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RESEARCH ARTICLE

Don't Deny Your Inner Environmental Physiologist: Investigating Physiology with Environmental Stimuli

Tilapia prolactin cells are thermosensitive osmoreceptors

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Abstract

Prolactin (PRL) cells within the *rostral pars distalis* (RPD) of euryhaline and eurythermal Mozambique tilapia, *Oreochromis mossambicus*, rapidly respond to a hyposmotic stimulus by releasing two distinct PRL isoforms, PRL_{188} and PRL_{177}. Here, we describe how environmentally relevant temperature changes affected mRNA levels of prl_{188} and prl_{177} and the release of immunoreactive prolactins from RPDs and dispersed PRL cells. When applied under isosmotic conditions (330 mosmol/kgH_2O), a 6°C rise in temperature stimulated the release of PRL_{188} and PRL_{177} from both RPDs and dispersed PRL cells under perifusion. When exposed to this same change in temperature, ~50% of dispersed PRL cells gradually increased in volume by ~8%, a response partially inhibited by the water channel blocker, mercuric chloride. Following their response to increased temperature, PRL cells remained responsive to a hyposmotic stimulus (280 mosmol/kgH_2O). The mRNA expression of *transient potential vanilloid 4*, a Ca^{2+}-channel involved in hyposmotically induced PRL release, was elevated in response to a rise in temperature in dispersed PRL cells and RPDs at 6 and 24 h, respectively; prl_{188} and prl_{177} mRNAs were unaffected. Our findings indicate that thermosensitive PRL release is mediated, at least partially, through a cell-volume-dependent pathway similar to how osmoreceptive PRL release is achieved.

fish; prolactin; osmoreceptor; thermoreceptor; thermosensitivity

INTRODUCTION

Prolactin (PRL) is a protein hormone secreted by the pituitary gland that exerts over 300 physiological functions in vertebrates (1). Despite its extensive functional characterization as a pleiotropic factor, less is known about the mechanisms underlying the environmental control of PRL release. In fish, PRL coordinates the activities of multiple osmoregulatory organs such as the gill, gastrointestinal tract, urinary bladder, and kidney (1–4). PRL is especially vital to physiological phenotypes that enable fish to inhabit dilute freshwater (FW) environments because it stimulates active ion absorption and inhibits passive water gain (2, 3). Consistent with facilitating FW acclimation via their secreted hormones, PRL cells of euryhaline fishes, such as the Mozambique tilapia (*Oreochromis mossambicus*), are highly sensitive to physiologically relevant changes in extracellular osmolality. A fall in extracellular osmolality initiates the rapid release of two isoforms of tilapia PRL (PRL_{188} and PRL_{177}) that are encoded by separate genes (5, 6). Hyposmotically induced PRL release is triggered by an increase in cell volume (7, 8) followed by the entry of extracellular Ca^{2+} into PRL cells through the stretch-gated transient potential vanilloid 4 (TRPV4) channel (9, 10). The operation of this osmosensory system to control hormone release continues to provide the basis for employing tilapia PRL cell as a model to investigate osmoreceptors in vertebrates (11–14). In mammals, the organum vasculosum of the lamina terminalis transduces changes in osmotic conditions into an endocrine response through vasopressinergic neurons (15); these neurons, however, reside among other cell types of the supraoptic and paraventricular nuclei with their axons projecting a considerable distance to the posterior pituitary where vasopressin is released into the bloodstream (16, 17). By contrast, tilapia PRL cells form a nearly homogeneous mass that comprises the *rostral pars distalis* (RPD), thereby making them highly amenable to in vitro experimental paradigms (18).

Euryhaline fish can tolerate large fluctuations in environmental salinity, whereas eurythermal fish can survive a wide range of ambient temperatures. Mozambique tilapia are both euryhaline and eurythermal given their tolerance of salinities ranging from FW to greater than double-strength

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seawater and temperatures between 14°C and 38°C (19, 20). An indispensable aspect of osmotically regulated hormone release from PRL cells is a change in cell volume, which increases and decreases as extracellular osmolality falls and rises, respectively (7–9, 21). The hyposmotically induced increase in PRL cell volume requires the operation of aquaporin 3 (AQP3) (22), an aquaglyceroporin that regulates the water permeability of cells comprising various vertebrate tissues (23). When expressed in *Xenopus* oocytes, tilapia AQP3 increases osmotic water permeability, which can be inhibited by mercuric chloride ($HgCl_2$) (24). $HgCl_2$ is a potent AQP blocker that also inhibits hyposmotically induced cell volume changes (2 and 10 μM) and PRL release (2 μM) from dispersed tilapia PRL cells (22).

Members of the transient receptor potential (TRP) family of channels, including TRPV4, are sensitive to changes in osmolality, mechanical stress, small molecules, and temperature (25). For instance, the thermal sensitivity of TRPV1 underlies mammalian thermoregulatory cooling through sweat production and is required for preemptive vasopressin-mediated renal water reabsorption (26). Moreover, TRPV4, which is also localized in thermosensitive regions of the brain, is activated by warming in *Xenopus* oocytes and HEK 293 expression systems (27). The gene expression of TRPV4 is elevated in the brain and pituitary of chum salmon (*Oncorhynchus keta*) following a rise in temperature (28). Knowing that TRPV4 is highly expressed in tilapia RPD (10), we hypothesized that PRL cells are directly thermosensitive. Under this scenario, PRL cells would respond to fluctuations in environmental (and internal) temperature to modulate hormone release. This would constitute an entirely new facet of how PRL cells are controlled in an ectothermic model. Moreover, inasmuch as cell volume changes provide a means to precisely regulate osmotically sensitive PRL release, we hypothesized that thermal stimulation of tilapia PRL cells may also trigger cell volume changes. A link between a rise in temperature and an increase in cell volume has been previously observed in various cell models. For example, hyperthermic conditions within mouse mastocytoma P815 cells and rapid transitions to higher temperatures in human leukemia cells promote increases in cell volume (29, 30). A connection between thermal stimuli and changes in cell volume in tilapia PRL cells would provide a mechanism that allows for the integration of thermal and osmotic stimuli to support adaptive patterns of PRL secretion.

