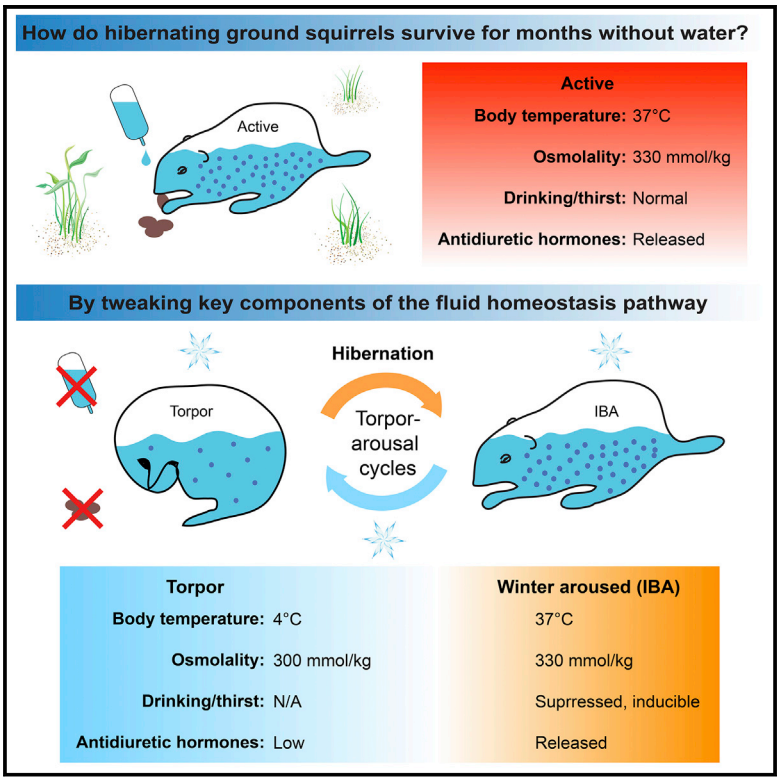
Report

Current Biology

Osmolyte Depletion and Thirst Suppression Allow Hibernators to Survive for Months without Water

Graphical Abstract



Highlights

- Hibernating squirrels employ several strategies to survive for months without water
- Hibernating squirrels have decreased blood osmolality despite water deprivation
- Basal thirst is inhibited in ground squirrels during hibernation
- Antidiuretic hormonal release and water seeking are uncoupled during hibernation

Authors

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In Brief

Feng et al. reveal strategies that allow hibernating ground squirrels to survive for months without water, including depletion of blood osmolytes, inhibition of thirst, and uncoupling of the circuits for antidiuretic hormonal release and water-seeking. These strategies help avoid the potentially detrimental drive to leave the burrow to seek water.

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CellPress

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Report

Osmolyte Depletion and Thirst Suppression Allow Hibernators to Survive for Months without Water

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SUMMARY

Thirteen-lined ground squirrels (*Ictidomys tridecemlineatus*) are obligatory hibernators who can survive over 6 months of the year in underground burrows or laboratory hibernaculum without access to food or water [1]. Hibernation consists of prolonged periods of torpor, lasting up to 18 days, which are characterized by low body temperature and suppressed metabolism. This torpidity is interspersed with short periods of interbout arousal, lasting up to 48 h, during which squirrels temporarily return to an active-like state and lose small amounts of water to urination and evaporation [2]. Water is also lost during torpor due to a positive vapor pressure difference created by the slightly higher temperature of the body compared to its surroundings [2, 3]. Here, we investigate the physiological mechanism of survival during prolonged water loss and deprivation throughout hibernation. By measuring hydration status during hibernation, we show that squirrels remain hydrated during torpor by depleting osmolytes from the extracellular fluid. During brief periods of arousal, serum osmolality and antidiuretic hormone levels are restored, but thirst remains suppressed. This decoupling of thirst and diuresis enables water retention by the kidney while suppressing the drive to leave the safety of the underground burrow in search of water. An acute increase in serum osmolality reinstates water-seeking behavior, demonstrating preservation of the physiological thirst circuit during hibernation. Better mechanistic understanding of internal osmolyte regulation and thirst suppression could translate to advancements in human medicine and long-term manned spaceflight.

RESULTS AND DISCUSSION

To understand how squirrels cope with months of water deprivation, we characterized changes in hydration status by measuring serum osmolality in active, prehibernation-torpor, torpor, and interbout arousal (IBA) states (Figures 1A and 1B). Blood osmolality is tightly linked to fluid homeostasis and is regulated by a physiological circuit that includes the subfornical organ (SFO), hypothalamus, pituitary gland, and kidney (Figure 1C) [4–7]. In mice, multiple hours of water deprivation can increase serum osmolality by 1%–2%. SFO neurons detect this increase and trigger both water-seeking behavior and the release of antidiuretic hormones to stimulate water retention by the kidney. We found that, despite not having access to water over the period of several months, squirrels undergo state-dependent changes in serum osmolality (Figure 1D). Compared to active animals, torpid squirrels experience a significant (~10%) drop in serum osmolality (334.2 ± 2.6 mmol/kg in active; 305.2 ± 1.9 mmol/kg in torpor; mean ± SE; Tukey's HSD, $p < 0.0001$; Figure 1D). A similar but less dramatic decrease in serum osmolality was observed in hibernating woodchucks [8] and black-tailed prairie dogs [9]. Osmolality starts to decrease during preparation for hibernation in prehibernation-torpor animals ($p = 0.01$) and returns to active levels during IBA (320.7 ± 3.4 mmol/kg in prehibernation-torpor, 331.5 ± 3.0 mmol/kg in IBA; $p < 0.0001$). We found no correlation between serum osmolality and the number of days spent in hibernation, the number of IBAs experienced across the hibernation season, or the length of torpor before blood collection (Figures S1A and S1B). Expectedly, there is a correlation between core body temperature and osmolality when states are grouped together. However, we found no correlation within each state, including the prehibernation-torpor state when the core body temperature range at the time of sacrifice was the largest (Figure S1C). These results strongly suggest that the prevailing physiological state, rather than body temperature, is a primary determinant of serum osmolality. Our data reveal that ground squirrels undergo cyclic changes between normal and increased hydration throughout the entire period of hibernation by relying exclusively on reversible internal mechanisms.

