Anti-Müllerian hormone receptor type 2 (AMHR2) expression in bovine oviducts and endometria: Comparison of AMHR2 mRNA and protein abundance among old Holstein cows and young and old Wagyu females

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Running head: AMHR2 expression in bovine oviducts and endometria

Abstract

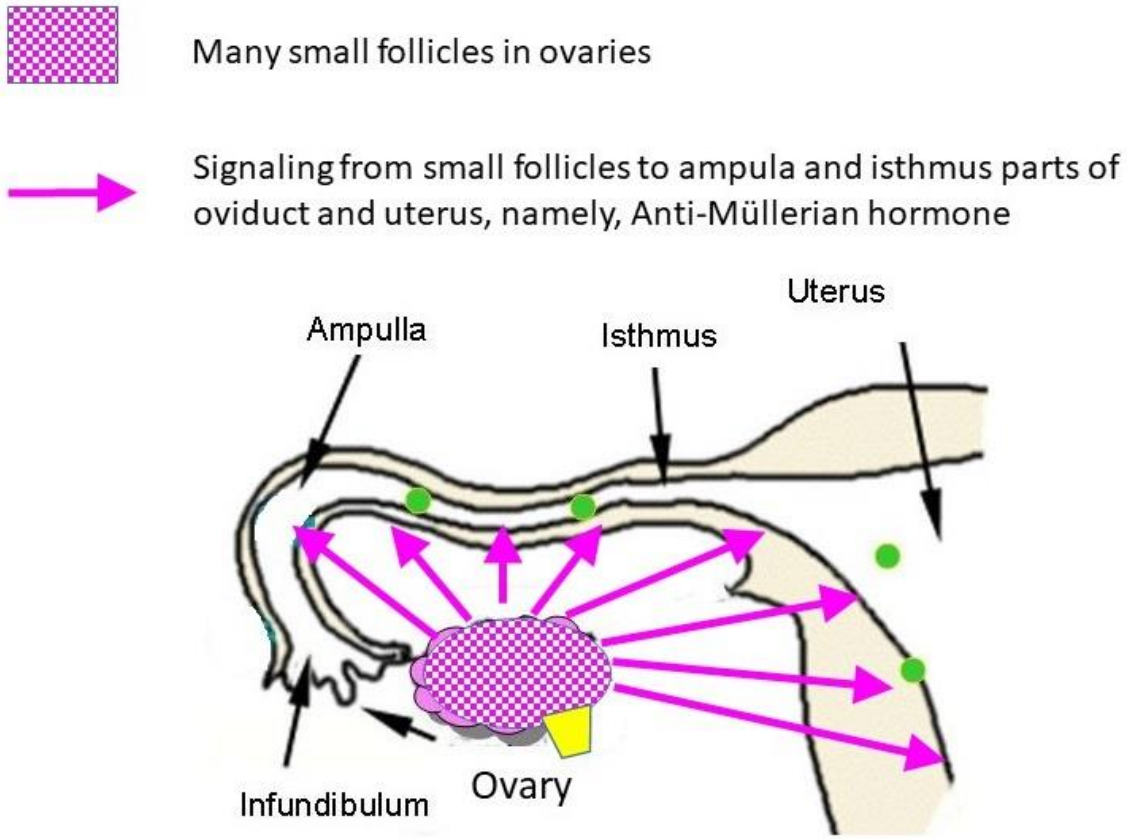
Anti-Müllerian hormone (AMH) is a glycoprotein produced by granulosa cells of the preantral and small antral follicles that has multiple, important roles in the ovaries. Recent studies have revealed the extragonadal AMH regulation of gonadotropin secretion from bovine gonadotrophs. In this study, we investigated whether the primary receptor for AMH, AMH receptor type 2 (AMHR2), is expressed in bovine oviducts and endometria. Reverse transcription-polymerase chain reaction detected expressions of *AMHR2* mRNA in oviductal and endometrial specimens. Western blotting and immunohistochemistry were performed to analyze AMHR2 protein expression using anti-bovine AMHR2 antibody. Immunohistochemistry revealed robust AMHR2 expression in the tunica mucosa of the ampulla and isthmus, and in the glandular and luminal epithelium of endometrium. AMHR2 mRNA (measured by real-time PCR) and protein expression in these layers did not significantly differ among estrous phases in adult Wagyu heifers ($P > 0.1$). The expression in these layers did not differ among old Holsteins (91.9 ± 6.4 months old) and young (26.6 ± 0.8 months old) and old (98.8 ± 10.2 months old) Wagyus. Therefore, *AMHR2* is expressed in bovine oviducts and endometria.

Additional keywords: age, breed, extragonadal role, Müllerian-inhibiting substance, ruminant.

Short summary

Anti-Müllerian hormone receptor 2 (AMHR2) is expressed in the tunica mucosa of ampulla and isthmus, and epithelium of uterine glands and luminal epithelium of the endometrium of heifers and cows. The receptor abundance did not differ among estrous phases and among old Holsteins and young and old Wagyu females.

Image of contents



Introduction

Ovaries regulate the oviducts and endometria through estradiol and progesterone secreted from mid-sized or large-sized follicles or corpora lutea (Pohler *et al.* 2012; Binelli *et al.* 2018). It is unclear, however, whether preantral and small antral follicles, the majorities in ovaries, secrete any hormone to regulate the oviducts and endometria. We questioned whether preantral and small antral follicles are the silent majority in ovaries.

Anti-Müllerian hormone (AMH) is a member of the transforming growth factor (TGF)-β family, primarily secreted by the preantral and small antral follicles in female animals (Bhide and Homburg 2016). Anti-Müllerian hormone has been well-characterized at the ovarian level, i.e., with respect to its roles in regulating follicular development (Hernandez-Medrano *et al.* 2012) and inhibiting follicular atresia (Seifer and Merhi 2014). Blood AMH concentration is a useful blood marker for antral follicle counts in the Wagyu breed (Hirayama *et al.* 2019), as well as in dairy cows and women (Arouche *et al.* 2015; Sefrioui *et al.* 2019). Mossa and Ireland (2019) have shown that dairy cows with a low antral follicle count (AFC) have lower concentrations of AMH and thinner endometrium than those in cows with high AFC. Circulating AMH concentrations can help predict the number of high-quality embryos produced by various animals, including women and cows (Arouche *et al.* 2015; Sefrioui *et al.* 2019). The number of high-quality embryos results from synchronous regulation by the sperm, ovum, oviduct, and endometrium. Further, plasma AMH concentrations are positively correlated with pregnancy rates in various animals, including women and cows (Ribeiro *et al.* 2014; Josso 2019). Recent studies have revealed extragonadal roles of AMH–by the activation of its primary receptor, AMH receptor type 2 (AMHR2), in gonadotrophs in the anterior

pituitaries of rats and bovines (Garrel *et al.* 2016; Kereilwe *et al.* 2018). Therefore, AMHR2 may be expressed in the oviducts and the endometria to mediate any yet unknown roles of AMH. Indeed, recent studies have shown an increased risk of miscarriage among women with low blood AMH concentrations (Tarasconi *et al.* 2017; Lyttle *et al.* 2018).

