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SHORT COMMUNICATION

Low glucose availability stimulates progesterone production by mouse ovaries *in vitro*

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ABSTRACT

Steroid production by the ovary is primarily stimulated by gonadotropins but can also be affected by biological cues that provide information about energy status and environmental stress. To further understand which metabolic cues the ovary can respond to, we exposed gonadotropin-stimulated mouse ovaries *in vitro* to glucose metabolism inhibitors and measured steroid accumulation in media. Gonadotropin-stimulated ovaries exposed to 2-deoxy-D-glucose increased progesterone production and steroidogenic acute regulatory protein mRNA levels. However, oocytes and granulosa cells in antral follicles do not independently mediate this response because targeted treatment of these cell types with a different inhibitor of glucose metabolism (bromopyruvic acid) did not affect progesterone production. Elevated progesterone production is consistent with the homeostatic role of progesterone in glucose regulation in mammals. It also may regulate follicle growth and/or atresia within the ovary. These results suggest that ovaries can regulate glucose homeostasis in addition to their primary role in reproductive activity.

KEY WORDS: 2-DG, Bromopyruvic acid, Glucose homeostasis, Steroidogenesis, Estradiol

INTRODUCTION

Reproduction is carefully regulated in most species to enable maximal reproductive success. Many of the environmental and physiological cues that animals use to time reproduction appropriately, including photoperiod, food availability and fat stores, are integrated by hypothalamic neural circuits that modulate gonadotropin production and release from the anterior pituitary gland. The gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH), in turn, control the activity and function of the gonads. In the ovary, LH stimulates steroid production by theca cells in the follicle [including progesterone (P_4) and other precursor steroids necessary for estradiol production by granulosa cells]. FSH promotes estradiol (E_2) production from granulosa cells of the follicle and stimulates follicle growth. Progesterone and estradiol exert paracrine action within the ovary to control follicle development (Hammes, 2004). These sex steroids also facilitate reproduction through their action on peripheral tissues (e.g. stimulating hypertrophy in the uterine epithelium or the oviduct; Ford, 1982; Liley, 1976; Spencer et al., 2004). They can also act in the brain to regulate other components of reproductive activity, including reproductive behaviors. Sex steroid production from the ovaries can therefore be indirectly controlled by physiological cues through central modulation of LH and FSH release.

Data collected over the last two decades demonstrate that physiological signals other than gonadotropins also meaningfully impact ovarian sex steroid production and follicle development. For example, the hormone leptin, which reflects fat stores, modulates estradiol and progesterone production by granulosa cells from human ovaries *in vitro* (Agarwal et al., 1999; Spicer and Francisco, 1997). Similarly, avian ovaries decrease estradiol production when treated *in vitro* with pharmacological agents that mimic low glucose and fatty acid availability (McGuire et al., 2013). However, we still know relatively little about the extent to which stimuli other than gonadotropins can modulate ovarian steroid production and/or follicular growth.

In light of data from avian ovaries (McGuire et al., 2013), we tested whether glucose availability could directly influence gonadotropin-induced sex steroid production from the mouse ovary. Glucose exhibits ultradian (<24 h) oscillations across a day in humans (Melanson et al., 1999; Simon et al., 2000), rodents (Tannenbaum et al., 1976) and horses (Evans, 1974), and more extreme decreases in blood glucose can occur naturally during acute fasting (Le Magnen et al., 1980) and exercise (Felig et al., 1982). Thus, low glucose is a salient biological cue to which the mammalian ovary could be sensitive. To test whether ovarian steroidogenesis in mice responds to low glucose, we utilized two different pharmacological agents that inhibit glucose metabolism: 2-deoxy-D-glucose (2-DG) and bromopyruvic acid (3BR). 2-DG enters cells via glucose transporters (e.g. Clarke et al., 1994), and therefore can be taken up by most cells in the ovary. In contrast, 3BR must be transported across the cell membrane by monocarboxylate transporter 1 (MCT1) (Birsoy et al., 2013), and expression of MCT1 is largely limited to granulosa cells and oocytes in antral follicles in the mouse ovary (Hérubel et al., 2002; Kuchiiwa et al., 2011). Thus, 2-DG can inhibit glucose utilization throughout the entire ovary, whereas 3BR targets a subset of cell types in antral follicles. Oocytes and cumulus granulosa cells, the targets of 3BR, employ extensive paracrine signaling that controls the development and maturation of the follicle and oocyte, in part by regulating glucose metabolism and steroid production (Brankin et al., 2003; Vanderhyden et al., 1993; Sutton-McDowell et al., 2010; Dumesic et al., 2014). Oocytes uptake relatively little glucose directly; they therefore rely on pyruvate produced by cumulus granulosa cells for metabolic substrate. Because maturation of follicles and oocytes is sensitive to glucose (Sutton-McDowall et al., 2010), we hypothesized that oocytes and/or granulosa cells would mediate any effect of low glucose availability on steroidogenesis.

