In vivo ovarian temperature promotes the *in vitro* growth and developmental competence of oocytes derived from bovine early antral follicles

Kohei Kawano[a], Kenichiro Sakaguchi[b,c] *,Yojiro Yanagawa[d], Seiji Katagiri[d]

Author affiliations

[a] Laboratory of Reproductive Physiology, Faculty of Environmental, Life, Natural Science and Technology, Okayama University, Okayama 700-8530, Japan

[b] Laboratory of Veterinary Theriogenology, Joint Department of Veterinary Medicine, Faculty of Applied Biological Sciences, Gifu University, Yanagito 1-1, Gifu 501-1193, Japan

[c] Division of Animal Medical Science, Center for One Medicine Innovative Translational Research (COMIT), Institute for Advanced Study, Gifu University, Yanagido 1-1, Gifu 501-1193, Japan

[d] Laboratory of Theriogenology, Department of Clinical Sciences, Division of Veterinary Medicine, Faculty of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan

*Corresponding author

Email address: sakaguchi.kenichiro.x3@f.gifu-u.ac.jp (K. Sakaguchi).

Abstract

In cattle, the culture temperature used for the *in vitro* growth (IVG) of immature oocytes is generally 38.5 or 39.0℃, which is close to the normal temperature in the vagina or rectum. However, the temperature in the *in vivo* ovarian tissue is approximately 1℃ lower (37.5℃) than that in the vagina or rectum. Therefore, the generally accepted culture temperature may not be optimal for the IVG of bovine oocytes. Herein, we investigated the effects of culture temperature on the IVG of oocyte-cumulus granulosa complexes (OCGCs) derived from early antral follicles (0.5–1 mm in diameter). OCGCs were subjected to 12 days of IVG at temperatures of 37.5, 38.5, and 39.0℃. OCGC viability and antrum formation were evaluated every 4 days. Estradiol-17β (E_2) and progesterone (P_4) production from OCGCs during the 1st, 2nd, and 3rd 4-day periods was evaluated by enzyme immunoassay. Viable OCGCs after IVG were subjected to *in vitro* maturation (IVM), *in vitro* fertilization, and embryo culture. Then, the nuclear status and diameter of oocytes after IVM, rates of cleavage and blastocysts, and cell number in blastocysts were evaluated. In addition, the mRNA expression of heat shock proteins (HSPs) in the granulosa cells and reduced glutathione (GSH) levels in oocytes after IVG were measured. The viability of OCGCs did not differ among the groups, whereas the rate of antrum formation on day 12 of IVG culture was highest in the 37.5℃ group ($P < 0.05$). P_4 production did not differ among the groups; however, E_2 production during days 8–12 tended to be higher in the 37.5℃ group than in the other two groups combined ($P < 0.1$). The mRNA expression of HSP70 and 90, and the GSH levels of oocytes, did not differ among the groups. The oocyte diameter after culture was larger in the 37.5℃ group than in the 39.0℃ group ($P < 0.05$), and that in the 38.5℃ group was intermediate between the other two groups. The rates of nuclear maturation and cleavage did not differ among the groups. However, the blastocyst rate was higher in the 37.5 and 38.5℃ groups than in the 39.0℃ group ($P < 0.05$). The cell number in the blastocysts in the 38.5℃ group was smaller than

the *in vivo*-grown oocytes, while that in the 37.5℃ group and the *in vivo*-grown oocytes did not differ. In summary, OCGCs in the 37.5℃ group showed healthy morphology and steroidogenesis, as well as better growth and developmental competence of oocytes. Therefore, culture conditions close to the *in vivo* ovarian tissue temperature would be optimal for the IVG of immature bovine oocytes.

(2544 / 2500 keystrokes (characters plus spaces))

Keywords: Bovine, Early antral follicle, *In vitro* growth, Temperature condition

1. Introduction

Temperature is a fundamental and critical factor for cell culture, and the optimal culture temperature depends on the type of cells. Optimal temperatures have been investigated in the conventional *in vitro* production (IVP) system for bovine embryos, which consists of *in vitro* maturation (IVM), fertilization (IVF), and culture (IVC). The nuclear maturation rate has been reported to be higher at culture temperatures between 37.0 and 39.0℃ than that between 33.0 and 35.0℃ during IVM [1], and the rate of oocytes developing to blastocysts did not differ between 37.0 and 38.5℃ during IVM [2]. In IVF, the rate of sperm penetration to oocytes was higher at 39.0℃ than at 35.0 or 37.0℃ [3]. In addition, it has been reported that the blastocyst rate was the highest at 39.0℃ during IVC in the temperature ranges between 36.0 and 39.0℃ [4]. Taken together, the temperature conditions of 38.5 or 39.0℃, which are close to the normal rectal or vaginal temperature [5,6], have been generally used for the conventional IVP system in cattle.

However, several studies have indicated that the *in vivo* ovarian temperature is lower than the rectal or vaginal temperatures. Using an infrared camera, it was found that the temperature of the ovarian surface (37.6℃) was approximately 1.0℃ lower than that of the rectum (38.7℃), and the temperature of the preovulatory follicle (36.1℃) was 1.5℃ lower than that of the ovarian surface [7]. Recently, the ovarian tissue temperature was directly measured by inserting a thermometer [8,9], and these reports also indicated that the temperatures of both the preovulatory follicle (37.4℃) and the ovarian parenchyma (37.6℃) were approximately 1℃ lower than those of the rectum (38.5℃) and vagina (38.6℃). Therefore, bovine oocyte growth and maturation *in vivo* is expected to occur at approximately 37.5℃, which is 1℃ lower than the normal rectal and vaginal temperatures.