Old age is associated with infertility in various animals, including women and cows (Osoro and Wright 1992; Scheffer *et al.* 2018); however, limited information is available regarding the exact mechanisms underlying this association among animals. Several studies in humans have linked aging to plasma AMH concentrations. Blood AMH concentration is highest in pubertal girls and gradually decreases starting at age 25 until it is undetectable after menopause (Dewailly *et al.* 2014), suggesting that low AMH is a marker for ovarian aging (Bhide and Homburg 2016). Studies on the relationship between age and plasma AMH concentration in adult female ruminants are not common, but one study showed that old Wagyu (syn. Japanese Black) cows have higher blood AMH concentrations than those in postpubertal heifers and young cows (Koizumi and Kadokawa 2017). Therefore, age may be a determinant of the AMHR2 expression levels in the oviducts and endometria, although there may be species differences.

Oviductal and endometrial AMHR2 expression are yet unknown in any species; however, endometriosis and uterine adenomyosis express AMHR2 in women (Signorile *et al.* 2014; Kim *et al.* 2018). In the present study, we, therefore, hypothesized that AMHR2 is expressed in bovine oviducts and endometria. A previous study showed breed-dependent differences in circulating AMH concentrations among dairy cow breeds (Ribeiro *et al.* 2014). Infertility in Holsteins is a critical issue in the dairy industry worldwide (Kadokawa and Martin 2006). Therefore, this study aimed to evaluate the

association between oviductal and endometrial AMHR2 expression and various physiological factors, i.e., stage of the estrous cycle, age, and breed.

Materials and Methods

All experiments were performed according to the Guiding Principles for the Care and Use of Experimental Animals in the Field of Physiological Sciences (Physiological Society of Japan) and were approved by the Committee on Animal Experiments of Yamaguchi University.

We obtained oviductal and endometrial samples from cattle managed by contracted farmers in western Japan. Since the disaster of bovine spongiform encephalopathy in 2002, all cattle born in Japan since 2003 are registered in a database at birth with an individual identification number. This number must be shown on an ear tag, carcass, and meat packages sold by butchers. Japanese farmers and consumers can obtain information, including breed, birth date, farm, movement from farm to farm, date and place of livestock auction, and slaughter, by querying the server of the National Livestock Breeding Center of Japan. We utilized both individual identification numbers and information given by the contracted farmers for the cattle in this study.

Experiment 1

Experiment 1 was conducted to evaluate whether AMHR2 is expressed in oviduct and endometrium in heifers utilizing reverse transcription-polymerase chain reaction (RT-PCR), western blotting, and immunofluorescence staining. We obtained the ampulla, isthmus, caruncular (CAR), and intercaruncular (ICAR) area of endometria from four post-pubertal (26 months of age) Wagyu heifers at a local abattoir. The four heifers were

126 at days 3, 8, 15, and 21 (day 0 = day of estrus), as determined via macroscopic
127 examination of the ovaries and uterus (Miyamoto *et al*. 2000). The ampulla, isthmus,
128 CAR, and ICAR samples collected were from the side ipsilateral to ovulation in the three
129 heifers from days 3, 8, or 15 but were from the side ipsilateral to the dominant follicle in
130 the remaining heifer at day 21. We collected ampullar samples from areas at least 3 cm
131 away from the fimbriated infundibulum as well as from the ampullary-isthmic junction
132 and the isthmus samples from areas also at least 3 cm away from the ampullary-isthmic
133 junction as well as from the utero-tubal junction. Half of the ampulla and half of the
134 isthmus were frozen in liquid nitrogen and preserved at -80°C until RNA or protein
135 extraction. The remaining halves of the ampulla and isthmus were stored in 4%
136 paraformaldehyde at 4°C for 16 h for immunohistochemistry studies. The middle area of
137 uterine horn was opened longitudinally using scissors, and CAR were carefully dissected
138 so as not to include ICAR; subsequently, ICAR areas were cut off. The collected CAR
139 and ICAR samples were frozen in liquid nitrogen and preserved at -80°C until RNA or
140 protein extraction or stored in 4% paraformaldehyde at 4°C for 16 h for
141 immunohistochemistry studies.
142 Since granulosa cells in small antral follicles express AMHR2 (Poole *et al.* 2016), we
143 harvested ovarian tissue specimens from the same heifers as positive controls of AMHR2
144 for RT-PCR and western blotting.
145
146 *RT-PCR, sequencing of amplified products, and homology search in gene databases*
147 Total RNA was extracted from the ovary, ampulla, isthmus, CAR, or ICAR from
148 the four heifers using RNAzol RT Reagent (Molecular Research Center Inc., Cincinnati,
149 OH, US) according to the manufacturer's protocol. The extracted RNA samples were

150 treated with ribonuclease-free deoxyribonuclease (Thermo Fisher Scientific, Waltham,
151 MA, US) to eliminate possible genomic DNA contamination. Using a NanoDrop ND-
152 1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA), the
153 concentration and purity of each RNA sample were evaluated to ensure the A260/A280
154 nm ratio was in the acceptable range of 1.8–2.1. The mRNA quality of all samples was
155 verified by electrophoresis of total RNA followed by staining with ethidium bromide, and
156 the 28S:18S ratios were 2:1. The cDNA was synthesized from 1 µg of the total RNA per
157 sample using SuperScript IV VILO Master Mix (Thermo Fisher Scientific) according to
158 the manufacturer's protocol. No reverse transcription controls (NRCs) were prepared for
159 RT-PCR; they were generated by treating the extracted RNA with the same
160 deoxyribonuclease but not with cDNA synthetase.

161 In order to determine the expression of *AMHR2* mRNA in the samples, PCR was
162 conducted using the primer pair reported by our group previously (Kereilwe *et al.* 2018),
163 which was designed by Primer3 based on reference sequence of bovine *AMHR2* [National
164 Center for Biotechnology Information (NCBI) reference sequence of bovine *AMHR2* is
165 NM_001205328.1], as one of PCR primers must span the exon-exon junction. Table 1
166 details the primers used for RT-PCR. Using a Veriti 96–Well Thermal Cycler (Thermo
167 Scientific), PCR was performed using 20 ng of cDNA, 20 ng RNA as the NRC or water
168 as the no template control (NTC), and polymerase (Tks Gflex DNA Polymerase, Takara
169 Bio Inc., Shiga, Japan) under the following thermocycles reported by our group (Kereilwe
170 *et al.* 2018): 94 °C for 1 min for pre-denaturing followed by 35 cycles of 98°C for 10 s,
171 60°C for 15 s, and 68°C for 30 s. PCR products were separated on 1.5% agarose gel by
172 electrophoresis with a molecular marker [Gene Ladder 100 (0.1-2kbp), Nippon Gene,
173 Tokyo, Japan], stained with fluorescent stain (Gelstar, Lonza, Allendale, NJ), and

observed using a charge-coupled device (CCD) imaging system (GelDoc; Bio-Rad, Hercules, CA, US). The PCR products were purified with the NucleoSpin Extract II kit (Takara Bio Inc.) and then sequenced with a sequencer (ABI3130, Thermo Fisher Scientific) using one of the PCR primers and the Dye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific). The sequences obtained were used as query terms with which to search the homology sequence in the DDBJ/GenBankTM/EBI Data Bank using the basic nucleotide local alignment search tool (BLAST) optimized for highly similar sequences (available on the NCBI website).

