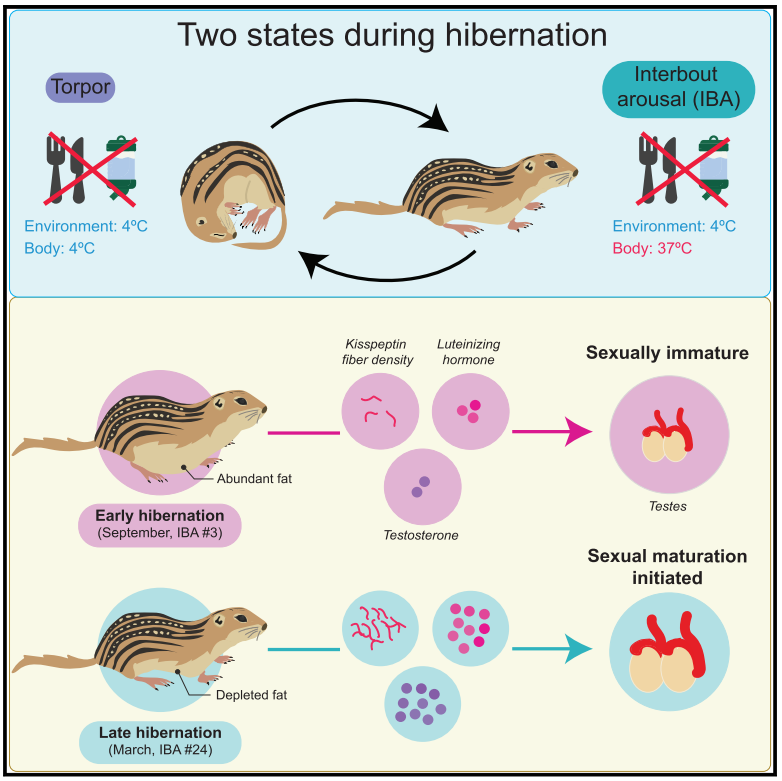
Report

Current Biology

Ground squirrels initiate sexual maturation during hibernation

Graphical abstract



Highlights

- Male hibernating squirrels initiate sexual maturation during hibernation
- Sexual maturation proceeds under conditions of negative energy balance
- The hypothalamic reproductive axis becomes active during interbout arousals
- Sexual maturation is circannually entrained

Authors

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In brief

Dai Pra et al. find that juvenile male ground squirrels initiate sexual maturation during their first hibernating season, despite depletion of internal reserves and long-term hypothermia. It occurs independently of physiological state and food availability and involves activation of central and peripheral components of the reproductive axis.

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Ground squirrels initiate sexual maturation during hibernation

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SUMMARY

Adequate nutrition is essential for normal reproductive function, which is vital for species to survive. In humans and other mammals, starvation and undernutrition deplete fat reserves and cause weight loss, attenuating the function of the reproductive axis and causing hypogonadism.[1–4] Thirteen-lined ground squirrels (*Ictidomys tridecemlineatus*) spend 7 months of every year in hibernation without food and water. Hibernating squirrels alternate between periods of torpor and interbout arousal (IBA), when animals temporarily return to an active-like state.[5] The physiological significance of IBA is unclear, but it is thought to be essential for hibernation in animals that drop their body temperature to 2°C–4°C during torpor. Here, we report that juvenile male ground squirrels initiate reproductive maturation during their first hibernation season, despite prolonged undernutrition and profound weight loss. We show that the hypothalamic reproductive axis undergoes activation during interbout arousals in the middle of hibernation, triggering production of luteinizing hormone and testosterone, and promoting testicular growth. Initiation of sexual maturation is circannually entrained and is independent of physiological state, ambient temperature, and food availability. Our study suggests a role for interbout arousals during hibernation and uncovers the neurophysiological mechanism of reproductive axis activation during conditions of extreme negative energy balance.

RESULTS AND DISCUSSION

Thirteen-lined ground squirrels (*Ictidomys tridecemlineatus*) inhabit a wide geographical area of North America, including northern territories with long winters.[1–5] To survive periods of low temperature and food scarcity, squirrels undergo solitary hibernation, spending up to 7 months of the year in underground burrows.[5] Pups are usually born during April and May[6–8] and enter hibernation in September as sexually immature juveniles.[9] Squirrels do not cache food but instead survive hibernation by utilizing internal fat reserves.[10] In most mammals, a depletion of energy resources limits sexual maturation and reproduction.[11] However, male ground squirrels begin to mate shortly after leaving the burrow, when they have burned most of their stored fat.[4,7–9] Furthermore, juvenile males appear capable of successful reproduction. Under our laboratory conditions, three out of seven breeding pairs that included juvenile males that had undergone a single hibernation season produced progeny. This led us to hypothesize that juvenile male ground squirrels initiate sexual maturation during hibernation.

