Bovine gonadotrophs express anti-Müllerian hormone (AMH): Comparison of AMH mRNA and protein expression levels among old Holsteins and young and old Japanese black females

Onalenna Kereilwe[A], and Hiroya Kadokawa[AB]

[A]Faculty of Veterinary Medicine, Yamaguchi University, Yamaguchi-shi, Yamaguchi-ken 1677-1, Japan

[B]Corresponding author: E-mail address: hiroya@yamaguchi-u.ac.jp

Faculty of Veterinary Medicine, Yamaguchi University, Yamaguchi-shi, Yamaguchi-ken 1677-1, Japan

Tel.: + 81 83 9335825; Fax: +81 83 9335938

Running head: AMH expression in bovine gonadotroph

Abstract. Anti-Müllerian hormone (AMH) is secreted from ovaries and stimulates gonadotropin secretion from bovine gonadotroph cells. Other important hormones for endocrinological gonadotroph regulation (e.g., gonadotropin-releasing hormone, inhibin, and activin) have paracrine and autocrine roles. Therefore, in this study, AMH expression in bovine gonadotroph cells and the relationships between AMH expression in the bovine anterior pituitary (AP) and estrous stage, age, and breed were evaluated. *AMH* mRNA expression was detected in APs of post-pubertal heifers (26 months old) by reverse transcription-polymerase chain reaction. Based on western blotting using an antibody to mature C-terminal AMH, the AMH protein was expressed in APs. Immunofluorescence microscopy utilizing the same antibody indicated that AMH is expressed in gonadotrophs. The expression of AMH mRNA and protein in APs did not differ among estrous phases ($P > 0.1$). We compared expression levels among old Holsteins (79.2 ± 10.3 months old) and young (25.9 ± 0.6 months old) and old Japanese black females (89.7 ± 20.3 months old). The APs of old Holsteins exhibited lower *AMH* mRNA levels ($P < 0.05$) but higher AMH protein levels than those of young Japanese black females ($P < 0.05$). In conclusion, bovine gonadotrophs express AMH, and this AMH expression may be breed-dependent.

Additional keywords: Age, Breed, Anterior pituitary, Müllerian-inhibiting substance, Ruminants, TGF-β superfamily

Short summary

Ovaries secrete anti-Müllerian hormone (AMH), which stimulates gonadotropin secretion from gonadotrophs in bovines. This study revealed that gonadotrophs themselves express AMH. The AMH expression levels in anterior pituitaries (APs) were not affected by the estrous phase. The APs of old Holsteins exhibited lower *AMH* mRNA levels but higher AMH protein levels than those in young Japanese black APs.

Introduction

Anti-Mullerian hormone (AMH) is a glycoprotein belonging to the transforming growth factor (TGF)-β superfamily. AMH is secreted by immature granulosa cells in the ovaries of humans and animals (Bhide and Homburg 2016). It has important roles in the ovaries for the regulation of gonadotropin-responsive pre-antral follicle development (Hernandez-Medrano *et al.* 2012) and the inhibition of follicular atresia (Seifer *et al.* 2014). Plasma AMH concentrations can predict the number of high-quality embryos produced by donor goats, ewes, or cows (Ireland *et al.* 2008; Monniaux *et al.* 2011; Pinto *et al*. 2018). These data suggest the importance of AMH for proper reproductive function in animals. In addition to the well-studied roles of AMH in the ovaries of female animals, recent studies have revealed that secreted AMH affects gonadotrophs in the anterior pituitary (AP). The main AMH receptor, i.e., AMH receptor type 2 (AMHR2), is colocalized with gonadotrophin-releasing hormone (GnRH) receptors on the surface of gonadotrophs (Kereilwe *et al*, 2018). AMH activates the synthesis and secretion of gonadotropins, e.g., luteinizing hormone (LH) and follicle stimulating hormone (FSH), in gonadotrophs of rodents and bovines (Bédécarrats *et al.* 2003; Garrel *et al*. 2016; Kereilwe *et al*, 2018). Therefore, AMH has important roles in controlling gonadotropin secretion.

GnRH secreted from the hypothalamus is a well-known endocrine mechanism to control gonadotropin synthesis and secretion in gonadotrophs. However, GnRH is also expressed in gonadotrophs themselves, with paracrine or autocrine roles in the control of gonadotropin synthesis and secretion (Pagesy *et al.* 1992; Miller *et al.* 1996). Inhibin and activin, other family members of the TGF-β superfamily, are secreted by the ovaries and affect gonadotrophs. It is important to note that gonadotrophs synthesize and secrete both inhibin and activin, with paracrine and autocrine effects on gonadotropin synthesis and secretion (Popovics *et al.* 2011). The coordination of the endocrine, paracrine, and autocrine control of these hormones is likely important for normal reproductive functions (De Kretser *et al.* 2002).

Old age is associated with decreased fertility in beef cows (Osoro and Wright 1992). However, little is known about the exact mechanisms underlying this association in domestic animals. Studies of AMH are promising for understanding these mechanisms. Blood AMH concentrations are highest in pubertal girls, and decrease gradually from the age of 25 years until the post-menopausal period (Dewailly *et al.* 2014). In contrast, old Japanese Black cows have higher blood AMH concentrations than those in postpubertal heifers and young cows (Koizumi and Kadokawa 2017). These data suggest that age may be a determinant of the blood AMH concentration.

77 AMH expression in gonadotrophs has not been evaluated in any species. However,
78 AMH is expressed in the APs of both male and female tilapia (Poonlaphdecha *et al.* 2011).
79 Therefore, we evaluated the hypothesis that AMH is expressed in bovine gonadotrophs
80 in AP tissues. Ribeiro *et al.* (2014) reported a difference in blood AMH concentrations
81 among dairy cow breeds. Infertility in Holsteins is an important issue in dairy industries
82 worldwide (Kadokawa and Martin 2006; Adamczyk *et al.* 2017; Gernand and König
83 2018). Therefore, we also evaluated the relationship between AMH expression in APs
84 and various physiological factors, i.e., stage of the estrus cycle, age, and breed.

85

86 **Materials and Methods**

87 *AP and ovary sample collection for RT-PCR and western blotting*

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

92 We obtained AP tissue from post-pubertal (26 months of age) Japanese Black heifers
93 at a local abattoir, using a previously described method (Kadokawa *et al.* 2014) for RNA

94 or protein analyses (n = 4) or immunohistochemistry (n = 5). The heifers were in the
95 middle luteal phase, i.e., 8 to 12 days after ovulation, as determined by macroscopic
96 examination of the ovaries and uterus (Miyamoto *et al*. 2000); the AP show the highest
97 LH, FSH and GnRHR concentrations in this phase (Nett *et al.* 1987).
98 Granulosa cells in preantral and small antral follicles express AMH (Campbell *et al.*
99 2012). Therefore, we also collected ovary tissue samples from the same heifers to use as
100 positive-control of AMH in western blotting (n = 4) and immunohistochemistry (n = 5)
101 assays.
102 The AP and ovary samples for RNA or protein extraction were immediately frozen
103 in liquid nitrogen and stored at −80°C. The AP and ovary samples for
104 immunohistochemistry were fixed with 4% paraformaldehyde at 4°C for 16 h.
105
106 *RT-PCR, sequencing of amplified products, and homology search in gene databases*
107 Total RNA was extracted from the AP samples (n = 4) using RNAzol RT Reagent
108 (Molecular Research Center Inc., Cincinnati, OH, US) according to the manufacturer's
109 protocol. The extracted RNA samples were treated with ribonuclease-free
110 deoxyribonuclease (Thermo Fisher Scientific, Waltham, MA, US) to eliminate possible
111 genomic DNA contamination. Using a NanoDrop ND-1000 spectrophotometer

112 (NanoDrop Technologies Inc., Wilmington, DE, USA), the concentration and purity of
113 each RNA sample were evaluated to ensure the A_{260}/A_{280} nm ratio was in the acceptable
114 range of 1.8–2.1. The mRNA quality of all samples was verified by electrophoresis of
115 total RNA followed by staining with ethidium bromide, and the 28S:18S ratios were 2:1.
116 The cDNA was synthesized from 1 µg of the total RNA per AP using SuperScript IV
117 VILO Master Mix (Thermo Fisher Scientific) according to the manufacturer's protocol.
118 In order to determine the expression of AMH mRNA in the AP, PCR was conducted
119 using a primer pair designed by Primer3 based on reference sequence of bovine AMH
120 [National Center for Biotechnology Information (NCBI) reference sequence of bovine
121 AMH is NM_173890]. The expected PCR-product size of AMH using the primer pair is
122 328 bp (nucleotides 1486-1813; forward primer: 5′-GCTCATCCCCGAGACATACC- 3′;
123 reverse primer: 5′-TTCCCGTGTTTAATGGGGCA-3′). Using a Veriti 96–Well Thermal
124 Cycler (Thermo Fisher Scientific), PCR was performed using 20 ng of cDNA and
125 polymerase (Tks Gflex DNA Polymerase, Takara Bio Inc., Shiga, Japan) under the
126 following thermocycles: 94 °C for 1 min for pre-denaturing followed by 35 cycles of
127 94°C for 60 s, 60°C for 15 s, and 68°C for 30 s. PCR products were separated on 1.5%
128 agarose gel by electrophoresis with a molecular marker [Gene Ladder 100 (0.1-2kbp),
129 Nippon Gene, 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 DNA Data Bank of Japan/GenBank/European Bioinformatics Institute Data Bank using the basic nucleotide local alignment search tool (BLAST) optimized for highly similar sequences (available on the NCBI website).

