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Cyclical DNA methyltransferase 3a expression is a seasonal and oestrus timer in reproductive tissues.

--Manuscript Draft--

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School of Biological Sciences
Zoology Building
Tillydrone Avenue
Aberdeen AB24 2TZ
Scotland
United Kingdom
Tel: +44 (0) 1224 274144

18th March, 2016

Endocrinology
2055 L Street NW, Suite 600
Washington, DC 20036

Dear Dr Hugh Taylor,

On behalf of my colleagues, thank you very much for the review of our manuscript entitled *Cyclical DNA methyltransferase 3a expression is a seasonal and oestrus timer in reproductive tissues* (EN-15-1988). We were pleased to read the positive response and the potential for publication in *Endocrinology.* We appreciate the constructive feedback from all three Reviewers and accordingly, we have thoroughly revised the manuscript. In the response to reviewer document, we have enumerated each reviewer comment and provide a detailed response. We feel the comments from the Reviewers have resulted in a much stronger manuscript. In brief, the major revisions in the manuscript include:

1) In response to Reviewer 1, we have completely revised the abstract and results according to their suggestion to separate testicular and ovarian analyses.
2) In addition to the preadsorption suggested by Reviewer 1, we have also conducted three negative control immunocytochemistry assays. All confirm the specificity of the DNMT3a antibody.
3) In response to Reviewer 2, we have conducted qPCR for a second reference gene, ribosomal 18s (*18s*). The revised analyses do not significantly change the findings or implications of the data compared to the initial submission.
4) Moreover, we have provided the uterine histology as requested by Reviewer 2 (see new Figure S3). The histology confirms that DNMT3a expression is primarily localized in the endometrium.
5) In response to Reviewer 3 we have conducted a hematoxylin and eosin stain on testicular tissue (new FigS2) as well as uterine tissue (FigS3). This stain provided the ability to confirm that DNMT3a is primarily localized to the spermatogonium in SD testes.

In addition, we have incorporated a series of minor revisions indicated by the Reviewers. Unfortunately, I am unable to accommodate the request by Reviewer 3 for an alternative method to examine global DNA methylation. Other techniques to confirm DNA methylation include chromatin-immunoprecipitation or sodium bisulfite conversion of DNA. Both of these approaches include bioinformatic analyses that are not financial feasible.

Thank you in advance for considering our revised manuscript for publication and we look forward to your response.

Sincerely,

Tyler John Stevenson, Ph.D.
Institute of Biological and Environmental Sciences
University of Aberdeen

General response:

We thank the reviewers for the objective and careful assessment of our manuscript. Below, we have enumerated each comment and provided a detailed response. We have endeavoured to incorporate each suggestion in the revised manuscript. There have been four major revisions:

1) The results have been revised using the average CT of 2 reference genes
2) We have conducted a hematoxylin and eosin stain on testicular tissue (new FigS2)
3) We have revised the statistical analyses and examined testicular and ovarian tissue separately.
4) We have conducted histological analyses on the uterine tissue to confirm DNMT3a expression is localized in the endometrium.

Reviewer #1:

Comment 1 - The abstract and results sections might benefit from a re-organization by sex. The comparison between testicular and ovarian methylation/dnmt expression is somewhat specious as it is unusual compare gene expression across tissues. The same holds true for comparison of testicular and ovarian mass by ANOVA as the magnitude of weight difference will differ by tissue. My suggestion would be to organize the abstract and results sections to discuss testicular methylation/dnmt expression and the testicular IHC results together. Then, discuss ovarian and uterine results, including those that point to hormonal changes contributing to seasonal changes. Conclude with the suggestion that melatonin might drive changes in both sexes based on the HEK293 cell culture work.

Response 1 – We agree with the Reviewers suggestion to reorganize the abstract and results section. We have revised both sections accordingly. Now, the abstract and result sections have been divided by sex and then followed by hormonal analyses in ovariectomized hamsters and HEK293 cells. We have also divided the male and female gonad data into two separate figures (new Fig1 and Fig2).

Comment 2 - The abstract is packed with a listing of findings without transitional elements to the sentences to highlight the impetus for the findings or their significance. The abstract might benefit from some consolidation of major findings and the addition of such transitional/supporting language.

Response 2 – We have considerably revised the abstract taking into consideration the suggestions raised in Comment 1 above. During the revision we endeavoured to include transitional language to consolidate the major findings.

Comment 3 - 9000 RPMs seems unusually high for spinning blood samples and has the potential to lyse RBCs. Is this a typo?

Response 3 – The plasma was centrifuged at 9000rpms. Indeed the speed has a significant impact on the ability to separate supernatant from RBCs; however the major impact is the diameter of the centrifuge. The important value is the g-force (indicated in the methods as 3622g) and is calculated based on the speed (i.e. rpms) and the diameter of the centrifuge. In our laboratory, the centrifuge has a relatively small diameter reducing the g-force. The 3622g is within the range previously used in the laboratory and reliably separates supernatant and RBC pellets. We are confident that the hormone analyses conducted in our laboratory minimized the amount of lysed RBC in the samples.

Comment 4 - I'm assuming that animals were deeply anesthetized before cervical dislocation. This point should be added to the methods where cervical dislocation is mentioned.

Response 4 – Female hamster used in the oestrus study were deeply anesthetized with isofluorane vapours (see lines: 102-104). This was conducted as we collected blood samples from the retroorbital sinus. We understand the Reviewers concern that other hamsters were not anesthetized before cervical dislocation. We anticipate that the Reviewer may not be familiar with ethical review procedures in the UK. The Home Office (UK Government) has outlined and maintains oversight over the procedures for the ethical treatment of research animals (i.e. Animal Scientific Procedures

Act 1986; Amended in 2002). In the document, a Schedule 1 method for approved euthanasia requires animals are killed by cervical dislocation. Only in conditions justified by scientific rationale (i.e. hormone sampling) are anaesthesia procedures approved before cervical dislocation.

Comment 5 - The testes are quite porous when sectioned and vulnerable to erroneous labeling using IHC. It would provide more confidence in the findings to have an LD and SD testis labeled with the antibody preadsorbed with the antigen. It's possible that necrotic SD testis tissue shows more non-specific labeling, necessitating the labeling of both LD and SD tissues with preadsorbed antibody.
Response 5 – We agree with the Reviewers concern. We have conducted four different ICC controls: with no primary antibody, no secondary antibody, no fluorescein and a dose-dependent response to pre-adsorption with a blocking peptide. Immmunoreactive signal was abolished when the primary, secondary or fluorscein was removed from the protocol. We conducted the preadsortion using either 5ug or 10ug of blocking peptide that targets the antibody sequence. Both concentrations significantly reduced ($p<0.05$ and $p<0.001$ respectively) the immunoreactive signal in testes. These data indicate that the DNMT3a antibody used in our analyses has a very high specificity for the endogenous DNMT3a protein expression. We have added the control assay to the method section (see lines: 181-186).

Comment 6 - It's possible that the negative results for ovarian methylation/dnmt expression result from the fact that LD females were not staged in this portion of the experiment in the same manner as they were for measuring cyclic changes. If the SD ovaries are compared to proestrus female ovaries, does a difference emerge? Basically, it's surprising that there are such pronounced changes across the cycle (and by E2P4 treatment), yet comparing SD animals with low E2P4 to LD animals does not show a difference. At the minimum, these points should be considered in the discussion.
Response 6 – We are not completely clear what the Reviewer is asking in this concern. It seems there is some mis-interpretation of the results; ovarian methylation was only assessed in the first study; and not across the oestrus study as indicated by the Reviewer. Because we did not detect a significant different in any measures, such as global methylation or methyltransferase, we opted to not investigate ovarian methylation in the oestrus study. It seems the Reviewer may have confused the uterine analyses in the oestrus study as ovarian analyses. In hindsight it would have been beneficial to collect the ovarian tissues to directly address the Reviewer's question of whether DNA methylation in the ovary changes across the female cycle. Unfortunately, we cannot examine the relationship proposed by the Reviewer at this time. However, we predict that any variation in ovarian methylation across the oestrus study would be extremely small, if present. In light of the robust increase in uterine *dnmt3a* between LD-SD; compared to the relatively smaller difference in uterine *dnmt3a* across the oestrus cycle, it is unlikely that a statistical difference would be detected for *dnmt3a* in the ovaries.

Reviewer #2:
Comment 1 - The Short Day (SD) acronym should be included earlier in the Abstract
Response 1 – We have included the SD acronym earlier in the revised Abstract.

Comment 2 - Line 64: delete 'and' after regulate
Response 2 – We have deleted 'and' accordingly.

Comment 3 - Line 72: include 'and cell culture' after 'uterine tissue'
Response 3 – We agree with the Reviewer that the cell cultures should be included in this sentence. Given the revised organization of the results, we have opted to state 'cell culture' after 'uterine tissue'. The sentence now states (see lines: 72-73): '*Here, we investigated the photoperiod and hormonal regulation of gonadal DNA methylation and dnmt1, dnmt3a and dnmt3b expression in testicular, ovarian and uterine tissue and cell culture'.*

Comment 4 - Line 135: More details of the cell line should be provided. In particular, it should be made clear that these cells are derived from human non-reproductive tissue (kidney) and why hamster cell lines couldn't be used; in the Discussion it should be made clear that extrapolation from these cells to in vivo reproductive tissues in rodents should be made with caution.
Response 4 – We agree with the Reviewer that hamster cells, such as the Chinese hamster ovary (CHO), could have been a suitable model. Once we observed the complete lack of photoperiodic change in global DNA methylation and DNA methyltransferases (*dnmt*), CHO cells were clearly inappropriate to investigate the role of melatonin for the regulation of *dnmt* expression. HEK293 cells were selected because they express all the key players; such as the melatonin receptor 1 (Conway et al., 1997; new citation #27) and *dnmt* enzymes. We have added the rationale for using HEK293 cells in the methods (see lines: 189-193) and a sentence in the discussion that states melatonin driven methylation in HEK293 should be interpreted with caution when extrapolating to other cells and animals (see lines: 334-336).