In cattle, early antral follicles (0.4–1 mm in diameter) are the smallest follicles capable of producing calf using *in vitro* cultured oocytes [10-12]. After two weeks of *in vitro* growth (IVG)

78 culture as oocyte-cumulus-granulosa complexes (OCGCs), oocytes from early antral follicles
79 acquire maturational and developmental competence and can be subjected to conventional IVP
80 [11,13]. Generally, the culture temperature for bovine IVG is 38.5 or 39.0°C, which is also used
81 for IVM, IVF, and IVC [11,14,15]. Given that the temperature of ovarian tissue measured *in viv*o
82 is approximately 37.5°C and the period of the IVG culture system is approximately two weeks,
83 which is relatively long compared with conventional IVP, lowering the temperature condition to
84 37.5°C during IVG may positively affect the growth and developmental competence of oocytes.
85 Estradiol-17β (E_2) concentrations in follicular fluids of healthy growing follicles
86 elevates as the follicles grew, and the degeneration of follicles led to increases in progesterone
87 (P_4) concentrations [16]. In *in vitro*, E_2 supplementation to IVG culture medium improved the
88 growth and maturational competence of oocytes derived from early antral follicles [17], and
89 OCGCs that produce higher E_2 showed higher growth and developmental competence [18]. Thus,
90 proper steroidogenesis is important for healthy follicular growth and the acquisition of
91 developmental competence of oocytes. On the other hand, we previously reported that
92 physiologically high temperatures (38.5°C: 5 h, 39.5°C: 5 h, 40.5°C: 5 h, and 39.5°C: 9 h) during
93 IVG impairs the developmental competence of oocytes through reduced glutathione (GSH) levels
94 [19]. Therefore, our interest is also whether *in vivo* ovarian temperature affects the GSH levels of
95 oocytes, and expressions of other heat stress makers including heat shock proteins (HSPs).
96 In the present study, we investigated the effects of *in vivo* ovarian temperature on the *in*
97 *vitro* growth and developmental competence of oocytes derived from early antral follicles. We
98 evaluated the effects of *in vivo* ovarian temperature on the morphology of OCGCs, nuclear status
99 and diameter of oocytes after IVM, and developmental competence after IVF. In addition, we also
100 examined steroidogenesis, gene expression of HSPs in the granulosa cells, and the GSH levels of
101 oocytes.

2. Materials and Methods

2.1. Reagents

All the reagents used were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated.

2.2. Collection of oocyte-cumulus-granulosa complexes (OCGCs) and the IVG culture

Ethical approval for animal work was not necessary for this study because all the bovine ovaries were derived from cattle slaughtered at a local slaughterhouse for commercial food production purposes only.

OCGCs derived from early antral follicles (0.5–1 mm in diameter) were subjected to IVG culture, as previously described [19]. Briefly, abattoir-derived ovaries were thinly sliced using a surgical blade (No. 11), and the ovarian cortical tissues were kept in the isolation media [20]. The diameter of follicles was judged using the grid lines on the 90-mm petri dish with 1-mm scales (FLAT Co., Ltd., Chiba, Japan). Then, early antral follicles were manually collected from the ovarian cortexes using fine forceps and a surgical blade (No. 20). Follicles were punctured to obtain OCGCs using fine forceps and an 18G needle. The growth medium was HEPES (25 mM)-buffered TCM-199 (12340-030, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 0.91 mM sodium pyruvate, 5% (v/v) fetal calf serum (FCS; Invitrogen, Waltham, MA, USA), 4 mM hypoxanthine, 4% (w/v) polyvinylpyrrolidone (PVP) (MW 360,000), 50 μg/mL gentamicin sulfate, and 10 ng/mL androstenedione as a precursor for E_2 [21]. Morphologically healthy OCGCs (Fig. 1a) were individually cultured in a 96-well culture plate (Primaria 353872, Corning Incorporated, Corning, NY, USA) with 200 μL of growth medium for 12 days in humidified air with 5% CO_2. OCGCs were cultured at 37.5, 38.5, or 39.0°C (Fig. 2).

126 The temperature condition of 37.5°C is based on the ovarian tissue temperature of cattle [9],
127 whereas 38.5 and 39.0°C are based on the rectal temperatures observed in cattle and often used
128 for bovine IVG [19,22]. Half of the spent IVG medium (100 μL) was collected and replaced with
129 the same volumes of fresh media every four days of culture (days 4, 8, and 12), and the samples
130 were stored at -30°C for subsequent measurements of the steroid hormones.
131
132 *2.3. Evaluation of the OCGC morphology*
133 The morphological appearance of the OCGCs was evaluated every four days during
134 IVG culture using an inverted microscope (CK 40, Olympus, Tokyo, Japan). OCGCs with an
135 evenly granulated ooplasm that were completely enclosed by several layers of healthy cumulus
136 and granulosa cells with or without antrum (Fig. 1b, c), which is an indicator of the healthy
137 development of OCGCs [14,23], were defined as normal. OCGCs with scattered cumulus and
138 granulosa cells or denuded oocytes were defined as abnormal (Fig. 1d).
139
140 *2.4. E_2 and P_4 assays of the IVG culture medium*
141 Spent IVG medium was assayed to determine the E_2 and P_4 concentrations using a
142 competitive double-antibody enzyme immunoassay as previously described [18]. Spent IVG
143 medium was subjected to 20- to 600-fold serial dilutions with assay buffer (145 mM NaCl, 40 mM
144 Na_2HPO_4, and 0.1% bovine serum albumin (BSA) (w/v), pH7.2). After dilution, samples (20 μL)
145 were incubated with 100 μL (E_2 and P_4) of the primary antisera and horseradish peroxidase-
146 labeled hormone in the wells of a 96-well microplate (Costar 3590; Corning, NY, USA) coated
147 with the secondary antiserum at 4°C for 16–18 h. The primary antisera used for the E_2 and P_4
148 assays were anti-estradiol-17β-6-carboxymethyloxime (CMO)-BSA (FKA204; Cosmo Bio,
149 Tokyo, Japan) and anti-progesterone-3-CMO-BSA (KZ-HS-P13; Cosmo Bio), respectively. Goat