Western Blotting for AMHR2 detection

Proteins were extracted from the ovary, ampulla, isthmus, CAR, or ICAR samples (used as positive controls) from the four heifers, and western blotting was performed as previously described (Kereilwe *et al.* 2018). The extracted protein sample (33.4µg of total protein in 37.5µl) was mixed in 12.5µl of 4x Laemmli sample buffer (Bio-rad) containing 10% (v/v) β-mercaptoethanol, then boiled for 3 min at 100 °C. The boiled protein samples were quickly cooled down in ice, and then the 12 µl of boiled protein samples (8 µg of total protein) were loaded onto a polyacrylamide gel along with a molecular weight marker and resolved by electrophoresis on sodium dodecyl sulfate polyacrylamide gels at 100 V for 90 min. Proteins were then transferred to polyvinylidene fluoride (PVDF) membranes. Blocking was done with 0.1% Tween 20 and 5% non-fat dry milk for 1h at 25 °C. Next, immunoblotting was performed with our original anti-AMHR2 chicken antibody (Kereilwe *et al.* 2018; 1:25,000 dilution) overnight at 4 °C. The anti-AMHR2 chicken antibody binds to the extracellular region located near the N terminus of the AMHR2, and spec and other details were published previously (Kereilwe *et al.* 2018).

After washing, the PVDF membrane was incubated with horseradish peroxidase (HRP)-conjugated anti- chicken IgG goat antibody (Bethyl laboratories, Inc., Montgomery, TX, USA; 1:50,000 dilution) at 25 °C for 1 h. Protein bands were visualized using an ECL-Prime chemiluminescence kit (GE Healthcare, Amersham, UK) and CCD imaging system (Fujifilm, Tokyo, Japan). Previous studies utilizing western blotting for AMHR2 reported that human, mouse, and bovine AMHR2 are present as dimers, full-length monomers, or cleaved monomers (Faure *et al.* 1996; Hirschhorn *et al.* 2015; Kereilwe *et al.* 2018). Thus, we defined bovine AMHR2 bands based on band size as one of these structure types. After antibodies were removed from the PVDF membrane with stripping solution, the membrane was used for immunoblotting with the anti-β-actin mouse monoclonal antibody (A2228, 1:50,000 dilution; Sigma-Aldrich, St. Louis, MO, USA).

Immunofluorescence staining and confocal microscopy

After storage in 4% paraformaldehyde PBS at 4°C for 16 h, tissue blocks were placed in 30% sucrose-PBS until the blocks were infiltrated with sucrose. Tissue blocks were then subjected to immunofluorescence staining using previously described protocols (Nahar *et al.* 2013; Kereilwe *et al.* 2018). Briefly, 15-µm sections were prepared and mounted on slides. The sections were treated with 0.3% Triton X-100-PBS for 15 min, then, blocking done by incubating with 0.5 mL of PBS containing 10% normal goat serum (Wako Pure Chemicals, Osaka, Japan) for 1 h at room temperature. The slides were incubated with the same anti-AMHR2 chicken antibody (Kereilwe *et al.* 2018; 1:1,000 dilution) for 12 h at 4°C, then followed by incubation with Alexa Fluor Alexa Fluor 488 goat anti-chicken IgG (Thermo Fisher Scientific and diluted as 1 µg/mL) and 1 µg/mL of 4′, 6′-diamino-2-phenylindole (DAPI; Wako Pure Chemicals) for 2 h at room temperature.

The stained sections on slides were observed by confocal microscopy (LSM710; Carl Zeiss, Göttingen, Germany) equipped with equipped with diode (405 nm), argon (488 nm) lasers. Images obtained by fluorescence microscopy were scanned with a 20× or 40× objective and recorded by a CCD camera system controlled by ZEN2012 black edition software (Carl Zeiss). In the confocal images obtained after immunohistochemistry analysis, the DAPI is shown in blue and AMHR2 is shown in green. To verify the specificity of the signals, we included several negative controls in which the primary antiserum had been omitted or pre-absorbed with 5 nM of the antigen peptide (Kereilwe *et al.* 2018) or in which normal chicken IgG (Wako Pure Chemicals) was used instead of the primary antibody.

Experiment 2

Experiment 2 was conducted to compare AMHR2 expression in oviductal and endometrial samples among stages of the estrous cycle utilizing real-time PCR and western blotting described later.

The ampulla, isthmus, CAR and ICAR tissues were harvested from adult (26-month-old) non-pregnant healthy Wagyu heifers in the pre-ovulatory phase (day 19-21; n = 5), day 1 to 3 (n = 5), day 8 to 12 (n = 5), or day 15 to 17 (n = 5), as determined via macroscopic examination of the ovaries and uterus (Miyamoto *et al*. 2000). Samples were obtained at the local abattoir and immediately frozen in liquid nitrogen and stored at −80°C until RNA or protein extraction.

Experiment 3

Experiment 3 was conducted to compare AMHR2 expression in oviductal and endometrial samples based on age or breed utilizing the real-time PCR and western blotting described later.

The ampulla, isthmus, CAR, and ICAR tissues were harvested during the luteal phase (day 8 to 12) from healthy post-pubertal Wagyu heifers (26.6 ± 0.8 months of age; n = 6; young Wagyu group), old Wagyu cows (98.8 ± 10.2 months of age; n = 6; old Wagyu group), and old Holstein cows (91.9 ± 6.4 months of age; n = 6; old Holstein group) from the local abattoir. We compared these three groups for four reasons. First, it was not possible to obtain samples from postpubertal Holstein heifers because they are kept in dairy farms for milking purposes. Second, in our previous study (Kereilwe and Kadokawa 2018), we compared expression levels of AMH in gonadotrophs between old Holsteins (about 80 months old) and young (about 26 months old) and old Wagyu females (about 90 months old), finding significant differences in *AMH* mRNA and AMH protein between them. Third, we previously discovered a significant difference in blood AMH concentrations between old Wagyu cows (81 months) and young Wagyu heifers (22 months) (Koizumi and Kadokawa 2017). Fourth, farmers slaughter old Wagyu cows after completing parturition a sufficient number of times to obtain beef, usually after 84 months of age. All heifers and cows in the three groups were non-lactating and non-pregnant, with no follicular cysts, luteal cysts, or other ovarian or uterine disorders upon macroscopic ovarian examination (Kamomae 2012). The old Holstein cows were slaughtered because they had not become pregnant after at least five artificial insemination attempts. Unlike Wagyu and Holstein, other breeds of cattle (such as Angus, Hereford, and Guernsey) are very rare in Japan; thus, we were unable to collect samples from these other breeds.