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MATERIALS AND METHODS

Animal use

All laboratory protocols were approved by the UC Berkeley Office of Laboratory Animal Care and were consistent with NIH guidelines for the care and use of laboratory animals. Six-week-old female C57BL/6J mice (*Mus musculus* Linnaeus 1758), purchased from Jackson Laboratory (Bar Harbor, ME, USA), were given a week to acclimate to the laboratory environment before the experiment began. The mice were group-housed under a 17 h:7 h light:dark cycle. A vaginal lavage was used to determine the stage of estrus for all animals (McLean et al., 2012). Animals were provided with *ad libitum* food and water. Mice were deeply anesthetized using isoflurane and rapidly decapitated prior to ovary collection. From each individual, one ovary was assigned to the control group and the other to the treatment group. Left versus right ovary assignment was randomized (see below).

Culture media and reagents

Culture media consisted of Eagle's MEM with Earle's balanced salts supplemented with 2 mmol l^{-1} L-glutamine, 100 IU ml^{-1} penicillin, 100 µg ml^{-1} streptomycin, 50 µmol l^{-1} ascorbic acid, 1% v/v insulin-transferrin-selenium (ITS+, Gibco Laboratories, Gaithersburg, MD, USA) and 0.3% bovine serum albumin. All media (control and treatment media) were supplemented with 2 IU ml^{-1} luteinizing hormone (L5269, Sigma-Aldrich, St Louis, MO, USA) and 1 IU ml^{-1} follicle-stimulating hormone (F2293, Sigma-Aldrich) immediately prior to use. LH and FSH concentrations used in these experiments are supraphysiological, but are routinely used to stimulate follicle growth and induce ovulation-related changes in gene expression *in vitro* (Fowler and Spears, 2004; Carletti and Christenson, 2009; also see citations in Devine et al., 2002). In addition, the LH and FSH concentrations used here stimulated steroid accumulation in media from cultured mouse ovaries in preliminary studies (compared with control media without gonadotropin supplementation). 2-DG (D8375, Sigma-Aldrich) was diluted in culture media to a concentration of 5 mmol l^{-1}, and 3BR (16490, Sigma-Aldrich) was similarly diluted in culture media to 5 mmol l^{-1}. These concentrations of 2-DG and 3BR reduce ATP production and inhibit glycolytic activity *in vitro* (Xiao et al., 2013; Tower, 1958; Ganapathy-Kanniappan et al., 2013; Xu et al., 2005; Ko et al., 2001).

Experimental design and ovarian culture

Data were collected in two experiments using identical tissue collection protocols. In experiment 1, ovaries were treated with 2-DG. Experiment 1 was independently repeated (n=8 in the first iteration, n=7 in the second); similar results were achieved in the two replications and data were pooled for presentation and analysis. In experiment 2, ovaries were treated with 3BR (n=11). 3BR treatments were carried out once.

For both experiments, ovarian culture was carried out immediately after collection. Ovaries cleaned of fat and accessory tissues were paired within an experiment such that one ovary from each mouse was cultured in control medium while the other was cultured in medium containing the experimental treatment. Ovaries were cultured in 24-well plates. Each well contained 500 µl of media (control, 2-DG or 3BR). Ovaries were placed in a sealed, humidified incubator for 6 h at 37°C with 5% carbon dioxide gas. Similar culture conditions have previously been used to evaluate the effects of other proteins, hormones and pharmacological agents on steroidogenesis from whole rodent ovaries *in vitro* (Devine et al., 2002; Funkenstein et al., 1980; Uilenbroek et al., 1983).