150 anti-rabbit serum (111–005-003; Jackson Immuno Research, West Grave, PA, USA) was used as
151 the secondary antiserum. After the washing of all wells four times with 300 μL of washing buffer
152 (0.05%Tween 80), 150 μL of 3,3′,5,5′-tetramethylbenzidine (TMB) solution (5 mM citric acid, 50
153 mM Na_2HPO_4, 500 mM urea hydrogen peroxide, 1 mM TMB, and 2% dimethyl sulfoxide) was
154 added to each well and incubated at 37°C for 40 min. The absorbance of the solution in the wells
155 was measured at 450 nm using a microplate reader (Model 550; Bio-Rad Laboratories, Tokyo,
156 Japan) after stopping the chromogenic reaction with 50 μL of $4NH_2SO_4$. All samples were assayed
157 in triplicate. The assay sensitivities were 7.1 pg/well for E_2 and 11.2 pg/well for P_4. The inter- and
158 intra-coefficients of variation were 5.6% and 1.7% for E_2 and 3.4% and 4.2% for P_4, respectively.
159 The steroid hormone production during each period (days 0–4, 4–8, and 8–12) was calculated as
160 previously described [23].
161
162 *2.5. Detection of GSH in oocytes*
163 The GSH levels in oocytes were assayed as previously described [19]. Briefly, denuded
164 oocytes were washed with Dulbecco's PBS (DPBS) (Nissui Pharmaceutical CO., LTD. Tokyo,
165 Japan) supplemented with 0.2% PVP (DPBS-PVP) and treated with 10 μM CellTracker Blue
166 (Thermo Fisher Scientific) for GSH staining at 38.5℃ for 30 min. The denuded oocytes were
167 then washed three times with 0.2% DPBS-PVP, and placed on a glass slide. Images of the oocytes
168 were acquired using a digital fluorescence microscope (BZ-9000; Keyence, Osaka, Japan) with
169 identical parameter settings for all experiments. The fluorescence intensity (arbitrary units) was
170 calculated using Image J software.
171
172 *2.6. Real-time PCR*
173 Granulosa cells used for the real-time PCR were collected from OCGCs after 12 days

174 of IVG culture. The remaining COCs derived from same OCGCs were subjected to IVM to
175 evaluate the nuclear status and diameter of oocytes. Total RNA was isolated using a ReliaPrep
176 RNA Cell Miniprep System (Promega Corporation, Madison, WI, USA), following the
177 manufacturer's instructions. Samples were stored at -80℃, and RNA extraction and cDNA
178 synthesis were performed within 1 month from the start of storage. RNA concentration and purity
179 were determined using a NanoDrop spectrophotometer (NanoDrop 2000; Thermo Fisher
180 Scientific). The purified total RNA (10 ng/μL) was treated as a template to synthesize cDNA using
181 the ReverTra Ace qPCR RT Master Mix (Toyobo Co., Ltd., Osaka, Japan). The cDNA samples
182 were stored at -30℃ until PCR analysis.
183 Real-time PCR was performed after preparing reaction mixtures in the
184 THUNDERBIRD SYBR qPCR Mix (Toyobo Co., Ltd., Osaka, Japan). The primers were
185 designed using Primer3 software based on the bovine sequences or adopted from a previous study
186 [24]. The reaction efficiency ranged between 90% and 110% with $R^2 > 0.993$. The amplification
187 program included preincubation at 95℃ for 1 min, followed by 40 amplification cycles of
188 denaturation at 95℃ for 15 s, annealing at 60℃ for 30 s, and elongation at 72℃ for 30 s. All
189 groups were evaluated in triplicate in 48-well plates. Melting-curve analysis was performed at the
190 end of the amplification to confirm single-gene specificity. Relative mRNA abundance was
191 calculated using the ΔΔCt method, with β-actin as the reference gene in each sample. All primer
192 sequences, PCR product sizes, and GeneBank accession numbers are shown in Table 1.
193
194 *2.7. Evaluation of the growth and nuclear maturation of in vitro-grown oocytes*
195 After IVG culture, OCGCs were subjected to IVM as previously described [25]. Briefly,
196 OCGCs were individually cultured in microwell plates (Mini Trays 163 118; NUNC, Roskilde,
197 Denmark) containing 6 mL of IVM medium at 38.5℃ under 5% CO_2 in air for 22 h. The IVM

198 medium consisted of HEPES-buffered TCM-199 supplemented with 0.2 mM sodium pyruvate,
199 20 μg/mL FSH (from porcine pituitary), 1 μg/mL E_2, 10% FCS, and 50 μg/mL gentamicin sulfate.
200 After IVM, the oocytes were denuded by pipetting, and then photographed, and their diameters
201 were measured. Oocyte nuclear maturation was assessed using 1% (w/v) aceto-orcein staining, as
202 previously described [26]. Oocytes with a polar body were defined as mature.
203
204 *2.8. Evaluation of developmental competence of in vitro-grown oocytes*
205 After IVM, some OCGCs were subjected to IVF as previously described [19]. Briefly,
206 after thawing frozen semen from a Holstein bull in a 37°C water bath for 40 s, motile sperm were
207 separated by centrifuging twice (300×g) for 5 min in a semen preparation medium (BO-
208 SemenPrep; IVF Bioscience, Cornwall, UK). OCGCs were cultured in a group with separated
209 sperm in 500 μL of fertilization medium (BO-IVF; IVF Bioscience) in 4-well dishes (11–17
210 zygotes/well, 2×10^6 sperm/mL) for 18 h at 38.5℃ in 5% CO_2 in air. After IVF, the presumptive
211 zygotes were cultured in a 30 μL droplet (10–16 zygotes/droplet) of culture medium (BO-IVC;
212 IVF Bioscience) with an overlay of paraffin oil at 5% O_2, 5% CO_2, and 90% N_2 at 39℃ for 150
213 h. The cleavage and blastocyst rates were examined at 48 ± 2 and 168 ± 2 h after IVF, respectively.
214 Cell numbers in the blastocysts were counted as previously described [27].
215
216 *2.9. IVM/IVF/IVC of in vivo-grown oocytes*
217 *In vivo*-grown oocytes were collected from antral follicles (2-8 mm in diameter) by
218 aspirating follicular fluid with a syringe equipped with an 18G needle. *In vivo*-grown oocytes
219 were cultured in droplets (approximately 10 COCs/50 μL) covered with paraffin oil at 38.5℃ in
220 5% CO_2 in air for 22-24 h. The IVM medium was same as the *in vitro*-grown oocytes described
221 above. After IVM, COCs were subjected to IVF and IVC. Frozen-thawed motile sperm were