Antibodies used in this study

Human AMH is secreted as a homodimeric precursor consisting of two identical monomers (560 amino acids; NCBI accession number AAA98805.1) (Mamsen *et al.* 2015). Each monomer consists of two domains, (i) a mature C-terminal region, which becomes bioactive after proteolytic cleavage and binds to AMHR2, and (ii) a pro-region, which is important for AMH synthesis and extracellular transport. The human AMH precursor is cleaved at amino acid 451 (arginine) between the two domains. The pro-region has another cleavage site at amino acid 229 (arginine), giving rise to three potential

148 cleavage products: pro-mid-mature, mid-mature, and mature (Mamsen *et al*. 2015). The
149 bovine AMH precursor monomer (575 amino acids; NCBI accession number
150 NP_776315.1) has 91% sequence similarity to the human protein [evaluated using
151 Genetyx ver. 11 (Genetyx, Tokyo, Japan)]. The bovine AMH precursor contains an
152 arginine cleavage site between the two domains at amino acid 466. However, the bovine
153 AMH precursor does not contain arginine at the residue corresponding to amino acid 229
154 of the human pro-region of AMH. A rabbit polyclonal anti-AMH antibody
155 (ARP54312_P050; Aviva Systems Biology, CA, USA) that recognizes the mature C-
156 terminal form of human AMH (corresponding to amino acids 468–517;
157 SVDLRAERSVLIPETYQANNCQGVCGWPQSDRNPRYGNHVVLLLKMQARG)
158 was used. This sequence had 98% homology to amino acids 483–532 of the mature C-
159 terminal form of bovine AMH but no homology to other bovine proteins, as determined
160 using protein BLAST.

161 We used also a specific guinea pig polyclonal anti-bovine GnRHR (Kadokawa *et al*.
162 2014), a specific mouse monoclonal anti-LH β (LHβ) subunit antibody (clone 518-B7;
163 Matteri *et al.* 1987; Iqbal *et al.* 2009), and a specific mouse monoclonal anti-FSH β
164 (FSHβ) subunit antibody (clone A3C12; Borromeo *et al.* 2004) for immunohistochemical
165 analysis of AP tissue. These antibodies are utilized for immunohistochemical analysis of

166 AP tissue in our previous papers (Kadokawa *et al*. 2014; Pandey *et al.* 2017a, 2017b;
167 Kereilwe *et al.* 2018)
168
169 *Western Blotting for AMH detection*
170 Proteins were extracted from the AP (n = 4) or ovary samples (n = 4, used as positive
171 controls) and western blotting was performed as previously described (Kereilwe *et al.*
172 2018). The extracted protein sample (33.4µg of total protein in 37.5µl) was mixed in
173 12.5µl of 4x Laemmli sample buffer (Bio-rad) containing 10% (v/v) β-mercaptoethanol,
174 then boiled for 3 min at 100 °C. The boiled protein samples were quickly cooled down in
175 ice, then the protein samples (2, 4, 8, or 16 µg of total protein) were loaded onto a
176 polyacrylamide gel along with a molecular weight marker (Precision Plus Protein All
177 Blue Standards; Bio-Rad), and resolved by electrophoresis on sodium dodecyl sulfate
178 polyacrylamide gels at 100 V for 90 min. Proteins were then transferred to polyvinylidene
179 fluoride (PVDF) membranes. Blocking was done with 0.1% Tween 20 and 5% non-fat
180 dry milk for 1h at 25 °C then immunoblotting was performed with the anti-AMH rabbit
181 antibody (1:25,000 dilution) overnight at 4 °C. After washing membrane with 10 mM
182 Tris–HCl (pH 7.6) containing 150 mM NaCl and 0.1% Tween 20, the PVDF membrane
183 was incubated with horseradish peroxidase (HRP)-conjugated anti- rabbit 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). Mamsen *et al.* (2015) utilized western blotting to detect AMH in the human testis or immature granulosa cells and identified 2 to 5 bands. In this previous study, the pro-mature form was detected as 3 or 4 bands (12.5, 16, 35, or 55 kDa) and the precursor was 98 kDa. We defined bovine AMH bands based on mobility as the AMH precursor or the pro-mature form (4 sizes) according to these previous studies (Pierre *et al.* 2016; Mamsen *et al.* 2015; Di Clemente *et al.* 2010). Antibodies were removed from the PVDF membrane with stripping solution (Nacalai Tesque Inc., Kyoto, Japan), then, 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).

Fluorescent immunohistochemistry and confocal microscopy

We collected tissue blocks of AP (n=5) in 4% paraformaldehyde PBS. The tissue blocks were stored at 4°C for 16 h, after which they were placed in 30% sucrose PBS until the blocks were infiltrated with sucrose. For the immunofluorescence analysis of AP tissues, previously described methods were used (Kadokawa *et al.* 2014). Briefly, 15-µm

202 sagittal sections were prepared and mounted on slides. The sections were treated with
203 0.3 % Triton X-100-PBS for 15 min, then, blocking done by incubating with 0.5 mL of
204 PBS containing 10% normal goat serum (Wako Pure Chemicals, Osaka, Japan) for 1 h at
205 room temperature. The slides were incubated with a cocktail of primary antibodies (anti-
206 GnRHR guinea pig antibody, anti-AMH rabbit antibody, and either anti-LHβ or anti-
207 FSHβ mouse antibody [all diluted as 1:1,000]) for 12 h at 4°C, then followed by
208 incubation with a cocktail of fluorochrome-conjugated secondary antibodies (Alexa Fluor
209 Alexa Fluor 488 goat anti-rabbit IgG, Alexa Fluor 546 goat anti-mouse IgG, and Alexa
210 Fluor 647 goat anti-guinea pig IgG [all from Thermo Fisher Scientific and diluted as 1
211 μg/mL]) and 1 μg/mL of 4′, 6′-diamino-2-phenylindole (DAPI; Wako Pure Chemicals)
212 for 2 h at room temperature.

213 The stained sections on slides were observed by confocal microscopy (LSM710; Carl
214 Zeiss, Göttingen, Germany) equipped with a diode lasers (405nm, argon laser 488nm,
215 HeNe laser 533nm, and HeNe laser 633nm). Images obtained by fluorescence microscopy
216 were scanned with a 40× or 63× oil-immersion objective and recorded by a CCD camera
217 system controlled by ZEN2012 black edition software (Carl Zeiss). GnRHR, AMH and
218 LHβ or FSHβ localization were examined in confocal images of triple-immunolabeled
219 specimens. In the confocal images obtained after immunohistochemistry analysis, the

GnRHR is shown in light blue, AMH is shown in red, and LHβ or FSHβ 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 same antigen peptide, or in which normal rabbit IgG (Wako Pure Chemicals) was used instead of the primary antibody. Percentage of single- and double–labeled AMH- and GnRHR-positive cells were determined from 12 representative confocal images per pituitary gland.

AP sample collection for comparisons among estrus stages

AP tissues were collected from the heads of adult (26-month-old) non-pregnant healthy Japanese Black heifers in the pre-ovulation [day 19 to 21 (day 0 = day of estrus); n = 5], early luteal (day 2 to 5; n = 5), mid-luteal (day 8 to 12; n = 5), or late luteal (day 15 to 17; n = 5) phase, as determined by the macroscopic examination of the ovaries and uterus (Miyamoto *et al.* 2000). Samples were obtained at a local abattoir as previously described (Rudolf and Kadokawa 2014) and immediately frozen in liquid nitrogen and stored at −80°C until RNA or protein extraction.

AP sample collection for comparisons among ages or breeds

AP tissues were obtained during the luteal phase from healthy post-pubertal Japanese Black heifers (25.9 ± 0.6 months of age; n = 5; young JB group), old Japanese Black cows (89.7 ± 20.3 months of age; 5.2 ± 0.5 parity; n = 5; old JB group), and old Holstein cows (79.2 ± 10.3 months of age; 6.6 ± 0.9 parity; n = 5; old Hol group) from the local abattoir. It was not possible to obtain AP samples from post-pubertal Holstein heifers because they are kept in dairy farms for milking purposes. All of the heifers and cows in the three groups were non-lactating and non-pregnant and they had no follicular cysts, luteal cysts, and other ovarian disorders based on macroscopic examinations of the ovaries (Kamomae 2012). All cows in the old Hol group had endometritis as determined by the macroscopic examination of the uterus with mucopurulent vaginal discharge (Kamomae 2012). All of their endometritis clinical scores (Sheldon and Dobson 2004) were 1, since the mucus character was clear or translucent with flecks of white pus, and the mucus odor was not unpleasant. The old Holstein cows were slaughtered owing to infertility, diagnosed after at least 5 artificial insemination attempts. The old Japanese Black cows were slaughtered after completing parturition a sufficient number of times, as planned by farmers to obtain beef.

Real-time PCR to evaluate the factors affecting AMH expression

255 Real-time PCR was performed to compare *AMH* expression among estrous phases or
256 among the young JB, old JB, and old Hol groups. The preparation of high-quality total
257 RNA and cDNA synthesis were performed as described above.
258 Table 1 shows the primers designed for real-time PCR using Primer Express
259 Software V3.0 (Thermo Fisher Scientific) based on the reference sequences. The amount
260 of gene expression was measured in duplicate by real-time PCR analyses with 20 ng
261 cDNA, using CFX96 Real Time PCR System (Bio-Rad, Hercules, CA, USA) and Power
262 SYBR Green PCR Master Mix (Thermo Fisher Scientific), together with a 6-point
263 relative standard curve, non-template control, and no reverse-transcription control.
264 Standard 10-fold dilutions of purified and amplified DNA fragments were prepared.
265 Temperature conditions for all genes were as follows: 95°C for 10 min for pre-
266 denaturation; five cycles each of 95°C for 15s and 66°C for 30s; and 40 cycles each of
267 95°C for 15s and 60°C for 60s. Melting curve analyses were performed at 95°C for each
268 amplicon and each annealing temperature to ensure the absence of smaller non-specific
269 products such as dimers. To optimize the real-time PCR assay, serial dilutions of a cDNA
270 template were used to generate a standard curve by plotting the log of the starting quantity
271 of the dilution factor against the C_q value obtained during amplification of each dilution.
272 Reactions with a coefficient of determination (R^2) >0.98 and efficiency between 95 and

105% were considered optimized. The concentration of PCR products was calculated by comparing C_q values of unknown samples with the standard curve using appropriate software (CFXmanagerV3.1, Bio-Rad). The gene expression levels for *AMH* genes were normalized to the geometric mean of the expression levels of two house-keeping genes, *GAPDH* and *RANBP10*. We selected these two housekeeping genes from among 20 that have been previously described (Rekawiecki et al. 2012; Walker et al. 2009) because they had the smallest inter-heifer coefficients of variation of reads per kilobase of transcript per million mapped reads value upon deep sequencing of the transcriptome (Pandey *et al.* 2017c).