In the present study, we employed RPDs and dispersed PRL cells in both perifusion and static incubation systems to determine how temperature impacts PRL release and whether cell volume changes are coincident with thermal PRL responses. Experiments using RPDs were carried out to initially investigate the presence and direction of a thermal response. We then used dispersed PRL cells to further investigate the effects of temperature on PRL release and cell volume over a shorter time interval. We also evaluated the effects of temperature on the gene expression of prl_{188}, prl_{177}, and *trpv4* in RPDs and PRL cells. Furthermore, we assessed whether thermally induced changes in cell volume are sensitive to $HgCl_2$ to probe the involvement of AQP3. Together, these experiments revealed a relationship between thermal sensitivity, cell volume changes, and hormone secretion in a vertebrate osmoreceptor.

MATERIALS AND METHODS

Animals

Mature Mozambique tilapia (*O. mossambicus*) of mixed sex and size (0.4–1.0 kg) were obtained from stocks maintained at the Hawai'i Institute of Marine Biology, University of Hawai'i (Kaneohe, HI) and at Mari's Garden (Mililani, HI). Fish were reared in outdoor tanks with a continuous flow of FW (Honolulu municipal water) with temperatures ranging between 24°C and 27°C under natural photoperiod and fed ~5% of their body weight per day with trout chow pellets (Skretting, Tooele, UT). Fish were anesthetized with 2-phenoxyethanol (0.3 mL/L; Sigma Aldrich, St. Louis, MO) and euthanized by rapid decapitation. All experiments were conducted in accordance with the principles and procedures approved by the Institutional Animal Care and Use Committee, University of Hawai'i.

Static Incubations of RPDs

Following euthanasia, RPDs of *O. mossambicus* were dissected from the pituitary gland and placed individually into a single well of 48-well plates containing 300 μL of isosmotic medium (330 mosmol/kgH_2O) (31). After preincubation for 2 h at 25°C, the RPDs were rinsed with incubation medium and incubated for 24 h at 20°C, 25°C, or 30°C under saturated humidity (8 RPDs per experimental group). At the end of 1, 6, and 24 h of incubation, media were collected, diluted 20 times with radioimmunoassay (RIA) buffer (0.01 M PBS containing 1% bovine serum albumin and 0.1% Triton X-100), and stored at −20°C until analysis. Following the final collection of media at 24 h, the RPDs were collected in 500 μL of TRI Reagent (MRC, Cincinnati, OH) and stored at −80°C before RNA extraction.

PRL Cell Dispersion

Dispersed PRL cells were prepared from RPDs dissected from *O. mossambicus* as previously described (9, 21, 31). Briefly, RPDs were treated with 0.125% (wt/vol) trypsin (Sigma-Aldrich) dissolved in PBS and placed on a gyratory platform set at 120 rpm for 30 min to allow for complete cell dissociation. The cells were centrifuged for 5 min at 1,200 rpm and the supernatant decanted and discarded; cells were resuspended and triturated in trypsin inhibitor (0.125% wt/vol; Sigma-Aldrich) to terminate the trypsin treatment. Cells were washed with PBS twice and then resuspended in isosmotic medium. A hemocytometer and the trypan blue exclusion test were used to detect cell yield and viability, respectively (31).

Static Incubations of PRL Cells

Dispersed PRL cells were preincubated in 100 μL of isosmotic media (~124,000 cells/well; 8 replicates per treatment) at 26°C. The cells were then rinsed with incubation media and incubated for 1 and 6 h at 26°C and 32°C under saturated humidity. At the end of 1 and 6 h of incubation, 10 μL of media were collected, diluted 20 times with RIA buffer, and stored at −80°C. Following the final collection of media

at 6 h, 100 μL of TRI Reagent (MRC, Cincinnati, OH) was added to each well. The cells and TRI Reagent were then transferred to 1.5-mL tubes containing 500 μL of TRI Reagent and stored at −80°C before RNA extraction and gene expression analyses.

Perifusion and PRL Release

Dispersed PRL cells were added to poly-L-lysine (Sigma; 0.1 mg/mL)-coated chambers as previously described (7, 31). The chamber consisted of two rectangular coverslips (22 × 50 mm) joined by 100% silicone around the edges with cut hypodermic needles (23 gauge) inserted at each end to form an inlet and outlet. The chamber volume ranged from 200 to 400 μL and accommodated an average of 300,000 ± 50,000 PRL cells (12 different chamber preparations). To stabilize perifusion conditions, the chambers were first submerged in a water bath at 26°C and pre-perifused with isosmotic media for 60 min. After pre-perifusion, the chambers for the control group were perifused for an additional 70 min under the same conditions to establish a baseline before exposure to hyposmotic media (280 mosmol/kgH_2O) for 35 min.

Following the pre-perifusion, temperature-exposed group chambers were perifused for 15 min under isosmotic media at 26°C to establish a baseline of PRL release, placed in a 32°C water bath with isosmotic media for 30 min, returned to 26°C with isosmotic media for 25 min, and perifused with hyposmotic media at 26°C for the final 35 min. The desired internal temperature of the chambers (26°C or 32°C) was reached within a minute. Both experimental runs were replicated six times. Media were perifused at a rate of 50 ± 4 μL/min using a peristaltic pump (Cole-Parmer 7618-60; Cole-Parmer, Chicago, IL). The perifusate was collected manually every 5 min in 1.5-mL microcentrifuge tubes and stored at −80°C until analysis.

Perifusion and PRL Cell Volume

Dispersed PRL cells were resuspended in isosmotic media and added to perifusion chambers similar to those used in the *Perifusion and PRL Release*. The chamber consisted of two rectangular coverslips (22 × 30 mm and 22 × 50 mm) joined with silicone around the edges, with cut hypodermic needles (23 gauge) serving as the inlet and outlet, and an insulated thermocouple (Omega 5SRTC-TT-K-40-36; Omega, Norwalk, CT) inserted and connected to a computer for the monitoring of temperature (Fig. 1). The chamber volume ranged from 200 to 400 μL and accommodated an average of 600,000 ± 100,000 PRL cells. The chamber was mounted on a heating stage with a controller for modulating temperature (BoliOptics SG13701211, Rancho Cucamonga, CA) on top of an inverted microscope (Nikon Diaphot TMD Inverted Microscope, Japan) stage equipped with a ×100 oil-immersion objective lens. The internal temperature of the chamber was maintained via manual operation of the heating stage. Heating and cooling the chamber to the desired internal temperature (26°C or 32°C) required ~8 min.