To test whether juvenile male thirteen-lined ground squirrels initiate sexual maturation during their first hibernation season, we performed a longitudinal study using animals undergoing their first hibernation season. First, we assessed the dynamics of total body weight, fat mass, and lean mass during hibernation. To mimic natural conditions, animals were placed in individual burrows at 4°C without access to food and water and in total darkness. Squirrels from this cohort spent up to 167 days in hibernation and underwent up to 27 torpor-interbout arousal (IBA) cycles (Figures 1A–1C; Table S1). During periods of torpor, which lasted for 2–3 weeks, animals ceased activity and their body temperatures decreased to ambient levels (Figures 1A and 1B).[12,13] During periods of IBA, which lasted for 24–48 h, squirrels temporarily restored their vital signs to active levels (Figures 1A and 1B).[12,13] Because hibernation is an energy-demanding state for ground squirrels, we used quantitative magnetic resonance to assess fat mass and lean mass content every three IBAs (Figures 1D and S1). As expected, body composition analyses revealed a steady reduction in total body mass from 248.2 ± 9.37 g (mean ± SEM, n = 8) in active animals before hibernation to 120.2 ± 6.45 g (mean ± SEM, n = 9) during IBA 24 (Figures 1E and 1F). This reduction corresponds to 48% of total body weight and was largely due to the depletion of fat reserves (loss of 84.91 g by IBA 24, n = 9; Figures 1G and 1H), with a smaller contribution from lean mass (loss of 40.56 g by IBA 24, n = 9; Figures 1I and 1J). Because squirrels do not eat or drink during the entire hibernation period, they undergo dramatic weight loss that is indicative of a profoundly negative energy balance.

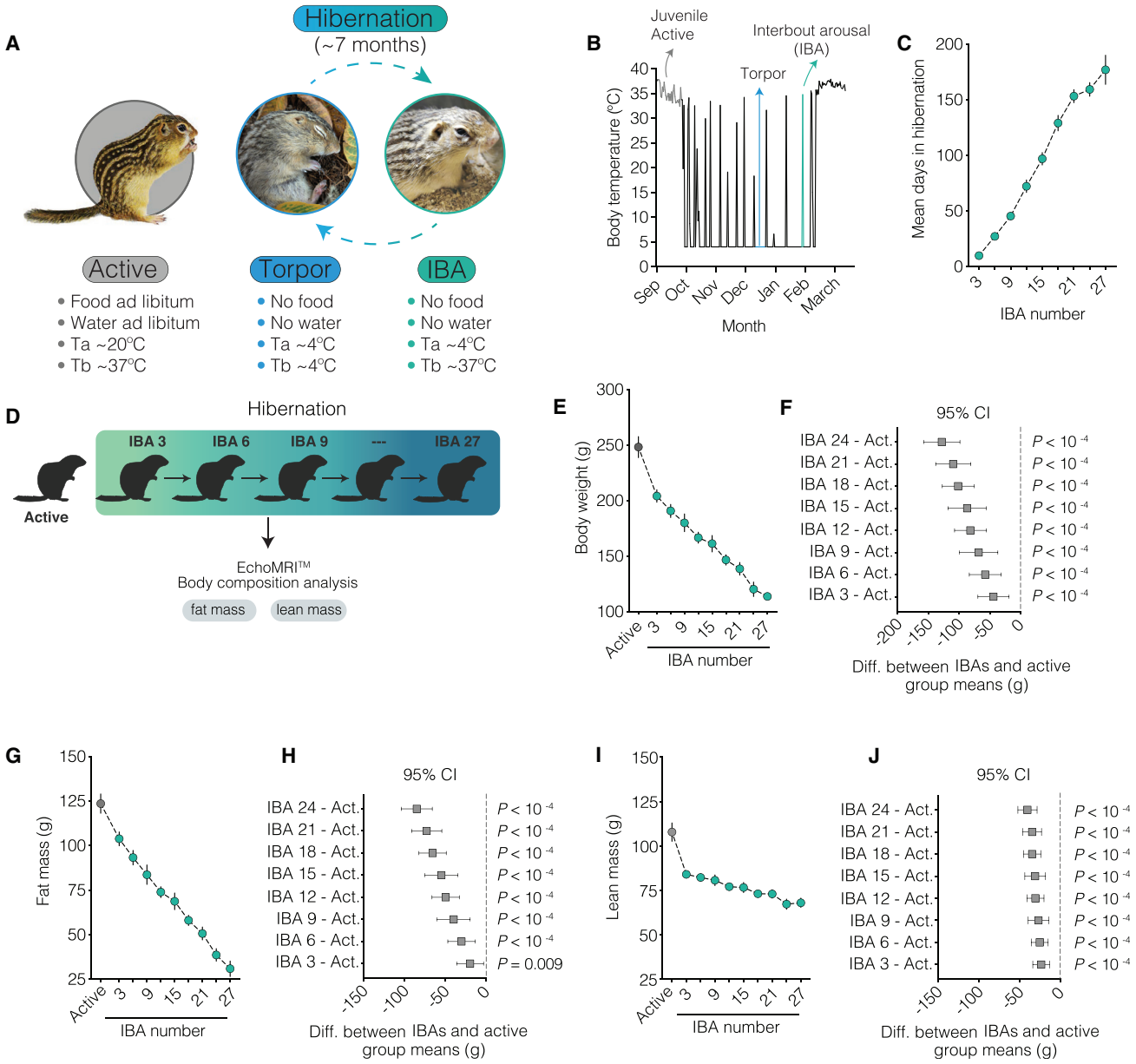

Figure 1. Squirrels decrease body weight, fat mass, and lean mass during hibernation

(A) Illustration of ground squirrels in different states. Ta, ambient temperature; Tb, body temperature; IBA, interbout arousal.

(B) Representative body temperature trace of a squirrel before, during, and after hibernation. Every temperature peak represents an IBA.