222 separated by centrifuging twice in a 90% Percoll for 10 min (760×g), and in a semen preparation
223 medium (BO-SemenPrep) for 5 min (550×g). Approximately 10 COCs were incubated in a 100
224 μL droplet (2×10^6 sperm/mL) of IVF medium (BO-IVF) covered with paraffin oil for 18-20 h at
225 38.5℃ in 5% CO_2 in air. After IVF, the presumptive zygotes were cultured in a 30 μL droplet
226 (12–17 zygotes/droplet) of culture medium (BO-IVC) with an overlay of paraffin oil at 5% O_2,
227 5% CO_2, and 90% N_2 at 39℃ for 150 h. The cleavage and blastocyst rates, and the cell number
228 in blastocysts were evaluated at the same timing and in the same methods as the *in vitro*-grown
229 oocytes.
230
231 *2.10. Experimental design*
232 A schematic drawing of the experimental design is shown in Fig. 2. In each experiment,
233 the OCGCs were evenly distributed among each group (37.5, 38.5, and 39.0°C groups) according
234 to the oocyte diameter and the thickness of the mural granulosa cell layer. However, in an
235 experimental session that was performed to evaluate the developmental competence of IVG
236 oocytes, the OCGCs were distributed only between the 37.5 and 38.5°C groups because the
237 number of collected OCGCs was insufficient for three groups.
238 A total of 524 OCGCs were cultured for 12 days, with the following sample distribution:
239 182 OCGCs in the 37.5°C group (10 culture replicates), 181 OCGCs in the 38.5°C group (10
240 culture replicates), and 161 OCGCs in the 39.0°C group (9 culture replicates). The percentage of
241 antrum formation in the granulosa cell layer was calculated based on the 343 OCGCs that survived
242 until day 12 (120 from the 37.5°C group, 120 from the 38.5°C group, and 103 from the 39.0°C
243 group). Both the viability and antrum formation of the OCGCs were evaluated on days 4, 8, and
244 12 of IVG culture. Some surviving OCGCs (32 from the 37.5°C group, 30 from the 38.5°C group,
245 and 32 from the 39.0°C group) were subjected to IVM culture to evaluate the nuclear status of the

oocytes. The diameters of these oocytes before IVG and after IVM were also evaluated. The production of E_2 and P_4 in the culture medium was assessed every 4 days (days 4, 8, and 12). The spent medium for E_2 and P_4 measurements (21 samples from the 37.5°C group, 19 samples from the 38.5°C group, and 19 samples from the 39.0°C group) was derived from two experimental replicates of the IVG culture in which OCGCs were used for IVM and checking the nuclear status of their oocytes. The granulosa cells (6 samples from each group) collected from surviving OCGCs after IVG were utilized for analyzing the mRNA expression of HSP70 and 90. These samples were also derived from two replicates of the IVG culture in which OCGCs were used for IVM and checking the nuclear status of their oocytes. Some surviving OCGCs (22 from the 37.5°C group, 20 from the 38.5°C group, and 21 from the 39.0°C group) were used for evaluating the GSH levels in oocytes. The other surviving OCGCs (63 from the 37.5°C group, 67 from the 38.5°C group, and 48 from the 39.0°C group) were subjected to IVM, IVF, and IVC to evaluate their developmental competence.

In vivo-derived COCs collected from antral follicles (2-8 mm in diameter: n = 94) were also subjected to IVM/IVF/IVC and served as an *in vivo* control for blastocyst development.

2.11. Statistical analysis

All statistical analyses were performed using software (JMP Pro 14.0.0, SAS Institute, Cary, NC, USA). The rates of viability, antrum formation, nuclear maturation, cleavage, and blastocysts were evaluated using chi-squared tests. The production of steroid hormones and diameters of the oocytes were evaluated by two-way ANOVA followed by the Tukey-Kramer test. The rate of OCGCs with increased E_2 production until the end of IVG culture was also compared using the chi-squared test. The GSH levels and mRNA expressions were analyzed by one-way ANOVA followed by the Tukey-Kramer test. The cell numbers in the blastocysts were analyzed

using Steel's test, considering the *in vivo*-grown oocytes as control. P values of less than 0.05 were considered significant.

3. Results

Effects of temperature during IVG on the viability and antrum formation of OCGCs

As shown in Fig. 3, the viability of OCGCs in the 37.5, 38.5, and 39.0℃ groups decreased throughout the IVG ($P < 0.05$) and did not differ among the three groups. The rate of antrum formation increased during the culture period ($P < 0.05$) and became higher in the 37.5℃ group (91.7%) than in the other two groups (38.5℃: 80.0%, 39.0℃: 78.6%, $P < 0.05$) on day 12 of IVG.

Effects of temperature during IVG on the steroidogenesis of OCGCs, GSH levels in oocytes, and mRNA expressions of HSP70 and 90

The two-way ANOVA for groups and culture days (days 0–4, 4–8, and 8–12) revealed only the main effects of culture days ($P < 0.01$) on the E_2 and P_4 production, and thereby the E_2/P_4 ratio (Fig. 4). In all three groups, E_2 production increased from days 0–4 to 4–8, and the levels were maintained until the end of IVG (days 8–12). P_4 production continuously increased during the culture period, and the E_2/P_4 ratio (E_2 / P_4) decreased. Conversely, E_2 production on days 8–12 of culture tended to be higher in the 37.5℃ group than in the other two groups combined ($P < 0.1$). In addition, the percentage of OCGCs with increased E_2 production throughout the IVG culture was significantly higher in the 37.5℃ group (66.7%) than in the other two groups combined (39.5%, $P < 0.05$) (Fig. 5). However, the intracellular GSH levels in oocytes and the mRNA expression of HSP70 and 90 did not differ among the three groups (Figs. 6 and 7).