Western blotting to evaluate the factors affecting AMH expression

Western blotting was performed to compare AMH protein levels in APs among different estrous phases or among the young JB, old JB, and old Hol groups. Sample collection and western blotting were performed as described above. Briefly, 15 µL (8 µg of total protein) of boiled sample was loaded on a polyacrylamide gel along with the molecular weight marker and four standard samples (2, 4, 8, and 16 µg total protein for each of five randomly selected AP samples diluted with protein extraction reagent). MultiGauge v.3.0 software (Fujifilm) was used to quantify the signal intensity of the

291 protein bands. The intensities of band of AMH (as mature C-terminal form) for 16-, 8-,
292 4-, and 2-μg AP protein samples were set as 100%, 50%, 25%, and 12.5%, respectively,
293 and the intensity of other samples was calculated as a percentage of these standards using
294 MultiGauge software. After antibodies were removed from the PVDF membrane with
295 stripping solution, the membrane was used for immunoblotting with the anti-β-actin
296 mouse monoclonal antibody. The intensities of the β-actin band for 16-, 8-, 4-, and 2-μg
297 AP protein samples were set as 100%, 50%, 25%, and 12.5%, respectively, and the
298 intensity of other samples was calculated as a percentage of these standards using
299 MultiGauge software. AMH expression level was normalized to that of β-actin in each
300 sample.
301
302 *Analysis of the* AMH *gene 5'-flanking region*
303 The 5000-nucleotide sequence of the 5'-flanking region of the *AMH* gene
304 (chromosome 7: 22691978-22696977) was obtained using the online Ensembl
305 (www.ensembl.org) and BLAT Search Genome program (http://genome.ucsc.edu) (Cow
306 Jun. 2014, Bos_taurus_UMD_3.1.1/bosTau8). The sequence was analyzed using Genetyx
307 software ver. 13 (Genetyx, Tokyo, Japan) for the presence of consensus response element
308 (RE) sequences for estrogen—i.e., ERE (5'-GGTCANNNTGACC-3') (Gruber *et al.*

309 2004), and half ERE (GGTCA, TGACC, or TGACT) (Liu et al. 1995), as well as for
310 progesterone—i.e., PRE (5'-G/A G G/T AC A/G TGGTGTTCT-3') (Geserick *et al.* 2005).
311
312 *Statistical analysis*
313 The statistical analysis was done by one-factor ANOVA followed by *post-hoc*
314 comparisons using Fisher's protected least significant difference (PLSD) test using
315 StatView version 5.0 for Windows (SAS Institute, Inc., Cary, NC, USA). The level of
316 significance was set at $P < 0.05$. Data are expressed as mean ± standard error of the mean
317 (SEM).
318
319 **Results**
320 *Expression of AMH mRNA in APs of post-pubertal heifers*
321 PCR products of the expected size (328 bp), indicating AMH, were obtained by
322 agarose gel electrophoresis (Fig. 1). Homology searching in the gene databases for the
323 obtained sequence of amplified products revealed that the best match alignment was
324 bovine AMH (NM_173890.1), which had a query coverage of 100%, an e-value of 0.0, and
325 a maximum alignment identity of 99%. There was no other bovine gene found to have a
326 homology for the obtained sequences of amplified products, which lead to the conclusion

that the sequences of the amplified products were identical with the sequence of bovine AMH.

AMH protein expression in APs

Western blotting confirmed the presence of AMH in both the AP and ovary samples, with differences in intensity between sample type (Fig. 2A). Weaker bands for the AMH precursor (70 kDa) were detected in the AP samples than in the ovary samples. Stronger bands for the mature C-terminal form were observed for the AP samples than the ovary samples, and there was a difference in molecular weights between AP (20 kDa) and ovary (25 kDa) samples. Figure 2B shows representative β-actin bands for both tissue types.

Immunofluorescence analysis of AMH expression in bovine small follicles and AP tissues

Figure 3 shows immunofluorescence signals in the granulosa cells of small follicles in the ovary tissues of post-pubertal heifers.

Also the expression of GnRHR, AMH, LHβ, and FSHβ in bovine AP tissues was investigated by immunohistochemistry. AMH was localized in the majority of LHβ-positive (Fig. 4a) and FSHβ-positive (Fig. 4b) cells. In AP samples, there were 53.2 ± 2.1 GnRHR-positive cells, 43.2 ± 2.5 AMH-positive cells, and 30.2 ± 1.6 double-positive

345 cells; 57.0% ± 3.4% of GnRHR-positive cells were AMH-positive, whereas 82.0% ±
346 5.5% of AMH-positive cells were GnRHR-positive.
347
348 *Relationship between AMH in APs and the estrous phase*
349 We used real-time PCR and western blotting to evaluate the relationship between the
350 estrous phase and AMH expression at the mRNA and protein levels in APs. There were
351 no differences among phases of the estrous cycle in AMH expression at the mRNA ($P > 0.05$; Fig. 5) or protein levels ($P > 0.05$; Fig. 6).
353
354 *AMH expression in APs of Holstein cows, Japanese black heifers, and Japanese black*
355 *cows*
356 Real-time PCR and western blotting were used to analyze AMH expression at the
357 mRNA and protein levels in the AP. The levels of *AMH* mRNA were lower in the old Hol
358 group than in young JB group ($P < 0.05$; Fig. 7). The old JB group had tendency to express
359 lower levels *AMH* mRNA than those in the young JB group ($P = 0.10$). AMH protein
360 levels were greater in the old Hol group than in the old JB and young JB groups ($P < 0.05$;
361 Fig. 8). There was no difference in AMH levels between the young JB and old JB groups
362 ($P > 0.10$).

363

364 *ERE, and PRE in the 5'-flanking region of bovine AMH gene*

365 The 5'-flanking region of the bovine *AMH* gene was analyzed for EREs, PREs, and

366 similar sequences. There were no ERE, no half ERE, nor PRE sequences.

367

368 **Discussion**

369 The APs of tilapia express AMH but the specific cells expressing AMH are unclear

370 (Poonlaphdecha *et al.* 2011). This study is the first to evaluate gonadotrophs expressing

371 AMH. AMH activates the synthesis and secretion of gonadotropins from the

372 gonadotrophs of rodents and bovines (Bédécarrats *et al.* 2003; Garrel *et al*. 2016;

373 Kereilwe *et al*, 2018). Therefore, the coordination of the endocrine, paracrine, and

374 autocrine control of AMH may be important for normal reproductive functions, similar to

375 GnRH, inhibin, and activin (Pagesy *et al*. 1992; Miller *et al*. 1996; Popovics *et al*. 2011).

376 Immunohistochemistry using an anti-AMH antibody has shown a strong signal in the

377 granulosa cells of small antral follicles, which express *AMH* mRNA (Campbell *et al*.

378 2012), consistent with AMH expression in granulosa cells of ruminants (Rocha *et al.*

379 2016). Additionally, the band patterns observed by western blotting were similar to those

380 of a previous study of AMH (Mamsen *et al*. 2015). Therefore, the anti-AMH antibody

381 can be used for immunohistochemical analyses of bovine samples. Gonadotrophs are a

382 heterogeneous cell population including LH and FSH monohormonal and bihormonal
383 subsets in rats, equines, and bovines (Townsend *et al.* 2004; Pals *et al.* 2008; Kadokawa
384 *et al.* 2014). Our results showed that both LHβ-positive cells and FSHβ-positive cells
385 express AMH, suggesting that AMH secreted from the three types of gonadotrophs have
386 paracrine or autocrine roles.

387 Western blotting showed differences in band strength or size between APs and
388 ovaries. The APs exhibited weaker bands for the AMH precursor (70 kDa) than those for
389 ovary samples, suggesting that APs store less AMH precursor than ovaries. The band size
390 for the mature C-terminal form was smaller for APs (20 kDa) than for ovaries (25 kDa).
391 We did not observe a 25-kDa band in the AP lane or a 20-kDa band in the ovary lane,
392 even after longer exposure periods (data not shown). A potential explanation for the band
393 size difference for the mature C-terminal form may be a difference in *O*-glycosylation
394 among organs (Medzihradszky *et al.* 2015; Skaar *et al.* 2011).

395 AMH expressed by gonadotroph has not been evaluated previously in any species;
396 accordingly, it was impossible to compare our data with the results of previous studies.
397 Our results that AMH expression at the mRNA and protein levels in APs differ between
398 the young JB and old Hol groups. Jerseys have higher blood AMH concentrations than
399 those of Holsteins (Ribeiro et al. 2014). Therefore, the difference in AMH expression in

APs may be explained by a difference among breeds or any factor related to breed. The APs of the old Hol group expressed lower levels of *AMH* mRNA than those of the young JB group. In contrast, the APs of the old Hol group exhibited higher AMH protein levels than those of the young and old JB groups. However, the results for the effects of breed and age on AMH expression in APs should be interpreted with caution because we could not obtain APs from young Holsteins. Further studies are required to clarify whether AMH secretion from APs is decreased in old Hol and results in higher AMH protein levels within APs.