After 4 h of incubation, 100 µl of culture medium was replaced with fresh medium. After 6 h of incubation, all culture media were transferred into clean Eppendorf tubes and heavily vortexed prior to aliquoting for storage. Tissues were snap-frozen on dry ice. Tissues and culture supernatant were stored at −80°C until analysis.

Hormone production measurements

Media collected from ovarian cultures were assayed using ELISAs for progesterone (P_4; item no. 582601, Cayman Chemical, Ann Arbor, MI, USA) and estradiol (E_2; item no. 582251, Cayman Chemical) in compliance with instructions for use with culture media.

All samples were assayed in triplicate. Intra-assay variation ranged from 1.9% to 6.2% for P_4 and from 4.1% to 5.5% for E_2. Inter-assay variation was 7.9% for P_4 and 9.9% for E_2.

Steady-state levels of mRNA

RNA was extracted from frozen ovaries (Bioline Isolate II RNA Mini Kit, Taunton, MA, USA) and 1 µg RNA was reverse-transcribed for downstream analysis (iScript Reverse Transcription Supermix, Bio-Rad, Hercules, CA, USA). Quantitative PCR was carried out using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) with 0.5 µmol l^{-1} primers for steroidogenic acute regulator protein (StAR; F: 5′-CTTGGCTGCTCAGTATTGAC, R: 5′-TGGTGGACAGTC-CTTAACAC; annealing temperature T_A: 55°C) and cholesterol side-chain cleavage enzyme (SCC; F: 5′-CGATACTCTTCTCATGCG-AG, R: 5′-CTTTCTTCCAGGCATCTGAAC; T_A: 55°C). Primers were validated prior to use and efficiencies for all primer pairs were between 90% and 105%. All data were corrected for efficiency, normalized to the geometric mean of two reference genes [tubulin beta 5 (TTUB5); F: 5′-GGACAGTGTGGCAACCAGAT, R: 5′-CC-CCAGACTGACCGAAAACG; T_A: 60°C; and beta-2-microglobulin (B2M); F: 5′-CTGCTACGTAACACAGTTCCACCC, R: 5′-CATG-ATGCTTGATCACATGTCTCG; T_A: 55°C] that did not change with treatment, and then analyzed for fold-change using the Pfaffl (2001) method. Because there was no difference in P_4 and E_2 production by 3BR-treated ovaries, RNA extraction and gene expression analyses were not carried out for this treatment group.

Statistical analyses

Statistical analyses were carried out in R 3.1.2. All statistical analyses use paired Wilcoxon rank-sum tests. Differences were considered statistically significant at P<0.05. All values are expressed as means±s.e.m.

RESULTS AND DISCUSSION

Accumulation of progesterone in the media increased in response to 2-DG treatment (Fig. 1A, V=5, P<0.001), whereas estradiol accumulation in the media did not change (Fig. 1A, V=35, P=0.169). Estrus stage at collection did not affect the qualitative response to 2-DG of hormone accumulation or mRNA levels, though we could not statistically evaluate this response. Pre-antral and antral follicles are found in the ovary at all stages of the estrous cycle in mature mice (McGee and Hsueh, 2000; Peters et al., 1975), and stimulation with gonadotropins *in vitro* may therefore explain the similar response of all ovaries to treatment. Treatment with 2-DG also increased StAR mRNA levels in mouse ovaries (Fig. 1B, V=1, P<0.002), though it had no effect on SCC mRNA levels (Fig. 1B, V=24, P=0.465). Taken together, our hormone accumulation and mRNA level data suggest that gonadotropin-stimulated mouse ovaries can increase steroid hormone production in direct response to pharmacologically induced hypoglycemia. These findings are novel because it was not previously known that