All treatments were pre-perifused with isosmotic media at 26°C for 60 min. The control group was perifused with isosmotic media at 26°C for 145 min followed by exposure to hyposmotic media at 26°C for the final 30 min. The temperature-exposed group was perifused in isosmotic media at 26°C for 25 min, exposed to 32°C in isosmotic media for 60 min, returned to isosmotic media at 26°C for 60 min, and exposed to hyposmotic media at 26°C for the final 30 min. Finally, the $HgCl_2$-treated group was perifused in isosmotic media at 26°C for 25 min, perifused with 10 μM $HgCl_2$ isosmotic media at 26°C for 10 min (not shown) before exposure to 32°C in 10 μM $HgCl_2$ isosmotic media for 60 min, returned to isosmotic media at 26°C for 60 min, and exposed to hyposmotic media at 26°C for the final 30 min. Each perifusion experiment for cell volume determination was replicated 2–3 times.

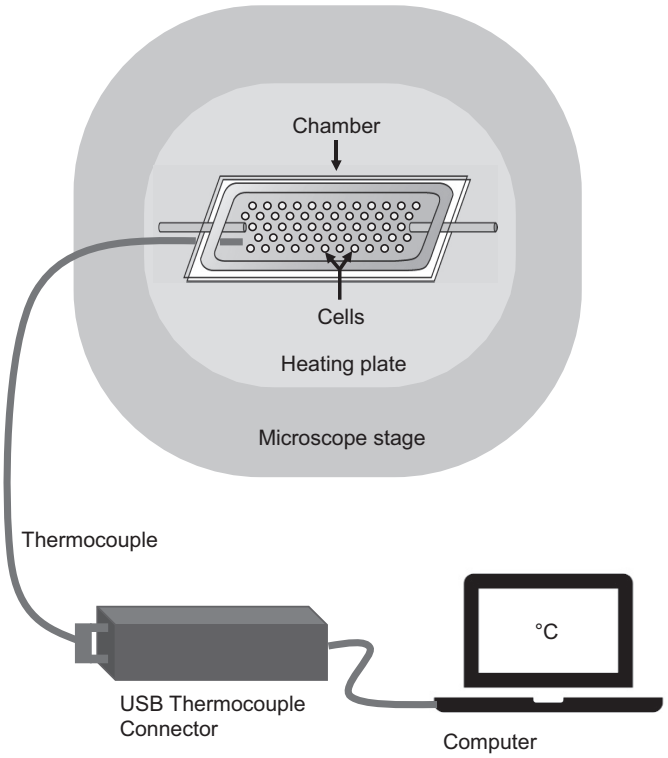

Figure 1. Diagram summarizing the experimental setup used for determining changes in PRL cell volume in responses to a rise in temperature. PRL, prolactin.

Cell Volume Determination

During the perifusion incubation period, cell images were captured every 5 min and cell volume was estimated as previously described (7) with minor modifications. The area of each cell was generated using the ImageJ software (Bethesda, MD) by tracing the perimeter of the cell from digitally captured images. Areas (A) were obtained in pixels and transformed into square micrometers. Cell volume (V) was estimated from the area shown in the equation below, assuming the cell to be a perfect sphere:

$$r = \sqrt{A/\Pi},$$

and

$$V = 4/3\Pi(r^3).$$

Cell volume was expressed as a percent change from the baseline set at 100%. The baseline value is the mean volume

calculated from the first six time points of the initial 25 min exposure period in isosmotic media at 26°C.

Radioimmunoassay

PRL_{188} and PRL_{177} levels in the collected media samples were measured by homologous radioimmunoassay (RIA) (32, 33) using primary antibodies (antisera) raised in rabbit against PRL_{188} and PRL_{177} (anti-PRL_{188} and anti-PRL_{177}, respectively) and secondary antibody raised in goat against rabbit IgG (anti-rabbit IgG; Sigma-Aldrich) as recently described and validated (31). Dilutions employed for anti-PRL_{188}, anti-PRL_{177}, and anti-rabbit IgG were 1:35,000, 1:8,000, and 1:100, respectively.

Quantitative Real-Time PCR

Total RNA was extracted from RPDs and PRL cells frozen in TRI Reagent following the manufacturer's protocol. Total RNA (150 ng from PRL cells and 400 ng from RPDs) was reverse transcribed using a High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA). The levels of reference and target genes were determined by the relative quantification method using a StepOnePlus real-time qPCR system (Thermo Fisher Scientific). The qPCR reaction mix (15 μL) contained Power SYBR Green PCR Master Mix (Thermo Fisher Scientific), 200 nmol/L forward and reverse primers, and 1–3.5 μL of cDNA. PCR cycling parameters were as follows: 2 min at 50°C, 10 min at 95°C followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Primer sequences are listed in Table 1. After verifying that levels did not vary across treatments, 18S ribosomal RNA was used to normalize target genes. Data are expressed as a fold-change ± SE (n = 8) from a given control group.

Statistics

Data for PRL release from RPDs were analyzed by two-way ANOVA with incubation temperature and time as main effects. Values were normalized by body weight as a surrogate marker for pituitary and RPD weight (31) and expressed as a fold-change relative to the 20°C group. There were no differences in the body weight of donor animals between treatment groups (data not shown). Data for PRL release from static incubations of dispersed PRL cells were analyzed by Student's t test. Data for PRL release from perifused PRL cells were analyzed by two-way ANOVA with treatment and time as main effects. Changes in cell volume were analyzed by two-way ANOVA with treatment and experimental group as main effects. Cell volume data were converted to net change in cell volume (NCCV) by subtracting the mean cell volume over the 25-min baseline from the mean cell volume change during subsequent incubation periods. Data on mRNA expression from 24 h RPD static incubations were analyzed by one-way ANOVA; data from 6-h PRL cell static incubations were analyzed by Student's t test. Significant effects were followed up by protected Fisher's least-significant different (LSD) test. When necessary, data were log-transformed to satisfy normality and homogeneity of variance requirements before statistical analysis. All statistics were performed using Prism 6 (GraphPad, La Jolla, CA) and data are reported as means ± SE.

RESULTS

Effects of Temperature on PRL_{188} and PRL_{177} Release from Static Incubations

A two-way ANOVA detected significant individual and interaction effects of incubation temperature and time on the release of both PRL_{188} and PRL_{177} from RPDs (Fig. 2, *A* and *B*). As early as 6 h, the release of both PRLs was elevated at 30°C compared with both 25°C and 20°C; there were no differences in the release of PRLs between the two lowest incubation temperatures at 6 h. By 24 h of incubation, however, a temperature-dependent pattern of PRL release was observed; the release of PRL_{188} and PRL_{177} was highest at 30°C followed by 25°C, which in turn, was higher than release at 20°C (Fig. 2, *A* and *B*). PRL_{188} release at 30°C was eightfold higher than release at 20°C at 24 h (Fig. 2*A*). Similarly, PRL_{177} release at 30°C was sixfold higher than release at 20°C over the same time course (Fig. 2*B*).