Mammals can prevent dehydration with anticipatory drinking [10], increased renal water retention [11, 12], and elevated metabolic water production [13]. We investigated whether these strategies could account for state-dependent changes in osmolality across the hibernation cycle. To test whether blood dilution in torpor is driven by increased water intake due to anticipatory thirst in preparation for hibernation, we video-monitored drinking

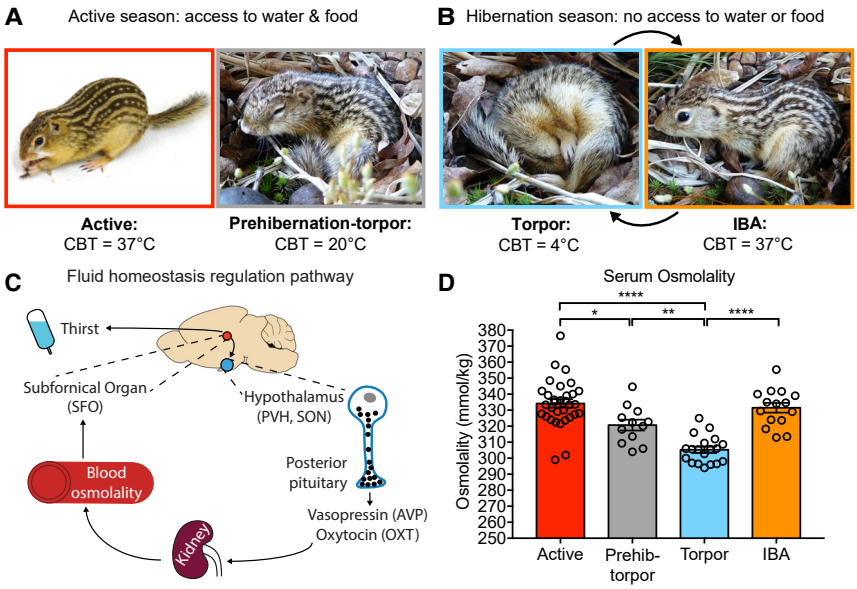

Figure 1. Torpid Squirrels Decrease Serum Osmolality Despite Water Deprivation

(A and B) Images of thirteen-lined ground squirrels in active and prehibernation-torpor (prehib-torpor) (A) and torpor and IBA (B) states. CBT, core body temperature. Photos courtesy of the Gracheva lab.

(C) A diagram of the physiological circuits regulating fluid-ionic balance. PVH, paraventricular hypothalamus; SON, supraoptic nucleus.

(D) Serum osmolality across physiological states (mean ± SEM). One-way ANOVA: a significant main effect of state ($F_{3,65} = 17.06$; $p < 0.0001$).

Each data point represents measurement from an individual animal. $n \geq 12$ per state. *$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$, Tukey's multiple comparisons test. See also Figure S1.

behavior over 24 h periods in active and prehibernation states. We found that prehibernation squirrels exhibited decreased, rather than increased drinking, suggesting the decrease in serum osmolality was not due to dilution by increased water intake. The duration of each drinking bout remained the same in active and prehibernation states, but prehibernation squirrels decreased the number of times they drank (linear mixed model, $p = 0.0028$) and the overall drinking time per hour (linear mixed model, $p = 0.026$) (Figure 2A).

Next, we tested whether decreased serum osmolality in torpor results from increased water retention due to elevated production of the antidiuretic hormones arginine-vasopressin (AVP) and oxytocin (OXT) [4, 14, 15]. We detected significant changes of plasma AVP and OXT between all states (Kruskal-Wallis test, $p = 0.0013$ for AVP, $p < 0.0001$ for OXT). However, plasma levels of AVP and OXT decreased, rather than increased, during torpor (Dunn's multiple comparisons test, $p = 0.0005$ for AVP, $p < 0.0001$ for OXT), often to below the assay detection limit (Figure 2B). As with blood osmolality, plasma levels of both hormones returned to active levels during IBA (Figure 2B). These results are consistent with reduced kidney function during torpor, when glomerular filtration reaches a minimum [16] and renders AVP and OXT action unnecessary. The antidiuretic actions of AVP and OXT are more likely needed during IBA, when blood pressure and kidney function return to active levels to produce and excrete urine [16]. In contrast to the plasma, pituitary peptide content did not change significantly across torpor-IBA cycles (Figure 2C). Due to the short half-life of AVP and OXT in circulation (on the order of minutes) [17], the inhibition of peptide release from the posterior pituitary during torpor is a likely mechanism for the decrease in plasma peptide levels. In summary, the observed decrease in blood osmolality in torpor is due neither to increased drinking in preparation for hibernation, nor AVP-OXT-dependent water retention.

Consistent with the idea that white adipose tissue (WAT) is the main energy source during hibernation (the respiratory quotient falls to 0.7 during torpor) [18–20], we detected a significant increase in plasma levels of β-hydroxybutyrate in torpor and IBA animals (Figure S2A). Because the metabolism of 100 g of fat yields ~110 g of water [9, 21–23], we calculated whether fat metabolism could theoretically produce enough water to cause the observed drop in plasma osmolality [9]. Assuming 50% body water, a squirrel weighing 275 g (Figure S2B) would need to metabolize 12.5 g WAT to reduce osmolality by 30 mmol/kg during each torpor-IBA cycle at the beginning of hibernation, and 7.5 g WAT during each torpor-IBA cycle at the end of hibernation due to a 40% body volume decrease (Figure S2C). An average of 200 g WAT would need to be metabolized for 20 torpor-IBA cycles (Figure S2D). However, it has been observed that thirteen-lined ground squirrels are 60% WAT by weight as they enter hibernation and only lose 30% of their WAT during hibernation, equivalent to ~50 g for a 275 g squirrel [23]. Further, we found that some of the metabolically produced water is lost during each IBA, mostly via urine excretion (Figure S2E) [2]. In addition, we found no evidence for a significant change in blood volume, which is inversely correlated with total protein levels across states (Figure S2F). Consequently, global dilution via WAT metabolism is insufficient to account for the observed decrease in osmolality during torpor [13, 24–26].