Comment 5 - The quantitative PCR analyses only employed one housekeeping gene (Gapdh). It is best practice to use a number (at least two) housekeeping genes whose expression is highly correlated for normalisation purposes to ensure that data aren't confounded by effects of the experimental manipulations on Gapdh expression.
Response 5 – We agree with the Reviewer that additional reference genes should be used to assess RNA expression. We had initially conducted qPCR for ribosomal 18s (*18s*) in a subset of tissues and observed a very high correlation with *gapdh* (r=.94). In order to address this concern, we have conducted *18s* qPCR for all experiments (see lines 161). We used the average CT of *gapdh* and *18s* for calculating the Fold expression. qPCR analyses have been revised with the new results. The addition of the *18s* did not impact the narrative of the findings or eliminate the significance of the results. Undoubtedly, there have been some slight modifications to F-values and p-values; however, the overall outcome of photoperiod, estrogen and melatonin driven changes in *dnmt3a* remains.

Comment 6 - The authors should explain why histological analyses were only performed in testes, and not in uterus/ovary tissue
Response 6 – Given the lack of photoperiodic variation in DNA methylation within the ovaries and the well defined localization of DNMT3a in the endometrium layer in the uterus, we opted to focus on identifying the specific cell types in the testes. We have added a supplementary figure (FigS3) from LD and SD uterine tissue to illustrate the robust change in DNMT3a expression. Uterine tissue was stained for the DNMT3a (FigS3 G,H) antibody and compared to sections stained with hematoxylin and eosin. DNMT3a immunoreactivity shows a clear increase in the SD endometrium.

Comment 7 - Lines 201-202: method of testing for normality should be included
Response 7 – We have revised the sentence and included the normality test used. The text now states: *'Shapiro-Wilk Normality tests were conducted on all data sets to ascertain whether parametric or non-parametric analyses were appropriate'* (see lines: 210-212).

Comment 8 - Line 205: 'mass' should be included after 'testes'
Response 8 – The section on 'Statistical analyses' was revised and this concern was incorporated (see lines: 210-212) specifically: '*T-test was conducted to examine photoperiod effects on testes, ovarian and uterine mass as well as global DNA methylation and DNA methyltransferase expression*.'

Comment 9 - Line 215: Dnmt3b expression increases ~2-fold rather than ~0.5-fold
Response 9 – In response to Reviewer 1, we have completely revised the results and separate testicular and ovarian data. In the revised section, we have not included a statement on the fold change in *dnmt3b* expression.

Comment 10 - Lines 218-220: data should be presented 'per unit volume' rather than simply as number of cells
Response 10 – The Reviewer is correct and we appreciate the notification of our oversight. The number of DNMT3a cells was summed across 10 seminiferous tubules and the average for each photoperiodic treatment is presented in Fig2B. In the interest of clarity, we have not included the unit volume on the y-axis. Instead we have included the per unit volume in the methods section as well as the figure legend. See lines: 179-180.

Comment 11 - Lines 221-228: Expression data might be included for an additional few genes to show whether melatonin-induced increase, or estrus cycle changes, in Dnmt3a/b expression are specific, or due to a general up-regulation of transcription e.g. Dnmt1, genes adjacent to Dnmt3a/3b, other genes involved in methylation processes such as Mecp2
Response 11 – We appreciate the Reviewer's concern that *dnmt3a/b* expression may represent a general up-regulation of transcription. However, existing data indicates that this is not the case. For example, photoperiod treatment did not affect the reference gene CT ($p=0.52$); indicating that there was not a general up-regulation of transcription. Moreover, *dnmt1* expression does not consistently change with *dnmt3a* expression, indicating a level of dissociation between the activation of gene transcription for the respective DNA methyltransferases. Given these two patterns, we feel the additional analyses of general (e.g. reference genes) or methylation genes (e.g. MeCP2) are not warranted.

Comment 12 - Lines 230-232: authors need to explain why DNA methylation in uterus was not examined.
Response 12 – We agree that it would be beneficial to confirm that SD uterine tissue exhibits a significant increase in global DNA methylation (and across the oestrus cycle [comment 14 below]; and driven by E2P4). Unfortunately, the cost for these assays is currently prohibitive. It would require additional animals and consumables to conduct the global DNA methylation assay that are presently not available. Given the robust photoperiodic change in the testes that is paralleled by the change in the uterus, it is likely that global DNA methylation increases in the SD uterine, oestrus and in response to E2P4. In addition to the other control studies were have conducted (e.g. uterine DNMT3a histology in LD/SD) that confirm the photoperiodic switch in DNA methylation, we feel that this addition work is not necessary. We have provided a sentence in discussion that highlights the importance of confirming global DNA methylation in the uterus; in addition to employing other methods (i.e. ChIP) (see lines 358-360).

Comment 13 - Line 242: change 'slight' to 'slightly'
Response 13 – The text has been changed accordingly.

Comment 14 - Lines 244-248: Need to clarify that expression data are from uterine tissue; reasons for not examining DNA methylation across estrus cycle need to be provided
Response 14 – A similar concern was raised above (i.e. Comment 12) and a response has been provided there.

Comment 15 - Figs 1 and 5B: need to indicate clearly which pairwise comparisons are significant
Response 15 – The results and figures have been significantly revised to address Comment 1 raised by Reviewer 1. In the revised analyses, we have conducted pairwise comparisons and these are indicated in the revised Figures.

Comment 16 - Fig 1: Tissues (testes and ovary) need to be included on the x-axis. y-axis should be labelled 'relative dnmt1 expression' etc. Often, with the delta delta Ct method, one control group is

assigned an arbitrary value of 1; perhaps this could be done considered here to make reading fold changes in expression easier to determine.
Response 16 – Similar to response 15; we have considerably revised the organization of the results to accommodate suggestions by Reviewer 1. The revised manuscript has addressed the concerns raised here. In the interest of clarity, we have kept the y-axis as '*dnmt* expression'. We have used 'relative expression' when discussing the patterns of *dnmt* in the figure legends.

Comment 17 - The wide age range of the experimental animals might be commented upon; presumably reproductive function and success varies considerably between the ages of 3 and 8 months
Response 17 – We have selected the age range because hamsters are considered 'adults' between the ages of 3-9 months. Reproductive senescence, particularly in females, does not occur until 9 months (at the earliest). It is common to maintain animals in long day conditions without detectable changes in reproductive function for over 12 months (e.g. Stevenson & Prendergast, 2013 PNAS). We have added a sentence in the methods that supports the age range selected for investigations. Specifically, lines: 83-85 state 'Adulthood in hamsters occurs between the age of 3-9m months (15). Hamsters are classified as aged at 14 months (16) and reproductive decline does not occur in females until after 9 months (15)'.

Reviewer #3:
Comment 1 - The animals used appear to live permanently before experimenting with long photoperiod condition. However, this species is in nature subjected to photoperiod changes. So is it possible to consider that animals used have normal physiology?
Response 1 – The photoperiodic conditions and age range used in this study is common for investigations of seasonal changes in reproductive physiology. It is common to be exposed to LD photoperiod for up to 1 year without significant effect to testicular volume/mass or reproduction function (e.g. 7). Moreover, aging and reproductive function has been examined in Siberian hamsters (15,16). Specifically, Horton & Yellon (2001; citation 15) demonstrated that it takes over 9 months for reproductive function to decline, even in constant photoperiodic conditions. Therefore, the photoperiodic conditions selected for this experiment are entirely suitable to assess normal physiology. We have added a sentence in the methods section that addresses this concern. Please see lines 83:85.

Comment 2 - Figure 1B shows a 4 fold variation of global methylation in male. This variation is very significant for DNA methylation. To consolidate this result, the authors should confirm it using another technology.
Response 2 – We feel that the additional measures including *dnmt3a* and DNMT3b in testicular tissue and the examination of *dnmt3a* across three other studies in females is sufficient to support the marked change in global DNA methylation in reproductive tissues (i.e. testes). Indeed Chromatin Immunoprecipitation assays for MeCP2 and/or sodium bisulfite sequencing of DNA would provide an alternative method; however, these approaches are not financially feasible at the current time. We have included a statement that indicates other methods should be conducted to confirm the variation in global methylation (see lines 358-360).

Comment 3 - The staining difference observed between figure 2C and 2D is highly visible. However, could this observation be the result of the changes of the organization of the testes. It is clear that the size and shape of the seminiferous tubules are not similar. Authors should also take the opportunity to identify the type of cell that expresses Dnmt3a.
Response 3 – We agree with the reviewer that the seminiferous tubules exhibit marked morphological changes; these include a massive reduction in the lumen and a decrease in the number of sertoli cells and spermatogonia (Type A and B) (see Meachem et al., 2005; Bio Reprod). Previous work has illustrated that *dnmt3a* is localized in the spermatogonia (see citation 12). In

order to confirm the cells that exhibit DNMT3a expression, we conducted hematoxylin and eosin stain of testes sections (See new Fig S2). It appears that in hamsters, DNMT3a is expressed in the leydig cells in both LD and SD conditions. However, the increased DNMT3a in SD appears to occur in the spermatogonium layer, consistent with the previous identified anatomical localization in testes (12).