(C–J) Mean days spent by male ground squirrels in hibernation (C), body composition analysis of active and hibernating squirrels (D, E, G, and I), and corresponding Bonferroni's multiple comparison tests of the difference between the active and IBA group means, representing effect sizes (F, H, and J). Active, n = 8; IBA 3, n = 19; IBA 6, n = 17; IBA 9, n = 7; IBA 12, n = 17; IBA 15, n = 7; IBA 18, n = 17; IBA 21, n = 10; IBA 24, n = 9; IBA 27, n = 2. One-way ANOVA, $F_{8,102} = 32.06$, $p < 0.0001$ (E); $F_{8,102} = 40.19$, $p < 0.0001$ (G); $F_{8,102} = 15.96$, $p < 0.0001$ (I). Statistical analysis did not include IBA 27 due to n = 2. Data are mean ± SEM.

See also Figure S1 and Table S1.

In mammals, sexual maturation requires the activation of hypothalamic kisspeptin neurons, which stimulate gonadotropin-releasing hormone neurons to trigger the production and pulsatile release of luteinizing hormone (LH) and follicle stimulating hormone from the anterior pituitary (Figure 2A).[6,12–16] LH activates testicular Leydig cells to stimulate secretion of testicular testosterone and promote testicular growth.[17–19] Using immunohistochemical analysis of the hypothalamic arcuate nucleus, we detected a significant increase in kisspeptin fiber density and area covered by kisspeptin-expressing fibers during IBA compared with that found in active juvenile animals pre-hibernation, suggesting that the hypothalamic center that drives sexual maturation becomes active during hibernation (Figures 2B–2D and S2D–S2F).

In well-studied mammals, including humans, long-term starvation and low environmental temperatures suppress sex

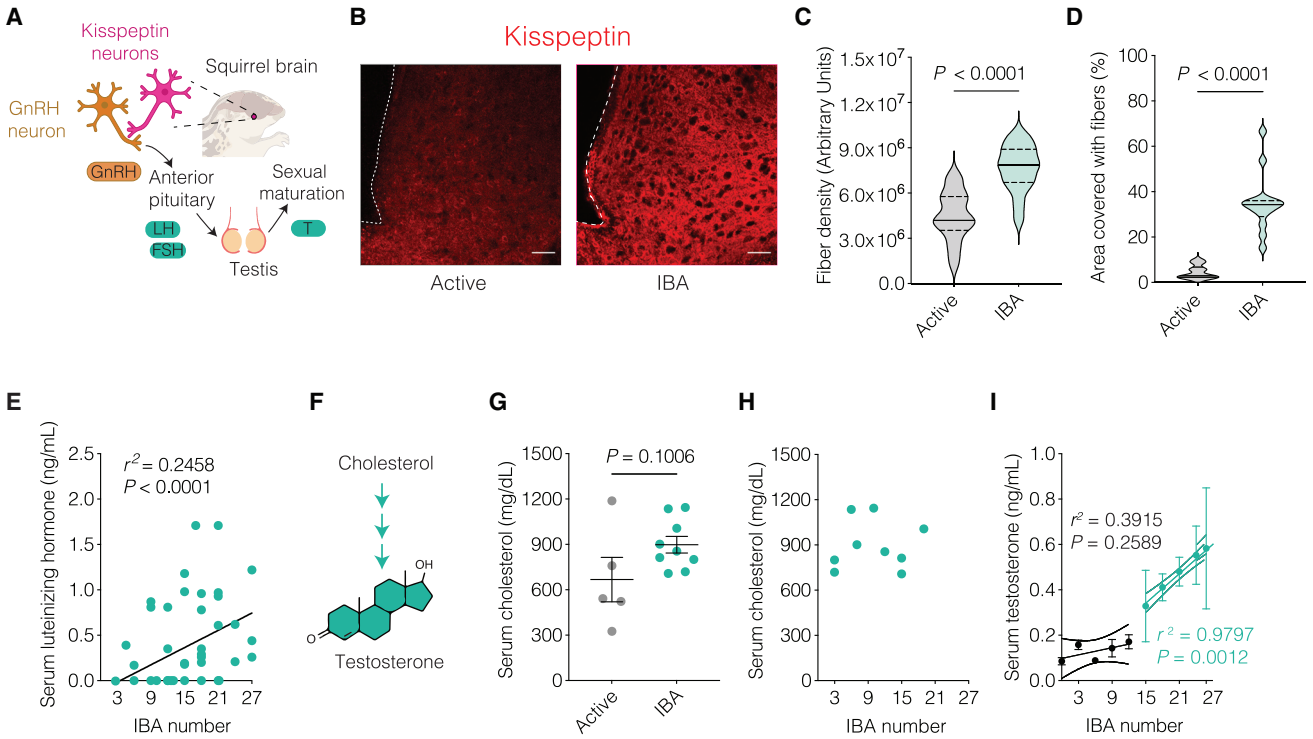

Figure 2. Central and peripheral components of the reproductive axis activate during hibernation

(A) Diagram of the physiological circuits responsible for sexual maturation in mammals.

(B) Representative immunohistochemistry images of the arcuate nucleus (20x) in juvenile squirrels pre-hibernation (active, tissues collected in August) and during IBA (IBA 24, 25, and 31; shown is IBA 25) using anti-kisspeptin antibody. Scale bar, 50 μm.

(C and D) Quantification of kisspeptin-positive fiber density (C) and area (D). Data combined from 12 to 18 slices per animal, from three animals for each state. Student's t test (C), Mann-Whitney U test, U = 0, p < 0.0001 (D). Solid and dashed lines are median and quartiles, respectively.