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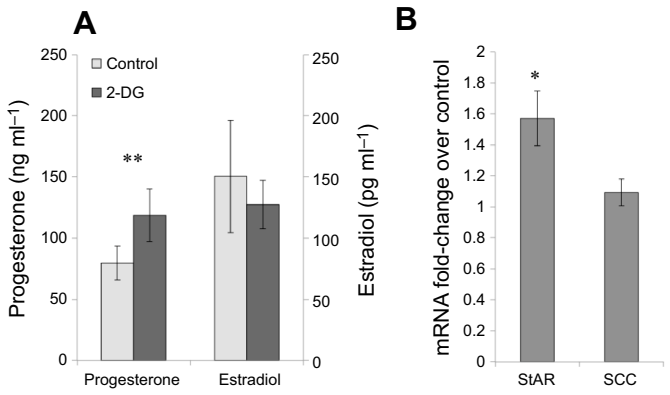

Fig. 1. 2-Deoxy-D-glucose increases progesterone release. (A) Exposure to 2-deoxy-D-glucose (2-DG) *in vitro* increased progesterone accumulation relative to controls; however, estradiol production did not change (*N*=15). (B) Exposure to 2-DG *in vitro* also increased mRNA levels of steroidogenic acute regulatory protein (StAR), but did not affect mRNA levels of cholesterol side-chain cleavage enzyme (SCC) (*N*=15). Values are expressed as fold-change over controls. Asterisks indicate *P*<0.002 (*) or *P*<0.001 (**).

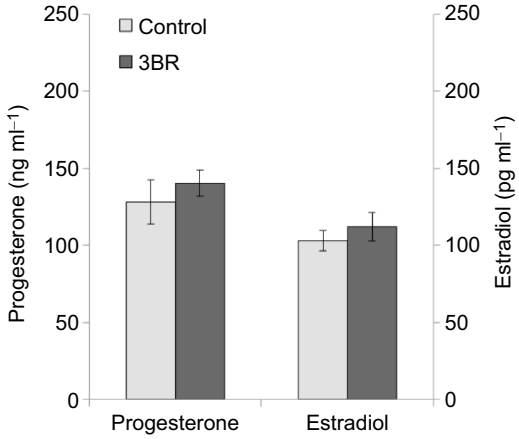

Fig. 2. Bromopyruvic acid has no effect on steroid production. Treatment of mouse ovaries with bromopyruvic acid (3BR) had no effect on progesterone or estradiol production (*N*=11).

ovarian steroidogenesis in mammals is directly responsive to glucose availability.

Although SCC acts as the rate-limiting step for steroid production, differential expression of StAR is involved in rapid changes in steroid production (Stocco and Clark, 1996; Ronen-Fuhrmann et al., 1998). StAR expression (mRNA and protein) increases in rodent gonads *in vitro* and *in vivo* within 4 h of gonadotropin stimulation (Espey and Richards, 2002), whereas SCC tends to be upregulated in response to gonadotropins over longer time periods, from 8 h to several days following exposure (Goldring et al., 1987; Oonk et al., 1989). Our data are consistent with acute upregulation of steroid hormone production (here, progesterone) via increased expression of StAR. Further quantification of mRNA and protein expression of steroidogenic enzymes will help us better understand how StAR modulation affects production of progesterone and other gonadal steroids.

Steroid production by the follicle is regulated, in part, by intra-follicular signaling, especially between the oocyte and granulosa cells (Eppig, 2001; Matzuk et al., 2002). Moreover, cumulus granulosa cells are responsible for metabolizing glucose into pyruvate for use by the oocyte (which takes up very little glucose by itself) (Dumesic et al., 2014). We therefore expected cumulus granulosa cells and oocytes to be responsible for the increased steroidogenesis that occurred in response to 2-DG. 3BR primarily inhibits glucose utilization by oocytes and granulosa cells within antral mouse follicles. 3BR therefore provides information about whether oocytes, granulosa cells and their interactions are independently responsible for the increased progesterone accumulation that occurs in response to 2-DG treatment. However, 3BR did not increase progesterone accumulation in the media (V=21, P=0.32), nor did it affect estradiol (V=22, P=0.37) (Fig. 2). Our data consequently suggest that elevated progesterone production in response to 2-DG is not mediated solely by oocyte–granulosa cell interactions. These experiments cannot provide information on the role of paracrine signaling from other sources or between theca cells and oocyte–granulosa cell complexes. Because outputs like steroid production are modulated by interactions among several cell types in the ovary, determining the precise mechanism by which 2-DG stimulates progesterone secretion requires further, cell-specific targeting.