To assess whether changes in osmolality across states are dependent on the internal regulation of major osmolytes, we measured serum levels of inorganic ions, glucose, blood urea nitrogen (BUN), and lactate. Sodium, glucose, and BUN are the main osmolytes that contribute to measured plasma osmolality and are used clinically to calculate osmolality. Consistent with the hypothesis that the apparent hydration in torpor is due to depletion of osmolytes from the extracellular fluid, we observed a significant decrease in serum levels of Na^+, K^+, BUN, and lactate during torpor (Figure 3). The levels of Na^+, K^+, and lactate rebounded back to active levels during IBA. Levels of Ca^{2+} and Mg^{2+} increased in prehibernation-torpor but remained stable across other states. Glucose concentration did not change across states (Figure 3). The state-dependent changes in osmolytes found in our study suggest that each is under independent regulation, affirming that

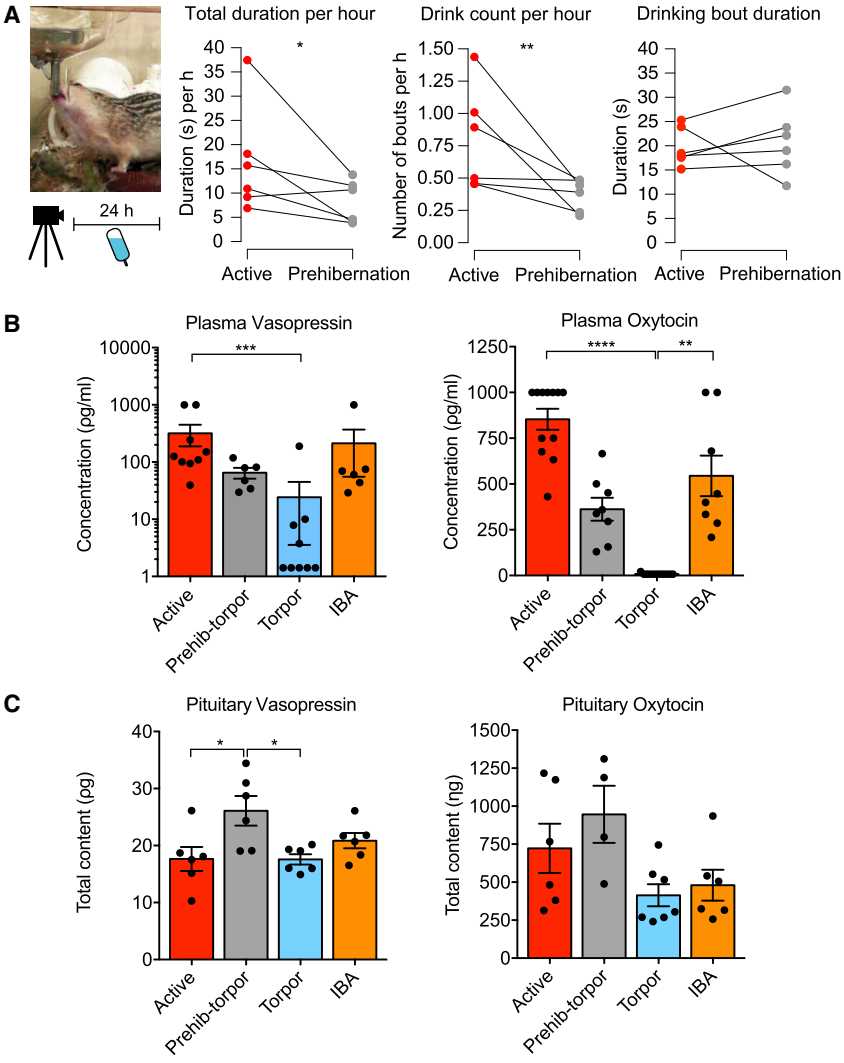

Figure 2. Decreased Blood Osmolality Is Not Due to Increased Water Intake or Retention

(A) Drinking behavior in each squirrel was recorded over 24 h periods in active and prehibernation states (n = 6 in each state). Data points represent daily means for each squirrel filmed over multiple days. *p < 0.05; **p < 0.01, linear mixed model.

(B) Plasma levels of vasopressin (left panel) and oxytocin (right panel) across physiological states. **p < 0.01; ***p < 0.001; ****p < 0.0001, Kruskal-Wallis one-way ANOVA with Dunn's correction for multiple comparisons. n ≥ 6 in each state.

(C) Whole pituitary content of vasopressin (left panel) and oxytocin (right panel) across physiological states. n ≥ 4 in each state. In (B) and (C), each dot represents measurement from an individual animal. *p < 0.05, one-way ANOVA. Prehib-torpor, prehibernation torpor. See also Figure S2.

the decrease in serum osmolality during torpor is not due to a global dilution effect. Instead, our data strongly suggest that hydration status is maintained during torpor by reversible depletion and redistribution of major osmolytes from the extracellular fluid, potentially into body compartments, such as the bladder, as previously shown for hibernating bears [27].

Thirst is a powerful instinct that drives water-seeking behavior [28, 29], but squirrels do not leave the safety of the underground burrow for the entire duration of the hibernation season. This suggests that thirst is suppressed even during IBA, when blood osmolality increases by 10% to a level that induces basal drinking in active animals (Figure 4A). To test this hypothesis, squirrels were provided with access to water during IBA and video-monitored for 24 h. We found that some IBA squirrels did not drink at all and others exhibited only minimal drinking behavior, both in terms of the number and duration of each drinking bout (Figure 4A). Thus, IBA animals do not experience thirst despite months of water deprivation and serum osmolality resembling that of active animals. Importantly, the decrease in basal thirst contrasts with active levels of AVP and OXT during IBA (Figure 2B), suggesting that the pathways that induce water seeking and release of antidiuretic hormones in response to elevated osmolality [30] are decoupled during hibernation.