(E) Serum LH levels during hibernation, fitted to the linear equation. n = 58.

(F–H) Serum cholesterol, a substrate for testosterone production, in active and IBA squirrels (active, n = 5; IBA 3–19, n = 9; unpaired Welch's t test).

(I) Serum testosterone in active and IBA squirrels, fitted to the linear equation (active is denoted as IBA 0, n = 19; IBA 3, n = 20; IBA 6, n = 17; IBA 9, n = 8; IBA 12, n = 17; IBA 15, n = 7; IBA 18, n = 17; IBA 21, n = 9; IBA 24, n = 11; IBA 27, n = 3). Data are mean ± SEM.

See also Figure S2 and Table S1.

hormone release, which delays sexual maturation and leads to reproductive quiescence and hypogonadism.[17–22] In juvenile grounds squirrels, however, we detected a progressive increase in serum LH during the first hibernation season (Figure 2E), consistent with the activation of the hypothalamic center. Several IBA samples contained near-zero LH concentrations, likely due to the pulsatile nature of the hormone's release.

Because LH stimulates testicular production of testosterone from cholesterol,[23] we measured serum cholesterol during IBAs to investigate whether the substrate for testosterone production is available during hibernation. Indeed, we found that cholesterol was present at a steady level across IBAs (Figures 2F–2H). We also measured the dynamics of serum testosterone in juvenile animals throughout the hibernation season. During the initial phase of hibernation (IBAs 3–12), serum testosterone levels remained low (mean = 0.128 ng/mL). However, between mid-December and the end of hibernation (IBAs 15–27), we observed a linear increase in serum testosterone at an average rate of ~0.022 ng/mL per IBA (Figure 2I; Table S1). Overall, the increase in testosterone positively correlated with LH (Figure S2A). These experiments demonstrate that hormones associated with sexual maturation increase during hibernation in juvenile male ground squirrels. Interestingly, the observed increase in testosterone concentration correlated with the decreased duration of torpor bouts during the second half of hibernation (Figures S2B and S2C), supporting the idea that testosterone may influence the duration of hibernation.[24]

We next asked whether testicular growth was associated with elevated hormone levels. Indeed, we observed a significant increase in testicular mass, which was more than doubled by the end of hibernation (Figure 3). Taken together, the increase in hypothalamic kisspeptin, LH, testosterone, and testicular mass demonstrate that the reproductive axis is activated during hibernation in juvenile squirrels, despite months of hypothermia, food deprivation, and negative energy balance. Because metabolic activity is profoundly decreased during torpor,[10] when body temperature remains close to 4°C for 2 to 3 consecutive weeks, the bulk of hormone production and testicular growth is most likely to occur during IBAs, when metabolism temporarily returns to active-like levels.[20] The extent to which this process takes place during torpor remains to be investigated.

To test whether activation of the reproductive axis is dependent on hibernation or is circannually entrained, we prevented juvenile squirrels from hibernating by keeping them on 12-h

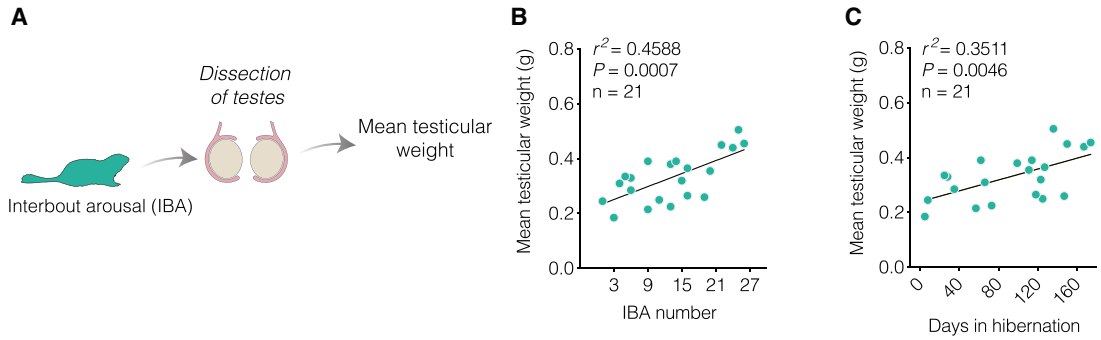

Figure 3. Testicular growth occurs during hibernation

(A) Testes of ground squirrels were collected during different IBAs.

(B and C) Mean testicular weight increases during hibernation (n = 21). The same data are plotted as a function of IBA (B) or the number of days in hibernation (C). Symbols represent measurements from an individual animal, fitted to the linear equation.

light/dark cycles at 20°C, with an *ad libitum* supply of food and water. Squirrels prevented from hibernating in this way, referred to herein as "winter active," continued to consume food and water, and therefore maintained a nearly constant body weight (Figures 4A and 4B). From October to January, serum testosterone in winter active animals remained low but increased significantly in February, rising to a peak in March when squirrels normally begin to emerge from hibernation to mate (Figures 4G and 4H). These changes are reminiscent of the biphasic testosterone dynamics observed in hibernating animals, even though in winter active squirrels the surge begins to occur about a month later (Figure 2I; Table S1). Elevated testosterone decreases fat and increases lean mass.[25] Consistently, we observed an increase in lean mass at the expense of fat mass between February and April (Figures 4C–4F and S3).[26] We also detected a significant increase in testicular mass to levels similar to those observed in hibernating squirrels in their final IBAs (Figures 4I–4K). Testosterone levels began to decrease in May in winter active squirrels (Figures 4G and 4H), consistent with the post-mating initiation of reproductive quiescence in seasonal breeders.[26] Thus, winter active squirrels activate the reproductive axis in a similar way to hibernating animals, revealing that the trigger is not food or water availability, energy balance, or hibernation per se, but rather entrainment via a circannual rhythm.