The effects of temperature on PRL release from PRL cells are shown in Fig. 2, *C* and *D*. In this experiment, we compared PRL release between cells incubated at 26°C and 32°C for 1 and 6 h. Temperature had no effect on the release of either PRL_{188} or PRL_{177} at 1 h (Fig. 2, *C* and *D*). Although there was no effect of temperature on PRL_{177} release at 6 h (Fig. 2*D*), PRL_{188} release rose by over twofold during the same period (Fig. 2*C*).

Effects of Temperature on PRL_{188} and PRL_{177} Release from Perifused PRL Cells

Two experimental treatments, distinguished by their exposure to either baseline temperature (26°C) or increased temperature (32°C), were compared. There were significant effects of treatment, time, and an interaction on PRL_{188} (Fig. 3*A*). A significant difference in PRL_{188} release between control and temperature-exposed cells was observed between 50 and 65 min (Fig. 3*A*). PRL_{188} release from the control group increased to ~364% relative to the average PRL release at the first 5 time points (baseline) when cells were exposed to hyposmotic media. PRL_{188} release from the temperature group increased by ~400% relative to baseline between 50 and 60 min, before subsiding to ~350% above baseline by 70 min. Finally, PRL_{188} release increased by ~500% relative to

Table 1. *Primer sequences used for qPCR analysis*

Target Gene	Forward Primer Sequence (5′–3′)	Reverse Primer Sequence (5′–3′)	Reference
18S ribosomal RNA	GCTACCACATCCAAGGAAGGC	TTCGTCACTACCTCCCCGAGT	34
prl$_{188}$	GGCCACTCCCCATGTTTAAA	GGCATAATCCCAGGAGGAGAC	34
prl$_{177}$	TGGTTTGGCTCTTTTAACACAGTG	AGACAATGAGGAGTCACAGAGATTTTAC	34
trpv4	AGTGGAGCCCATCAATGAG	TGTGGTATGTGGGTATGGAG	10

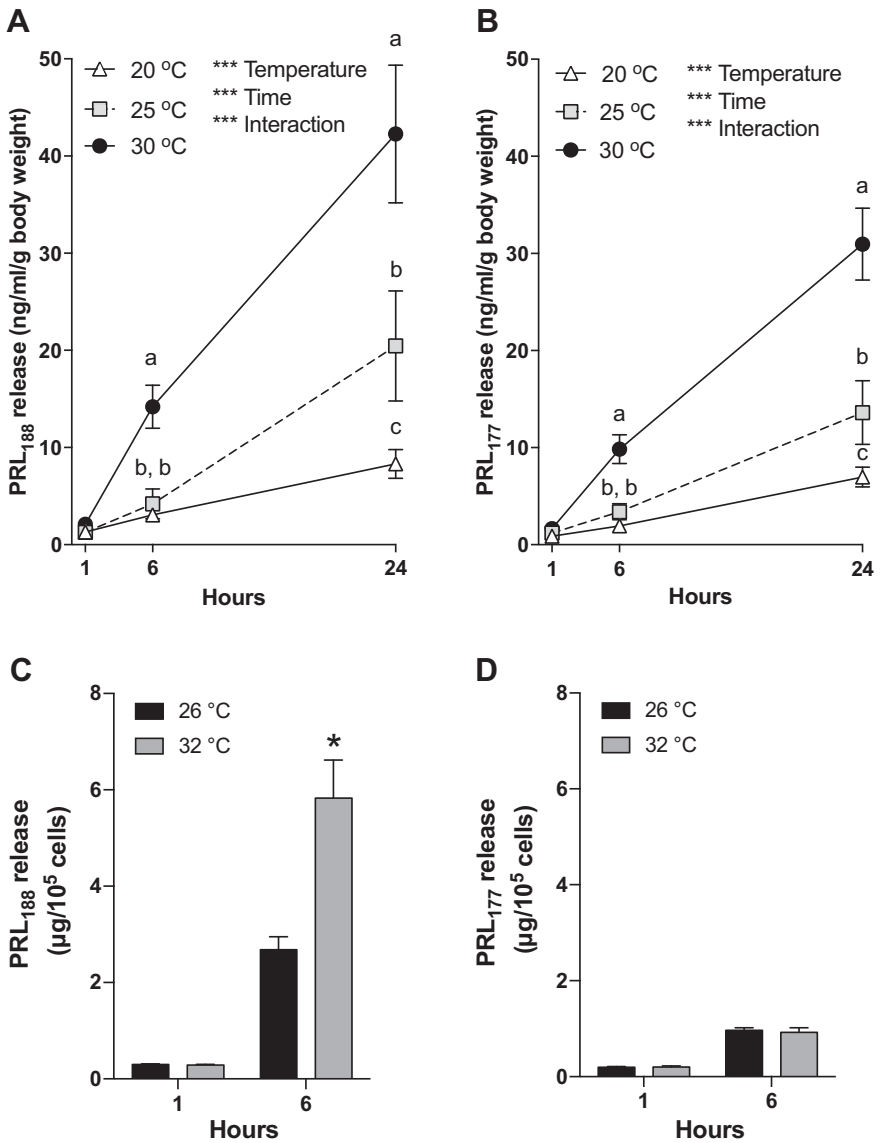

Figure 2. Effects of temperature on the release of PRL_{188} (*A*) and PRL_{177} (*B*) from RPDs at 1, 6, and 24 h and on the release of PRL_{188} (*C*) and PRL_{177} (*D*) from PRL cells at 1 and 6 h. *A* and *B*: data are expressed in ng/mL/g body wt ± SE (n = 7 or 8). Effects of incubation time and temperature were analyzed by two-way ANOVA (***$P < 0.001$). Incubation temperature effects were followed up by protected Fisher's LSD test. Symbols not sharing the same letter are significantly different across temperatures at $P < 0.05$. *C* and *D*: data are expressed in µg/10^5 cells ± SE (n = 7 or 8). Effects of incubation time and temperature were analyzed by Student's t test at each time point (*$P < 0.05$). LSD, least-significant different; PRL, prolactin; RPDs, *rostral pars distalis*.

baseline 10 min after cells were exposed to hyposmotic media.