Comment 4 - The use of melatonin is relevant. However, it is necessary to justify the move to an ex vivo model (the cells HEK293) and not to continue the analysis in vivo.
Response 4 – We agree with the Reviewer and have added a couple sentences in the methods section that justify the move to ex vivo model and the choice for HEK293 cells. The text now states (see lines 334-336): In order to assess the potential direct effects of melatonin on *dnmt3a* and *dnmt3b* expression, we conducted a melatonin dose-dependent study using cell culture. Given the low levels of DNA methylation and absence of *dnmt3a/3b* plasticity in the ovary (see results below) we selected HEK293 to examine the role of melatonin dependent regulation of *dnmt* expression as these cells are known to express melatonin receptor 1a (27).

Comment 5 - In the discussion lane 287 to 290, the observations made with CONSITE are too speculative to be specified using "indicates that it is likely". It is only one track to test experimentally. The authors should consider removing this part or perform the experiments suggesting that their hypothesis is true.
Response 5 – We have removed the text from the discussion.

Comment 6 - In the conclusion, the authors indicate that the data have direct applications to human fertility. It may be a bit exaggerated given the results.
Response 6 – We have removed the text from the discussion.

Comment 7 - The presentation of results in the abstract is poorly organized.
Response 7 – A similar concern was raised by Reviewer 1 above. We have considerably revised the abstract with a focus on reorganization of the results.

Comment 8 - Figure 1A and 2A seem very similar. It is not clear what is the difference between the two results.
Response 8 – Figure 1A and 2A both depict photoperiodic regulation in testes mass from two different studies. Figure 1A shows SD induce gonadal involution and these testes were used for the *dnmt1, 3a* and *3b* expression analyses (Fig1B-D). Figure 2A is a separate group of males (see lines xxx) that were used to provide confirmation that SD induced gonadal involution. These testes were used for the DNMT3a histology (Fig 2C,D).

Comment 9 - In figure 1C, the expression of dnmt1 slightly decreases in male. Is it possible to reach significance by increasing the number of samples?
Response 9 – In the revised results, the t-test revealed that SD significantly reduced *dnmt1* expression (see new FigS1a). This observation has likely developed from the addition of the second reference gene as recommended by Reviewer 2 (see comment #2 above).

Comment 10 - In figure 5, the authors should clarify in the legends, the meaning of white bars, gray bars and black bars.
Response 10 – We thank the Reviewer for this suggestion and we agree this would increase the clarity of the figure legend. The text now includes the distinction between white, gray and black bars. We have also indicated what the bars represent in Figure 6.

Comment 11 - There is no figure legend for supplementary figure 1.

Response 11 – We apologise for this oversight. Figure legends have been added to all supplementary figures.

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Cyclical DNA methyltransferase 3a expression is a seasonal and oestrus timer in reproductive tissues.

Eloise WJ Lynch, Chris S Coyle, Marlene Lorgen, Ewan M Campbell, Alan S Bowman & Tyler J Stevenson*

Institute for Biological and Environmental Sciences, University of Aberdeen, Aberdeen, UK

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**Corresponding author:*
Tyler J Stevenson
Institute for Biological and Environmental Sciences
University of Aberdeen
Aberdeen, UK
AB24 2TZ
Email: tyler.stevenson@abdn.ac.uk

Disclosure statement: The authors have nothing to declare

Abstract

It is becoming clear that epigenetic modifications such as DNA methylation can be dynamic and in many cases, reversible. Here, we investigated the photoperiod and hormone regulation of DNA methylation in testes, ovaries and uterine tissue across multiple time scales. We hypothesized that DNA methyltransferase 3a (*dnmt3a*) is driven by photoperiodic treatment, exhibits natural variation across the female reproductive cycle and that melatonin increases whereas estrogen reduces DNA methylation. We used Siberian hamsters (*Phodopus sungorus*) due to their robust changes in reproductive physiology across seasonal and oestrus time scales. Our findings indicate that short day (SD) – winter like conditions significantly increased global DNA methylation and *dnmt3a* expression in the testes. Using immunohistochemistry, we confirm that increased *dnmt3a* expression was primarily localized to spermatogonium. Conversely, the ovaries did not exhibit variation in DNA methylation or *dnmt3a/3b* expression. However, exposure to SD significantly increased uterine *dnmt3a* expression. We then determined that *dnmt3a* was significantly decreased during the oestrus stage. Next, we ovariectomized females and subsequently identified that a single estrogen+progesterone injection was sufficient to rapidly inhibit *dnmt3a* and *dnmt3b* expression. Finally, we demonstrate that treatment of HEK293 cells with melatonin significantly increased both *dnmt3a* and *dnmt3b* expression suggesting that long-duration nocturnal signalling in SD may be involved in the regulation of DNA methylation in both sexes. Overall, our data indicate that *dnmt3a* shows marked photoperiod and oestrus plasticity that likely has broad downstream effects on the timing of the genomic control of reproductive function.

Introduction

Biological rhythms in reproductive physiology are common across vertebrates; from fish and reptiles to bird and mammalian species (1-3). Our understanding of a role for epigenetic modifications, such as DNA methylation, in regulating biological rhythms is in its infancy. Daily rhythms in metabolism and food intake are strongly associated with cyclical changes in histone acetylation (4,5). Moreover, daily changes in the amount of DNA methylation in a number of gene promoter regions are involved in timing circadian locomotor behavior (6). Despite these advances, the role of epigenetic rhythms during reproductive cycles is not well described. In seasonally breeding species such as the Siberian hamster (*Phodopus sungorus*), the hypothalamus exhibits photoperiod-dependent reduction in global DNA methylation and enzymes involved in the methylation of DNA (7). Whether similar changes in DNA methylation occur in the timing of reproductive physiology in peripheral tissues, such as the testes, ovary and/or uterus, is poorly understood.

In mammals, the key enzymes that catalyze the methylation of DNA consist of three distinct isoforms: DNA methyltransferase 1 (*dnmt1*), 3a (*dnmt3a*) and 3b (*dnmt3b*). *dnmt1* is critical for maintenance methylation of DNA during cell division; whereas both *dnmt3a* and *dnmt3b* are involved in *de novo* methylation primarily in post-meiotic cells (8,9). *dnmt1, 3a* and *3b* enzymes have been identified in testes, ovary and uterine tissue. *dnmt1, dnmt3a* and *dnmt3b* are predominantly localized in the spermatogonia (10), epithelial layer in ovaries (11) and endometrium cells in the uterus (12). The localization of *dnmts* in peripheral reproductive tissues indicates the potential for timing cyclical changes within molecular pathways involved in fertility (13). Indeed the mechanisms that regulate the functional role of methyltransferases are well described in germline cells and during development (reviewed by 14). The objective of this paper was to examine photoperiod and hormone dependent changes in DNA methylation and *dnmt1, dnmt3a* and *dnmt3b* mRNA expression in adult testes, ovary and uterine tissues.

Given the massive seasonal and oestrus changes in testicular and uterine tissue in Siberian hamsters, we tested the hypotheses that peripheral reproductive tissues exhibit significant variation in global gonadal DNA methylation and DNA methyltransferase expression. Here, we investigated the photoperiod and hormonal regulation of gonadal DNA methylation and *dnmt1, dnmt3a* and *dnmt3b* expression in testicular, ovarian and uterine tissue and cell culture. Using adult male and female Siberian hamsters, we identified marked naturally

occurring plasticity in *dnmt3a* methyltransferase expression that is regulated by photoperiod, melatonin and ovarian hormones. Increased *dnmt3a* in short day (SD) testes results in a substantial accumulation of global DNA methylation. The findings reported herein reveal robust plasticity in key DNA methylation enzymes and indicated epigenetic reorganization within peripheral reproductive tissues across multiple time scales. Overall, this work has significant implications for reproductive timing and fertility in mammalian species.

Methods

Animals

Adult male and female Siberian hamsters (Total N=100; 3-8 month old) were randomly selected from a colony maintained at the University of Aberdeen. Adulthood in hamsters occurs between the age of 3-9m months (15). Hamsters are classified as aged at 14 months (16) and reproductive decline does not occur in females until after 9 months (15). Hamsters were housed in polypropylene cages in a long day (LD) photoperiod (15L:9D). Food and water were provided *ad libitum* and hamsters were provided cotton-nesting material. All procedures were approved by the Animal Welfare and Ethics Review Board at the University of Aberdeen and conducted under the Home Office licence (70/7917).

Experimental designs

Photoperiod regulation of reproductive physiology and gonadal DNA methylation

Thirty-six adult male and female hamsters (3-8 months) were used in this study. Male (n=8) and female (n=8) hamsters were group housed in long day (15L:9D) conditions prior to the experiment. Baseline measures of body weight were recorded and measured for the duration of the experiment. A group of males (n=10) and females (n=10) were transferred from LD to short day cabinets (Arrowmight; SD 9D:15L) for 8 weeks. At the termination of the study animals were sacrificed by cervical dislocation and testes, ovary and uterine mass was determined using aeADAM scales (Adam Equipment PGL2002) and measured to ±0.1g. Tissues were frozen in powdered dry ice and stored at -80°C until global gonadal DNA methylation and RNA expression analyses (see below).

Naturally occurring changes in uterine DNA methyltransferase enzymes across the oestrus cycle

Female hamsters (N=33) were group housed and maintained in LD. On the final day of the experiment, animals were sacrificed from 1500-1700 in order to capture the proestrus surge in prolactin (17). Females were lightly anaesthetized with isofluorane gas (4%) and 500 µl whole blood was collected via the right retro-orbital sinus using Natelson tubes coated with sodium heparin. The blood samples were kept on ice and then centrifuged at 9000rpm (3622g) in 4°C for 20 minutes. Plasma was removed and stored at -20°C until prolactin levels were determined by ELISA assay (see below). Females were sacrificed by cervical dislocation and uterine mass was measured and subsequently frozen in powdered dry ice. Samples were kept at -80°C until RNA extraction.