Effects of temperature during IVG on the growth, maturation, and developmental competence of

oocytes

The two-way ANOVA for groups (37.5, 38.5, and 39.0℃) and culture days (before or after) showed the significant effects of culture days ($P < 0.05$) and interaction ($P < 0.01$) on the oocyte diameters (Fig. 8). The oocytes diameters became larger after IVG than before culture in all three groups ($P < 0.05$). The oocyte diameters before IVG did not differ among the three groups; however, the oocyte diameter after IVG and IVM was larger in the 37.5℃ group than in the 39.0℃ group ($P < 0.05$), and that in the 38.5℃ group was intermediate between the other two groups. In addition, the mean diameter of oocytes in the 37.5℃ group reached 120 μm, which is close to the final diameter of oocytes developed *in vivo* [28,29]. The nuclear maturation rate (Table 2) did not differ among the three groups. Whereas the diameters of oocytes both before and after culture was larger in mature oocytes than in immature oocytes (Supplementary Table 1). The cleavage rate did not differ among the four groups derived from the *in vitro*-grown oocytes and the *in vivo*-grown oocytes. However, the blastocyst rate was higher in the 37.5 and 38.5℃ groups, and *in vivo*-grown oocytes than in the 39.0℃ group ($P < 0.05$) (Table 3 & Fig. 9). The number of cells in the blastocysts in the 38.5℃ group was smaller than the *in vivo*-grown oocytes, while those in the 37.5 and 39.0℃ group did not differ with that in the *in vivo* control (Table 3).

4. Discussion

In previous reports, the temperature condition based on the *in vivo* ovarian temperature of bovine during IVM did not positively affect the nuclear maturation and blastocyst rates [1,2]. Therefore, the physiological significance of the ovarian temperature being lower than the rectum or vagina had not been elucidated. However, we demonstrated that maintaining the temperature condition based on the ovarian temperature *in vivo* (37.5℃) improves the steroidogenesis, growth,

318 and developmental competence of OCGCs derived from early antral follicles cultured *in vitro*.
319 Therefore, the lower ovarian temperature may be significant for the oocyte and follicle growth,
320 which is necessary for a relatively long period, compared with that during oocyte maturation.
321 To investigate the mechanisms by which the slight reduction in the culture temperature
322 improves the growth and developmental competence of OCGCs, we evaluated the steroidogenesis
323 of OCGCs, GSH contents in oocytes, and mRNA expression of HSP70 and 90 in the granulosa
324 cells. Although the GSH levels in oocytes and HSP expression did not differ among the 37.5, 38.5,
325 and 39.0℃ groups, the E_2 production of OCGCs on days 8–12 of culture tended to be higher in
326 the 37.5℃ groups. In addition, the proportion of OCGCs with increased E_2 production throughout
327 IVG was significantly higher in the 37.5℃ group. Moreover, the antrum formation, which is
328 associated with healthy steroidogenesis and follicular growth, was promoted in the 37.5℃ group.
329 The E_2 concentration in the follicular fluid of healthy antral follicles generally increases as they
330 grow, with a peak at estrus in cattle [16]. It has also been demonstrated that E_2 supplementation
331 in the IVG culture medium increased the number of transzonal projections between oocytes and
332 surrounding cumulus cells and improved the growth and maturational competence of oocytes [17].
333 Moreover, OCGCs that showed higher E_2 production during IVG have better growth and
334 developmental competence [18]. Therefore, higher E_2 production suggests that 37.5℃ is more
335 suitable for the growth of OCGCs. In contrast, the slight difference in culture temperature in the
336 present study may not alter the oxidative stress or the markers of heat stress. A previous study
337 also reported that the reactive oxygen species (ROS) level and HSP70 expression did not differ
338 between the bovine granulosa cells cultured at 38.0 and 39.0℃. However, 142 differentially
339 expressed genes (DEGs) were identified between the granulosa cells cultured at 38.0 and 39.0℃,
340 and the pathways, including ovarian steroidogenesis and E_2 signaling, were detected in the DEG
341 analysis [30]. Therefore, even the slight temperature difference can affect the function of

342 granulosa cells without inducing changes in the ROS levels or HSP expression.

343 In the present study, the blastocyst rate (per inseminated oocytes) reached over 40%
344 even in the 39.0℃ group, exhibiting the highest rates observed for *in vitro*-grown oocytes derived
345 from bovine early antral follicles with diameters of 0.4–1 mm (i.e., blastocyst rates ranged
346 between 3.7% and 38.9% in the previous studies) [10-13,15,18,19,22,31-40]. One of the possible
347 reasons for the high blastocyst rates in the present study is the difference in the type of complexes
348 subjected to IVM compared with other studies. Usually, cumulus-oocyte-complexes (COCs) that
349 are removed from OCGCs after the IVG culture are subjected to IVM [10-13,15,18,19,22,31-40].
350 However, we subjected the whole intact OCGCs after IVG culture to the IVM culture in the
351 present study. It has been reported that co-culture with granulosa cells collected from antral
352 follicles larger than 10 mm in diameter during IVM significantly improves the developmental
353 competence of COCs compared with those collected from 2–8 mm antral follicles [41-43].
354 Therefore, large amounts of factors secreted from abundant granulosa cells may be favorable
355 during IVM and enhance the developmental competence of oocytes.

356 In the 37.5℃ group, the culture temperatures were 37.5℃ for IVG, 38.5℃ for IVM and
357 IVF, and 39.0℃ for IVC. We cannot deny the possibility that this temperature elevation during
358 the *in vitro* growth and development could cause stress or heat shock for oocytes and embryos.
359 However, the highest developmental competence was obtained in the 37.5℃ group among the *in*
360 *vitro*-grown oocytes. In addition, the blastocyst rate and cell numbers in blastocysts did not differ
361 between the 37.5℃ group and the *in vivo*-grown oocytes. To our knowledge, *in vivo* temperature
362 in bovine oviducts has not been investigated yet, but optimal temperatures for IVF and IVC have
363 been reported to be 38.5 or 39.0℃ [3,4]. Therefore, the elevation of temperature from ovary to
364 oviduct could happen in *in vivo*.