Real-time PCR analysis to evaluate factors affecting AMHR2 expression

After preparation of high-quality total RNA and cDNA synthesis using a previously described protocol, *AMHR2* mRNA expression was compared among estrous phases or among the young Wagyu, old Wagyu, and old Holstein groups via real-time PCR and data analyses as described previously (Nahar and Kadokawa 2017; Kereilwe *et al.* 2018).

The primers for *AMHR2* were designed using Primer Express Software v3.0 (Thermo Fisher Scientific), based on reference sequences. Table 2 details the primer sequences. Anti-Müllerian hormone receptor type 2 expression levels were normalized to the geometric mean of the expression levels of two house-keeping genes, *C2orf29* and *SUZ12*, selected using Normfinder program (Vandesompele *et al*. 2002) and amplified using previously reported primers (Rekawiecki *et al.* 2012; Nahar and Kadokawa 2017), since they are the most stable and reliable housekeeping genes in the bovine oviducts and endometria (Walker *et al.* 2009; Nahar and Kadokawa 2017).

The amount of gene expression was measured in duplicate by real-time PCR analyses with 20 ng cDNA, using CFX96 Real Time PCR System (Bio-Rad) and Power SYBR Green PCR Master Mix (Thermo Fisher Scientific), together with a 6-point relative standard curve, non-template control, and no reverse-transcription control. Standard 10-fold dilutions of purified and amplified DNA fragments were prepared. Temperature conditions for all genes were as follows: 95°C for 10 min for pre-denaturation; five cycles each of 95°C for 15s and 66°C for 30s; and 40 cycles each of 95°C for 15s and 60°C for 60s. Melting curve analyses were performed at 95°C for each amplicon and each annealing temperature to ensure the absence of smaller non-specific products such as dimers. To optimize the real-time PCR assay, serial dilutions of a cDNA

293 template were used to generate a standard curve by plotting the log of the starting quantity
294 of the dilution factor against the C_q value obtained during amplification of each dilution.
295 All the C_q values of the unknown samples (22.85 ± 0.15) were between the highest (8.00)
296 and lowest (30.33) standards for AMHR2 in real-time PCR. Further, all the C_q values of
297 the unknown samples were between the highest and lowest standards for *C2orf29* or
298 *SUZ12* in real-time PCR. Reactions with a coefficient of determination (R^2) > 0.98 and
299 efficiency between 95 and 105% were considered optimized. The coefficients of variation
300 of real-time PCRs were less than 6%. The concentration of PCR products was calculated
301 by comparing C_q values of unknown samples with the standard curve using appropriate
302 software (CFXmanagerV3.1, Bio-Rad). Then the *AMHR2* amount was divided by the
303 geometric mean of *C2orf29* and *SUZ12* in each sample.
304
305 *Western blotting to evaluate factors affecting AMHR2 expression*
306 The protein expression levels of AMHR2 were compared in the ampulla, isthmus,
307 CAR and ICAR among estrous phases or among young Wagyu, old Wagyu, and old
308 Holsteins, using previously reported protocols for western blotting and data analyses
309 (Kereilwe *et al.* 2018; Kereilwe and Kadokawa 2018). Briefly, boiled samples (8 μg total
310 protein of each sample) were loaded on a polyacrylamide gel along with the molecular
311 weight marker and four standard samples (2, 4, 8, and 16 μg total protein for each of five
312 randomly selected samples diluted with protein extraction reagent). MultiGauge v.3.0
313 software (Fujifilm) was used to quantify the signal intensity of the protein bands. The
314 intensities of band of AMHR2 (as full-length form) for 16, 8, 4, and 2 μg protein samples
315 were set as 100%, 50%, 25%, and 12.5%, respectively, and the intensity of other samples
316 was calculated as a percentage of these standards using MultiGauge software. After

antibodies were removed from the PVDF membrane with stripping solution, the membrane was used for immunoblotting with the anti-β-actin mouse monoclonal antibody. The intensities of the β-actin band for 16-, 8-, 4-, and 2-μg protein samples were set as 100%, 50%, 25%, and 12.5%, respectively, and the intensity of other samples was calculated as a percentage of these standards using MultiGauge software. AMHR2 expression level was normalized to that of β-actin in each sample.

Statistical analysis

The statistical analyses were performed using StatView version 5.0 for Windows (SAS Institute, Inc., Cary, NC, USA). Grubb's test verified the absence of outliers. The Shapiro-Wilk's test and Kolmogorov-Smirnov Lilliefors test verified the normality of distribution of each variable. The F-test verified homogeneity of variance of all of variables between stages of estrous and ages. One-factor ANOVA followed by *post-hoc* comparisons using Fisher's protected least significant difference (PLSD) test were performed for the four stages comparisons. One-factor ANOVA followed by Fisher's PLSD test were performed for the differences between old Holstein cows, young Wagyus, and old Wagyus. The level of significance was set at $P < 0.05$. Data are expressed as mean ± standard error of the mean (SEM).

Results

Experiment 1

Polymerase chain reaction products of the expected size (320 bp), indicating *AMHR2*, were obtained from the ovary, ampulla, isthmus, CAR, and ICAR, as revealed through agarose gel electrophoresis (Fig. 1a). Bands of PCR products from ovary, CAR, and ICAR were stronger than bands from the ampulla. Neither the NTC nor any of the NRCs yielded any PCR amplified products (Fig. 1b). A homology search against gene databases for the sequenced amplified products revealed bovine *AMHR2* (NM_001205328.1) as the best match, with a query coverage of 100%; e-value, 0.0; maximum alignment identity, 99%. No other bovine gene displayed homology with the PCR product herein, thus indicating that the amplified product was indeed AMHR2.

Anti-Müllerian hormone receptor type 2 expression in the ampulla, isthmus, CAR, ICAR, and in positive control ovarian specimens were analyzed via western blot analysis, using the anti-AMHR2 antibody (Fig. 2). The western blot displayed similar protein bands for AMHR2 among all tissue samples (Fig. 2a). Full length bands and cleaved bands, however, were stronger in ovary, CAR and ICAR, than ampulla and isthmus. No protein bands were observed for the negative control membranes, wherein the primary antiserum was pre-absorbed with the antigen peptide.

Fig. 3 shows the results of immunofluorescence staining for AMHR2 in the ampulla, isthmus, CAR, and ICAR. Robust, high-intensity fluorescent signals of AMHR2 localized in the tunica mucosa of ampulla (Fig. 3a) and isthmus (Fig. 3b) The strong AMHR2 signals were also localized in the luminal epithelium of endometrium (Fig. 3c, 3d), and the epithelium of endometrium glands (Fig. 3e, 3f). Also uterine stroma showed the AMHR2 signals (Fig. 3c, 3d).