To test whether the thirst circuit retains functionality during IBA, we acutely increased blood osmolality in active and IBA squirrels by intraperitoneal injection of a hypertonic solution (3 M NaCl or 1.18 M mannitol) and video-monitored drinking behavior for 2 h (Figure 4B). Additionally, we water deprived active squirrels for 24 h and video-monitored drinking behavior for 2 h after the return of water to measure chronically induced thirst (Figure 4B). Both hypertonic solution injection and water deprivation in active squirrels resulted in elevation of blood osmolality by ~30 mmol/kg (Figure S3) and significantly increased drinking behavior (Figures 4B and 4C). Similarly, hypertonic solution injection strongly stimulated drinking behavior in IBA squirrels. Although the duration of each drinking bout was shorter in IBA squirrels, there was compensation by an increased number of drinking bouts such that total drinking duration was the same in IBA and active squirrels injected with 3 M NaCl (Figure 4B). Thus, our data show that thirst can be induced during IBA, suggesting functional preservation of this physiological circuit during hibernation (Figures 4B and 4C).

Conclusions

Homeostatic pathways regulating fluid balance are highly conserved and essential for survival. We have shown how ground squirrels who experience months of water deprivation are able to avoid dehydration and escape the drive of thirst during hibernation, even though the thirst circuitry remains functional and sensitive to perturbations in fluid balance. When serum osmolality returns to active levels during IBA, the levels of AVP and OXT increase too, whereas baseline thirst remains suppressed. This reveals that when the animal

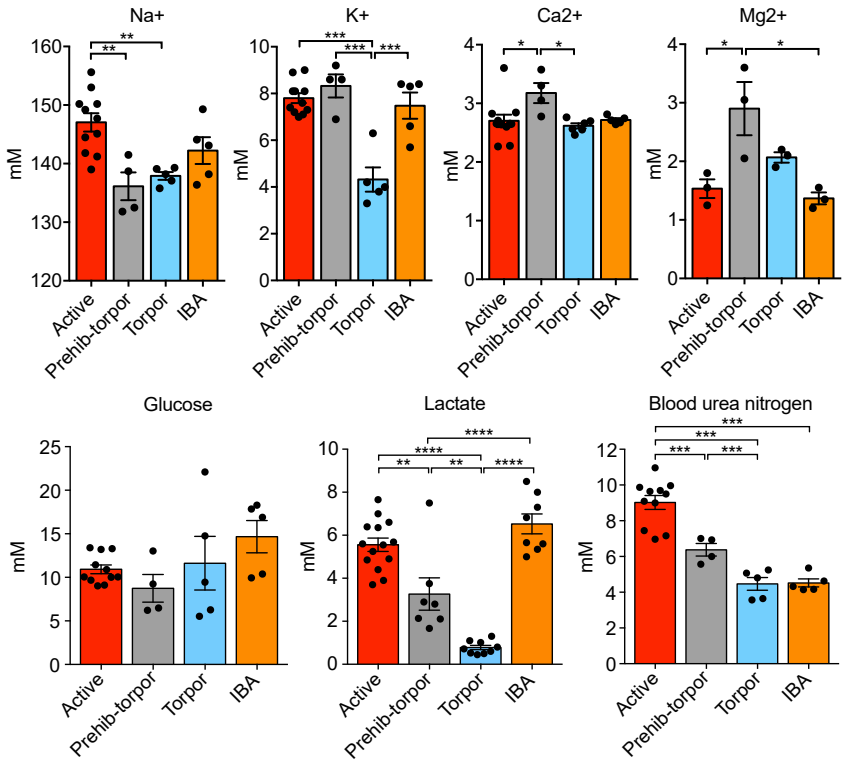

Figure 3. Internal Regulation of Serum Electrolytes and Metabolites across States

Concentrations of serum electrolytes and metabolites across states (mean ± SEM). *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001, one-way ANOVA and Tukey's multiple comparisons test. Each data point represents measurement from an individual animal; n ≥ 3 per analyte for each state. Prehib-torpor, prehibernation torpor. See also Figure S2.

enters a transient active-like state, the neural pathway controlling antidiuretic hormone release becomes functional, but the pathway that controls thirst remains suppressed. As a result, squirrels preserve the capability of retaining water by the kidney while avoiding the aversive drive of thirst so that they can remain in the safety of the underground burrow. One possibility is that osmolytes accumulate in the bladder and return to circulation during IBA by reabsorption via the urothelium, as previously shown in hibernating bears [27]. However, the exact mechanism of how hibernators cyclically redistribute osmolytes between body compartments, how the generation of thirst is suppressed at the level of neural circuits, and how cells and tissues of hibernators cope with the large changes in serum osmolality warrant future investigation using tools currently employed for standard animal models. It will also be interesting to determine whether changes in body fluid composition act as a global signal that prepares cells and major physiological systems for hibernation. Mechanistic insights gleaned from hibernators shed light on how fundamental physiological processes are tuned to allow life to persist and thrive under conditions that are currently not tolerated by humans and other non-hibernators.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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  - Drinking behavior
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- DATA AND CODE AVAILABILITY

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.cub.2019.07.038.

A video abstract is available at https://doi.org/10.1016/j.cub.2019.07.038#mmc3.