PRL_{188} release at *time 0* was compared with all other time points within each treatment for the experiments shown in Fig. 3*A*. PRL_{188} release from the control cells increased during hyposmotic-stimuli (80–95 min), whereas PRL_{188} release of temperature-exposed cells increased earlier, between 50 and 75 min of perifusion and during exposure to a hyposmotic stimulus (75–105 min; Fig. 3*A*).

There were significant effects of treatment and time on PRL_{177} (Fig. 3*B*). Significant differences in PRL_{177} between control and temperature-exposed cells were observed at 50 and 55 min (Fig. 3*B*). PRL_{177} release from the control group increased by ~445% relative to baseline when cells were exposed to 280 mosmol/kgH_2O. PRL_{177} release within the temperature-exposed group increased by ~240% relative to baseline at 50 min before subsiding to ~165% above baseline by 65 min. Finally, PRL_{177} release increased by ~400% relative to baseline when cells were exposed to hyposmotic media for 10 min.

PRL_{177} release at *time 0* was compared with all other time points within each treatment for the experiments shown in Fig. 3*B*. PRL_{177} release from the control cells increased during exposure to a hyposmotic stimulus (70–100 min), whereas PRL_{177} release from temperature-exposed cells increased earlier, at 50–55 min of perifusion and during exposure to a hyposmotic stimulus (80–100 min; Fig. 3*B*).

Effects of Temperature on PRL Cell Volume

Three experimental groups, distinguished by their exposure to either baseline temperature (26°C; control group), high temperature (32°C; high temperature group), or high temperature in the presence of a water channel blocker ($HgCl_2$ group), were compared. Although 100% of the viable cells responded to a hyposmotic stimulus, ~50% of the cells exposed to a thermal stimulus responded in an identical fashion as the control cells whereas ~50% responded to the thermal stimulus. The ~50% of cells that responded to a thermal stimulus were used for comparing controls versus

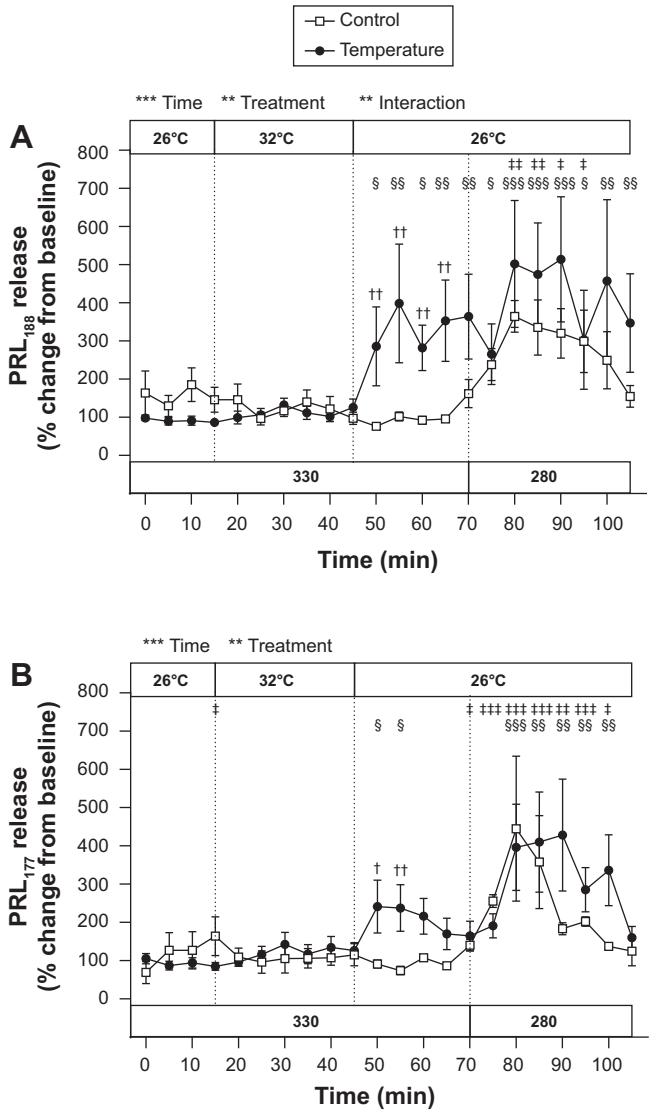

Figure 3. Effects of temperature increase (from 26°C to 32°C) and reduction in osmolality (from 330 to 280 mosmol/kgH_2O) on the release of PRL_{188} (*A*) and PRL_{177} (*B*) from dispersed PRL cells within 105 min. Vertical lines indicate a change in temperature or osmotic conditions. Symbols represent mean percent change PRL release ± SE (n = 5 or 6). Treatment and time effects were analyzed by two-way ANOVA (***$P < 0.001$; **$P < 0.01$) followed by protected Fisher's LSD test. Means with daggers (†) indicate significant differences from parallel controls at each time point (†$P < 0.05$, ††$P < 0.01$). Control means with double daggers (‡) indicate differences from control *time 0* (Fisher's protected LSD; ‡$P < 0.05$, ‡‡$P < 0.01$, ‡‡‡$P < 0.001$). Temperature means with silcrows (§) indicate differences from temperature *time 0* (Fisher's protected LSD; §$P < 0.05$, §§$P < 0.01$, §§§$P < 0.001$). LSD, least-significant different; PRL, prolactin.

high temperature and $HgCl_2$ groups. Cell volumes of the control group increased by ~20% relative to the average cell volume from the first six time points (cell volume baseline) when cells were exposed to hyposmotic media at 26°C (Fig. 4*A*, white circles). Cell volumes in the high temperature group gradually increased and peaked at ~8% relative to baseline between 30 and 70 min (Fig. 4*A*, black squares). Cell volume remained at ~8% above baseline up to 15 min following re-exposure to baseline conditions. Then, at 105 min, cell volume decreased from ~8% to ~5% above baseline, where it remained stable until 135 min before decreasing further to ~3% above baseline between 135 and 140 min. Finally, cell volume increased by ~20% relative to baseline when cells were exposed to hyposmotic media. When cells were