365 In conclusion, maintaining the culture temperature based on the *in vivo* ovarian tissue

temperature improves the steroidogenesis, growth and developmental competence of OCGCs derived from bovine early antral follicles. As with the *in vitro* culture of testicular tissue [44], even a slight difference in culture temperature can affect the growth and acquisition of developmental competence of oocytes cultured *in vitro*. Nonetheless, the optimal temperature conditions for the IVG culture system have not been fully elucidated, and investigations that include additional factors, such as the diurnal changes in ovarian temperature, may further improve the current IVG culture system.

Funding

The present study was supported by the Japan Society for the Promotion of Science (JSPS) KAKENHI Grant Numbers JP20K15672 to Kenichiro Sakaguchi and JP19H00964 to Seiji Katagiri, the Hokkaido University DX Doctoral Fellowship Grant Number JPMJSP2119 from the Japan Science and Technology (JST) Agency to Kohei Kawano, and the World-leading Innovative and Smart Education (WISE) Program from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) to Kohei Kawano.

Author contributions

Kohei Kawano: conceptualization, data curation, formal analysis, investigation, methodology, visualization, writing – original draft. **Kenichiro Sakaguchi**: investigation, methodology, funding acquisition, project administration, supervision, writing – review & editing. **Yojiro Yanagawa**: investigation, methodology, writing – review & editing. **Seiji Katagiri**: funding acquisition, resources, supervision, writing – review & editing.

Declaration of interest

The authors declare no conflicts of interest.

Acknowledgements

We thank Dr. Koji Kimura at Okayama University for providing materials to perform additional experiment. We also thank Mr. Yosuke Sugino, Ms. Sayaka Kubota, Mr. Yuki Uehata, and Ms. Miyu Takahashi at Okayama University for their technical support to perform additional experiment. We thank the Genetic Hokkaido Association for the donation of frozen bull spermatozoa and Nichiro Chikusan Co., Ltd. for providing bovine ovaries. This manuscript was edited by Medical English Service, Kyoto, Japan.

Figure legends

Figure 1. Morphology of oocyte-cumulus-granulosa complexes (OCGCs) before and after 12 days of *in vitro* growth (IVG) culture.

The OCGCs during IVG culture was observed using an inverted microscope. (a) Isolated OCGC before IVG culture. (b) Surviving OCGC with antrum formation (white arrowhead) in the granulosa cell layer after 12 days of IVG culture. (c) Surviving OCGC without antrum formation in the granulosa cell layer after 12 days of IVG culture. (d) Degenerated OCGCs after 12 days of IVG culture. Scale bar = 100 μm.

Figure 2. Schematic illustration of the experimental design.

Oocyte-cumulus-granulosa complexes (OCGCs) derived from early antral follicles (0.5–1 mm in diameter) were subjected to 12 days of *in vitro* growth (IVG) culture under three different temperature conditions (37.5, 38.5, or 39.0℃). Oocyte diameter was evaluated on day 0 of IVG culture. The morphology of OCGCs (viability of OCGCs and antrum formation in granulosa cell layers) was evaluated every 4 days during IVG culture (days 4, 8, and 12). After 12 days of IVG, some surviving OCGCs were subjected to *in vitro* maturation (IVM). After IVM, the diameter and nuclear status of some oocytes (32 from the 37.5°C group, 30 from the 38.5°C group, and 32 from the 39.0°C group) were evaluated. Then, some oocytes (63 from the 37.5°C group, 67 from the 38.5°C group, and 48 from the 39.0°C group) were subjected to *in vitro* fertilization (IVF) and *in vitro* culture (IVC) to evaluate developmental competence. The production of estradiol-17β (E_2) and progesterone (P_4) in the IVG media during the 1st, 2nd and 3rd 4-day periods was measured by enzyme immunoassay. The mRNA expression of heat shock protein (HSP) 70 and HSP90 in the granulosa cells and the reduced glutathione (GSH) levels in oocytes were evaluated on day 12 of IVG culture.

Figure 3. Effects of temperature conditions on the viability and antrum formation of oocyte-cumulus-granulosa complexes (OCGCs).

The viability of OCGCs was calculated based on 524 OCGCs that were cultured until the end of the *in vitro* growth culture (12 days) (182 from the 37.5℃ group, 181 from the 38.5℃ group, and 161 from the 39.0℃ group). The percentage of antrum formation in the granulosa cell layer was calculated based on 343 surviving OCGCs (120 from the 37.5℃ group, 120 from the 38.5℃ group, and 103 from the 39.0℃ group). [ab] Different letters indicate a significant difference among

438 the days of culture in the same group ($P < 0.05$). * An asterisk indicates a significant difference
439 between the 37.5℃ group and the other groups ($P < 0.05$).
440
441 Figure 4. Effects of temperature conditions on the production of estradiol-17β (E_2) and
442 progesterone (P_4) by oocyte-cumulus-granulosa complexes (OCGCs), and the E_2/P_4 ratio in
443 culture media.
444 The culture media used for E_2 and P_4 assays were derived from some OCGCs employed to
445 evaluate nuclear status after *in vitro* maturation (two replicates). [a-c] Different letters indicate
446 significant differences among the days of culture in the same group ($P < 0.05$). [†] A dagger indicates
447 the tendency of difference between the 37.5℃ group and the other two groups combined ($P <$
448 0.1). Error bars indicate SEM.
449
450 Figure 5. Effects of temperature conditions on the proportion of oocyte-cumulus-granulosa
451 complexes (OCGCs) with increased estradiol-17β (E_2) production throughout *in vitro* growth.
452 The culture media for hormone assays were derived from some OCGCs employed to evaluate
453 nuclear status after *in vitro* maturation (two replicates). * An asterisk indicates a significant
454 difference between the 37.5℃ group and the other two groups combined ($P < 0.05$).
455
456 Figure 6. Effects of temperature conditions on the reduced glutathione (GSH) levels in oocytes
457 after 12 days of *in vitro* growth culture.
458 The fluorescent intensity of GSH was measured using a total of 63 oocytes (22 from the 37.5℃
459 group, 20 from the 38.5℃ group, and 21 from the 39.0℃ group) and two experimental replicates.
460 Error bars indicate SEM.
461