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AUTHOR CONTRIBUTIONS

N.Y.F., E.O.G., and S.N.B. conceptualized the study. N.Y.F., M.S.J., E.O.G., and S.N.B. designed and performed experiments. N.Y.F., M.S.J., E.O.G.,

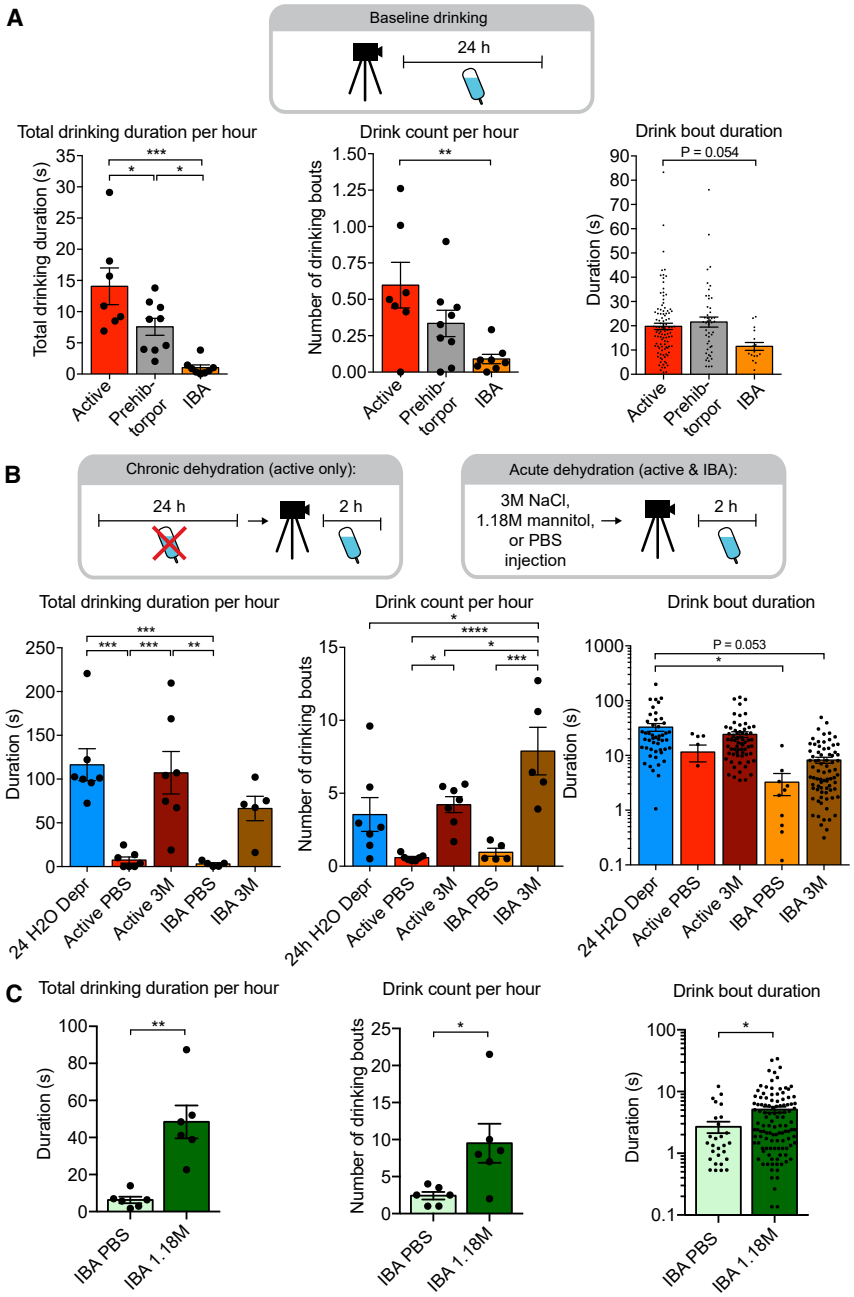

Figure 4. Baseline Thirst Is Reduced in IBA but Can Be Induced by Acute Dehydration

(A) Drinking behavior in active, prehibernation, and IBA squirrels recorded over 24 h. Each dot represents data from an individual animal (left and middle panel) or the duration of a single drinking bout (right panel), n ≥ 7 per treatment condition.

(B) Drinking behavior of squirrels in the indicated states video monitored for 2 h after 24 h water deprivation or injection with 3 M NaCl (3M) or phosphate-buffered saline (PBS). Each dot represents data from an individual animal (left and middle panel) or the duration of a single drinking bout (right panel), n ≥ 5 per treatment condition.

(C) Drinking behavior of IBA squirrels monitored for 2 h after injection with 1.18 M mannitol (1.18 M) or PBS. Each dot represents data from an individual animal (left and middle panel) or the duration of a single drinking bout (right panel), n = 6 per treatment condition.

Data are shown as mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001 by one-way ANOVA with Tukey's multiple comparison's test (left and middle panels in A, B) or t test (left and middle panels in C) or linear mixed model (right panels in A–C). See also Figure S3.

and S.N.B. collected and analyzed data. N.Y.F. and M.S.J. provided data visualization. D.K.M. supplied squirrels and provided advice on animal husbandry. N.Y.F., S.N.B., and E.O.G. wrote the manuscript with contributions from M.S.J. and D.K.M. E.O.G. and S.N.B. provided guidance and supervision throughout the project.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, Peptides, and Recombinant Proteins		
Aprotinin	Millipore Sigma	Cat# 9087-70-1
Critical Commercial Assays		
Oxytocin ELISA kit	Enzo Life Sciences, Inc.	Cat# ADI-901-153A-0001
Vasopressin ELISA kit	Enzo Life Sciences, Inc.	Cat# ADI-900-017A
Experimental Models: Organisms/Strains		
Thirteen-lined ground squirrel: *Ictidomys tridecemlineatus*	University of Wisconsin Oshkosh	N/A
Software and Algorithms		
Prism 7.0	GraphPad	RRID:SCR_002798
MATLAB	MathWorks	RRID:SCR_001622
R Project for Statistical Computing version 3.4.4	R Project for Statistical Computing	RRID:SCR_001905
Python Programming Language	Python Programming Language	RRID:SCR_008394
VLC Media Player with "Jump to time (Previous frame)" extension	VideoLAN Organization	https://www.videolan.org/vlc/index.html
Other		
Clinical Metabolism Core	Yale University	https://medicine.yale.edu/intmed/drc/cores/metabolism.aspx
Magnesium Test	Antech Diagnostics	Cat# T170
IPTT-300 Temperature Transponder	Bio Medic Data Systems	https://bmds.com/
Vapro 5600 Vapor Pressure Osmometer	Wescor, Inc.	https://www.elitechgroup.com/benelux/product/vapro-vapor-pressure-osmometer/
Spectramax 384 Plus plate reader	Molecular Devices	www.moleculardevices.com

LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Elena Gracheva (elena.gracheva@yale.edu). This study did not generate new unique reagents.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

All animal procedures were performed in compliance with the Office of Animal Research Support of Yale University (protocol 2018-11497). Thirteen-lined ground squirrels were housed in temperature and humidity controlled facilities (hibernaculum) at Yale University. During the active season (Summer-Fall), animals were held in a vivarium with room temperature of 18-20°C, a photoperiod of 12h:12h light:dark, and maintained on a diet of dog food (Iams) supplemented with sunflower seeds, superworms, and fresh vegetables, with *ad libitum* access to water. During the hibernation season, hypothermic animals are moved to a hibernaculum with 2-4°C room temperature, constant darkness (except for red light during temperature measurements or behavioral monitoring), and 50%–60% humidity. All squirrels were implanted with a temperature transponder (BMDS). In this study, "active" squirrels are those who hold a constant core body temperature (CBT) of ~37°C held in the vivarium, "prehibernation" squirrels are those who experience a drop in their CBT to 20-23°C at the start of video monitoring (see below), and "prehibernation-torpor" squirrels are those whose CBT was measured to be 20-23°C in the vivarium within 1 h of sacrifice (at the time of sacrifice, CBT was 24 ± 0.8°C, mean ± SE). Torpor squirrels are those whose CBT in the hibernaculum are below 5°C, while IBA squirrels are those whose CBT in the hibernaculum are above 35°C for at least 1.5 h. Active squirrels were collected from June to October; prehibernation and prehibernation-torpor squirrels were collected from August to December; torpor squirrels were collected mainly from August to February; and IBA animals were collected from August to March. Both males and females were used in these studies and combined in analyses as fluid homeostasis is essential for the basic survival of both sexes.

METHOD DETAILS

Serum, plasma, and pituitary collection

Animals were euthanized by CO2 and decapitated with a guillotine. 2 mL of trunk blood was first collected in K2 EDTA tubes (Fisher Scientific, Waltham, MA), according to standard procedures used in the field [31, 32] with 50 μL of 5 mg/ml aprotinin (Sigma). Plasma was collected by spinning at 4°C for 15 min at 1600xg, supernatant removed, and flash frozen on dry ice, and stored in −80°C until use for hormone measurement. The rest of the trunk blood was collected and allowed to coagulate at room temperature for 30 min before spinning at 4°C for 15 min at 2000xg. Serum was removed, 30 μL was used for measurement of osmolality on a Wescor EliTechGroup Vapro 5600 Vapor Pressure Osmometer (Wescor Inc., Locan, UT) and the rest was flash frozen on dry ice and stored at −80°C until use for measurement of osmolytes. The pituitary was removed with fine forceps under a light microscope from the base of the skull after removing the rest of the brain, and stored in eppendorf tubes at −80°C until use.

Plasma vasopressin and oxytocin measurement

Plasma vasopressin (AVP) and oxytocin (OXT) levels were measured by enzyme linked immunoassay (ELISA) kits (Enzo Life Sciences, Inc., Farmingdale, NY). 500 μL of plasma from each animal was extracted using 100 mg C18 Sep-Pak columns (Waters Corporation, Milford, MA) on a vacuum manifold (Waters Corporation) following procedures outlined in Cool and DeBrosse, 2003 (Cool and DeBrosse, 2003). Briefly, plasma was thawed on ice, combined with 500 μL 1% Trifluoroacetic acid (TFA), and centrifuged at 4°C for 20 min at maximum speed 15,000 rpm. Columns were activated with 500 μL methanol and washed three times with 1 mL 1% TFA. 1mL of the spun plasma sample was loaded into columns and flowed through slowly over ~2 min. The OXT fraction was eluted and collected with 3 mL 98% acetone, then the AVP fraction was eluted and collected with 3 mL 80% containing 0.1% TFA (v/v). Collected fractions were evaporated to dryness under nitrogen. For AVP ELISA, 500 μL of the assay buffer was added to the dried fraction and the rest of the assay followed the manufacturer's instructions. For OXT ELISA, the dried fraction was either stored at −20°C until use or assayed immediately following manufacturer's instructions. All standards and samples were run in duplicate. Optical density at assay-specific wavelengths were read by a Spectramax 384 Plus plate reader (Molecular Devices). The standard curves were fit with 4 parameter logistic regression in GraphPad Prism 7.0 (GraphPad Software). Values above the standard curve limit were assigned with the maximum value (1000 pg/ml). The samples that were below the detection limit of the assay were assigned a value of 1/2 of the manufacturer's reported detection limit. A non-parametric Kruskal-Wallis test followed by Dunn's multiple comparisons test was performed using GraphPad Prism 7.0 to assess state-dependent differences in AVP and OXT.

Pituitary AVP and OXT measurement

In order to extract and measure AVP and OXT from the pituitary, 200 μL of the appropriate ELISA assay buffer and 20 μL of aprotinin were added to each tube, and the pituitary was mechanically dissociated on ice using a pestle (Corning Inc., Corning, NY). The rest of the assay followed the manufacturer's instructions. One-way ANOVA was performed using GraphPad Prism 7.0 to assess state-dependent differences in pituitary AVP and OXT content. For OXT, we diluted initial dissociated pituitary 10,000 times to stay within assay detection limits.

Drinking behavior

Baseline drinking was measured by continuous video monitoring over 24 h periods in active, prehibernation (CBT at room temperature of ~20-23°C at the start of video recording), and IBA (CBT above 35°C for at least 1.5 h in the hibernaculum at the start of video recording) states. Food and water was provided to active and prehibernation animals in the vivarium *ad libitum*. While IBA animals normally do not have access to food or water in the hibernaculum, water was provided to animals *ad libitum* during video recording experiments. Videos were captured by Microsoft LifeCam Studio 1080p HD Webcam at the lowest frame rate (7.5 FPS) and written to disk at 8X real time speed using MATLAB. Raw video analysis for occurrence of drinking bouts was performed manually using VLC Media Player with Jump to time (Previous frame) extension. Total drinking duration, duration of each drinking bout, and the number of drinking bouts normalized by length of video recording (lights on period for vivarium animals) was calculated using Python. Animals from 24 h water deprivation and intraperitoneal injection experiments were video monitored for 2 h post treatment.