Unlike mice and rats, female hamsters do not exhibit marked cyclical changes in vaginal cell types resulting in the inability for external tracking of the oestrus stages. In order to determine stage of oestrus cycle we took advantage of a well described method that uses convergent measures consisting of uterine mass and plasma prolactin concentrations (17). The combinations of reproductive measures permit the identification of diestrus (low prolactin; small uteri), proestrus (high prolactin; intermediate uteri) and oestrus (low prolactin; engorged uteri) stages of the female cycle. Plasma prolactin concentrations were determined using a Hamster Prolactin ELISA (2BScientific Ltd, Oxfordshire, UK). Samples were assayed in duplicate and compared to a standard curve. The intra-assay coefficient was 4.4%. Analyses of the uterine weight and plasma prolactin values resulted in the identification of diestrus (n=13), proestrus (n=12) and oestrus (n=8) females.

The sufficiency of ovarian steroids to regulate DNA methylation enzymes

In order to assess the sufficiency of ovarian hormones on uterine DNA methyltransferase expression, females were ovariectomized (N=21) and maintained in LD for 8 weeks to reduce circulating levels of gonadal steroids. In brief, ovariectomies were conducted while hamsters were under deep anaesthesia (5% isofluorane gas). The ovaries are externalized via bilateral incisions to the dorsum (lateral to the spine, caudal to the ribcage). The ovary was localized at the distal end of the uterine horn and ligated with sterile sutures (4-0, non-absorbable monofilament nylon). The ovary was then excised and repeated for the other ovary. The abdominal wall and skin were closed separately with sterile sutures (5-0 non- absorbable and 4-0 non-absorbable, respectively; monofilament nylon). After ovariectomy, female body mass decreased on average 7.5g (±0.9

SEM); a reliable long term indicator of reduced ovarian steroids (18). Estrogen and progesterone (E2P4) injections were prepared by dissolving diethylstilbestrol (Sigma Aldrich, UK) and progesterone (Sigma Aldrich, UK) in sterile vegetable oil to a final concentration of 5µg E2 and 500µg P4 in 100µL vegetable oil (OIL). These values were selected based on previous work in female hamsters (19). Females received an intraperitoneal injection at 1700 with 100µl of the hormone cocktail. Control hamsters were injected with 100µl of OIL. The following day hamsters were sacrificed at 12h (n=6) and 24h (n=6) post-injection by cervical dislocation and uterine weights were measured and frozen in powdered dry ice. We selected 12hr and 24hr time points to control for potential daily variation in *dnmt3a* or *dnmt3b* expression. OIL controls were counterbalanced across the 12h and 24h collection periods.

Assessment of global gonadal DNA methylation

DNA was extracted from tissues using DNeasy kits (QIAGEN, UK) following the manufacturer's directions. 1µg of DNA was digested using nuclease P1 (5 units; Sigma Aldrich, UK) and then incubated at 70ºC for 30 minutes. 1 µl of alkaline phosphatase (5 units; Sigma Aldrich, UK) was added and the samples were incubated at 37ºC for 30 minutes. The samples were then transferred to 65ºC for 15 min and then placed at -20ºC until assayed. Global DNA methylation levels were measured using a 5'-methyl-2'-deoxycytidine quantitation ELISA kit (Cell Biolabs Inc.). The kit is a competitive assay used for the quantification of 5-methyl-2'-deoxycytidine and has previously been used in Siberian hamsters (7). Samples were run in duplicates and the intra-assay CV was 10%.

Quantification of RNA expression

RNA was extracted from tissues using Trizol (ThermoFisher Scientific). Nucleic acid concentration and quality were determined by spectrophotometer (Nanodrop, Thermo Scientific). cDNA was synthesized using Superscript III (Invitrogen) and cDNA was stored at -20ºC until quantitative PCR was performed. All cDNA tissue samples were run in triplicate; cDNA from HEK293 cell culture and oestrus study were assayed in duplicate. qPCRs were performed using a BIORAD CFX96 system using the following steps i) an initial denature at 95°C for 30 secs, then 39 cycles of ii) 95°C for 10 sec, iii) annealing dependent on target mRNA

(See Table S1) for 30 secs and then iv) an extension at 72°C for 30 sec. The specificity of select samples was established by resolving PCR products in 2.5% agarose gel. A melting curve analysis was added to determine the quality and specificity of each reaction. Quantification of mRNA expression levels was accomplished with iQ Sybr Green Supermix (BIORAD, UK). We used PCR Miner (22) to calculate reaction efficiencies (E) and cycle thresholds (CTs). According to the MIQE guidelines, samples that had efficiency values below 0.8 or above 1.2 were excluded from analyses (23). The expression of each target gene of interest was measured in relation the average cycling time (CT) for two reference targets: glyceraldehyde 3-phosphate dehydrogenase (*gapdh* 7,24) and 18S ribosomal RNA (*18s; 25*) and calculated using 2-(delta-deltaCt).

Histological analyses of dnmt3a

Male hamsters (n=10) were divided into LD and SD conditions (n=5 each) for 8 weeks. Testes length and width were measured, weighed and frozen in powdered dry ice. Testes were sectioned at 30 µm with a cryostat (Reichert-Jung) in series of three. Microscope slides were then placed at -80°C until the immunocytochemistry (ICC) procedure. Testes sections were washed three times for 5 minutes in 0.1% tween-20 (Sigma-aldrich) in 1M PBS (PBSt). Then tissues were incubated in 3% hydrogen peroxide for 20 minutes followed by three 5 minute washes in PBSt. Tissues were incubated in 10nM sodium citrate at 83ºC for 30 minutes followed by three 5 minute washes. Tissues were then incubated in 5% normal goat serum for 1 h at room temperature, and then with the DNMT3a primary antibody (PA3-16557, ThermoFisher) at 4ºC for 48 hours. The sections were then washed 3 times, incubated in a biotinyated goat anti-rabbit second antibody (Vector Labs) for 1 h at room temperature, washed 3 times, incubated in avidin biotin horseradish peroxidase complex (Vectastain ABC Elite, 1:200) for 1 h, and then washed 3 times. DNMT3a was visualized by incubating tissue sections in fluorescein (Vector Labs) for 5 minutes. Sections were then washed 3 times, serially dehydrated and cover slipped using Vectashield mounting medium with DAPI (Vector Labs). Sections were examined using fluorescence light microscopy (Zeiss), and photomicrographs were captured using Zeiss Slide scanner AxioScan.Z1. Photomicrographs were analysed using ImageJ and cell counts were conducted using unbiased stereology (26) as previously described (27). The number of DNMT3a cells determined taking the sum of immunoreactive cells from 10 randomly selected seminiferous tubules for each hamster.

The specificity of the ICC signal was tested using four controls. We conducted the ICC protocol in the absence of the primary antibody, no secondary antibody and no fluorescein. In all cases, the immunoreactivity signal was abolished. Preadsorption of the primary antibody with 5ug or 10ug blocking peptide (3227BP; Cambridge Bioscience Ltd) for 2 hours resulted in a dose-dependent decrease in staining intensity ($p<0.05$ and $p<0.001$, respectively). Overall, these data indicate that the primary antibody used here is specific for the endogenous DNMT3a antigen.

Sufficiency of melatonin to drive DNA methylation enzymes

In order to assess the potential direct effects of melatonin on *dnmt3a* and *dnmt3b* expression, we conducted a melatonin dose-dependent study using cell culture. Given the low levels of DNA methylation and absence of *dnmt3a/3b* plasticity in the ovary (see results below) we selected HEK293 to examine the role of melatonin dependent regulation of *dnmt* expression as these cells are known to express melatonin receptor 1a (28). Cells of the HEK293 cell line were grown in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% Fetal bovine serum, 1% Penicillin/Streptomycin and sodium pyruvate (complete medium) in a T75 flask in a humidified atmosphere at 37°C with 5% CO_2. HEK293 cells were selected for melatonin assay as these cells express *dnmt3a* and *dnmt3b* as well as the melatonin receptor involved in the neural control of the seasonal photoperiodic response (20,21). When confluent, HEK293 cells were plated in 24 well plates as follows. Media was removed and cells were rinsed with Phosphate buffered saline (PBS at 37°C) and 5ml of trypsin was added to detach cells. 2ml of media was added before pipetting up and down to mix and transferring to a 15ml tube to pellet cells by centrifugation. Supernatant was removed and cells re-suspended in 7ml complete medium (at 37°C). 150µl of cell suspension (~$1x10^6$ cells) was added to each well in a total volume of 1.5ml complete medium. Cells were allowed to settle for 48 hours in a humidified atmosphere at 37°C with 5% CO_2 before stimulation. In order to examine the sufficiency of melatonin (Sigma Aldrich, UK) to induce RNA expression, HEK93 cells were assigned to four treatment groups: (1) saline controls; (2) 1nM melatonin (3) 10nM melatonin or (4) 100nM melatonin. Stimulated cells were incubated for 4 hours in a humidified atmosphere at 37°C with 5% CO_2. Wells containing cells and medium were then transferred to -80°C.

Statistical analyses

SigmaStat 13.0 was used for all statistical analyses and significance was determined at $p < 0.05$. Shapiro-Wilk Normality tests were conducted on all data sets to ascertain whether parametric or non-parametric analyses were appropriate. T-test was conducted to examine photoperiod effects on testes, ovarian and uterine mass as well as global DNA methylation and DNA methyltransferase expression. One-way ANOVA was conducted to examine the effect of hormone (i.e. E2P4, melatonin) treatment on *dnmt* expression. Dunnett's *post hoc* analyses were performed to compare hormone treatment versus untreated control conditions. Fishers Least Square Difference (LSD) was conducted to determine significant difference in uterine mass, plasma prolactin concentrations and *dnmt* expression across the oestrus cycle. Log-transformation was conducted on qPCR data when violations in normality were detected.