One of the general tenets of life is that during scarcity of resources the survival of an organism is prioritized over growth and reproduction.[27–29] The implementation of this principle is conditional upon the inability to escape the period of energy shortage via geographical migration, a behavioral strategy utilized by many species.[30] Hibernation constitutes a common physiological solution to this problem by enforcing long periods of decreased metabolic activity in an animal's native environment. Thirteen-lined ground squirrels are obligatory hibernators that do not cache food but spend the entire season using internal resources that are accumulated during the summer.[10] This study shows that when male squirrels have no access to food and water during hibernation, they lose nearly half of their total body weight and almost completely deplete their fat reserves. In most mammals, negative energy balance caused by anorexia, starvation, excessive exercise, or cold exposure inhibits the hypothalamic reproductive axis, causing a delay in sexual maturation and hypogonadism.[17,19,21,22,31] We have found that juvenile male ground squirrels more than double their testicular mass during the first hibernation season, despite nearly complete depletion of internal reserves, long-term hypothermia, and cold exposure. Although we do not rule out that some tissue growth may occur during torpor, the majority of this process likely happens during IBAs, when metabolic functions temporarily return to near-active levels.[20] Gonadal growth is accompanied by an increase in kisspeptin production in the arcuate nucleus of the hypothalamus, as well as an increase in LH and testicular testosterone, indicative of a general activation of the central and peripheral components of the reproductive axis.[16]

A number of seminal works have investigated gonadal growth and hormone dynamics during hibernation in thirteen-lined squirrels, golden-mantled ground squirrels (*Spermophilus lateralis*), and other hibernators. Many such studies used field-caught ground squirrels, or were conducted under conditions with food and water supplied at room temperature or on a dark/light cycle instead of constant darkness.[9,32–36] Studies of mixed age golden-mantled ground squirrels receiving *ad libitum* food and water during the hibernation season have shown that most of the hormonal surge (including testosterone and LH) and testicular growth occur only after the end of heterothermy.[20,24,35–37] Similar observations were made in European ground squirrels (*Spermophilus citellus*) kept in hibernation without food but with *ad libitum* water.[38] Thus, although gonadal growth was reported during hibernation, it remained unclear whether upstream effectors; i.e., hypothalamic activation and hormonal release, corresponded with gonadal growth, particularly in young animals. In this study, we investigated the neuronal and hormonal components of the reproductive axis specifically using only age-matched juvenile male thirteen-lined ground squirrels that were kept in darkness, in the cold, and in the absence of food and water during hibernation. We showed that in these conditions, which closely resemble natural hibernation, juvenile squirrels initiate sexual maturation in the middle of their first hibernation season, despite dwindling energy reserves. We also revealed that activation of the reproductive axis occurs at a similar time of year in squirrels that were prevented from undergoing hibernation and had an unlimited supply of food and water, suggesting that this process is triggered