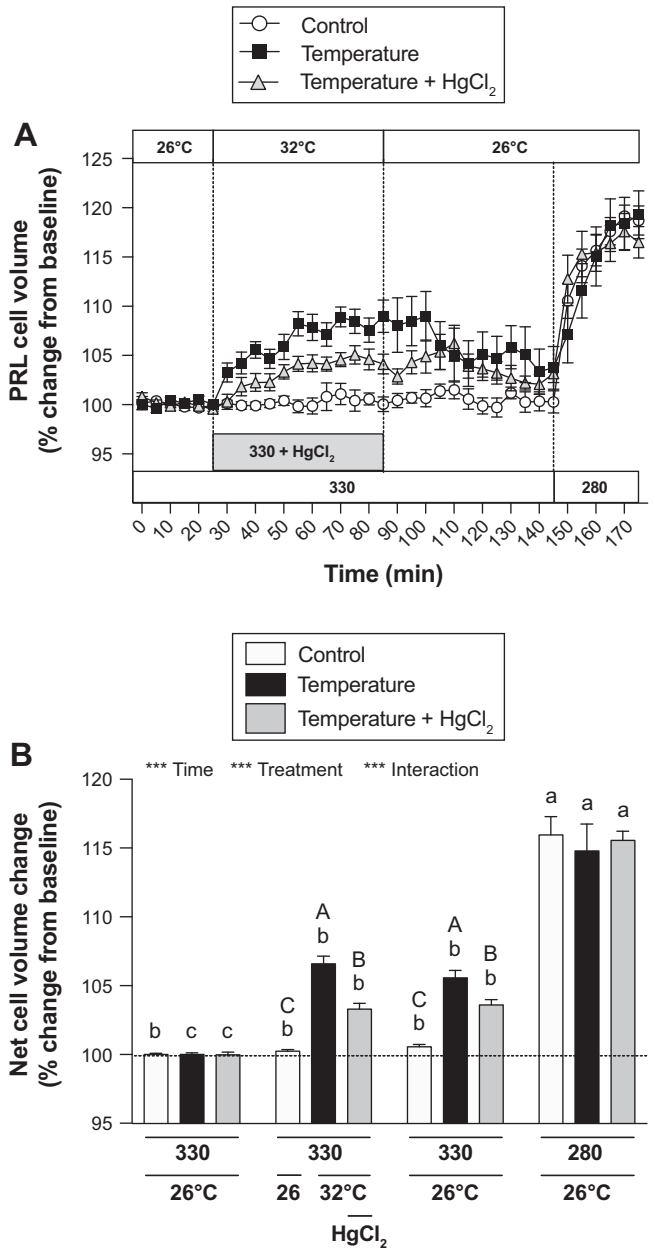

Figure 4. Effects of temperature and osmolality on the change in volume (*A*) and NCCV (*B*) of dispersed PRL cells within 175 min. *A*: symbols represent means expressed as percent change from an average cell volume of the first 6 time points ± SE (n = 10–12). Vertical lines indicate a change in treatment. *B*: the bars represent NCCV during control (26°C, 330 mosmol/kgH_2O), temperature (32°C, 330 mosmol/kgH_2O), recovery (26°C, 330 mosmol/kgH_2O), and hyposmotic treatments (26°C, 280 mosmol/kgH_2O) as a % change from the baseline. White, black, and gray bars represent control, temperature, and temperature + $HgCl_2$ experimental groups, respectively. Effects of treatment and experimental group were analyzed by two-way ANOVA (***$P < 0.001$), followed by protected Fisher's LSD test. Means not sharing the same letter (lowercase, between treatments; uppercase, between experimental groups) are significantly different at $P < 0.05$. LSD, least-significant different; NCCV, net change in cell volume; PRL, prolactin.

exposed to 32°C for 60 min in the presence of $HgCl_2$, cell volume remained steady for the first 5 min and then gradually increased, peaking at ~5% above baseline between 35 and 75 min and maintaining at ~5% above baseline for the remainder of the temperature exposure with $HgCl_2$ (Fig. 4*A*, gray triangles). Cell volume was maintained at ~5% above baseline between 90 and 110 min, before gradually decreasing to ~2% above baseline by 140 min. Lastly, cells exposed to hyposmotic media increased in cell volume by ~20% relative to baseline (Fig. 4*A*, gray triangles).

The cell volume responses reported in Fig. 4*A* were combined according to the treatments within each experimental group for statistical analysis and expressed as the NCCV. There were significant effects of treatment, experimental group, and an interaction on NCCV (Fig. 4*B*). Temperature-induced NCCV was significantly different between all experimental groups (Fig. 4*B*). The high-temperature group had a NCCV of ~7% above baseline, a value that was significantly higher than that of control and $HgCl_2$ groups. The $HgCl_2$ group had a NCCV of ~3% above baseline during exposure to high temperature, a value intermediate to that of the control and high-temperature groups. NCCV was significantly different between all experimental groups during the recovery period (Fig. 4*B*); the NCCV of the high temperature group was higher than that of both control and $HgCl_2$ groups. There were no significant differences in NCCV between experimental groups during the final 30 min of the perifusion with hyposmotic media; the NCCV of all three experimental groups increased by ~15% relative to baseline during that period (Fig. 4*B*). The NCCV induced by exposure to 32°C was higher than the baseline but lower than the NCCV induced by hyposmotic media (Fig. 4*B*).

Effects of Temperature on prl_{188}, prl_{177}, and *trpv4* mRNA Levels

Unlike release, the expression of *prl* mRNAs in RPDs was not altered by elevations in incubation temperature at 24 h (Fig. 5, *A* and *B*). By contrast, the mRNA expression of *trpv4* had increased by over twofold in RPDs incubated at 30°C compared with those held at 20°C and 25°C (Fig. 5*C*). Consistent with the 24-h RPD incubations, prl_{188} and prl_{177} expression in dispersed PRL cells was not affected by incubation temperature during a 6-h static incubation (Fig. 4, *D* and *E*). *trpv4* expression in PRL cells increased twofold at 32°C compared with cells incubated at 26°C (Fig. 5*F*).