462 Figure 7. Effects of temperature conditions on the mRNA expression of heat shock protein (HSP)
463 70 and HSP90 in the granulosa cells after 12 days of *in vitro* growth culture.
464 Granulosa cells were derived from 6 OCGCs in each group, using two experimental replicates.
465 Error bars indicate SEM.
466
467 Figure 8. Effects of temperature conditions on the *in vitro* growth of oocytes.
468 Lines on the boxes in the box-and-whisker plot delineate the 25^{th}, 50^{th}, and 75^{th} percentiles,
469 whereas the whiskers depict the 10^{th} and 90^{th} percentiles. Oocytes were derived from OCGCs
470 subjected to *in vitro* maturation. Values on the box-and-whisker plot indicate the mean diameters
471 (μm) of oocytes. $^{a-c}$ Different letters indicate significant differences ($P < 0.05$).
472
473 Figure 9. Representative photographs of bovine blastocysts.
474 Blastocysts developed from *in vitro*-grown oocytes (a: 37.5℃ group, b: 38.5℃ group, c: 39.0℃
475 group), and *in vivo*-grown oocytes. Scale bar = 100 μm.
476
477
478 **References**

479 [1] Katska L, Smorag Z. The influence of culture temperature on in vitro maturation of bovine oocytes.
480 Animal Reproduction Science 1985;9:205-12. https://doi.org/10.1016/0378-4320(85)90003-X.
481 [2] Shi DS, Avery B, Greve T. Effects of temperature gradients on in vitro maturation of bovine
482 oocytes. Theriogenology 1998;50:667-74. https://doi.org/10.1016/s0093-691x(98)00171-x.
483 [3] Lenz RW, Ball GD, Leibfried ML, Ax RL, First NL. In vitro maturation and fertilization of bovine
484 oocytes are temperature-dependent processes. Biol Reprod 1983;29:173-9.
485 https://doi.org/10.1095/biolreprod29.1.173.
486 [4] Wang WL, Jiang HS, Shi DS, Lu KH, Gordon I, Polge C. The effect of temperature on the in vitro
487 maturation, fertilization and development of bovine oocytes. Proc 7th Scientific meet Eur
488 Embryo Transfer Assoc 1991. p. 216.
489 [5] Rivera RM, Hansen PJ. Development of cultured bovine embryos after exposure to high

temperatures in the physiological range. Reproduction 2001;121:107-15. https://doi.org/10.1530/rep.0.1210107.

[6] Nabenishi H, Ohta H, Nishimoto T, Morita T, Ashizawa K, Tsuzuki Y. The effects of cysteine addition during in vitro maturation on the developmental competence, ROS, GSH and apoptosis level of bovine oocytes exposed to heat stress. Zygote 2012;20:249-59. https://doi.org/10.1017/s0967199411000220.

[7] Grøndahl C, Greve T, Schmidt M, Hunter RHF. Bovine pre-ovulatory follicles are cooler than ovarian stroma and deep rectal temperature. Theriogenology 1996;45:289 (Abstr).

[8] López-Gatius F, Hunter RHF. Pre-ovulatory follicular cooling correlates positively with the potential for pregnancy in dairy cows: Implications for human IVF. J Gynecol Obstet Hum Reprod 2019;48:419-22. https://doi.org/10.1016/j.jogoh.2019.03.005.

[9] Morita Y, Ozaki R, Mukaiyama A, Sasaki T, Tatebayashi R, Morishima A, et al. Establishment of long-term chronic recording technique of in vivo ovarian parenchymal temperature in Japanese Black cows. J Reprod Dev 2020;66:271-5. https://doi.org/10.1262/jrd.2019-097.

[10] Yamamoto K, Otoi T, Koyama N, Horikita N, Tachikawa S, Miyano T. Development to live young from bovine small oocytes after growth, maturation and fertilization in vitro. Theriogenology 1999;52:81-9. https://doi.org/10.1016/s0093-691x(99)00111-9.

[11] Hirao Y, Itoh T, Shimizu M, Iga K, Aoyagi K, Kobayashi M, et al. In vitro growth and development of bovine oocyte-granulosa cell complexes on the flat substratum: effects of high polyvinylpyrrolidone concentration in culture medium. Biol Reprod 2004;70:83-91. https://doi.org/10.1095/biolreprod.103.021238.

[12] Huang W, Kang SS, Nagai K, Yanagawa Y, Takahashi Y, Nagano M. Mitochondrial activity during pre-maturational culture in in vitro-grown bovine oocytes is related to maturational and developmental competences. Reprod Fertil Dev 2016;28:349-56. https://doi.org/10.1071/rd14023.

[13] Huang W, Nagano M, Kang SS, Yanagawa Y, Takahashi Y. Effects of in vitro growth culture duration and prematuration culture on maturational and developmental competences of bovine oocytes derived from early antral follicles. Theriogenology 2013;80:793-9. https://doi.org/10.1016/j.theriogenology.2013.07.004.

[14] Endo M, Kawahara-Miki R, Cao F, Kimura K, Kuwayama T, Monji Y, et al. Estradiol supports in vitro development of bovine early antral follicles. Reproduction 2013;145:85-96. https://doi.org/10.1530/rep-12-0319.

[15] Nagai K, Yanagawa Y, Katagiri S, Nagano M. The relationship between antral follicle count in a bovine ovary and developmental competence of in vitro-grown oocytes derived from early antral follicles. Biomed Res 2016;37:63-71. https://doi.org/10.2220/biomedres.37.63.

[16] Kruip TA, Dieleman SJ. Steroid hormone concentrations in the fluid of bovine follicles relative to size, quality and stage of the oestrus cycle. Theriogenology 1985;24:395-408.

https://doi.org/10.1016/0093-691x(85)90046-9.