Our findings contrast with McGuire et al.'s (2013) study that showed gonadotropin-stimulated avian ovaries decrease steroid hormone production in response to metabolic substrate depletion. McGuire et al. (2013) considered inhibition of steroid production to be consistent with an adaptive suppression of reproductive activity in response to metabolic stress (indicative of poor environmental conditions for breeding). Our data demonstrate an alternative response that is not consistent with an adaptive suppression of reproduction.

The differential response to low substrate availability could reflect differences in breeding strategy in the studied species. McGuire et al. (2013) showed an inhibitory effect of low glucose on ovarian steroids in European starlings, which are a strongly photoperiodic (seasonal) breeder. C57BL/6J mice, in contrast, are continuous breeders. Analogous to the starling work, seasonally breeding mammals such as deer mice (*Peromyscus maniculatus*) will readily inhibit reproductive attempts in energetically demanding conditions (Perrigo, 1987); the continuously breeding house mouse (*Mus musculus domesticus*) will continue to produce litters under energetically demanding conditions, but cannibalizes young during lactation (Perrigo, 1987). Comparable to these mammalian examples, opportunistically breeding zebra finches will maintain a partially activated reproductive system in spite of a low body condition (Perfito et al., 2007). The ability to breed opportunistically or continuously is therefore thought to reflect weak inhibition and increased sensitivity to positive environmental cues in both mammals and birds. The failure of low glucose availability to inhibit ovarian steroid production, as shown here, may therefore reflect a continuous breeder's lower sensitivity to inhibitory signals.

However, avian and mammalian physiology differs more broadly in ways that might also explain different ovarian responses to low energy availability. In mammals, progesterone is a well-established modulator of blood glucose (e.g. Bailey and Ahmed-Sorour, 1980; Kalkhoff et al., 1970), but similar regulation has not been shown in birds. In mammals, progesterone promotes an increase in circulating blood glucose. Though progesterone has primarily been recognized for its chronic role in glucose homeostasis during pregnancy (Kalkhoff et al., 1970; Lain and Catalano, 2007), progesterone signaling broadly inhibits the action of insulin (thus buffering blood glucose) by inhibiting storage and metabolism of glucose in adipocytes and muscle (Leturque et al., 1989; Sutter-Dub, 2002; Sutter-Dub et al., 1981; Wada et al., 2010). Progesterone can also

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promote the production of other hormones that increase blood glucose via action in the central nervous system: infusion of progesterone into the forebrain increases plasma noradrenaline (norepinephrine), presumably through modulation of sympathetic drive (Sandoval et al., 2007). Elevating progesterone in an acute response to low glucose availability may therefore stimulate known homeostatic processes through multiple mechanisms.

Our finding that the ovary increases progesterone production in response to low glucose availability is also consistent with work demonstrating that, *in vivo*, low glucose is associated with elevated progesterone in circulation. For example, bolus insulin injections used to induce hypoglycemia result in rapid increases in circulating progesterone in women (Gennarelli et al., 1999), and exercising women exhibit concomitant (<15 min apart) increases in circulating progesterone and blood glucose (Bonen et al., 1983). In addition, young men infused with 2-DG exhibit a relatively rapid (<1 h) elevation of serum progesterone (Breier and Buchanan, 1992; Elman and Breier, 1997; George et al., 1994). The acute increase in progesterone production measured *in vivo* has previously been attributed entirely to adrenal gland activity (Carroll et al., 1996; Provencher et al., 1990); however, our data indicate that the ovary acts as an additional source of progesterone following acute decreases in blood glucose concentration.