Results

SD induced gonadal involution facilitated testicular DNA methylation

Exposure to SD significantly reduced testes mass (t=14.33; p<0.001; Fig1a). Regressed testes were observed to have a robust and significant effect on global DNA methylation levels (t=3.17; p<0.005; Fig1b); indicating that the timing of testicular involution may be controlled by increased DNA methylation. Next, we assessed the levels of DNA methyltransferase expression in order to identify the enzymes involved in the catabolism of increased DNA methylation in regressed testes. *dnmt1* expression was found to have significantly greater levels in LD compared to SD conditions (t=2.77; p<0.01; Fig S1a). Increased *dnmt1* in LD testes may be due to the production of sperm during the breeding periods. *dnmt3a* expression was observed to exhibit the predicted increase in SD testes, regressed testes had significantly greater levels compared to LD (t=2.80; p<0.01; Fig1c). *dnmt3b* expression was found to remain constant across photoperiodic conditions (t=0.79; p=0.22; Fig1d).

Regressed testes have more DNMT3a expressing cells

A *t*-test was conducted to evaluate the effect of SD on the number of DNMT3a expressing cells in the testes. SD significantly reduced testes mass (t=9.05; p<0.001; Fig2a). There was a significant increase in the

number of DNMT3a cells in the SD compared to LD testes (t=2.159; p<0.05; Fig 2b-d). The SD increase in DNMT3a appears to be localized to spermatogonium (Fig S2).

Ovarian DNA methylation remains constant across photoperiodic conditions.

Ovary mass showed a relatively small, yet significant decrease in SD compared to LD hamsters (t=2.17; p<0.05; Fig3a). Unlike the testes, there was no significant photoperiodic effect on ovarian global DNA methylation (t=0.81; p=0.21; Fig3b). Not surprisingly, there was no significant difference between LD and SD levels of *dnmt1* (t=1.39; p=0.09; FigS1b), *dnmt3a* (t=0.27; p=0.39; Fig3c) or *dnmt3b* expression (t=0.62; p<0.27; Fig3d).

SD significantly increased uterine dnmt3a and dnmt3b

Exposure to SD significantly reduced uterine mass (t=3.388; P<0.005; Fig4a). Photoperiodic condition did not significant effect on *dnmt1* expression (t=0.95; p<0.18; FigS1c). The decrease in uterine mass was paralleled by a significant increase in *dnmt3a* expression (t=3.103; P<0.05; Fig4b) and *dnmt3b* expression (t=10.0; P<0.01; Fig4c). Histological analyses indicate that DNMT3a expression in SD shows a robust immunoreactive signal in the endometrium layer in the uterus (12, FigS3).

dnmt3a expression is reduced during oestrus

As previously established (Dodge *et al*., 2002), the oestrus cycle in female hamsters can be determined using the combined uterine mass and plasma prolactin measures. A one-way ANOVA indicated that uterine mass exhibits significant variation across the cycle (F=15.623; P<0.001; Fig5a). LSD *post-hoc* analyses confirmed that diestrus females have significantly lower uterine mass compared to proestrus (P<0.01) and oestrus (P<0.001) stages. Furthermore, the uterine mass during oestrus was significantly engorged and greater compared to proestrus (P<0.005). Plasma prolactin exhibited significant variation across the oestrus cycle (F=24.202; P<0.001; Fig5a). LSD analyses indicated that plasma prolactin concentrations significantly increased from diestrus to proestrus (P<0.001). Plasma prolactin concentrations then decreased during the oestrus phase (P<0.001). Oestrus females were found to have slightly higher levels of plasma prolactin compared to diestrus females (P<0.05).

A one-way ANOVA revealed a significant difference in *dnmt3a* (F=3.53; P<0.05; Fig5b) expression across the oestrus cycle. LSD analyses indicated that *dnmt3a* expression significantly decreased during the

transition from proestrus to oestrus (P=0.01). Diestrus females had intermediate levels as *dnmt3a* levels were not significantly different compared to oestrus (P=0.44) or proestrus (P=0.06) hamsters. There was no significant variation in *dnmt3b* expression observed across diestrus, proestrus or oestrus phases (F=2.22; P=0.33; Fig5c). There was no significant change in *dnmt1* expression across the oestrus cycle (F=0.26; P=0.77; FigS1d).

E2P4 is sufficient to inhibit DNA methyltransferase expression

Kruskal-Wallis ANOVA revealed that a single bolus injection of E2P4 was sufficient to significantly increase uterine mass (H=8.34; p<0.05; Fig6a). Dunnett's method identified that uterine mass was significantly greater than OIL treated controls 12hr (p<0.05) and 24hrs (p<0.05) post-injection. These data confirm that E2P4 was capable of inducing engorged uterine and oestrus within 24hrs. A one-way ANOVA revealed a significant difference in *dnmt3a* expression after administration of E2P4 (F=13.57; P<0.001; Fig6b). Dunnett's Method indicated that E2P4 induced a rapid inhibition in *dnmt3a* expression with a significant reduction after 12hr (P<0.001) and 24hr (P<0.001) compared to OIL treated females. Similarly, there was a significant difference in *dnmt3b* across treatment groups (F=32.35; P<0.001; Fig6c). E2P4 significantly reduced *dnmt3b* expression in uterine tissue 12hr (P<0.001) and 24hrs (P<0.001) after administration. *dnmt1* expression was also found to be significantly reduced by E2P4 treatment (F=8.79; P<0.005; FigS1e). Dunnett's method revealed that *dnmt1* expression was significantly lower 24hr after injection (P<0.005), but not 12hrs (P=0.51).

Melatonin is sufficient to increase dnmt3a and dnmt3b

Melatonin treatments categorically increased *dnmt3a* and *dnmt3b* expression in HEK293 cells. A one-way ANOVA revealed a significant difference in *dnmt3a* expression across treatment groups (F=17.207; P<0.001; Fig7a). Dunnett's Method for *post-hoc* analyses established that all doses of melatonin induced a significant increase in *dnmt3a* compared to control cells (P<0.001), but *dnmt3a* expression was similar across all melatonin concentrations (P>0.05). Similarly, the one-way ANOVA revealed a significant difference in *dnmt3b* expression (F=39.207; P<0.001; Fig7b). All doses of melatonin were observed to have significantly greater *dnmt3b* expression compared to controls (P<0.001).

Discussion

In this paper, we show marked photoperiod dependent regulation of DNA methylation in testes. The increased methylation appears to be driven by *dnmt3a* and likely *dnmt3b,* albeit to a lesser extent. One potential driver for the short day induced increase in DNA methylation may be a lengthening of nocturnal melatonin duration. Incubation of HEK293 cells with various concentrations of melatonin was sufficient to elicit a categorical increase in both *dnmt3a* and *dnmt3b* expression. Surprisingly, the ovary failed to show photoperiodic variation in DNA methylation, indicating a marked gonadal difference in the role of DNA methylation across the seasonal reproductive cycle. Instead, seasonal variation in DNA methylation may act in the uterus for reproductive timing. Further examination of *dnmt3a* and *dnmt3b* expression revealed significant plasticity during the oestrus cycle, with inhibition during the oestrus stage due to the increased secretion of oestrogen and progesterone. We conclude that seasonal and oestrus variation in testicular and uterine *dnmt3a* expression enhanced DNA methylation, triggered reproductive involution and reduced fertility.

Cyclical patterns in epigenetic modifications are gradually being uncovered. Recent work has identified marked daily (6) and seasonal (7) changes in DNA methylation. In the hypothalamus, there is a decrease in global DNA methylation and *dnmt3b* expression in adult Siberian hamsters after prolonged exposure to SD compared to LD (7). Here we show SD stimulated an increase in DNA methylation and *dnmt3a* and *dnmt3b* expression in peripheral tissues (i.e. testes and uterus). These findings suggest that DNA methylation patterns show opposite cyclic changes in the central nervous system (7) compared to peripheral systems, such as reproductive tissue and immune cells (23). DNMT expression in the brain is widely distributed and located in several hypothalamic nuclei (7). Peripheral tissues (e.g. testes, ovary) consist of a relatively homogenous cell population compared to the complex networks and diverse cells located in the hypothalamus. The increased DNMT expression in LD hypothalamus likely reflects the outcome of multiple localized changes and not the result of a single brain region. A greater resolution of anatomically localized changes in DNMT expression in the hypothalamus will help resolve the opposite patterns observed in neuroendocrine nuclei and peripheral reproductive tissues. It is clear that melatonin and ovarian hormones are involved in the regulation of *dnmt3a* and *dnmt3b* expression. Given the categorical increase in *dnmt3a* and *dnmt3b* after exposure to melatonin and the rapid change in response to a single bolus of E2P4, it is likely that these hormones could be acting directly on promoter regions or in the recruitment of transcription binding factors. Altered hormonal regulation of cell

autonomous timing of DNA methylation may be one potential molecular mechanism that underlies seasonal disruption in animal health (29).

Epigenetic modifications during gamete development are well described (30). Conditional knockout *dnmt3a* mice exhibit severe reproductive deficits; males exhibit impaired spermatogenesis and lack DNA methylation in parentally imprinted genomic regions (31). In females, conditional knockouts of *dnmt3a* are lethal and also have an absence of DNA methylation at parentally imprinted genomic regions (31). These data support a model in which *dnmt3a* signalling in males and females is vital for the generation of viable gametes and ultimately, fertility. In our study, we observed relatively low levels of *dnmt3a* in testes and ovaries in LD compared to non-breeding, SD conditions. The increase in *dnmt3a* expression in the regressed testes likely provides an inhibitory signal that arrests spermatogenesis. Whether enhanced melatonin or reduced gonadal steroids (i.e. E2P4) provide a hormonal signal that permits the greater *dnmt3a* expression requires further exploration. Regardless, the molecular outcome was a massive increase in DNA methylation that results in broad methylation across the entire genome resulting in reproductive involution. Given that the seasonal pattern in DNA methylation occurs over multiple annual oscillations, we propose that cyclical DNA methylation in reproductive tissues provides a single trigger with broad implications for the timing of gene transcription that enables yearly switches in gamete development and fertility.

