tb). Here, we developed a system to quantify natural patterns of huddling behavior and Tb in the home cage of laboratory mice at the resolution of seconds. We identified active and quiescent huddling substates that are associated with distinct thermal profiles. Moreover, we found that these huddling substates are affected by group size, sex, ambient temperature, and the genes *Shank3b* and *Trpm8*.

Our analysis of hundreds of 48-h recordings revealed that huddling is a far more effective thermoregulatory strategy in female groups than in male groups. These findings extend previous reports that, in a cold environment, female pups maintain warmer surface temperatures and have more effective thermoregulatory huddling strategies than male pups[3,28]. We found that adult female Tb was lower at 23 °C (i.e., below thermoneutrality) than at 30 °C (i.e., near the thermoneutral zone). Moreover, solo-housed females at 23 °C had lower Tb, decreased diurnal variability in Tb, and increased physical activity compared to group-housed females. These results are consistent with the observation that solo-housed females have greater thermal conductance and energy expenditure than their group-housed counterparts[30]. We then illuminated how this change in thermal biology was associated with huddling in group-housed females.

At 23 °C, active and quiescent huddling in female groups, but not male groups, was associated with strong decreases in Tb (approximately −0.45 °C). Notably, this Tb was lower than that of quiescence in solo-housed females. These huddling substates are also associated with a drastic reduction in Tb variance. In accordance with this observation, group-housed females, but not males, have lower total energy expenditure than their solo-housed counterparts at 23 °C[30]. Because females huddled less at 30 °C, our results suggest that active huddling at 23 °C is primarily a motivated behavior to thermoregulate and save energy. Conversely, huddling among females at 30 °C suggests possible social functions of this behavior.

Intriguingly, although active huddling in female groups was associated with lower Tb, it was associated with bidirectional changes in Tb. Specifically, active huddling epochs that came immediately before quiescent huddling displayed Tb decreases, whereas epochs immediately after quiescent huddling displayed Tb increases. This further suggests that active huddling is a motivated thermoregulatory behavior that, when aligned with other physiological processes (e.g., brown fat thermogenesis and cardiovascular pathways), facilitates significant modulation of Tb.

Taken together, our results suggest that sustained physical contact among females at 23 °C facilitates rapid thermoregulatory responses. This observation has important implications for the study of prosocial interactions because physical contact will result in heat exchange through conduction, increased insulation, and a reduction in the surface area to volume ratio of each individual[2,68].

We investigated *Shank3b* on the premise that humans and mice with mutations in this gene show deficits in social behavior[34,58,59] and predicted that *Shank3b* mutants would huddle less than wildtypes. Indeed, *Shank3b*$^{-/-}$ female, but not male, groups spent less time quiescent huddling than wildtypes. Surprisingly, despite being hypoactive, group-housed Shank3b$^{-/-}$ females were characterized by low-variance, hyperthermic Tb compared to wildtypes. This result suggests that group-housed *Shank3b*$^{-/-}$ females have disrupted thermal physiology and may near a Tb "ceiling". The hyperthermia we observed in group-housed *Shank3b* mutants might indicate that these animals experience psychosocial stress associated with being housed together. Psychosocial stress is associated with elevated Tb in mice and humans[31,69–72], and many neuropsychiatric disorders, including ASD, are linked to changes in efferent[31] and afferent thermoregulatory pathways[73]. Considering *Shank3b* mutants are an animal model of ASD[32], and that around half of individuals with ASD experience social anxiety[74–76], it is possible that *Shank3b* mutants experience social stress-induced hyperthermic Tb.

Although huddling in group-housed *Shank3b*$^{-/-}$ mice caused robust decreases in Tb and Tb-variance, these mutants spent less time quiescent huddling, and, as a result, less time in a low-Tb state. These results support the notion that *Shank3b*$^{-/-}$ mice have social deficits but illustrate new associations with elevated Tb and impairments in huddling, suggesting that studies of rodent models of ASD should consider how thermoregulatory changes might interact with and contribute to social deficits. For example, mutation of the oxytocin gene in mouse pups results in reduced BAT thermogenesis and less cohesive huddling[77].

We investigated *Trpm8* and *Trpv1* on the premise that these genes are associated with thermosensation[39,63] and thermoregulation[38]. Consistent with the observation that *Trpm8* deletion increases heat loss and reduces Tb[38], we found that, compared to wildtypes, solo-housed *Trpm8*$^{-/-}$ females displayed hypothermic Tb. Surprisingly, some animals even had Tb resembling torpor, with Tb reaching below 29 °C, despite having *ad libitum* food. Intriguingly, these deficits were attenuated in a group-housed setting. Solo-housed *Trpm8*$^{-/-}$ females had hypothermic Tb during quiescence, whereas group-housed *Trpm8*$^{-/-}$ females had normal Tb during quiescent huddling. These observations suggest that housing density is an important consideration for studies of *Trpm8* mutants. Furthermore, investigation of social thermoregulation as a mechanism of coping with thermoregulatory dysfunctions in animal models is warranted.

Active and quiescent huddling substates at standard room temperature (23 °C) are powerful and dynamic thermoregulatory behaviors for group-housed females. Studies of rodent social behavior are often conducted at room temperature, including studies of social homeostasis, or the ability of individuals to detect and regulate the quantity of social connections[78]. Our observation that mice are more likely to make physical contact at room temperature to thermoregulate suggests that both internal and external temperature should be an important experimental design consideration. Finally, our findings reveal mutations in *Shank3b* and *Trpm8*—two genes commonly used in studies of social interaction and energy balance, respectively—are associated with alterations in social thermoregulation. This study contributes to the idea that thermoregulation can be an important regulator of social interaction[77].

Limitations of the study

In this study, we examine how core body temperature (Tb) is associated with huddling substates in wildtype and mutant animals. Although we identify changes in Tb, our study does not test which thermoregulatory effector pathways (e.g., brown adipose fat thermogenesis and vasodilation) drive these changes. One possible limitation of the study is that we arbitrarily set thresholds on activity level to define categorical behavioral states in group-housed and solo-housed animals. However, the fact that we could identify distinct thermal states for all of these states lends support to the notion that they are in fact distinct. Although we find that *Shank3b* mutation is associated with both a decrease in huddling and an increase in Tb when animals are group housed, our study does not investigate whether the increase in Tb is a driver of decreased huddling. Although this study used a longitudinal design to examine thermal profiles of the same individuals in different housing contexts with and without siblings, this required that animals were isolated for 72 h at a time, which may have introduced physiological or behavioral changes. To mitigate these possible changes, mice were returned to their home cage for four days between experiments. Nevertheless, we did not determine how this cage rotation design affected the animals per se. Finally, it should be noted that for some experiments the sample size was rather low (e.g., 3 or 4 animals per experiment), and some conclusions could be due to insufficient statistical power.

Data availability

Raw data can be found on Mendeley. https://data.mendeley.com/preview/nwyfsn2bym?a=91f3a487-6543-490d-8176-444eaa9b0a8a.

Code availability

Code can be found on Mendeley. https://data.mendeley.com/preview/g29xwvhszj?a=02f3bf00-ad8b-42d3-893b-e01dcef1e4bd.

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Led the investigation: J.G.L. and A.C.N. Conceived and performed experiments: J.G.L., M.V., S.K., N.L.B., A.C.N. Wrote the manuscript: J.G.L and A.C.N. Secured the funding: A.C.N. and N.L.B. Provided expertise and feedback: A.C.N. and N.L.B.

Competing interests

The authors declare no competing interests.

Additional information

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