361

362 *Experiment 2*

363 Real-time PCR and western blotting revealed no significant differences in AMHR2
364 mRNA (Fig. 4) and protein (Fig. 5) expression levels among various estrous phases in the
365 ampulla, isthmus, CAR, and ICAR.

366

367 *Experiment 3*

368 Real-time PCR and western blotting revealed no significant differences in AMHR2
369 mRNA (Fig. 6) and protein (Fig. 7) expression levels in the ampulla, isthmus, CAR, and
370 ICAR among old Holsteins cows, and young and old Wagyus.

371

372 **Discussion**

373 To our knowledge, this study is the first to report *AMHR2* expression in the
374 oviducts in all species. Immunofluorescence analysis using anti-bovine AMHR2 antibody
375 revealed robust high-intensity signals in the tunica mucosa of ampulla and isthmus and
376 the glandular and luminal epithelium of endometria, leading to the speculation of the
377 potential roles of AMHR2 in these layers.

378 Anti-Müllerian hormone contributes to regression of the Müllerian duct to prevent the
379 development of the oviducts and uterus in male fetuses (Rey *et al.* 2003), thereby
380 potentially clarifying why the role of AMH in the oviduct has not been studied. In recent,
381 after completion of our experiment, Kim *et al.* (2019) reported AMHR2 expression in
382 healthy human endometrial tissues. Although Kim *et al.* (2019) did not study fluctuations
383 in endometrial AMHR2 expression during the menstrual cycle, no significant differences
384 were reported between proliferative and secretory endometria. Furthermore, in the present

study, no significant difference in AMHR2 expression was reported in the ampulla, isthmus, CAR, and ICAR among estrous stages in heifers. In addition, previous studies have not reported considerable changes in circulating AMH concentrations during the estrous cycle in ruminants *in vivo*, including Wagyu (El-Sheikh Ali *et al*. 2013; Pfeiffer *et al*. 2014; Koizumi and Kadokawa 2017). Therefore, constitutively expressed AMHR2 in the tunica mucosa of ampulla and isthmus and the glandular and luminal epithelium of endometrium may not play a temporal role, e.g., during sperm capacitation and fertilization (Croxatto 2002; Hunter 2005). These layers in the oviducts and endometria provide growth factors and nutrients for embryogenesis in various animals including cattle (Hugentobler *et al.* 2010; Besenfelder *et al.* 2012). Women with low blood AMH concentrations are at an increased risk of miscarriage (Tarasconi *et al.* 2017; Lyttle *et al.* 2018). Therefore, further studies are required to clarify whether AMHR2 in these tissue layers play critical roles in embryo development and conception.

Endometria and oviducts are subject to pathogens presented in the spermatozoa and seminal plasma (Kelly et al. 1997; Quayle 2002), and pathogens and endotoxins penetrate the uterus via the cervix (Herath *et al.* 2007; Tang *et al.* 2013). Circulating AMH levels are significantly lower in women with lymphoma than in healthy women and have been highly correlated with some cytokines, suggesting potential associations with the cytokine network (Paradisi *et al.* 2016). Therefore, further studies are required to clarify whether AMHR2 in the tunica mucosa of ampulla and isthmus and the glandular and luminal epithelium of endometrium may play important roles for immune system.

Fertility is decreased with old age in beef cows (Osoro and Wright 1992). In the present study, no significant difference was observed in AMHR2 expression in the

oviducts and the endometria among old Holsteins and young and old Wagyus. The blood AMH is, however, influenced by age in cows (Koizumi and Kadokawa 2017); old Wagyu cows have significantly higher blood AMH concentrations (100 pg/ml level) than young cows (1–10 pg/ml level) throughout the postpartum period (Koizumi and Kadokawa 2017). ~~Therefore, the AMHR2 in oviducts and endometria in old Wagyu cows may contribute to infertility among old cows. The association of age with oviductal and endometrial AMHR2 expression has not been reported previously in any species; hence, the present results cannot be compared with previous results.~~ The present results regarding the effects of breed and age on AMHR2 expression should be interpreted with caution because we could not obtain specimens from young Holsteins. Therefore, further studies are required to clarify whether AMHR2 in oviducts and endometria ~~has~~ have important roles for infertility in old cows.

Herein, multiple protein bands were observed for AMHR2 upon western blotting, concurrent with previous reports on various animals, including bovine (Faure *et al.* 1996; Hirschhorn *et al.* 2015; Kereilwe *et al.* 2018) showing that AMHR2 is present as a full-length and cleaved monomer (Faure *et al.* 1996; Hirschhorn *et al.* 2015) and because AMHR2 is glycosylated (Faure *et al.* 1996). In conclusion, AMHR2 is expressed in the oviducts and endometria of heifers and cows.

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Conflicts of Interest

The authors declare no conflicts of interest.

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601

602 **Table 1.** Details of the primers used for RT PCR.

603

Gene	Primer	Sequence 5'-3'	Position		Size (bp)
			Nucleotide	Exon	
AMHR2	Forward	AGATTTGCGACCTGACAGCAG	1272–1292	9-10	320
	Reverse	CTTCCAGGCAGCAAAGTGAG	1572–1591	11	

604

605

606 **Table 2.** Details of the primers used for real-time PCR.

607

Gene	Primer	Sequence 5'-3'	Position		Size (bp)
			Nucleotide	Exon	
AMHR2	Forward	TGGGAGATTATGAGTCGCTGC	1249–1269	9	52
	Reverse	GTGGTGGTCTGCTGTCAGGT	1281–1300	9-10	
C2orf29	Forward	TCAGTGGACCAAAGCCACCTA	928–948	3	170
	Reverse	CTCCACACCGGTGCTGTTCT	1077–1097	4	
SUZ12	Forward	CATCCAAAAGGTGCTAGGATAGATG	1441–1465	13	160
	Reverse	TTGGCCTGCACACAAGAATG	1581–1600	14	