DISCUSSION

The regulation of PRL and other pituitary hormones by environmental stimuli has remained understudied. The paucity of information stems from the lack of suitable model systems that are normally subject to wide and frequent excursions in the stimulus being studied and that allow for the homogeneous isolation of specialized cells with known secretory output. In mammals, tight homeothermic and osmoregulatory control ensures that homeostasis is rigorously maintained; deviations in internal temperature and blood osmolality are minimized. Many fishes, on the other hand, are capable of withstanding acute internal variations in osmolality and/or temperature. In Mozambique tilapia,

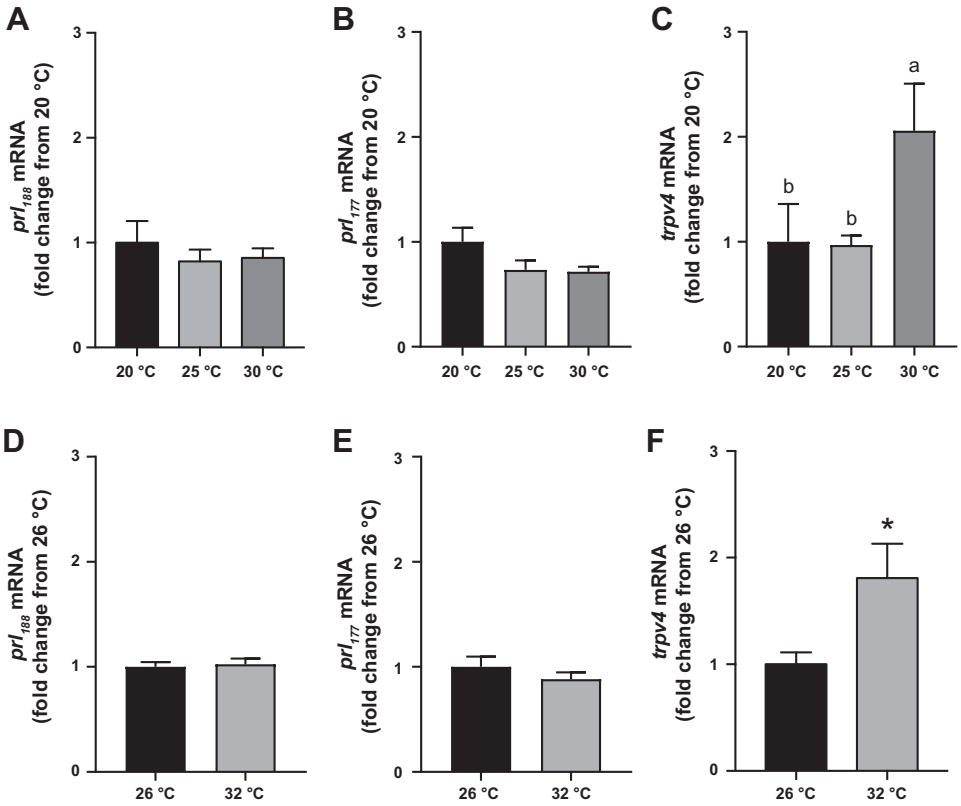

Figure 5. Effects of temperature on the mRNA expression of prl_{188} (*A*), prl_{177} (*B*), and *trpv4* (*C*) in RPDs at 24 h and prl_{188} (*D*), prl_{177} (*E*), and *trpv4* (*F*) in PRL cells at 6 h. RPDs and PRL cells were incubated in 330 mosmol/kgH_2O media in temperatures ranging from 20°C to 32°C. *A*–*C*: data are expressed as fold-change from 20°C ± SE (n = 6–8) and analyzed by one-way ANOVA followed by protected Fisher's LSD test. Bars not sharing the same letters are significantly different at $P < 0.05$. *D*–*F*: data are expressed as fold-change from the 26°C group ± SE (n = 7 or 8) and analyzed by Student's t test (*$P < 0.05$). LSD, least-significant different; PRL, prolactin; RPDs, *rostral pars distalis*.

for example, nonlethal deviations in plasma osmolality can occur between 290 and 450 mosmol/kgH_2O (35). In light of PRL's diverse actions in fishes and the ectothermic nature of Mozambique tilapia, we hypothesized that PRL cells are thermosensitive and may integrate thermal fluctuations in the environment with one or more of the physiological actions that PRL imparts. Here, we report for the first time that release of both tilapia PRLs (PRL_{188} and PRL_{177}) from the pituitary is induced by an environmentally relevant increase in temperature. Moreover, our findings indicate that PRL cell sensory mechanism(s), which respond to thermal stimuli, share similarities to those that transduce osmotic stimuli into PRL release. In light of the established role of PRL cells as osmoreceptors, these findings suggest that the integration of osmotic and thermal stimuli is carried out in a single cell type that secretes products with pleiotropic actions.

Previous studies established the direct osmotic control of PRL cell function (9, 35–39). We have also provided evidence that PRL_{188} and PRL_{177} responses to extracellular osmolality are modulated by a series of endocrine factors (40). In the current study, we discovered that the regulation of PRL cells is directly affected by temperatures that are within the range of environmental conditions (20°C–32°C) normally experienced by Mozambique tilapia (20). Although specific physiological drivers underlying PRL's release in a temperature-dependent manner remain subject to further investigation, there are a number of temperature-dependent processes that are regulated, at least in part, by PRL. For example, in addition to exerting hyperosmoregulatory actions required for survival in FW, PRL directs aspects of growth and reproduction (41, 42). In tilapia, growth is enhanced by elevations in water temperature (43) and PRL_{177} exhibits somatotropic activity (44). In several species of fish, PRL affects migration, binds to gonads, varies with sex steroids and breeding cycle, stimulates steroidogenesis and gonadogenesis, and induces reproductive behaviors such as mouth brooding, nesting, nest fanning, and fry defense (42). In *O. mossambicus*, PRL receptors are expressed in the gonads (45) and serum PRL levels change across the reproductive cycle (46). In the congeneric Nile tilapia (*O. niloticus*), pituitary and plasma PRL levels are highest during vitellogenesis (47). Given that spawning in tilapia is induced by increased temperatures (48), we propose that a direct transduction mechanism links environmental temperature with the release of a hormone, in this instance PRL, to support reproduction. In mammals, heat-induced PRL secretion plays physiological roles that have yet to be resolved (49).