DNMT3a/b has a high enzymatic activity and can rapidly methylate cytosine residues (e.g. 3hrs; 32). In this paper, we have shown that melatonin can increase *dnmt3a* and *dnmt3b* expression in cell culture within 4hrs and a single bolus of E2P4 can inhibit uterine levels within 12hrs. These data indicate that key seasonal and reproductive hormones can have a significant impact on *dnmt3a/b* expression and ultimately lead to a lasting-effect on the epigenomic landscape. It is important to note that caution should be exercised when extrapolating the melatonin-dependent increase of *dnmt3a/b* in HEK293 results to seasonal regulation of DNA methylation in hamster reproductive tissues. Given the large variation in gene transcription during spermatogenesis (33); seasonal and oestrus patterns in *dnmt3a* likely function to secure the inhibition of select genes leading to the successful timing of RNA expression required for optimal fertility. It is likely that several other hormones with links to reproduction function (e.g. leptin) will also impact the probability of *dnmt3a/b* expression. Overall, the rapid and long-term effects of melatonin and ovarian steroids reveal a novel and robust effect on

methyltransferase expression and illustrate that hormone driven changes in the epigenomic landscape are probably more common than previously thought.

The comparison of ovarian and testicular DNA methylation permitted the identification of significant sex differences in the levels of *dnmt1* and *dnmt3a* expression. The higher levels of *dnmt1* expression in testes is likely attributable to gamete production (i.e. spermatogenesis; 34). Several testicular genes exhibit reversible, seasonal variation in expression and these changes are proposed to enhance fertility during the breeding periods (35). Since seasonal variations in sperm parameters are common across mammalian species, including humans, (36,37) the patterns in *dnmt1* and *dnmt3a* may represent an evolutionarily ancient molecular signalling mechanism for the timing of reproduction. A role for *dnmt1* in the timing of reproductive physiology in the uterus is less clear (FigS1). *dnmt3a* has been shown to be important for decidualization, exhibiting transient estrogen-dependent decrease (38). Similarly, we found that E2P4 significantly reduced *dnmt3a* and *dnmt3b*. The specific role of reduced *dnmt3a* during decidualization is unknown; but may permit stromal vascularity and/or glandular epithelial secretion.

In conclusion, we present novel and robust findings that *dnmt3a* expression is dynamic and propose that variation in *dnmt3a* is involved in the local timing of reproductive physiology in key tissues. These data have significant implications for our understanding of the potential effects of DNA methylation for fertility in a rodent species. One particularly important finding was the significant increase in global DNA methylation in the male testes during reproductive involution. Future work that includes alternative methods, such as chromatin immunoprecipitation for DNMT3a will be important to confirm the large photoperiodic variation in DNA methylation and identify the genomic regions targeted in both testicular and uterine tissue. Uncovering the mechanism that underlies this natural pattern could have a significant impact for developing alternative methods for contraceptives. Moreover, these data provide further evidence that epigenetic modifications exhibit dynamic and cyclical patterns in expression and indicate DNA methylation is a key characteristic of timing biological rhythms.

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Figure Legends

Figure 1 – Photoperiod induced variation in testicular DNA methylation. A) SD significantly reduced testicular mass in males. B) regressed testes were observed to have a robust increase in global DNA methylation. C) the photoperiodic induced change in DNA methylation appears to develop from increased relative expression of *dnmt3a* and to a lesser extent *dnmt3b* (D). Significance is depicted by *** $P<0.001$; ** $P<0.01$; * $P<0.05$.

Figure 2 – Short days increase DNMT3a expression in the testes. A) SD significantly reduced testicular mass. B) the number of DNMT3a expressing cells (summed across 10 seminiferous tubules) in SD regressed testes exhibited a significant increase compared to LD controls. C) and D) exemplar photomicrographs of LD and SD DNMT3a expression in testicular tissue. Note the large decrease in testicular lumen in SD compared to LD samples. White scale bar represents 3mm.

Figure 3 – Ovarian tissue lacks photoperiod-dependent changes in DNA methylation. A) SD hamsters were observed to display a slight decrease in ovarian mass. Unlike the testes, the ovary did not exhibit a significant difference in global DNA methylation (B). Moreover, both *dnmt3a* (C) and *dnmt3b* (D) relative expression remained constant across LD and SD conditions.

Figure 4 – Photoperiod-induced variation in relative *dnmt3a* and *dnmt3b* uterine expression. Female hamsters transferred to SD exhibit a significant decrease in uterine mass (A). SD uterine had significantly more relative *dnmt3a* (B) and *dnmt3b* (C) expression compared to LD controls. Significance is depicted by *** $P<0.001$; ** $P<0.01$; * $P<0.05$.

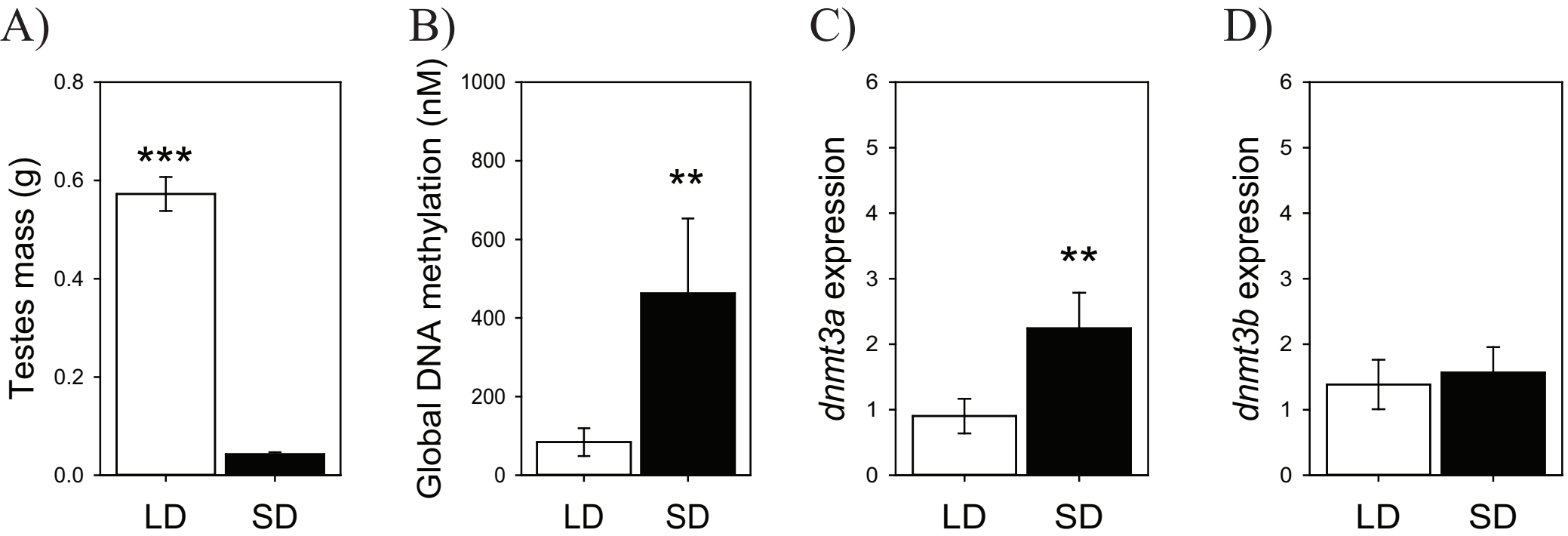
Figure 5 – Oestrus significantly decreased relative *dnmt3a* expression. A) combined uterine mass and plasma prolactin reliably indicate diestrus (DI), proestrus (PRO) and oestrus phases (EST). Uterine mass is low in diestrus and significantly increased during proestrus and again during oestrus. Plasma prolactin is low in diestrus, significantly increased during proestrus and then decreases during oestrus. B) the levels of relative *dnmt3a* expression are significantly reduced during oestrus. C) there was a non-significant decrease in relative *dnmt3b* expression during oestrus. White, grey and black bars indicate DI, PRO and EST stages respectively. Significance is depicted by *** $P<0.001$; ** $P<0.01$; * $P<0.05$.

Figure 6 – Rapid inhibition of relative *dnmt3a* and *dnmt3b* uterine expression. A) Ovariectomized females treated with a single bolus injection of E2P4 exhibit a significant increase in uterine mass after 24 hrs compared to OIL treated controls. There was a trend for heavier uterine in females after 12hrs. E2P4 injections significantly reduced both relative *dnmt3a* (B) and *dnmt3b* (C) uterine expression. White bars depict OIL, grey bars depict 12hr and black bars depict 24hr treatment groups. Significance is depicted by *** $P<0.001$; * $P<0.05$.

Figure 7 – Melatonin is sufficient to stimulate relative *dnmt3a* and *dnmt3b* expression. HEK293 cells incubated for 4 hours with melatonin exhibited a categorical increase in relative *dnmt3a* (A) and *dnmt3b* (B) expression. Since both *dnmt3a* and *dnmt3b* displayed a stepwise increase and not a dose dependent change in response to increased melatonin, it is likely that melatonin is acting indirectly via other genomic/molecular pathways. Significance is depicted by *** $P<0.001$.