We previously reported that old Japanese Black cows have significantly higher blood AMH concentrations (100 pg/mL) than those of young Japanese Black cows (1–10 pg/mL) throughout the postpartum period (Koizumi and Kadokawa 2017). However, we observed a small difference in AMH expression at the mRNA and protein levels in APs between the young and old JB groups in this study. Therefore, AMH secreted by the ovary, rather than AMH secreted by the AP, may explain the difference in blood AMH concentrations between young and old cows.

Previous studies have not detected significant changes in the blood AMH concentration during the estrous cycle in ruminants (Pfeiffer *et al*. 2014; Koizumi and Kadokawa 2017; El-Sheikh Ali *et al*. 2013). Our data also showed no significant changes

418 in AMH at the mRNA and protein levels in APs during the estrous cycle. The 5'-flanking
419 region of the bovine *AMH* gene does not contain ERE, half ERE, or PRE sequences.
420 Therefore, AMH expression in APs, similar to the blood AMH concentration, is unlikely
421 to change during the estrous cycle. In conclusion, bovine gonadotrophs express AMH,
422 and the AMH expression may be breed-dependent. Further studies are needed to
423 determine the precise effects of age and breed on AMH expression.
424
425 **Acknowledgments**
426 Onalenna Kereilwe was supported by MEXT (Ministry of Education, Culture, Sports,
427 Science, and Technology) with the provision of a scholarship. This research was partly
428 supported by a Grant-in Aid for Scientific Research (JSPS Kakenhi Grant Number
429 15K07693) from Japan Society for the Promotion of Science (Tokyo, Japan) to Hiroya
430 Kadokawa.
431
432 **Conflicts of Interest**
433 The authors declare no conflicts of interest.
434
435 **References**

436 Adamczyk, K., Makulska, J., Jagusiak, W., and Weglarz, A. (2017). Associations between
437 strain, herd size, age at first calving, culling reason and lifetime performance
438 characteristics in Holstein-Friesian cows. *Animal*. **11**, 327-334. doi:
439 10.1017/S1751731116001348
440 Bhide, P., and Homburg, R. (2016). Anti-Müllerian hormone and polycystic ovary
441 syndrome. *Best. Pract. Res. Clin. Obstet. Gynaecol.* **37**, 38-45. doi:
442 10.1016/j.bpobgyn.2016.03.004
443 Borromeo, V., Amsterdam, A., Berrini, A., Gaggioli, D., Dantes, A., and Secchi, C.
444 (2004). Characterization of biologically active bovine pituitary FSH purified by
445 immunoaffinity chromatography using a monoclonal antibody. *Gen. Comp.*
446 *Endocrinol.* **139**, 179-189. doi: 10.1016/j.ygcen.2004.09.005
447 Bédécarrats, G. Y., O'Neill, F. H., Norwitz, E. R., Kaiser, U. B., and Teixeira, J. (2003).
448 Regulation of gonadotropin gene expression by Müllerian inhibiting substance. *Proc.*
449 *Natl. Acad. Sci. U. S. A.* **100**, 9348-9353. doi: 10.1073/pnas.1633592100
450 Campbell, B.K., Clinton, M., and Webb, R. (2012). The role of anti-Müllerian hormone
451 (AMH) during follicle development in a monovulatory species (sheep).
452 *Endocrinology* **153**, 4533-4543. doi: 10.1210/en.2012-1158.
453 De Kretser, D. M., Hedger, M. P., Loveland, K. L., and Phillips, D. J. (2002). Inhibins,

activins and follistatin in reproduction. *Hum. Reprod. Update* **8**, 529-541. doi: 10.1093/humupd/8.6.529

Dewailly, D., Andersen, C. Y., Balen, A., Broekmans, F., Dilaver, N., Fanchin, R., Griesinger, G., Kelsey, T. W., La Marca, A., Lambalk, C., Mason, H., Nelson, S.M., Visser, J. A., Wallace, W. H., and Anderson, R. A. (2014). The physiology and clinical utility of anti-Müllerian hormone in women. *Hum. Reprod. Update* **20**, 370-385. doi: 10.1093/humupd/dmt062

Di Clemente, N., Jamin, S. P., Lugovskoy, A., Carmillo, P., Ehrenfels, C., Picard, J.Y., and Cate, R. L. (2010). Processing of anti-Müllerian hormone regulates receptor activation by a mechanism distinct from TGF-β. *Mol. Endocrinol.* **24**, 2193–2206. doi: 10.1210/me.2010-0273.

El-Sheikh Ali, H., Kitahara, G., Nibe, K., Yamaguchi, R., Horii, Y., Zaabel, S., and Osawa, T. (2013). Plasma anti-Müllerian hormone as a biomarker for bovine granulosa-theca cell tumors: Comparison with immunoreactive inhibin and ovarian steroid concentrations. *Theriogenology* **80**, 940-949. doi: 10.1016/j.theriogenology.2013.07.022

Garrel, G., Racine, C., L'Hôte, D., Denoyelle, C., Guigon, C. J., di Clemente, N., and Cohen-Tannoudji, J. (2016). Anti-Müllerian hormone: a new actor of sexual

472 dimorphism in pituitary gonadotrope activity before puberty. *Sci. Rep.* **6**, 23790. doi:
473 10.1038/srep23790
474 Gernand, E., and Konig, S. (2017). Genetic relationships among female fertility disorders,
475 female fertility traits and productivity of Holstein dairy cows in the early lactation
476 period. *J. Anim. Breed. Genet*. **134**, 353-363. doi: 10.1111/jbg.12274
477 Geserick, C., Meyer, H. A., and Haendler, B. (2005). The role of DNA response elements
478 as allosteric modulators of steroid receptor function. *Mol. Cell. Endocrinol.* **236**, 1-
479 7. doi: 10.1016/j.mce.2005.03.007
480 Gruber, C. J., Gruber, D. M., Gruber, I. M., Wieser, F., and Huber, J. C. (2004). Anatomy
481 of the estrogen response element. *Trends Endocrinol. Metab.* **15**, 73-78. doi:
482 10.1016/j.tem.2004.01.008
483 Hernandez-Medrano, J. H., Campbell, B. K., and Webb, R. (2012). Nutritional influences
484 on folliculogenesis. *Reprod. Domest. Anim. Suppl.* **4**, 274-282. doi: 10.1111/j.1439-
485 0531.2012.02086
486 Iqbal, J., Latchoumanin, O., Sari, I. P., Lang, R. J., Coleman, H. A., Parkington, H. C.,
487 and Clarke, I. J. (2009). Estradiol-17beta inhibits gonadotropin-releasing hormone-
488 induced Ca2+ in gonadotropes to regulate negative feedback on luteinizing hormone
489 release. *Endocrinology* **150**, 4213-4220. doi: 10.1210/en.2009-0092

490 Ireland, J. L., Scheetz, D., Jimenez-Krassel, F., Themmen, A. P., Ward, F., Lonergan, P.,
491 Smith, G. W., Perez, G. I., Evans, A. C., and Ireland, J. J. (2008). Antral follicle
492 count reliably predicts number of morphologically healthy oocytes and follicles in
493 ovaries of young adult cattle. *Biol. Reprod.* **79,** 1219-1225. doi:
494 10.1095/biolreprod.108.071670
495 Kadokawa, H., and Martin, G. B. (2006). A new perspective on management of
496 reproduction in dairy cows: the need for detailed metabolic information, an
497 improved selection index and extended lactation. *J. Reprod. Dev.* **52**, 161-168. doi:
498 10.1262/jrd.17088
499 Kadokawa, H., Pandey, K., Nahar, A., Nakamura, U., and Rudolf, F. O. (2014).
500 Gonadotropin-releasing hormone (GnRH) receptors of cattle aggregate on the
501 surface of gonadotrophs and are increased by elevated GnRH concentrations. *Anim.*
502 *Reprod. Sci.* **150**, 84-95. doi: 10.1016/j.anireprosci.2014.09.008
503 Kamomae, H. (2012). Reproductive disturbance. In ‘Veterinary Theriogenology’ . (Eds
504 T. Nakao, S. Tsumagari and S. Katagiri.) pp. 283-340. (Buneidou Press: Tokyo,
505 Japan) (in Japanese)
506 Kereilwe, O., Pandey, K., Borromeo, V., and Kadokawa, H. (2018). Anti-Müllerian
507 hormone receptor type 2 is expressed in gonadotrophs of postpubertal heifers to

508 control gonadotrophin secretion. *Reprod. Fertil. Dev.* **30**, 1192-1203. doi:
509 10.1071/RD17377
510 Koizumi, M., and Kadokawa, H. (2017). Positive correlations of age and parity with
511 plasma anti-Müllerian hormone concentrations in Japanese Black cows. *J. Reprod.*
512 *Dev.* **63**, 205-209. doi: 10.1262/jrd.2016-088
513 Mamsen, L. S., Petersen, T. S., Jeppesen, J. V., Møllgard, K., Grøndahl, M. L., Larsen,
514 A., Ernst, E., Oxvig, C., Kumar, A., Kalra, B., and Andersen, C. Y. (2015).
515 Proteolytic processing of anti-Mullerian hormone differs between human fetal testes
516 and adult ovaries. *Mol. Hum. Reprod.* **21**, 571–582. doi:10.1093/molehr/gav024
517 Matteri, R. L., Roser, J. F., Baldwin, D. M., Lipovetsky, V., and Papkoff, H. (1987).
518 Characterization of a monoclonal antibody which detects luteinizing hormone from
519 diverse mammalian species. *Domest. Anim. Endocrinol.* **4**, 157-165. doi:
520 10.1016/0739-7240(87)90011-7
521 Medzihradszky, K. F., Kaasik, K., and Chalkley, R. J. (2015). Tissue-specific
522 glycosylation at the glycopeptide level. *Mol. Cell. Proteomics* **14**, 2103-2110. doi:
523 10.1074/mcp.M115.050393
524 Miller, G. M., Alexander, J. M., and Klibanski, A. (1996). Gonadotropin-releasing
525 hormone messenger RNA expression in gonadotroph tumors and normal human