608

609

Figures

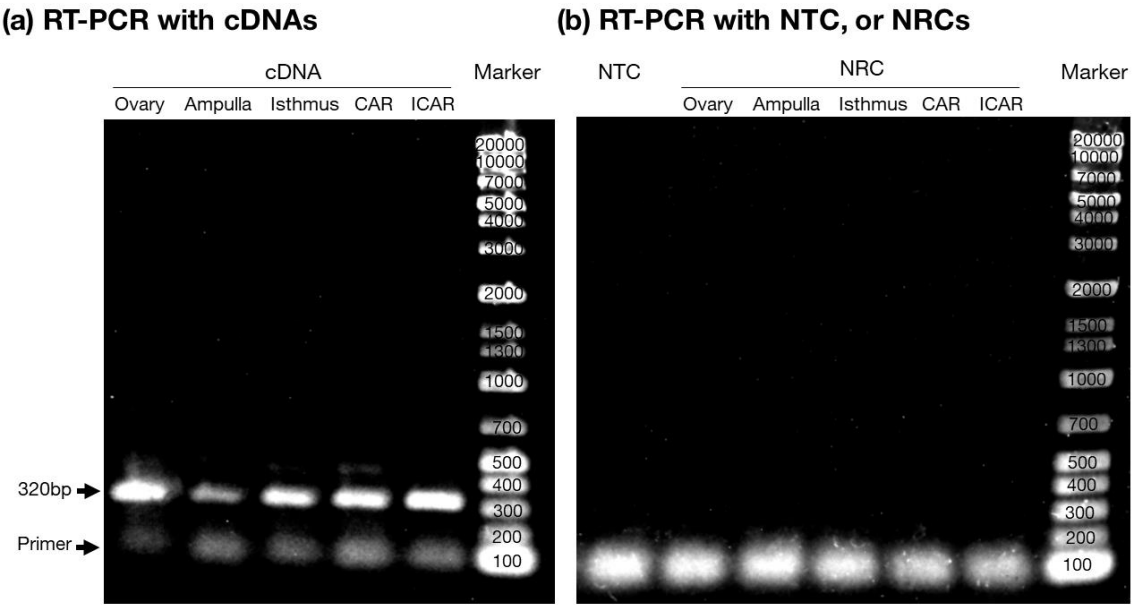
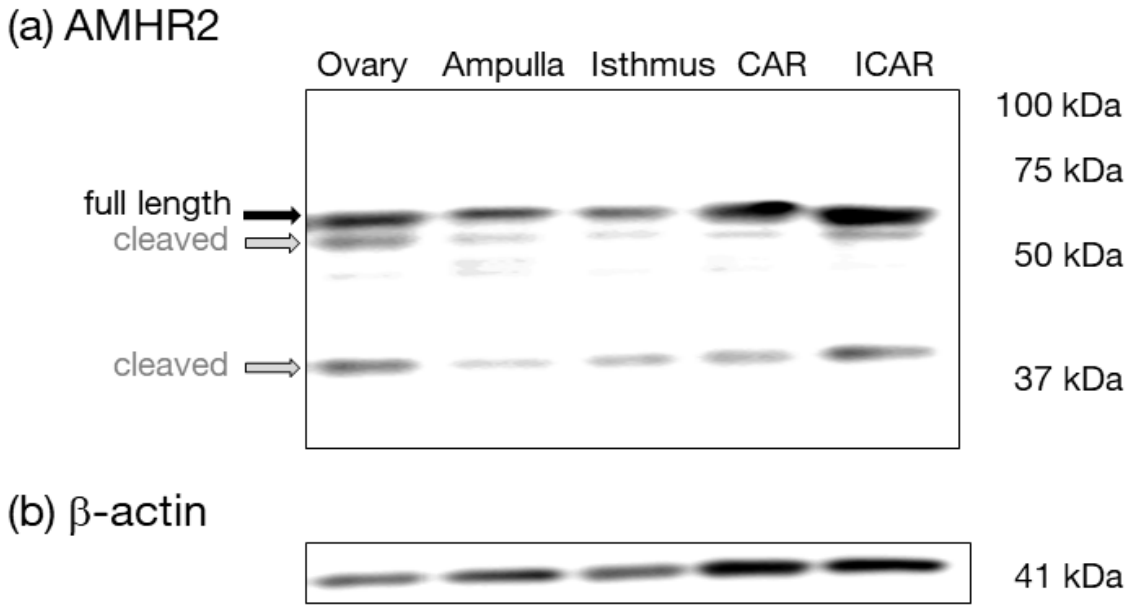


Fig. 1. Expression of anti-Müllerian hormone (AMH) receptor type 2 (*AMHR2*) mRNA, as detected via RT-PCR analysis. The electropherogram shows the expected size (320 bp) of PCR products of bovine *AMHR2* in the ovary, ampulla, isthmus, and caruncular (CAR) and intercaruncular (ICAR) area of the endometrium in healthy post-pubertal heifers (Fig. 1a) but not of the no template control (NTC) and no reverse transcription controls (NRCs) in the ovary, ampulla, isthmus, CAR, and ICAR (Fig. 1b).