The PRL cell is equipped with a sensory mechanism to transduce osmotic stimuli into hormone release via the cell-volume-dependent control of Ca^{2+} entry through TRPV4 channels. Hence, we hypothesized that PRL cells employ a similar mechanism to couple thermal stimuli with a change in cell volume. It is important to note that different methodological approaches (static incubations of RPDs/PRL cells and perifusion of PRL cells) were employed to account for patterns of PRL release under different experimental paradigms (31). Hence, some differences in the timing and magnitude of thermal responses can be attributed to the multiple incubation systems employed. In the current study, both thermal and hyposmotic stimuli led to an increase in PRL release and cell volume; however, the timing of the responses to these stimuli varied. The difference in osmotic and thermal responses may be attributed to the distinct nature of the stimuli and their subsequent effects on the cell membrane. In static incubations, both RPDs and dispersed PRL cells responded to a rise in temperature by 6 h (Fig. 2). By contrast, PRL cells in static incubation responded to a hyposmotic stimulus as early as 1 h (21). Similarly, in perifusion incubations, PRL release increased 35 min after initial exposure to a rise in temperature, whereas that response occurred within 5 min following exposure to hyposmotic conditions (Fig. 3). Interestingly, ~50% of PRL cells gradually increased in cell volume in response to the rise in temperature, peaking at ~8% after 30 min of exposure to an elevated temperature, whereas all PRL cells exposed to a hyposmotic stimulus increased in volume by ~15% within 15 min (Fig. 4*A*). Weber et al. (8) showed that the magnitude of PRL release is related to the rate of cell volume increases. Media containing urea, a solute that permeates the cell membrane at a slower rate than water, led to slower rates of cell volume increase and PRL release compared with standard hyposmotic media (8). Therefore, PRL release from dispersed PRL cells is tied to a cell-volume increase via water moving across the cell membrane rather than a direct response to extracellular osmolality per se. Similarly, the delayed PRL response to a rise in temperature was consistent with a gradual and protracted thermally induced change in cell volume. Notably, cell volume remained increased for ~20 min after temperature was decreased from 32°C to 26°C (Fig. 4), a pattern consistent with the delayed rise in PRL release following thermal stimulation in perifused PRL cells (Fig. 3). These findings provide the motivation for future studies targeting PRL thermal responses in vivo.

Thermal effects on cell volume have been previously reported in human leukemia K562 cells (29). Although a rise in temperature also elicited an increase in cell volume, the magnitude of thermal stimulation differed from the current experiment given that the thermal shock applied, from 37°C to 46°C, far exceeded the physiological range and resulted in a gradual lowering of cell viability (29). Irrespective of differences in experimental approach, temperature-induced cell volume changes may be attributable to the sensitivity of the plasma membrane and/or cytoskeleton to heat. Supraphysiological increases in temperature lead to modifications in the cytoskeleton, changes in membrane fluidity, and increased membrane surface due to loss of membrane invaginations in mammalian cells (50, 51). We propose these thermally induced cellular modifications may also underlie changes in the volume of tilapia PRL cells exposed to a rise in temperature. Although future investigations are warranted to resolve whether these modifications do in fact occur in tilapia PRL cells, our current findings suggest that subtle and gradual changes in cell volume play a role in thermally stimulated PRL secretion. This role was further supported by the attenuation of thermally induced increases in cell volume by $HgCl_2$ (Fig. 4*B*). Partial suppression of cell volume increases by $HgCl_2$, an AQP3 blocker shown to inhibit hyposmotically induced cell volume increases in PRL cells (22), suggests a role for the AQP3 water channel in thermally induced cell volume increases. Together with the established role that PRL cell volume plays as a trigger for hyposmotically induced PRL release, the current findings indicate that

thermally induced PRL release is also activated, at least in part, by a cell-volume dependent mechanism.

Heterologously expressed TRPV4 mediates Ca^{2+} influx in response to both hyposmotic and hyperthermal stimuli in *Xenopus* oocytes and HEK 293 T cells (27, 52). The activation of Ca^{2+} influx through TRPV4 channels is identical to the mechanism described in hyposmotically induced PRL release (9, 10). The expression of *trpv4* mRNA, however, is downregulated by hyposmotic stimuli and by pharmacological induction of Ca^{2+} influx, responses that reflect negative feedback loops involved in both the maintenance of intracellular Ca^{2+} homeostasis and the attenuation of osmosensitivity (53). Here, *trpv4* expression increased following an elevation in temperature (Fig. 5) indicating further regulation of PRL cell sensitivity to thermal stimuli. Interestingly, patterns of *prl* gene expression did not generally follow hormone release patterns; prl_{188} and prl_{177} expression was unresponsive to a rise in temperature. This observation suggests that a thermally induced change in cell volume and the associated release of PRLs, while delayed relative to hyposmotically induced PRL release, may be short-lived. Further studies combining hyposmotic and thermal stimuli in PRL cells will further clarify the disparate effects of these stimuli on the expression of prl_{188}, prl_{177}, and *trpv4*.

Perspectives and Significance

PRL_{188} and PRL_{177} differ in the magnitude of their responses to environmental osmolality (32, 35, 54, 55). This can be partly attributed to isoform-specific regulation of their associated gene transcripts (35). Similar to osmotic responses, PRL_{188} responded to rises in temperature in a more robust fashion than PRL_{177} under all incubation conditions. Although the effect of environmental stimuli on PRL cells is intricate and not fully understood, the current study is the first to link an established cell model of osmoreception with thermosensitivity, expanding our understanding of the sensory capacity of tilapia PRL cells. The underlying cellular mechanisms responding to these stimuli, in turn, manifest physiological consequences at the organismal scale through the myriad actions of PRL. One such consequence may include the enhanced control of osmoregulatory organs by PRL when its release is thermally stimulated. Indeed, elevated environmental temperatures are known to impact osmotic homeostasis (56, 57). Other metabolically demanding functions that are stimulated by both rises in temperature and PRL, such as growth and reproduction, may be impacted by the thermal responsiveness of PRL cells. Moreover, given that salinity acclimation histories can modulate the osmosensitivity of tilapia PRL cells (13, 58, 59), studies on fish acclimated to higher salinities, such as seawater, may reveal distinct thermosensitive responses. The current study paves the way to resolve the molecular mechanisms that enable integration of osmotic and thermal stimuli by the pituitary of ectotherms.

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DISCLAIMERS

The views expressed herein are those of the authors and do not necessarily reflect the views of the aforementioned granting agencies.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Y.Y., J.P.B., and A.P.S. conceived and designed research; D.W.W., G.H.T.M., F.T.C.-B., Y.Y., J.P.B., and A.P.S. performed experiments; D.W.W., G.H.T.M., F.T.C.-B., Y.Y., and A.P.S. analyzed data; D.W.W., G.H.T.M., F.T.C.-B., Y.Y., and A.P.S. interpreted results of experiments; D.W.W., G.H.T.M., F.T.C.-B., Y.Y., and A.P.S. prepared figures; D.W.W., J.P.B., and A.P.S. drafted manuscript; D.W.W., F.T.C.-B., J.P.B., and A.P.S. edited and revised manuscript; D.W.W., G.H.T.M., F.T.C.-B., Y.Y., J.P.B., and A.P.S. approved final version of manuscript.

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