526 pituitary. *J. Clin. Endocrinol. Metab.* **81**, 80-83. doi: 10.1210/jcem.81.1.8550798
527 Miyamoto, Y., Skarzynski, D. J., and Okuda, K. (2000). Is tumor necrosis factor alpha a
528 trigger for the initiation of endometrial prostaglandin F(2alpha) release at luteolysis
529 in cattle? *Biol. Reprod*. **62**, 1109-1115. doi: 10.1095/biolreprod62.5.1109
530 Monniaux, D., Baril, G., Laine, A. L., Jarrier, P., Poulin, N., Cognié, J., and Fabre, S.
531 (2011). Anti-Müllerian hormone as a predictive endocrine marker for embryo
532 production in the goat. *Reproduction* **142**, 845-854. doi: 10.1530/REP-11-0211
533 Nett, T. M., Cermak, D., Braden, T., Manns, J., and Niswender, G. (1987). Pituitary
534 receptors for GnRH and estradiol, and pituitary content of gonadotropins in beef
535 cows. I. Changes during the estrous cycle. *Domest. Anim. Endocrinol.* **4**, 123-132.
536 doi: 10.1016/0739-7240(87)90006-3
537 Osoro, K., and Wright, I. A. (1992). The effect of body condition, live weight, breed, age,
538 calf performance, and calving date on reproductive performance of spring-calving
539 beef cows. *J. Anim. Sci*. **70**, 1661-1666. doi: 10.2527/1992.7061661x
540 Pagesy, P., Li, J. Y., Berthet, M., and Peillon, F. (1992). Evidence of gonadotropin-
541 releasing hormone mRNA in the rat anterior pituitary. *Mol. Endocrinol.* **6**, 523-528.
542 doi: 10.1210/mend.6.4.1374837
543 Pals, K., Roudbaraki, M., and Denef, C. (2008). Growth hormone-releasing hormone and

544 glucocorticoids determine the balance between luteinising hormone (LH) beta- and
545 LH beta/follicle-stimulating hormone beta-positive gonadotrophs and somatotrophs
546 in the 14-day-old rat pituitary tissue in aggregate cell culture. *J. Neuroendocrinol.*
547 **20**, 535-548. doi: 10.1111/j.1365-2826.2008.01698.x
548 Pandey, K., Kereilwe, O., Borromeo, V., and Kadokawa, H. (2017a). Heifers express G-
549 protein coupled receptor 61 in anterior pituitary gonadotrophs in stage-dependent
550 manner. *Anim. Reprod. Sci.* **181**, 93-102. doi: 10.1016/j.anireprosci.2017.03.020
551 Pandey, K., Kereilwe, O., and Kadokawa, H. (2017b). Heifers express G-protein coupled
552 receptor 153 in anterior pituitary gonadotrophs in stage-dependent manner. *Anim.*
553 *Sci. J.* **89**, 60-71. doi: 10.1111/asj.12920
554 Pandey, K., Mizukami, Y., Watanabe, K., Sakaguti, S., and Kadokawa, H. (2017c). Deep
555 sequencing of the transcriptome in the anterior pituitary of heifers before and after
556 ovulation. *J. Vet. Med. Sci.* **79**, 1003-1012. doi: 10.1292/jvms.16-0531
557 Pfeiffer, K. E., Jury, L. J., and Larson, J. E. (2014). Determination of anti-Müllerian
558 hormone at estrus during a synchronized and a natural bovine estrous cycle. *Domest.*
559 *Anim. Endocrinol.* **46**, 58-64. doi: 10.1016/j.domaniend.2013.05.004
560 Pierre, A., Racine, C., Rey, R. A., Fanchin, R., Taieb, J., Cohen-Tannoudji, J., Carmillo,
561 P., Pepinsky, R. B., Cate, R. L., and di Clemente, N. (2016). Most cleaved anti-

562 Müllerian hormone binds its receptor in human follicular fluid but little is competent
563 in serum. *J. Clin. Endocrinol. Metab.* **101**, 4618-4627. doi: 10.1210/jc.2016-1742
564 Pinto, P. H. N., Balaro M. F. A., Souza-Fabjan, J. M. G., Ribeiro, L. D. S., Bragança, G.
565 M., Leite, C. R., Arashiro, E. K. N., de Moraes, S. K., Da Fonseca, J. F., and Brandão,
566 F. Z. (2018). Anti-Müllerian hormone and antral follicle count are more effective for
567 selecting ewes with good potential for in vivo embryo production than the presence
568 of FecGE mutation or eCG pre-selection tests. *Theriogenology* **113**, 146-152. doi:
569 10.1016/j.theriogenology.2018.02.018.
570 Poonlaphdecha, S., Pepey, E., Huang, S. H., Canonne, M., Soler, L., Mortaji, S., Morand,
571 S., Pfennig, F., Melard, C., Baroiller, J. F., and D'Cotta, H. (2011). Elevated AMH
572 gene expression in the brain of male tilapia (*Oreochromis niloticus*) during Testis
573 Differentiation. *Sex. Dev.* **5**, 33-47. doi: 10.1159/000322579
574 Popovics, P., Rekasi, Z., Stewart, A. J., and Kovacs, M. (2011). Regulation of pituitary
575 inhibin/activin subunits and follistatin gene expression by GnRH in female rats. *J.*
576 *Endocrinol.* **210**, 71-79. doi: 10.1530/JOE-10-0485
577 Rekawiecki, R., Rutkowska, J., and Kotwica, J. (2012). Identification of optimal
578 housekeeping genes for examination of gene expression in bovine corpus luteum.
579 *Reprod. Biol.* **12**, 362–367. doi: 10.1016/j.repbio.2012.10.010

580 Ribeiro, E. S., Bisinotto, R. S., Lima, F. S., Greco, L. F., Morrison, A., Kumar, A.,
581 Thatcher, W. W., and Santos, J. E. (2014). Plasma anti-Müllerian hormone in adult
582 dairy cows and associations with fertility. *J. Dairy Sci.* **97**, 6888-6900. doi:
583 10.3168/jds.2014-7908
584 Rocha, R. M., Lima, L. F., Carvalho, A. A., Chaves, R. N., Bernuci, M. P., Rosa-e-Silva,
585 A. C., Rodrigues, A. P., Campello, C. C., and Figueiredo, J. R. (2016).
586 Immunolocalization of the anti-Müllerian hormone (AMH) in caprine follicles and
587 the effects of AMH on in vitro culture of caprine pre-antral follicles enclosed in
588 ovarian tissue. *Reprod. Domest. Anim*. **51**, 212-219. doi: 10.1111/rda.12668
589 Rudolf, F.O., and Kadokawa, H. (2014). Effects of STX, a novel estrogen membrane
590 receptor agonist, on GnRH-induced luteinizing hormone secretion from cultured
591 bovine anterior pituitary cells. *J. Vet. Med. Sci.* **76**, 1623–1625. doi:
592 10.1292/jvms.14-0179
593 Seifer, D. B., and Merhi, Z. (2014). Is AMH a regulator of follicular atresia? *Assist.*
594 *Reprod. Genet.* **31**, 1403-1407. doi: 10.1007/s10815-014-0328-7
595 Sheldon, M., and Dobson, H. (2004). Postpartum uterine health in cattle. *Anim. Reprod.*
596 *Sci.* **82-83**, 295-306. doi.org/10.1016/j.anireprosci.2004.04.006
597 Skaar, K. S., Nobrega, R. H., Magaraki, A., Olsen, L. C., Schulz, R. W., and Male, R.

598 (2011). Proteolytically activated, recombinant anti Mullerian hormone inhibits
599 androgen secretion, proliferation, and differentiation of spermatogonia in adult
600 zebrafish testis organ cultures. *Endocrinology* **152**, 3527-3540. doi:
601 10.1210/en.2010-1469
602 Townsend, J., Sneddon, C. L., and Tortonese, D. J. (2004). Gonadotroph heterogeneity,
603 density and distribution, and gonadotroph-lactotroph associations in the pars distalis
604 of the male equine pituitary gland. *J. Neuroendocrinol.* **16**, 432-440. doi:
605 10.1111/j.1365-2826.2004.01174.x
606 Walker, C.G., Meier, S., Mitchell, M.D., Roche, J.R., and Littlejohn, M. (2009).
607 Evaluation of real-time PCR endogenous control genes for analysis of gene
608 expression in bovine endometrium. *BMC Mol. Biol.* **10**, 100. doi: 10.1186/1471-
609 2199-10-100
610

611 Table 1. Name, accession number, and details of the primers used for real-time PCRs

Gene name	Accession number	Primer	Sequence 5'-3'	Size (bp)
GAPDH	NM_001034034	Forward	TGGTGAAGGTCGGAGTGAAC	91
		Reverse	ATGGCGACGATGTCCACTTT	
RANBP10	NM_001098125	Forward	CCCAGTCCTACCAGCCTACT	133
		Reverse	CCCCCAGAGTTGAATGACCC	
AMH	NM_173890	Forward	GGGTTAGCCCTTACCCTGC	121
	Exon 3 4	Reverse	GTAACAGGGCTGGGGTCTTT	