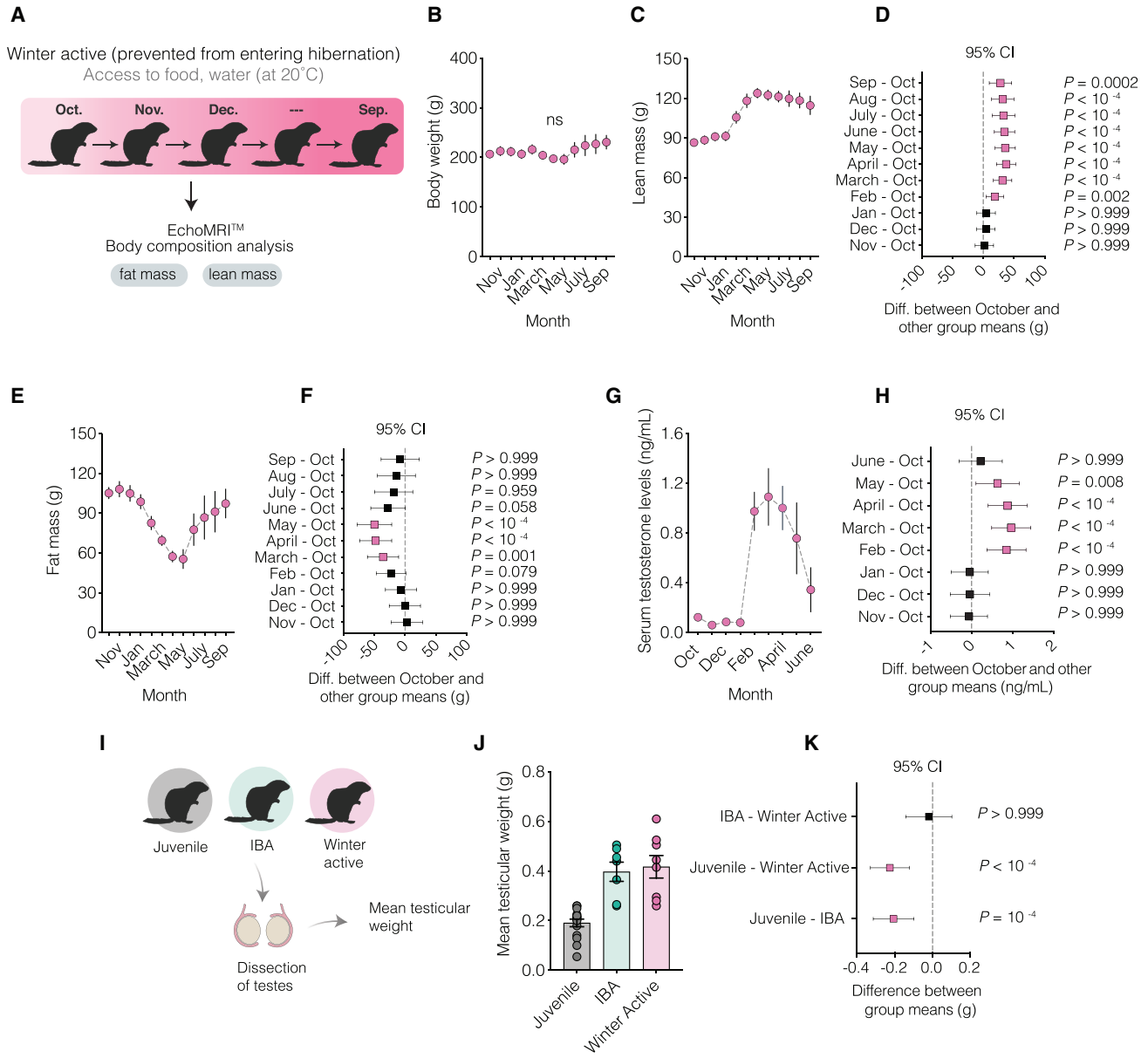

Figure 4. Activation of the reproductive axis in male ground squirrels is circannually entrained

(A) Winter active male ground squirrels were kept at 20°C with *ad libitum* food and water during the hibernation season.

(B–F) Body composition analysis (B, C, and E) and corresponding Bonferroni's multiple comparison tests of the difference between October and other group means (D and F). October, n = 15; November, n = 11; December, n = 11; January, n = 11; February, n = 14; March, n = 11; April, n = 10; May, n = 8; June, n = 8; July, n = 6; August, n = 6; September, n = 6. One-way ANOVA, $F_{11,105} = 0.8790$, p = 0.5632 (B); $F_{11,105} = 6.846$, p < 0.0001 (C); $F_{11,105} = 13.48$, p < 0.0001 (E).

(G and H) Serum testosterone levels (G) and Bonferroni's multiple comparison tests of the difference between October and other group means (H). October, n = 17; November, n = 13; December, n = 11; January, n = 13; February, n = 11; March, n = 11; April, n = 11; May, n = 8; June, n = 8. One-way ANOVA, $F_{8,94} = 12.14$, p < 0.0001.

(I–K) Mean testicular weight of juvenile, IBA (March to April), and winter active squirrels (January to April) (I and J) and corresponding Bonferroni's multiple comparison test between group means (K). Juvenile, n = 15; IBA, n = 7; winter active, n = 8. One-way ANOVA, $F_{2,27} = 20.89$, p < 0.0001. CI, confidence interval. Data are mean ± SEM.

See also Figure S3.

by a circannual rhythm rather than food availability, body temperature, or hibernation.

Our study has uncovered a potent neurophysiological mechanism that supports sexual maturation in the face of an extreme negative energy balance. The early onset of sexual maturation may serve the purpose of shortening the time between arousal from hibernation and mating, thus providing the benefit of "first male advantage," in which the first males to emerge have greatest access to receptive females. This strategy also lengthens the time for offspring to prepare for the next hibernation season

during the short period of resource availability.[39,40] The molecular mechanisms that initiate sexual maturation during hibernation, and whether this phenomenon is applicable to juvenile female squirrels, remains to be explored.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.cub.2022.02.032.

A video abstract is available at https://doi.org/10.1016/j.cub.2022.02.032#mmc3.