Figure

Click here to download Figure Figure 1 Testes Methylation.eps



Figure

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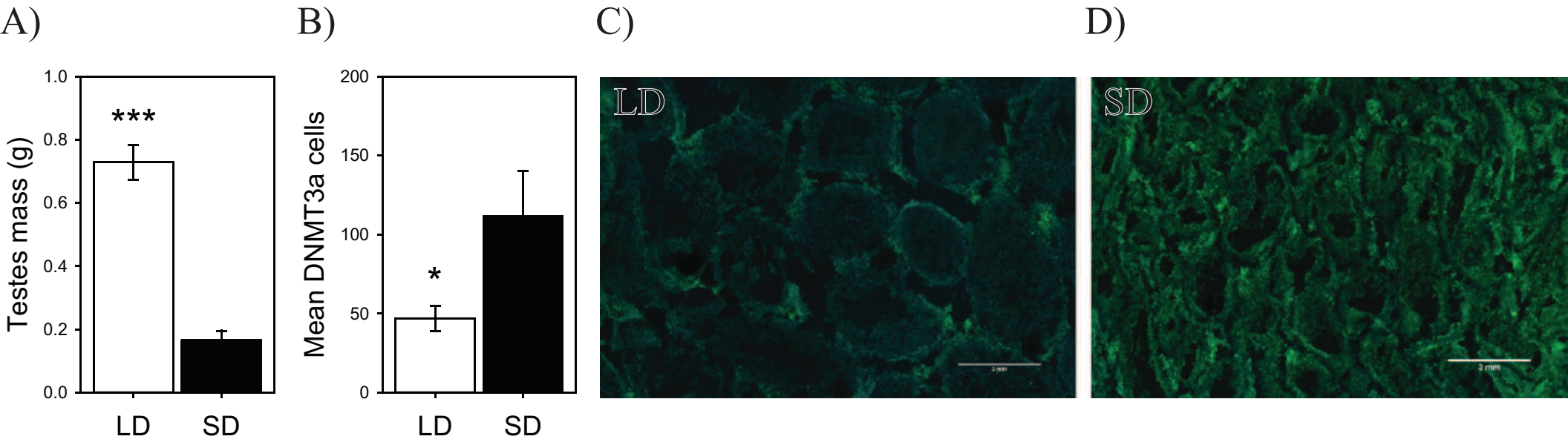

Figure

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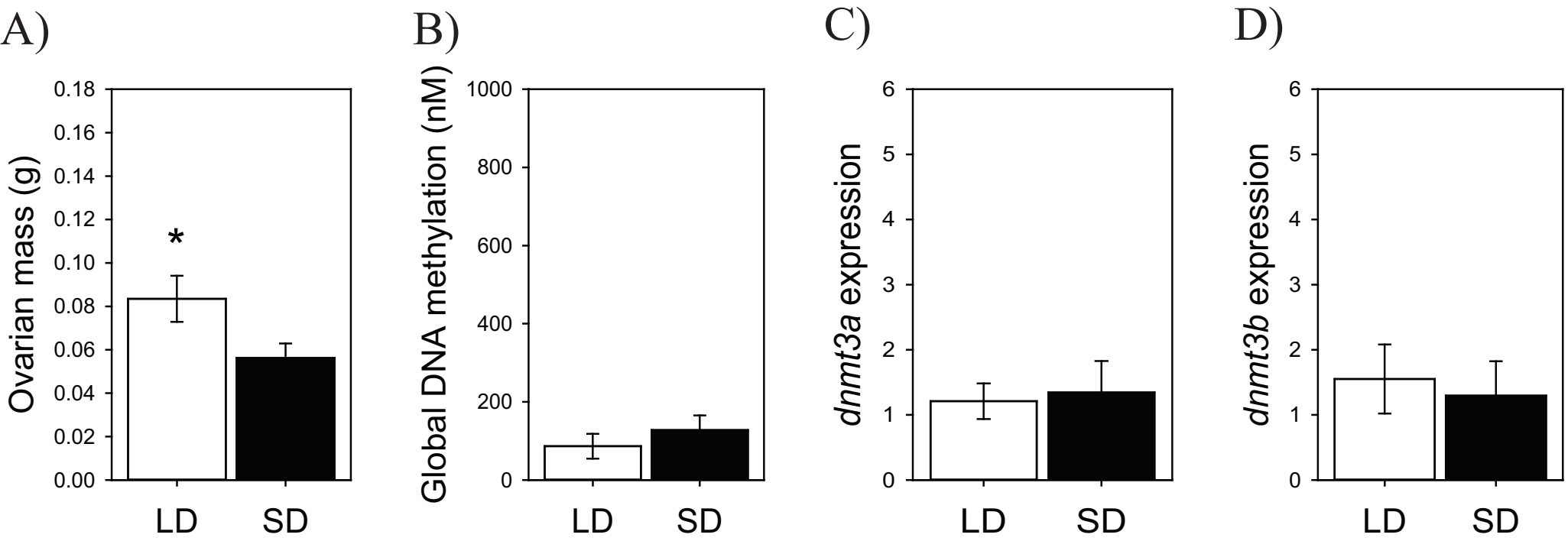

Figure

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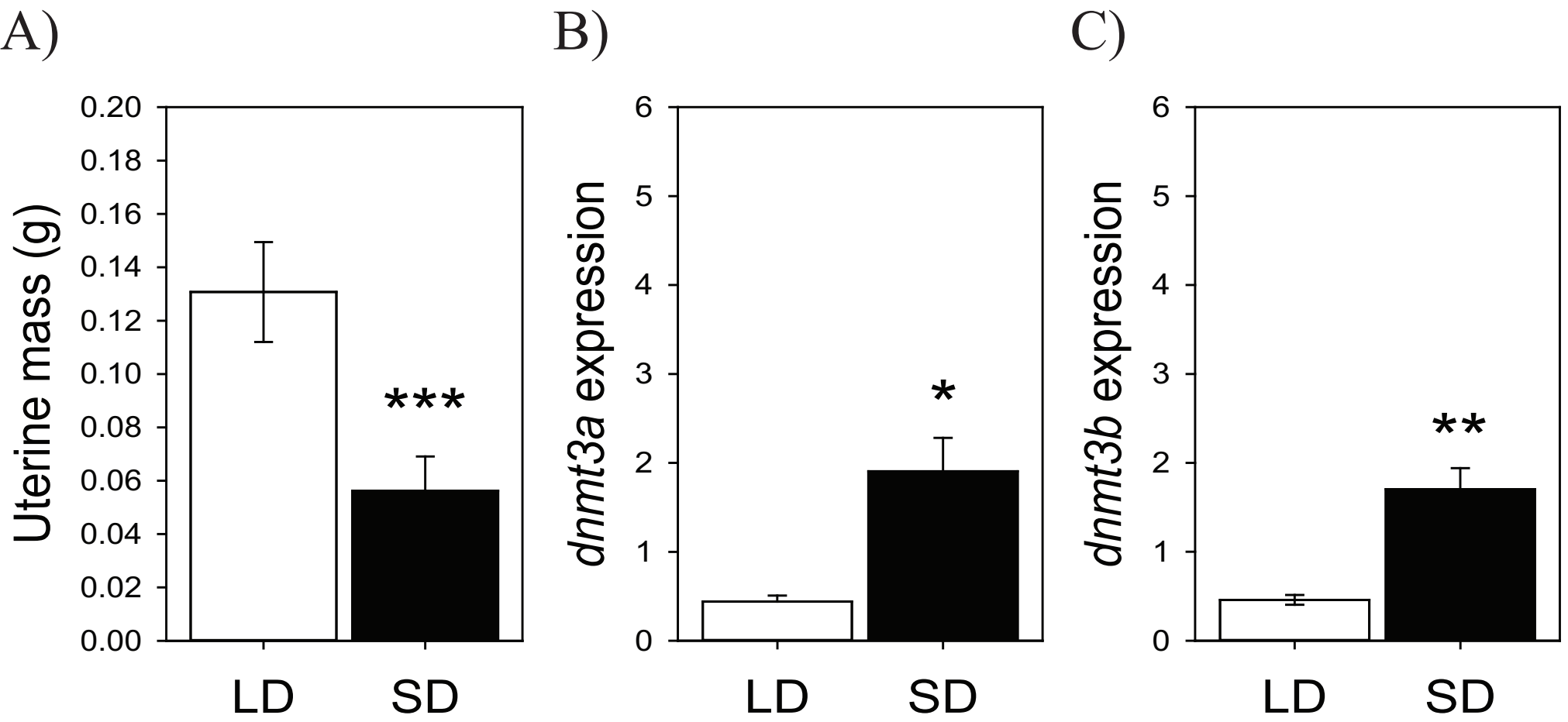