612

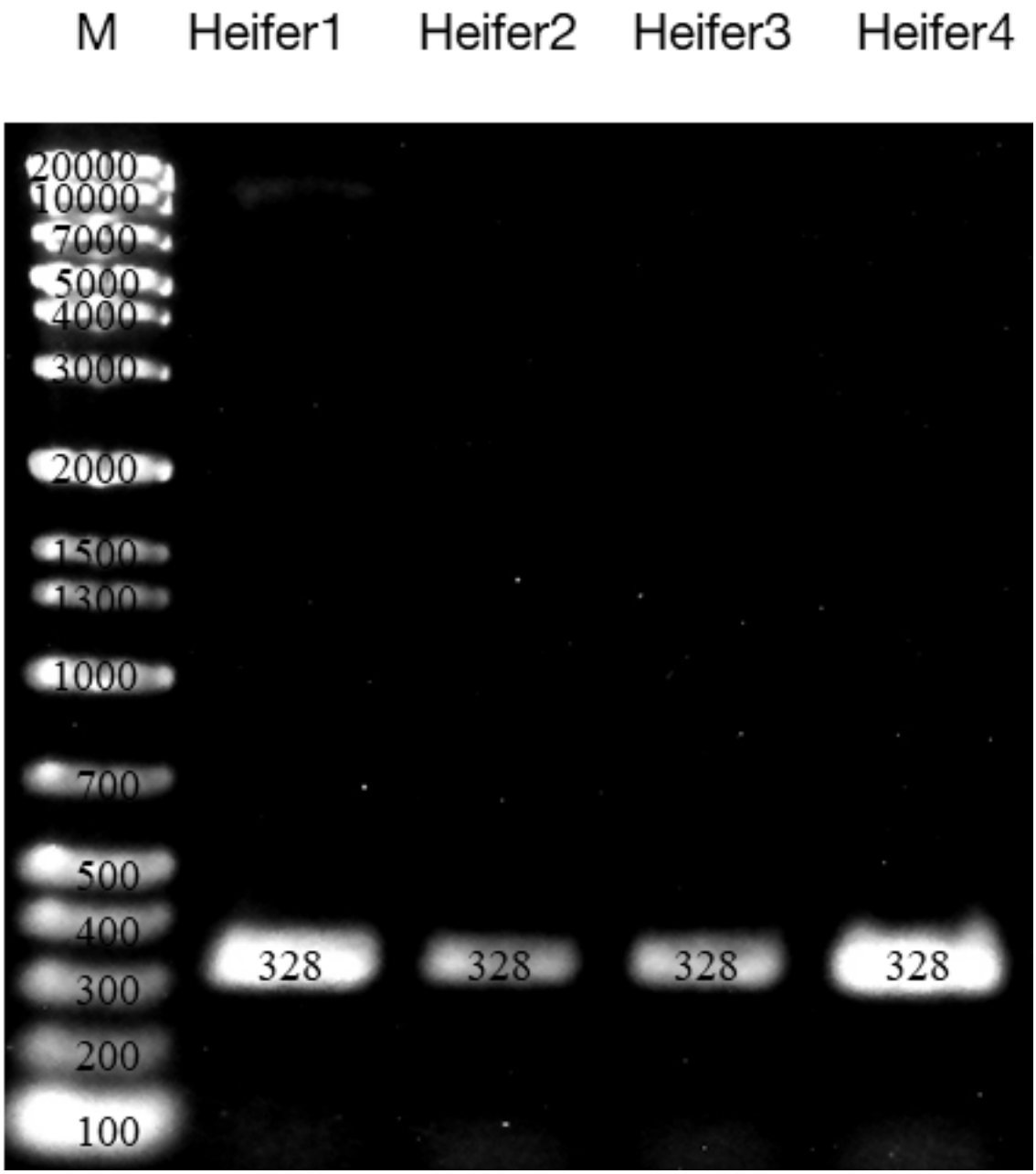


Fig. 1. Expression of anti-Müllerian hormone (*AMH*) mRNA, as detected by RT-PCR. Electrophoresis of PCR-amplified DNA products using the primer pair for bovine *AMH* and cDNA derived from the anterior pituitary (AP) of post-pubertal heifers. The lanes labeled Heifers 1 to 4 demonstrate that the DNA products were of the expected size, i.e., 328 bp. Lane M showed the band sizes of the DNA marker.

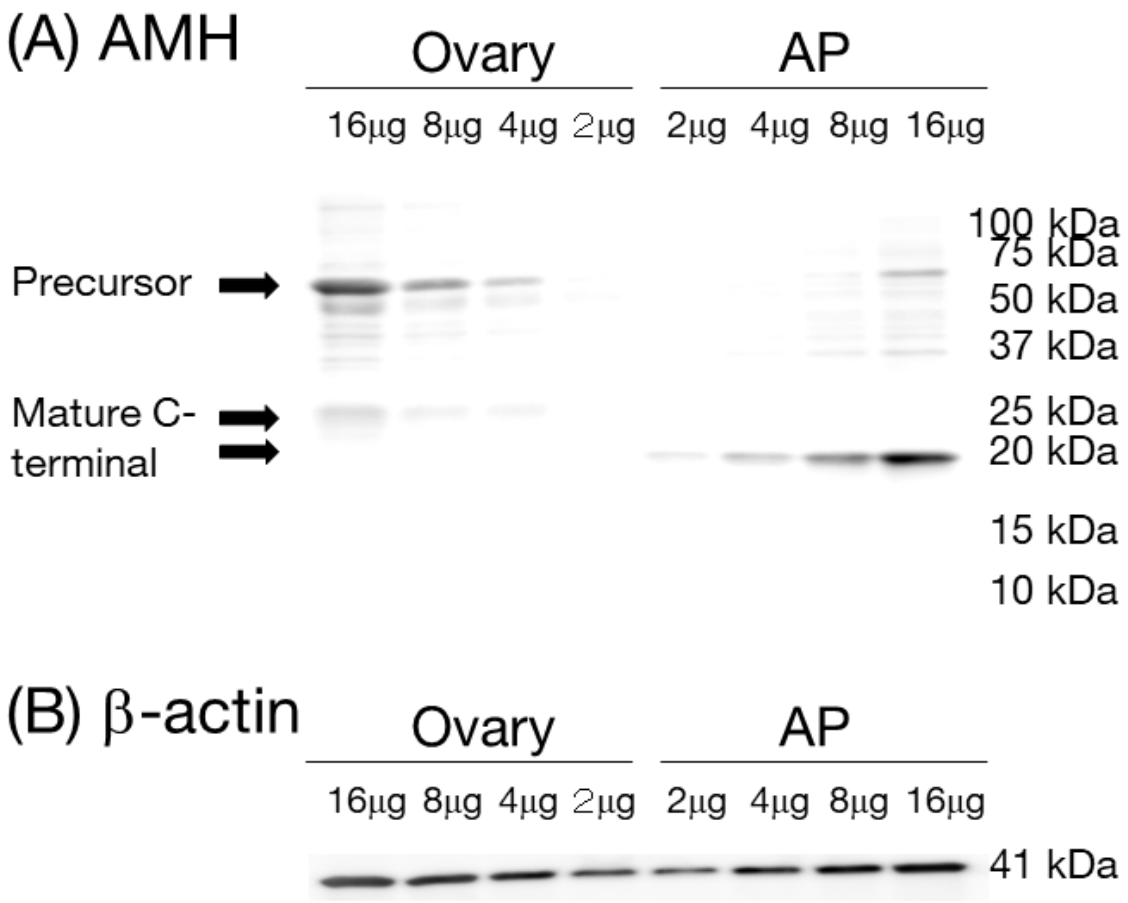


Fig. 2. Western blotting results using extracts (2, 4, 8, or 16 μg of total protein) from the APs or ovaries of post-pubertal heifers and an anti-AMH rabbit antibody (A) or anti-β–actin mouse antibody (B).