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AUTHOR CONTRIBUTIONS

R.D.P., S.M.M., S.N.B., and E.O.G. conceptualized the study. R.D.P. and S.M.M. performed experiments and collected data. R.D.P., S.M.M., S.N.B., and E.O.G. designed experiments and analyzed data. D.K.M. supplied squirrels and provided advice on animal husbandry. R.D.P., S.M.M., S.N.B., and E.O.G. wrote the manuscript with contributions from D.K.M. E.O.G. and S.N.B. conceived the project and provided guidance and supervision.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit polyclonal antibody anti-kisspeptin 10	INRAe (France)	Cat#AC566; RRID: AB_2314709
Goat Anti-rabbit secondary antibody Alexa 555	Abcam	Cat#Ab150086
Chemicals, peptides, and recombinant proteins		
Paraformaldehyde (PFA) solution 4% in PBS 1x	Santa Cruz Biotechnology	Cat#sc-281692
1x Phosphate-buffered saline (PBS) solution	Teknova	Cat#P5275
Bovine serum albumin (BSA)	Sigma Aldrich	Cat#A8806
Tween 20	American Bioanalytical	Cat#AB02038-00500
Sucrose	Sigma Aldrich	Cat#S0389
Hydrogen Peroxide (H_2O_2)	EMD Millipore	Cat#386790
Glycine	Calbiochem	Cat#4810
Sodium hydroxide (NaOH)	Macron Fine Chemicals	Cat#7708-10
Sodium Dodecyl Sulfate (SDS)	Fisher Chemical	Cat#O2674-25
Normal goat serum	Abcam	Cat#Ab138478
Critical commercial assays		
Rat testosterone ELISA kit	Crystal Chem	Cat#80550
Ultrasensitive rat luteinizing hormone (LH) ELISA kit	The Ligand Assay & Analysis Core of the Center for Research in Reproduction (CRR), University of Virginia	https://med.virginia.edu/research-in-reproduction/ligand-assay-analysis-core/
Cholesterol test	Antech Diagnostics	T125
Experimental models: Organisms/strains		
Thirteen-lined ground squirrel: *Ictidomys tridecemlineatus*	University of Wisconsin Oshkosh	N/A
Software and algorithms		
Prism 9.0 or above	GraphPad	RRID: SCR_002798
MATLAB 2018b or above	MathWorks	RRID: SCR_001622
Adobe Illustrator	Adobe	RRID: SCR_010279
ImageJ version 1.52k	NIH	RRID: SCR_003070
Other		
Fisherbrand Superfrost Plus Microscope Slides	Fisher Scientific	Cat#12-550-15
VECTASHIELD Antifade Mounting Medium with DAPI	Vector laboratories	Cat#H-1200
DAS 8001 Temperature Reader	Bio Medic Data Systems (BMDS)	https://bmds.com/
IPTT-300 Temperature Transponder	Bio Medic Data Systems (BMDS)	https://bmds.com/
EchoMRI-500	EchoMRI	http://www.echomri.com/Default.aspx

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Anesthesia Machine	VetEquip	Model V-10, cat. #901804
Isoflurane (Isothesia)	Henri Schein	Yale Veterinary Clinical Services (VCS)
Spectramax 384 Plus plate reader	Molecular Devices	https://www.moleculardevices.com/
Cryostat	Leica	CMS3050S
Confocal Microscope	Zeiss	LSM-780

RESOURCE AVAILABILITY

Lead contact

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).

Materials availability

This study did not generate new unique reagents.

Data and code availability

Data reported in this paper will be shared by the lead contact upon request. This paper does not report original code. Additional information required to reanalyze the data reported in this paper is available upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

All animal procedures involved in this study were performed in compliance with the Office of Animal Research Support of Yale University (protocol 2021-11497). Juvenile male thirteen-lined ground squirrels (*Ictidomys tridecemlineatus*) were single housed in temperature and humidity-controlled facilities at Yale University. During the 'active season' (May-August), animals were kept in a vivarium at a room temperature of 20°C and a 12h:12h light:dark photoperiod, and maintained on a diet of dog food (IAMS) supplemented with sunflower seeds, superworms, and fresh vegetables (celery and carrots), with *ad libitum* access to water. During the hibernation season (September-April), hypothermic squirrels (body temperature ~20°C) were moved to a hibernaculum at 2-4°C temperature, constant darkness and 50-60% humidity without food and water. All squirrels were implanted with a temperature transponder (BMDS). Throughout hibernation season, to detect all possible IBAs, core body temperature measurements were performed three times a day. Animals were moved from the hibernaculum to vivarium (left hibernation) if they were in IBA for more than 72 hours. In this study, 'active' squirrels were those with a constant core body temperature (CBT) of ~37°C held in the vivarium; 'IBA' squirrels were those whose CBT in the hibernaculum are above ~31°C; 'winter active' squirrels were those animals prevented from hibernating during winter. Winter active squirrels were kept in the vivarium at 20°C and a 12h:12h light:-dark photoperiod, with *ad libitum* food (only dog food, IAMS) and water. Active juvenile squirrel samples were collected in September 2020 and August 2021. IBA samples were collected from September 2020 to April 2021. Winter active samples were collected from October 2020 to September 2021.

METHOD DETAILS

Blood collection

Squirrels were anesthetized with isoflurane, and blood (~500 μL) was collected from the tail artery using a 27-G needle. Blood samples were allowed to coagulate at room temperature for 30 minutes, and centrifuged at 4°C for 10 min at 2000 rcf. Serum was collected, flash frozen on dry ice, and stored at -80°C until further use for measurements of testosterone, cholesterol, and luteinizing hormone. After blood collection, animals were rapidly weighed and returned to their home cage (if active) or returned to the hibernaculum (if hibernating). Blood was collected several times per squirrel. For hibernating squirrels, blood was collected every 3-6 IBAs. For 'winter active' squirrels, blood was collected once a month.

Body composition analyses

Juvenile male ground squirrels were briefly anesthetized with isoflurane and moved to the EchoMRI-500 body composition analysis device (EchoMRI). Three replicate measurements of fat mass and lean mass were taken, and squirrels were returned to their home cage. For hibernating squirrels, measurements were collected every 3-6 IBAs. For 'winter active' squirrels, measurements were taken once a month.

Collection and dissection of testes

For tissue collection, animals were sacrificed by isoflurane overdose, perfused with PBS followed by fixative (4% paraformaldehyde in PBS). Both testes were collected and epididymis and epididymal fat were removed. Both testes were weighed separately, and the mean testicular weight was calculated.