The contribution of progesterone to homeostatic glucose regulation is thought to depend on its concentration relative to circulating estrogens (Mauvais-Jarvis, 2015; Oosthuyse and Bosch, 2010; Varlamov et al., 2015). Elevated progesterone stimulates feeding behavior, but only when estrogens are simultaneously low (Butera, 2010; Gong et al., 1989). Despite finding no increase in estradiol production in response to 2-DG treatment, the ratio of P_4 to E_2 production did not differ between 2-DG and untreated ovaries (mean change in P_4:E_2=0.12±0.11; V=36, P=0.19). Whether ovarian progesterone production shown here is sufficient to stimulate homeostatic responses *in vivo* will depend on dynamics we were not able to investigate in this context. For example, progesterone and estradiol are released in pulsatile rhythms (Bäckström et al., 1982; Filicori et al., 1984; Genazzani et al., 1991), but surprisingly little is known about the physiological importance of these pulses to their targets. In women, pulsatile estradiol production by the ovary is delayed by about 20 min relative to progesterone peaks (Bäckström et al., 1982), and thus the temporal separation of pulsatile progesterone and estradiol may allow ovarian progesterone to positively regulate glucose homeostasis on acute time scales.

In addition to its role in glucose regulation, progesterone exerts paracrine effects within the ovary on follicle development (Peluso and Pru, 2014). For example, elevated progesterone in the follicular fluid of early-stage follicles is associated with atresia (Young and McNeilly, 2010). In later-stage follicles, progesterone may also positively regulate oocyte maturation and ovulation and exert anti-degenerative effects on granulosa cells (Borman et al., 2004; Hammes, 2004). Glucose availability also impacts follicle maturation and oocyte quality (Boland et al., 1994; Sutton et al., 2003). However, the effects of steroids and glucose availability on follicle maturation have been largely studied in the context of chronic exposure, so it is difficult to speculate as to whether acute changes in intra-ovarian glucose and progesterone availability meaningfully impact these outcome measures.

In summary, increasing steroidogenesis in response to pharmacologically induced hypoglycemia is not consistent with the expected response of the rest of the reproductive system under low energy availability (downregulation) (Hill et al., 2008). Our findings instead are consistent with a positive, homeostatic action of progesterone on blood glucose concentration in mammals, and perhaps also with the opportunistic breeding strategies in the lab mouse. Though the underlying mechanism and *in vivo* physiological importance of these changes require further investigation, the connection between ovarian steroidogenesis and glucose availability presents an intriguing new aspect of ovarian regulation (Fig. 3). Finally, even if hypoglycemia-induced progesterone production by the ovary plays a limited homeostatic role, dysregulated blood glucose likely impacts ovarian function via modulation of steroidogenic activity. We suggest that evaluating organ function in isolation can provide novel insight into organ interactions *in vivo* and further explain whole-organism responses. This is particularly true when examining hormone systems, which usually exhibit pleiotropic effects across tissues.

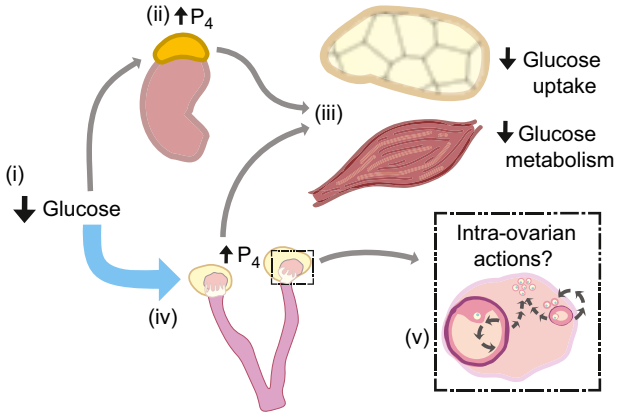

Fig. 3. Schematic diagram illustrating the connection between ovarian steroidogenesis and glucose availability. (i) Low blood glucose is known to stimulate progesterone production from (ii) the adrenal gland, which can relatively rapidly (iii) inhibit glucose uptake and metabolism in adipose tissue and muscle (see Discussion). (iv) We show that low blood glucose also stimulates progesterone production from the ovary (blue arrow). Stimulation of ovarian progesterone production could also contribute to (iii) the inhibition of glucose uptake in adipose tissue and muscle. (v) Progesterone production could also impact follicle metabolism and development through paracrine signaling within the ovary. Gray arrows: known pathways of control; blue arrow: novel pathway.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: K.W., G.E.B.; Methodology: K.W., A.P., G.E.B.; Validation: K.W., A.P.; Formal analysis: K.W.; Investigation: K.W.; Resources: G.E.B.; Writing - original draft: K.W.; Writing - review & editing: A.P., G.E.B.; Supervision: G.E.B.

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