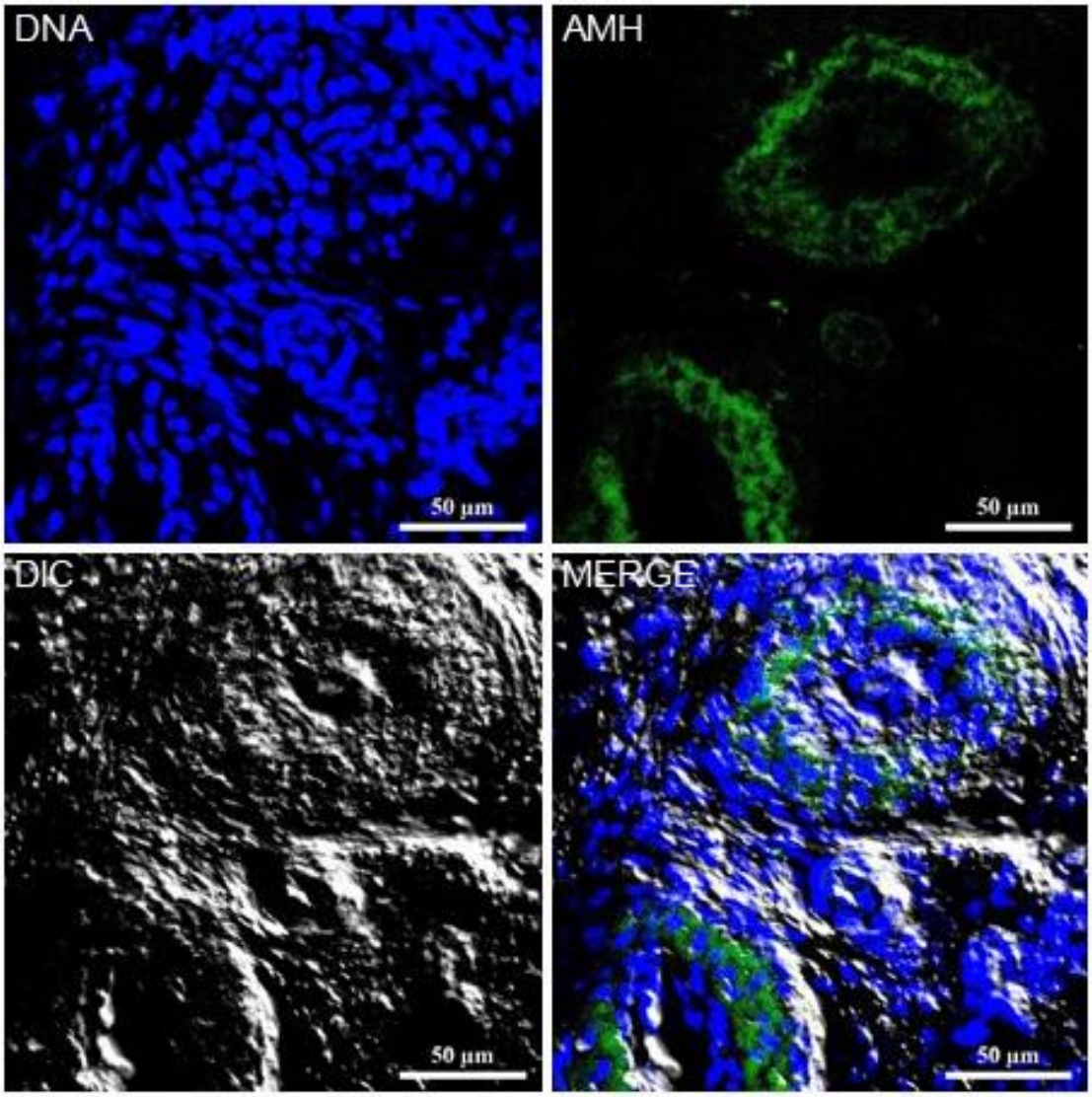
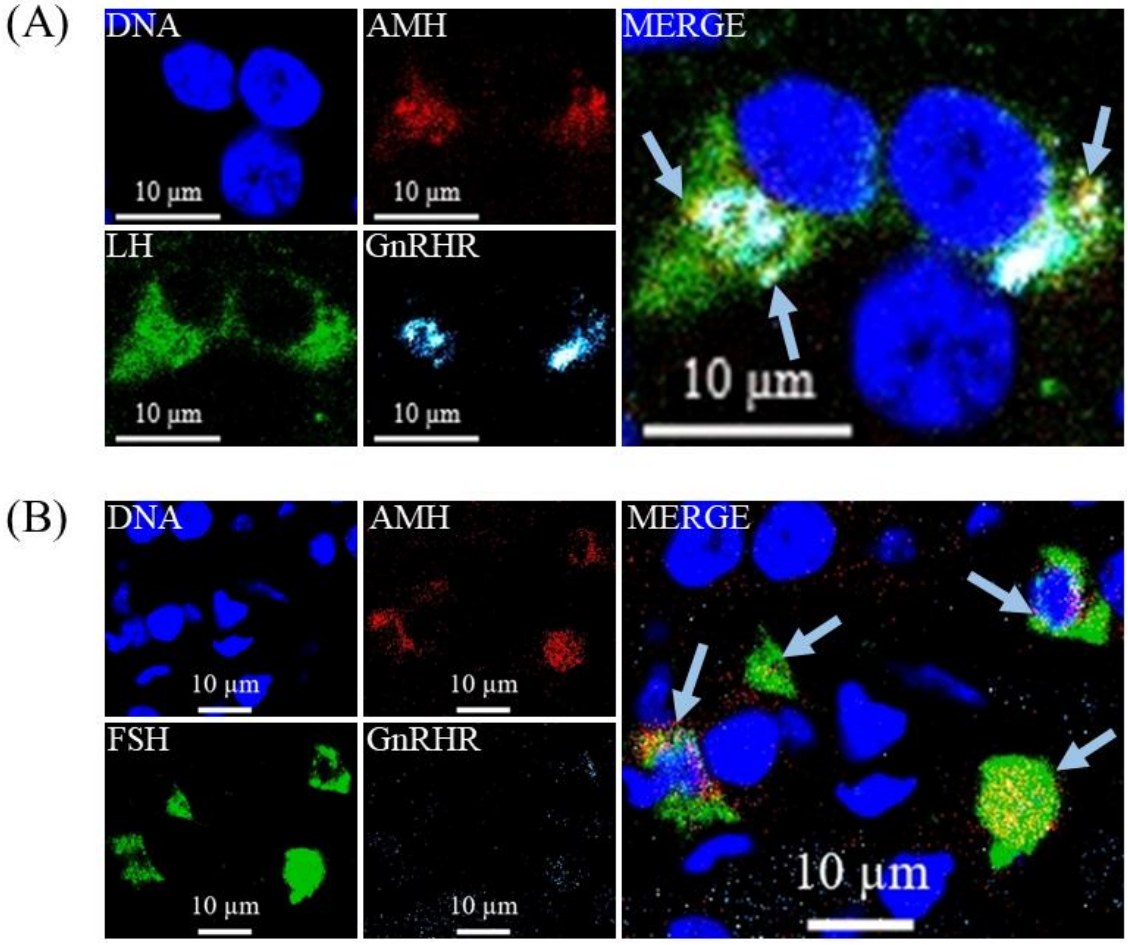


Fig. 3. Fluorescence immunohistochemical analysis of AMH on small antral follicles in ovaries of post-pubertal heifers. Images were captured by laser confocal microscopy for AMHR2 (green) with counter-staining by DAPI (dark blue, indicating DNA) and differential interference contrast (DIC). Scale bars represent 50 µm.



641

642

643 **Fig. 4.** Triple-fluorescence immunohistochemistry of AP tissues in post-pubertal heifers

644 for the detection of gonadotropin-releasing hormone receptor (GnRHR), AMH, and LH

645 (A) or FSH (B). Images were captured by laser confocal microscopy for GnRHR (cyan),

646 AMH (red), and LH or FSH (green) with counter-staining by DAPI for DNA (dark blue).

647 Yellow indicates the colocalization of AMH and LH/FSH, indicated by blue arrows. Scale

648 bars represent 10 μm.

649

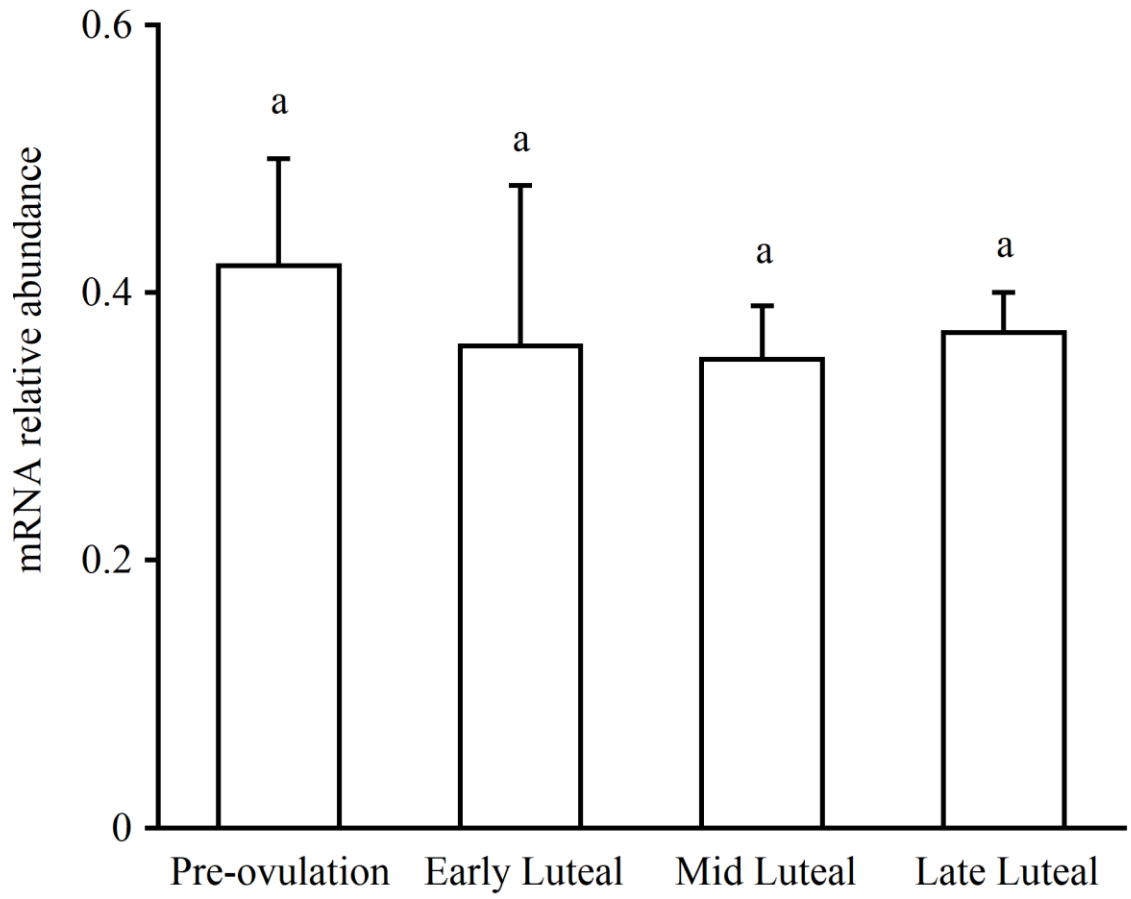


Fig. 5. Relative *AMH* mRNA levels (mean ± SEM) in bovine APs during pre-ovulation [day 19 to 21 (day 0 = day of estrus)], early luteal (day 2 to 5), mid-luteal (day 8 to 12), or late luteal (day 15 to 17) phases, as determined by real-time PCR. Data were normalized to the geometric means of *GAPDH* and RAN-binding protein (*RANBP10*) levels. The same letters indicate no significant differences (P>0.05) across phases.