Measurement of serum testosterone levels

Blood was collected as described above. Serum testosterone from juvenile male ground squirrels was measured by enzyme linked immunoassay (ELISA) kit (Crystal Chem), following manufacturer's instructions. All standards and samples were run in duplicates. Optical density at assay-specific wavelengths were read by the Spectramax 384 Plus plate reader (Molecular Devices). The standard curves were fit with 4 parameter logistic regression in GraphPad Prism 9 (GraphPad Software).

Measurement of serum luteinizing hormone levels

Blood and serum were collected as described above. Samples were pre-diluted 1:10 in assay buffer (0.2% BSA in PBS with 0.05% Tween 20). 12 μL of serum was diluted in 108 μL of assay buffer. Samples were frozen at -80°C. All standards and samples were run in duplicates. Serum luteinizing hormone levels were measured by enzyme linked immunoassay (ELISA) developed by the Ligand Assay and Analysis Core - Center for Research in Reproduction, University of Virginia.[41] Values below the detection limit (0.160 ng/mL) were scored as zero.

Measurement of serum cholesterol levels

Male juvenile ground squirrels were deeply anesthetized with isoflurane. The body cavity was opened and the right atrium snipped. Blood was collected and allowed to coagulate at room temperature for 30 mins. Serum was collected after centrifugation at 2000 rcf at 4°C for 10-15 minutes. Serum was stored at -80°C in small aliquots until ready for processing. Cholesterol measurement was performed by Antech Diagnostics (Fountain Valley).

Immunohistochemistry

Juvenile male active and IBA ground squirrels were deeply anesthetized by isoflurane inhalation and were submitted to intracardiac perfusion with PBS followed by fixative [4% paraformaldehyde in PBS]. Brains were dissected and kept in fixative [4% paraformaldehyde in PBS] overnight. The brains were moved to 10% sucrose solution in PBS until sinking. Next, brains were moved to 20% sucrose solution in PBS. After sinking, they were moved to 30% sucrose solution in PBS. Brains were frozen on dry ice and kept at -80°C until ready to be sectioned. Coronal brain sections of the arcuate nucleus were cut at a thickness of 50 μm on a cryostat (Leica CM3050S). Sections were mounted onto SuperFrost Plus slides and moved to -80°C until the day of the immunohistochemistry procedure. Sections were dried in an incubator at 37°C for 30 minutes. Slides were washed three times with PBS for 10 minutes. Next, slides were washed with 1% H_2O_2 and 1% NaOH in PBS for 10 minutes. Slides were moved to 0.3% glycine in PBS 1x for 10 minutes. Lastly, slides were washed with 0.03% SDS in PBS. Sections were blocked for 2 hours at room temperature with 5% normal goat serum in 0.5% PBST. Next, sections were incubated with the rabbit polyclonal antibody (1:5000, from Isabelle Franceschini and Massimiliano Beltramo, INRae - France, #AC566 - Rabbit Polyclonal Kp-10[42]) in 2% goat serum 0.1% PBST at 4°C for 24 hours. After incubation with the primary antibody, sections were washed four times with 0.1% PBST for 15 minutes. Sections were incubated with secondary antibody (1:1000, Abcam, Alexa Fluor 555, ab150086) for 2 hours at room temperature. After 2 hours, sections were washed four times with 0.1% PBST for 15 minutes. For the secondary antibody only control condition, the same protocol was used, except that the primary antibody was replaced by the 2% goat serum in 0.1% PBST. Last, sections were washed in PBS and mounted using Vectashield with DAPI. Sections were imaged the next day on a Zeiss LSM-780 Confocal Microscope.

Quantification of fiber density and area covered by fibers

Images of the left and right arcuate nucleus were captured at 20x magnification using a Zeiss LSM-780 Confocal Microscope. The light intensity, camera and resolution (512 x 512 pixels) settings were kept constant across the images. For each squirrel (3 squirrels per group), 12-18 sections of the anterior, middle and posterior portion of the arcuate nucleus were evaluated. Fiber density in the arcuate nucleus was analyzed using ImageJ (version 1.52k, National Institutes of Health, United States) software. Images were converted to grayscale and the same threshold was applied to each image. A circular region of interest (ROI, 326 pixels diameter, covers ~1/3 of total image area) was applied adjacent to the third ventricle. The mean intensity of the ROI was measured as a proxy of kisspeptin fiber density. The same procedure was used to measure the area covered by kisspeptin fibers, which corresponds to the percentage of the ROI covered by pixels above the threshold.

Squirrel breeding in laboratory conditions

Breeding protocol was performed as described elsewhere.[3] Prior to setting up breeding pairs, juvenile and adult female ground squirrels that were in IBA for 3 consecutive days were moved from the hibernaculum to the vivarium and housed individually at 22°C with active food diet and water *ad libitum* for 2 weeks (males) or 2 days (females). Of the seven breeding pairs, three pairs produced progeny.

QUANTIFICATION AND STATISTICAL ANALYSIS

Matlab (2018a or above) and Prism 9.0 were used to analyze data and plot figures. Final figures were assembled in Adobe Illustrator. Data were first subjected to the Shapiro-Wilk normality test. When homogeneity was assumed, a parametric analysis of variance (-ANOVA) test was used. The Student's t test was used to compare two groups. Welch's correction was used when standard deviations were unequal between groups. ANOVA was used to compare multiple groups. Bonferroni's multiple comparisons test was used to find post hoc differences among groups and calculate 95% confidence intervals to report effect size. Statistical data are provided in text and in the figures. In the text, values are provided as mean ± SEM, $p < 0.05$ was considered statistically significant.