Serum measurements

Serum analyses for Na^{+}, Ca^{2+}, K+, glucose, lactate, and blood urea nitrogen (BUN) were performed by the Clinical Metabolic Core at Yale School of Medicine. Serum analyses for Mg2+ were performed by Antech Diagnostics (Fountain Valley, CA).

Intraperitoneal injections

Active and IBA animals were immobilized with decapicones, weighed, and subjected to intraperitoneal injections of vehicle (PBS), 3 M NaCl using an injection volume of 4.5 μl/g body weight. For mannitol experiments, IBA animals were immobilized with decapicones, weighed, and subjected to intraperitoneal injections of PBS or 1.18 M mannitol using an average injection volume of 10.8 μl/g body weight.

QUANTIFICATION AND STATISTICAL ANALYSES

Statistical analyses were performed in GraphPad Prism and R (version 3.4.4). When multiple measurements were taken per animal as in the drink bout duration comparisons, linear mixed model was performed with animal as a random effect and state or treatment as main effects in R using the lme4 package and subsequent pairwise comparisons were performed using the lsmeans package. Tests were chosen based on data distribution and experimental design, and detailed accordingly in the Results section and figure legends. Sample sizes for each experiment are noted in figures and figure legends.

DATA AND CODE AVAILABILITY

This study did not generate/analyze datasets.

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Supplemental Information

Osmolyte Depletion and Thirst Suppression Allow Hibernators to Survive for Months without Water

Ni Y. Feng, Madeleine S. Junkins, Dana K. Merriman, Sviatoslav N. Bagriantsev, and Elena O. Gracheva

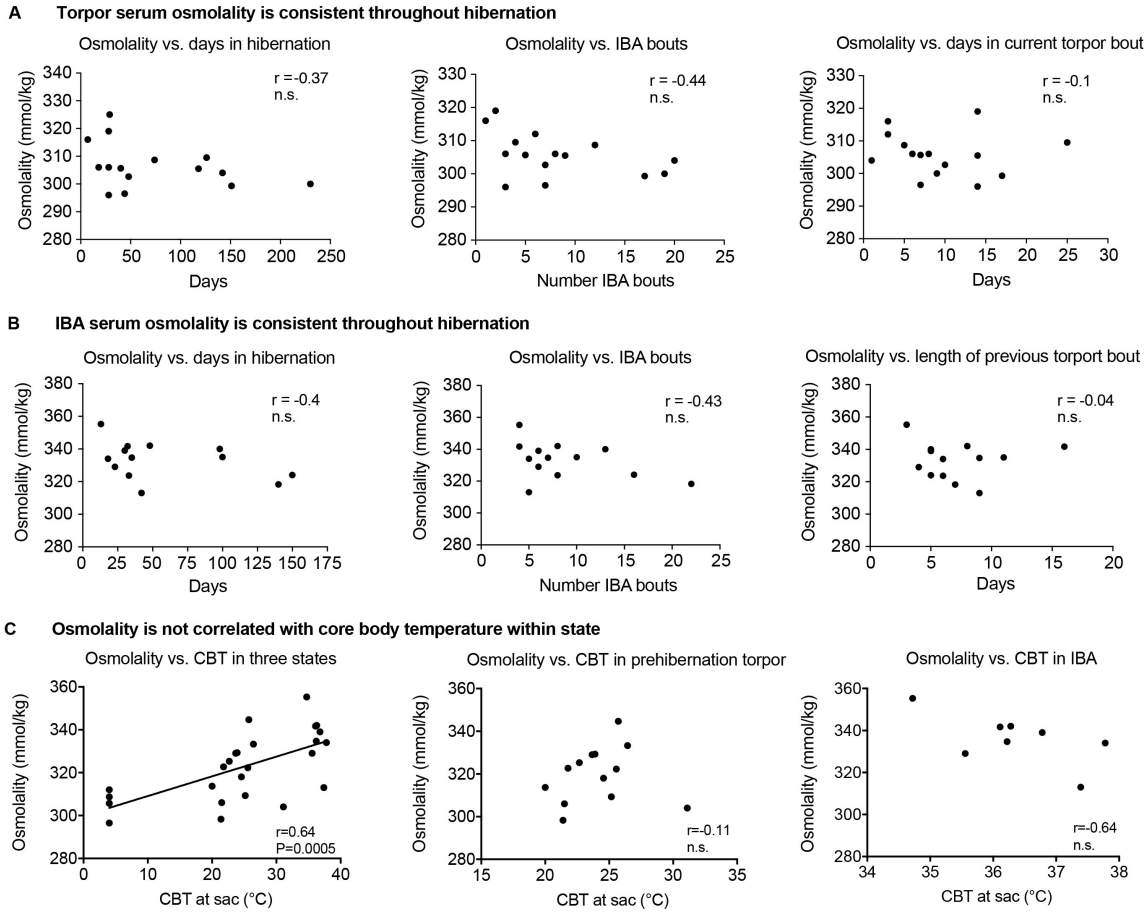

Figure S1. Physiological state is a primary determinant of serum osmolality. Related to Figure 1.

(A and B) Linear correlation analysis between serum osmolality in torpid (A, n = 15) and IBA (B, n = 13) squirrels and the number of days spent in the hibernaculum, number of IBA bouts, or the number of days in the current torpor bout in the case of torpid animals, or the length of the previous torpor bout before blood collection in the case of IBA animals. (C) Serum osmolality correlation with core body temperature (CBT) measured at the time of blood collection from animals in torpid, prehibernation torpor, and IBA states (left panel, n = 25). Each dot represents a measurement from an individual animal. r, Pearson correlation coefficient.