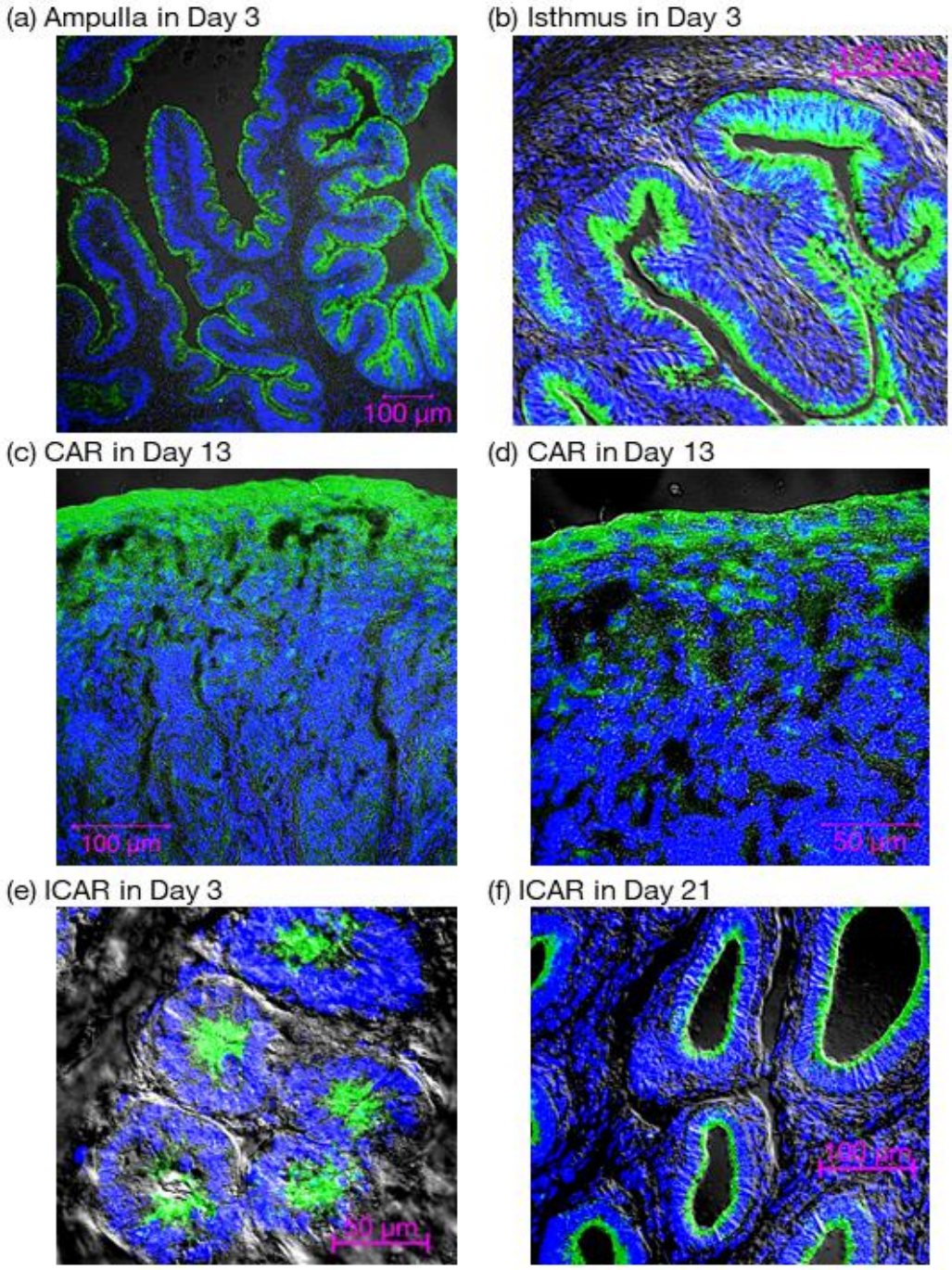
619

620

621 **Fig. 2.** Western blotting analysis of extracts from the ovary, ampulla, isthmus, CAR, or

622 ICAR in healthy post-pubertal heifers and the anti-AMHR2 antibody (a) or anti-β–actin

623 mouse antibody (b).



624

625 **Fig. 3.** AMHR2 (green) expression in the ampulla (a), isthmus (b), CAR (c and d), and

626 ICAR (e and f) of healthy post-pubertal heifers. Specimens shown in panels a, b, and e

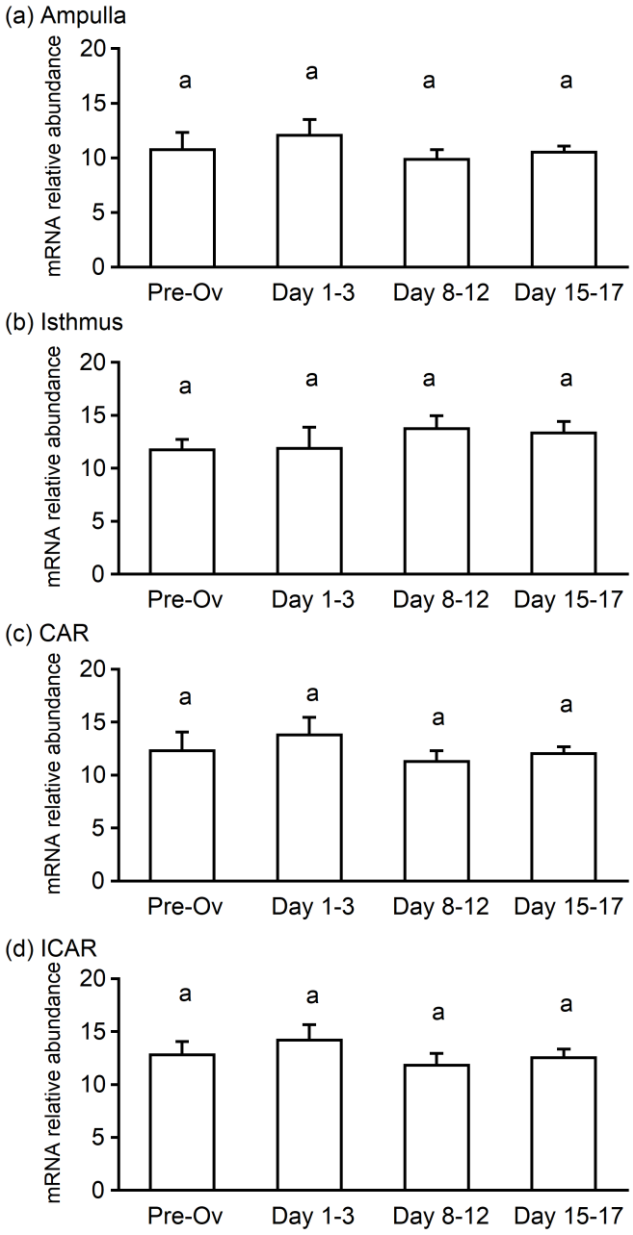
627 were collected on day 3 of the estrous cycle. Specimens shown in panels c (low

628 magnification) and d (high magnification) were collected on day 15. Specimen shown in

629 panel f was collected on day 21. Nuclei are counterstained with DAPI (dark blue) and

630 differential interference contrast microscopy was used to produce the grayscale image.

631 Scale bars: 100 μm in a, b, c, and f; 50 μm in d and e.



632

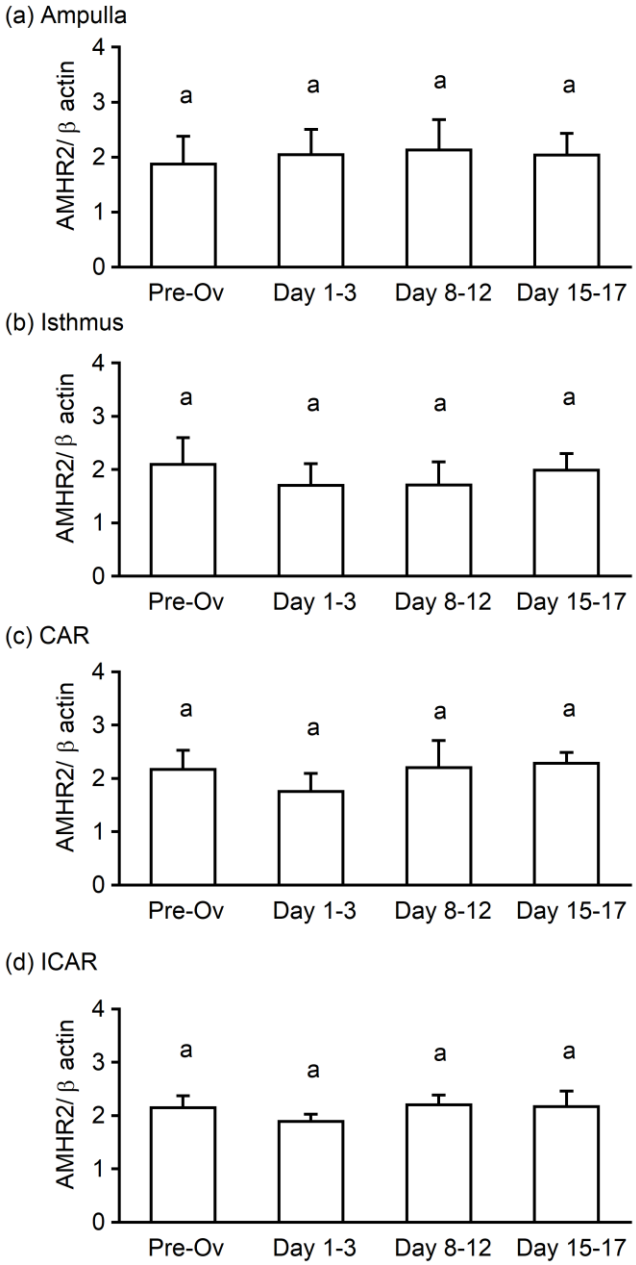
633 **Fig. 4**. Relative *AMHR2* mRNA levels (mean ± SEM) in the ampulla (a), isthmus (b),

634 CAR (c), or ICAR (d) in healthy post-pubertal heifers during the pre-ovulatory phase (day

635 19 to 21), day 1 to 3, day 8 to 12, or day 15 to 17, as determined via real-time PCR. Data

636 were normalized to the geometric means of *C2orf29* and *SUZ12* levels. The same letters

637 indicate no significant difference ($P > 0.05$) across phases.