Figure

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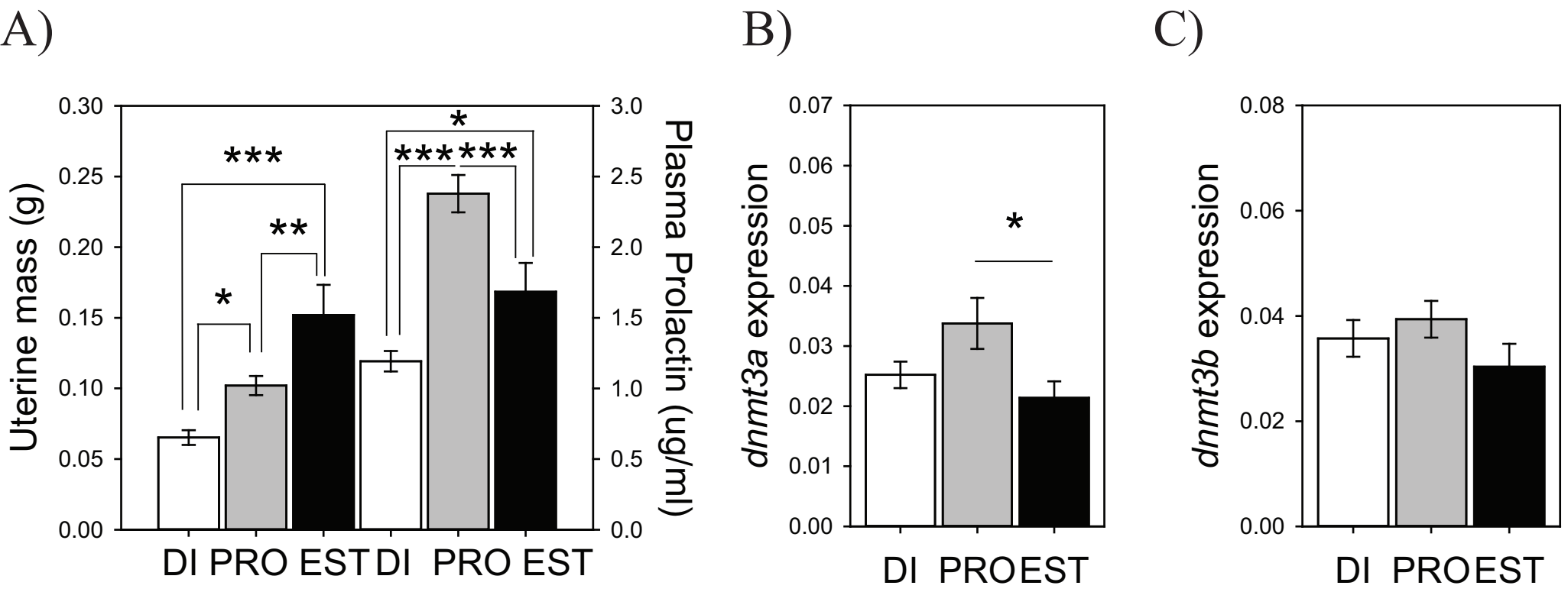

Figure

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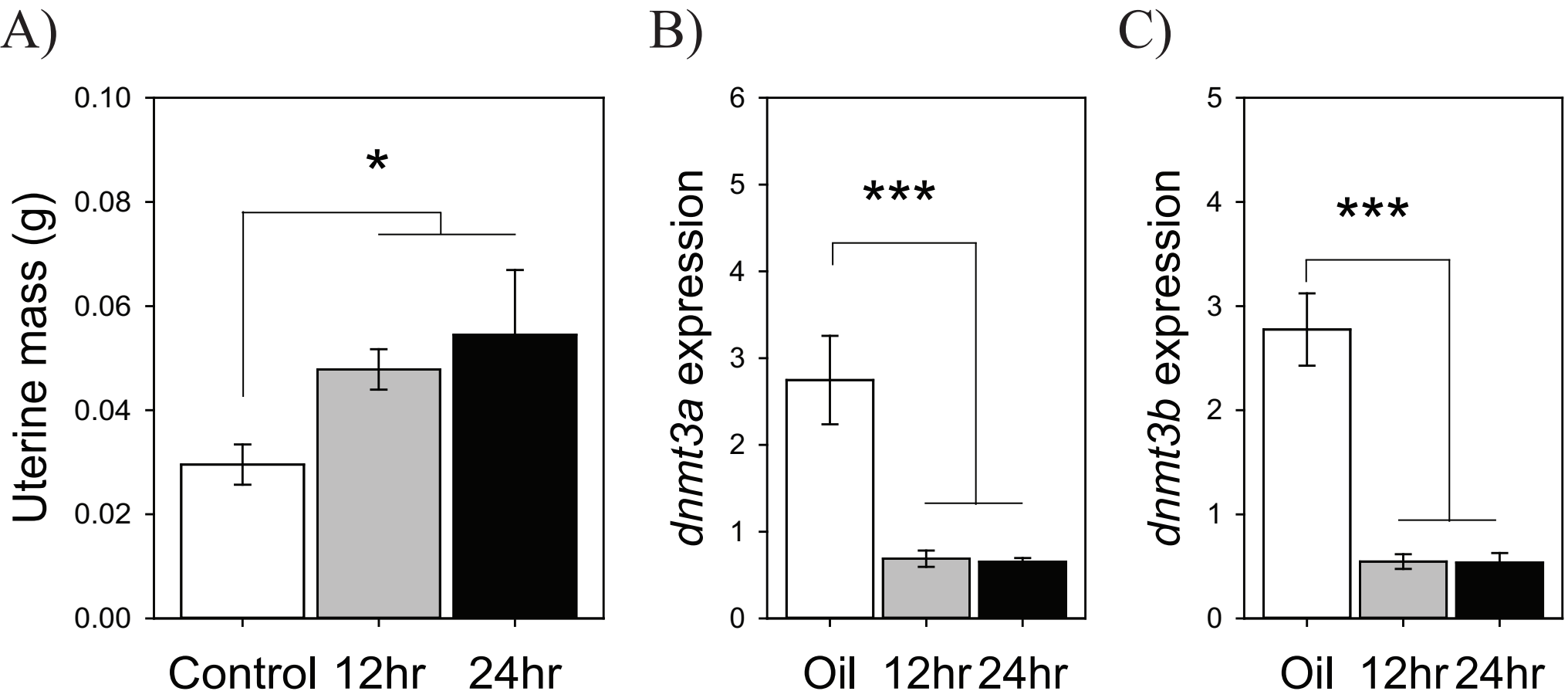

Figure

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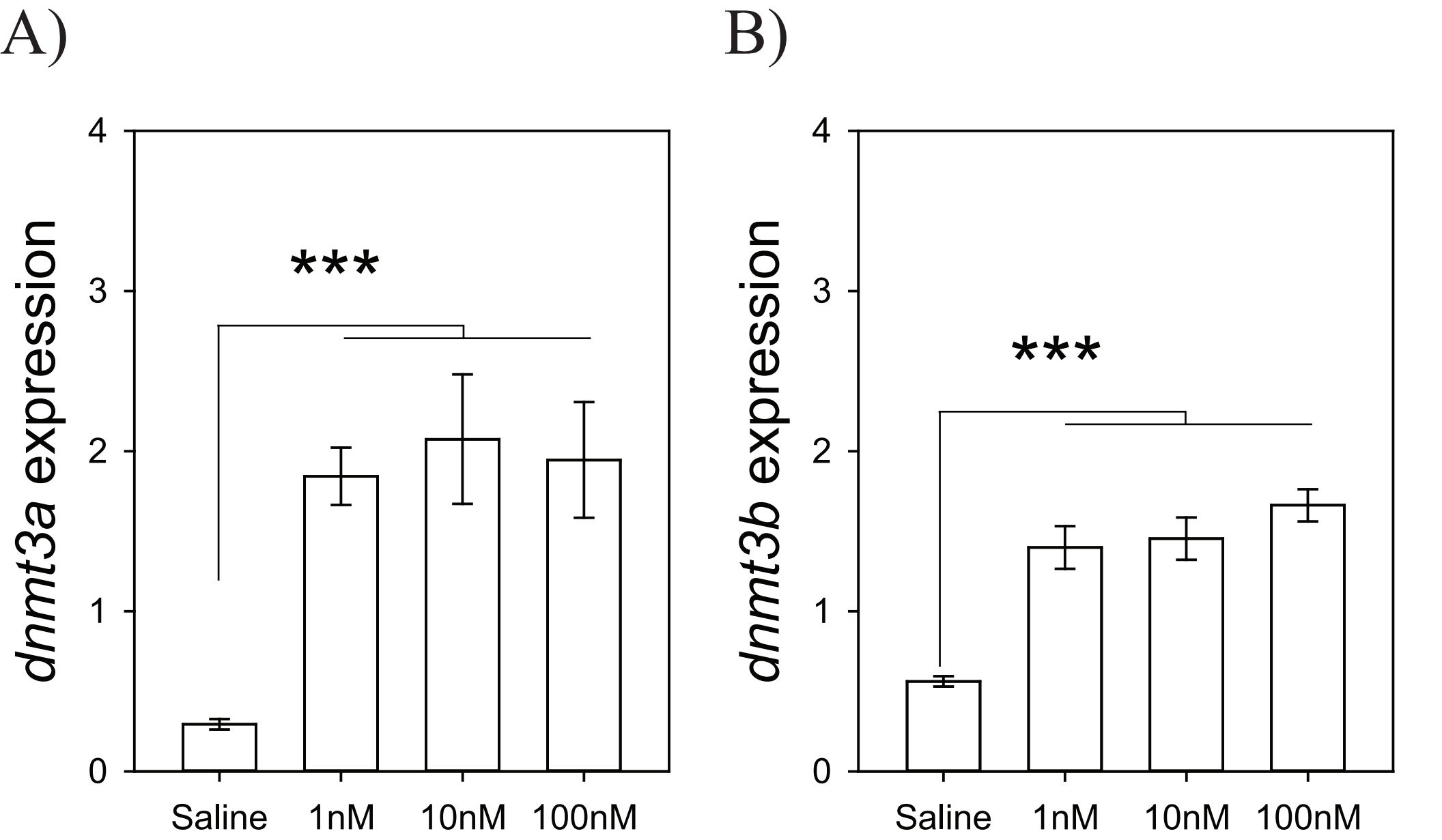

Figure S1

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Figure S1 - Revised DNA methyltransferase 1.eps

Figure S2

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Figure S2 - Testes histology.eps

Figure S3

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Figure S3 - Uterine histology.eps

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Antibody Table

Peptide/protein target	Antigen sequence (if known)	Name of Antibody	Manufacturer, catalog #, and/or name of individual providing the antibody	Species raised in; monoclonal or polyclonal	Dilution used
DNMT3a	AA 10-118 of human DNMT3a	Anti-DNA Methyltransferase 3a Antibody	ThermoFisher/Invitrogen, PA3-16557	Rabbit polyclonal	1:500