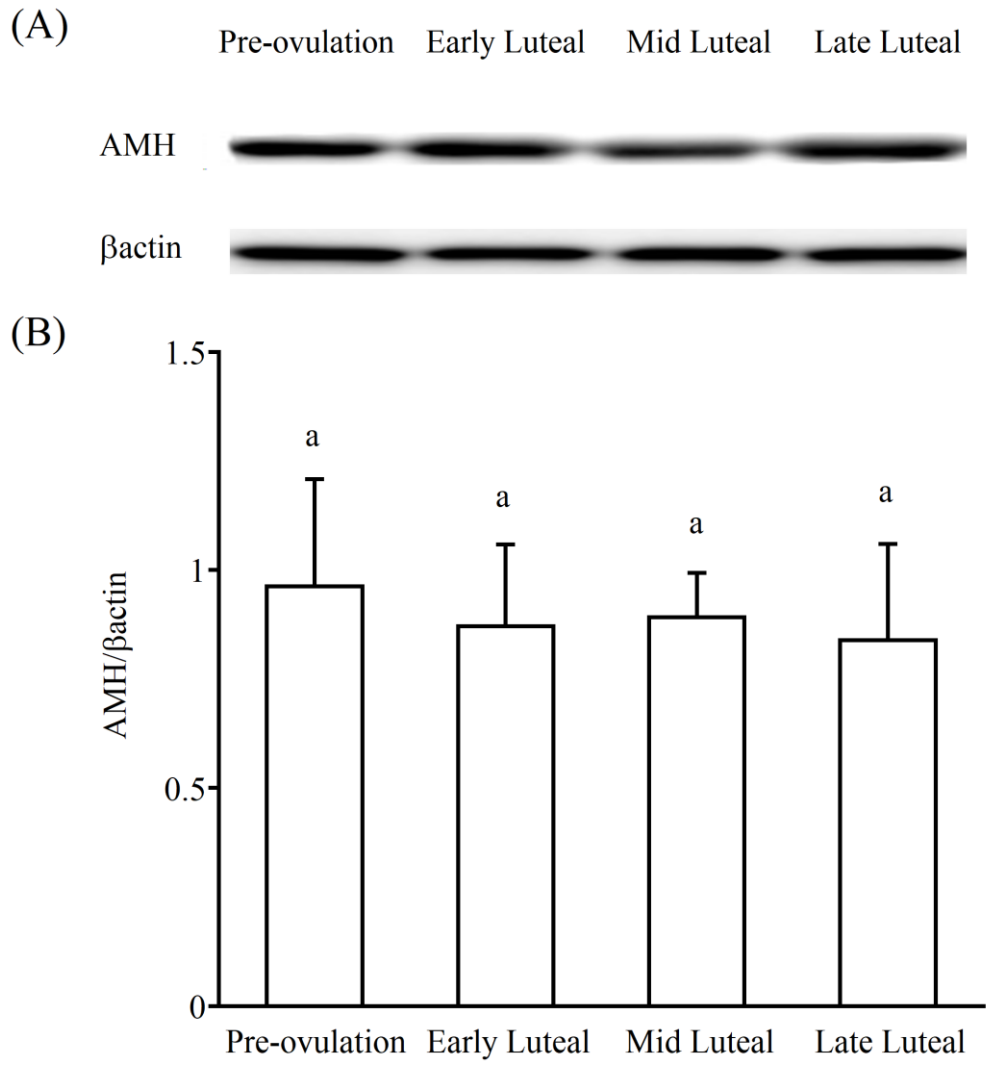
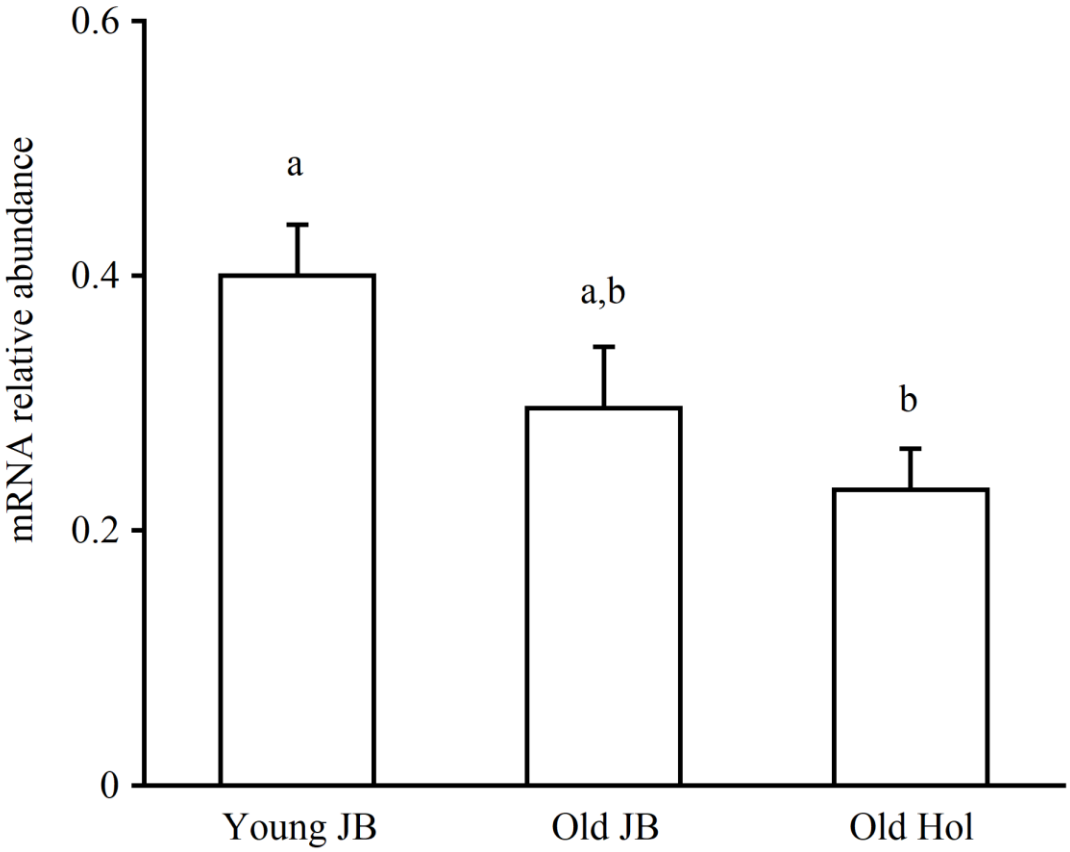


Fig. 6. (A) Representative AMH (as mature C-terminal form) and β-actin protein expression in bovine AP tissues obtained during the pre-ovulation, early luteal, mid luteal, or late luteal phases of estrous, as detected by western blotting. (B) AMH protein expression level of AMH normalized to that of β-actin in healthy post-pubertal heifer AP tissues obtained during pre-ovulation (n =5), early luteal (n =5), mid-luteal (n =6), and late luteal (n = 5) phases. The same letters indicate no significant differences ($P > 0.05$) across phases.



669

670

671

672

673 **Fig. 7.** Relative *AMH* mRNA levels (mean ± SEM) in bovine AP tissues obtained from

674 healthy young Japanese heifers (young JB), old Japanese black cows (old JB), and old

675 Holsteins cows (old Hol), as determined by real-time PCR. Data were normalized to the

676 geometric means of *GAPDH* and *RANBP10* levels. Letters (a vs. b) indicate significant

677 differences ($P < 0.05$) among groups.

678

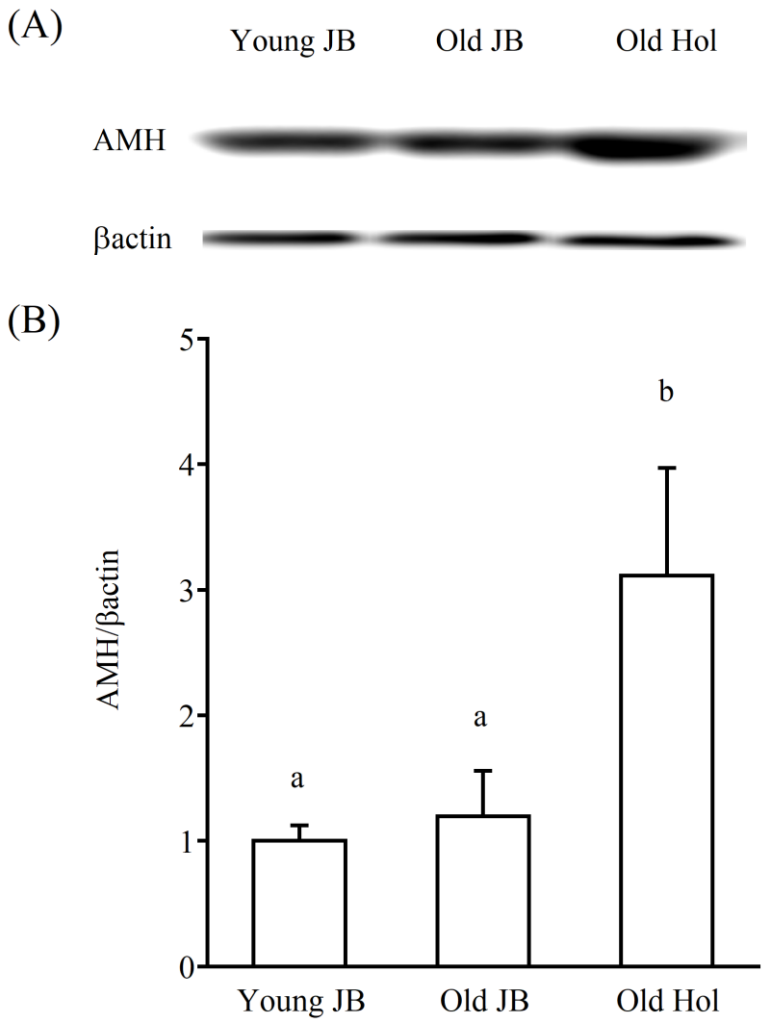


Fig. 8. (A) Representative AMH (in the mature C-terminal form) and β-actin protein expression in bovine AP tissues obtained from young JB, old JB, and old Hol. (B) Protein expression level of AMH normalized to that of β-actin in bovine AP tissues obtained from young JB, old JB, and old Hol. Letters (a vs. b) indicate significant differences ($P < 0.05$) between groups.