638

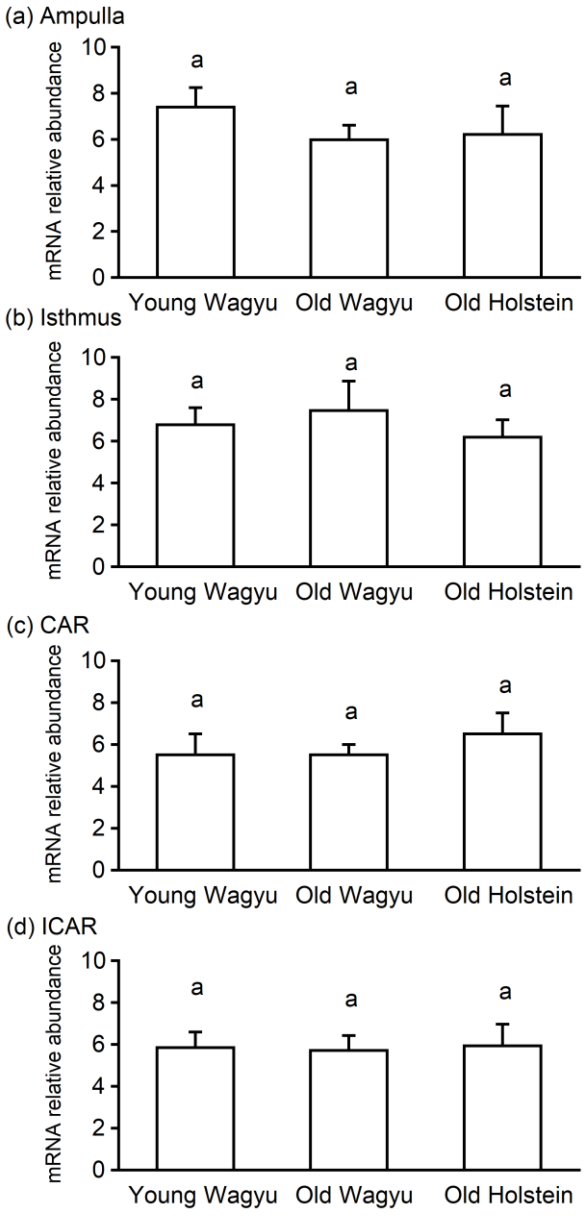
639

640 **Fig. 5.** Relative AMHR2 protein expression levels normalized to that of β-actin in the

641 ampulla (a), isthmus (b), CAR (c), or ICAR (d) in healthy post-pubertal heifers during

642 pre-ovulatory phase, day 1 to 3, day 8 to 12, or day 15 to 17, as determined via western

643 blotting. The same letters indicate no significant difference ($P > 0.05$) across phases.



644

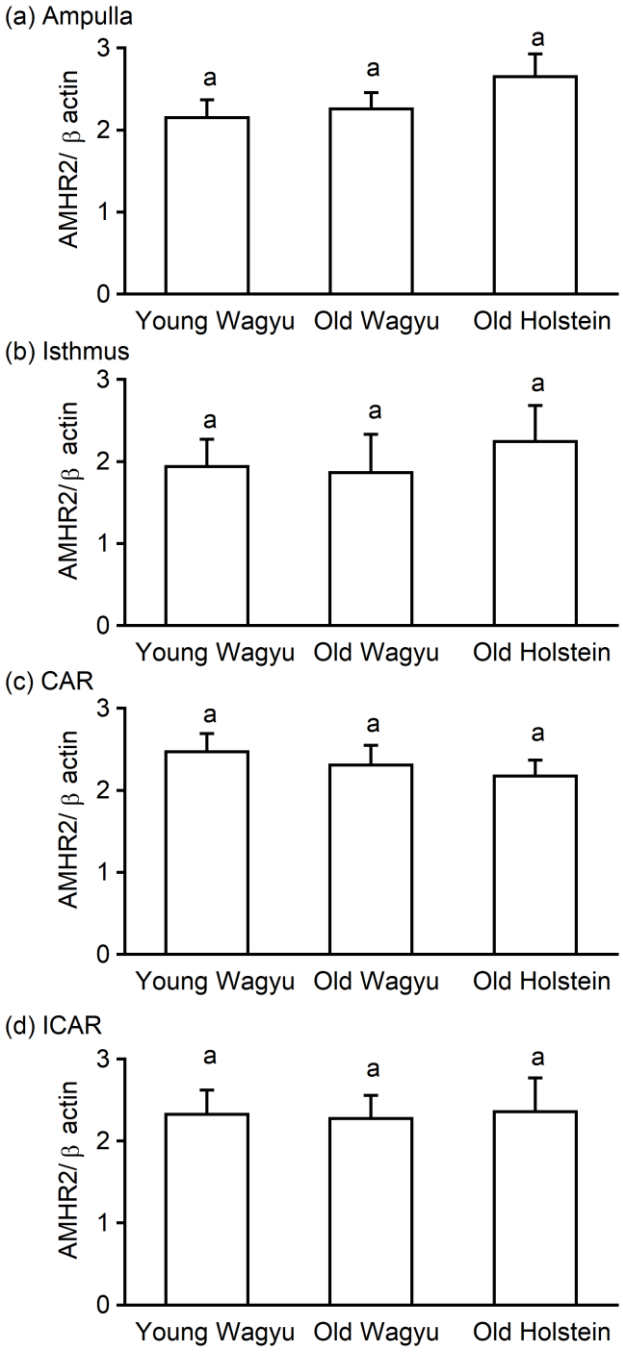
645 **Fig. 6.** Relative *AMHR2* mRNA levels (mean ± SEM) in the ampulla (a), isthmus (b),

646 CAR (c), or ICAR (d) in healthy young adult Wagyu heifers, old Wagyu cows, and old

647 Holsteins cows, as determined via real-time PCR. Data were normalized to the geometric

648 means of *C2orf29* and *SUZ12* levels. The same letters indicate no significant difference

649 ($P > 0.05$) among groups.



650

651 **Fig. 7.** Relative AMHR2 protein expression levels normalized to that of β-actin in the

652 ampulla (a), isthmus (b), CAR (c), or ICAR (d) in healthy young Wagyu heifers, old

653 Wagyu cows, and old Holsteins cows, as determined via western blotting. The same letters

654 indicate no significant difference ($P > 0.05$) across phases.