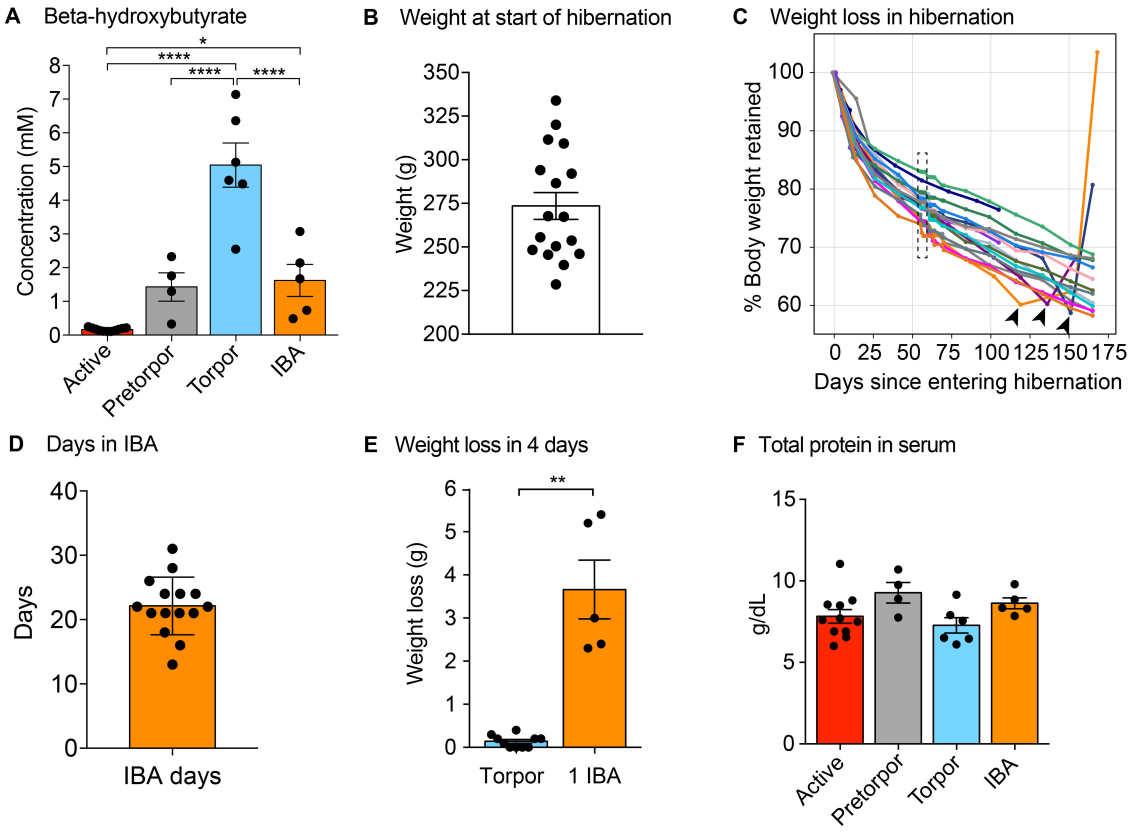

Figure S2. Metabolic and physical changes in squirrels across physiological states. Related to Figures 2 and 3.

(A) Serum levels of β-hydroxybutyrate across states. *P<0.05; ****P<0.0001, one-way ANOVA and Tukey's multiple comparisons test. (B) Squirrel body weight at the onset of hibernation. n = 17. (C) Percent weight retained during hibernation measured every two weeks. Each line represents weight measurement from an individual animal, n = 17. Dashed box highlights a four day period when squirrels were weighed daily (see E). Arrowheads indicate exit from hibernation. (D) Average number of days spent in IBA, n = 17. (E) Weight loss across four days in squirrels who stayed in torpor (n = 12) or squirrels who experienced one IBA bout (n = 5). (F) Total serum protein levels across states. All bar plots are mean ± SEM. n ≥ 4. Each dot represents a measurement from an individual animal.

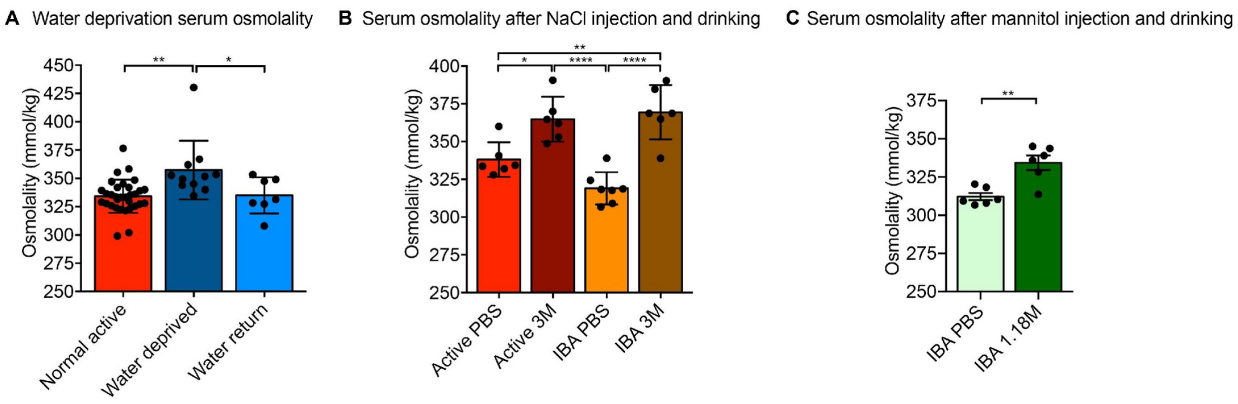

Figure S3. Water deprivation, NaCl and mannitol injection increase serum osmolality. Related to Figure 4.

(A-C) Serum osmolality measurements in active squirrels water deprived for 24 h (A) or in active or IBA squirrels injected with 3 M NaCl (3 M) or phosphate-buffered saline (PBS) (B), or 1.18 M mannitol (C). Data shown as mean ± SEM. *P<0.05; **P<0.01; ****P<0.0001, Tukey's multiple comparisons test (A, B), or t-test (C). Each dot represents a measurement from an individual animal, n ≥ 6.