[17] Makita M, Miyano T. Steroid hormones promote bovine oocyte growth and connection with granulosa cells. Theriogenology 2014;82:605-12. https://doi.org/10.1016/j.theriogenology.2014.05.020.

[18] Sakaguchi K, Tanida T, Abdel-Ghani MA, Kanno C, Yanagawa Y, Katagiri S, et al. Relationship between the antral follicle count in bovine ovaries from a local abattoir and steroidogenesis of granulosa cells cultured as oocyte-cumulus-granulosa complexes. J Reprod Dev 2018;64:503-10. https://doi.org/10.1262/jrd.2018-080.

[19] Kawano K, Sakaguchi K, Madalitso C, Ninpetch N, Kobayashi S, Furukawa E, et al. Effect of heat exposure on the growth and developmental competence of bovine oocytes derived from early antral follicles. Sci Rep 2022;12:8857. https://doi.org/10.1038/s41598-022-12785-2.

[20] Harada M, Miyano T, Matsumura K, Osaki S, Miyake M, Kato S. Bovine oocytes from early antral follicles grow to meiotic competence in vitro: effect of FSH and hypoxanthine. Theriogenology 1997;48:743-55. https://doi.org/10.1016/s0093-691x(97)00298-7.

[21] Kawano K, Sakaguchi K, Ninpetch N, Yanagawa Y, Katagiri S. Physiological high temperatures alter the amino acid metabolism of bovine early antral follicles. J Reprod Dev 2024;70:184-91. https://doi.org/10.1262/jrd.2023-096.

[22] Yang Y, Kanno C, Sakaguchi K, Katagiri S, Yanagawa Y, Nagano M. Theca cells can support bovine oocyte growth in vitro without the addition of steroid hormones. Theriogenology 2020;142:41-7. https://doi.org/10.1016/j.theriogenology.2019.09.037.

[23] Sakaguchi K, Huang W, Yang Y, Yanagawa Y, Nagano M. Relationship between in vitro growth of bovine oocytes and steroidogenesis of granulosa cells cultured in medium supplemented with bone morphogenetic protein-4 and follicle stimulating hormone. Theriogenology 2017;97:113-23. https://doi.org/10.1016/j.theriogenology.2017.04.030.

[24] Singh AK, Upadhyay RC, Malakar D, Kumar S, Singh SV. Effect of thermal stress on HSP70 expression in dermal fibroblast of zebu (Tharparkar) and crossbred (Karan-Fries) cattle. J Therm Biol 2014;43:46-53. https://doi.org/10.1016/j.jtherbio.2014.04.006.

[25] Nagano M, Kang SS, Koyama K, Huang W, Yanagawa Y, Takahashi Y. In vitro maturation system for individual culture of bovine oocytes using micro-volume multi-well plate. Jpn J Vet Res 2013;61:149-54. https://doi.org/10.14943/jjvr.61.4.149.

[26] Nagano M, Katagiri S, Takahashi Y. ATP content and maturational/developmental ability of bovine oocytes with various cytoplasmic morphologies. Zygote 2006;14:299-304. https://doi.org/10.1017/s0967199406003807.

[27] Takahashi Y, First NL. In vitro development of bovine one-cell embryos: Influence of glucose, lactate, pyruvate, amino acids and vitamins. Theriogenology 1992;37:963-78. https://doi.org/10.1016/0093-691x(92)90096-a.

[28] Fair T, Hyttel P, Greve T. Bovine oocyte diameter in relation to maturational competence and transcriptional activity. Mol Reprod Dev 1995;42:437-42. https://doi.org/10.1002/mrd.1080420410.

[29] Otoi T, Yamamoto K, Koyama N, Tachikawa S, Suzuki T. Bovine oocyte diameter in relation to developmental competence. Theriogenology 1997;48:769-74. https://doi.org/10.1016/s0093-691x(97)00300-2.

[30] Khan A, Dou J, Wang Y, Jiang X, Khan MZ, Luo H, et al. Evaluation of heat stress effects on cellular and transcriptional adaptation of bovine granulosa cells. J Anim Sci Biotechnol 2020;11:25. https://doi.org/10.1186/s40104-019-0408-8.

[31] Makita M, Ueda M, Miyano T. The fertilization ability and developmental competence of bovine oocytes grown in vitro. J Reprod Dev 2016;62:379-84. https://doi.org/10.1262/jrd.2016-001.

[32] Sugiyama M, Kawahara-Miki R, Kawana H, Shirasuna K, Kuwayama T, Iwata H. Resveratrol-induced mitochondrial synthesis and autophagy in oocytes derived from early antral follicles of aged cows. J Reprod Dev 2015;61:251-9. https://doi.org/10.1262/jrd.2015-001.

[33] Hirao Y, Shimizu M, Iga K, Takenouchi N. Optimization of oxygen concentration for growing bovine oocytes in vitro: constant low and high oxygen concentrations compromise the yield of fully grown oocytes. J Reprod Dev 2012;58:204-11. https://doi.org/10.1262/jrd.11-132m.

[34] Yang Y, Kanno C, Sakaguchi K, Yanagawa Y, Katagiri S, Nagano M. Extension of the culture period for the in vitro growth of bovine oocytes in the presence of bone morphogenetic protein-4 increases oocyte diameter, but impairs subsequent developmental competence. Anim Sci J 2017;88:1686-91. https://doi.org/10.1111/asj.12841.

[35] Huang W, Nagano M, Kang SS, Yanagawa Y, Takahashi Y. Prematurational culture with 3-isobutyl-1-methylxanthine synchronizes meiotic progression of the germinal vesicle stage and improves nuclear maturation and embryonic development in in vitro-grown bovine oocytes. J Reprod Dev 2014;60:9-13. https://doi.org/10.1262/jrd.2013-082.

[36] Huang W, Kang SS, Yanagawa Y, Yang Y, Takahashi Y, Nagano M. Effects of in vitro-growth culture duration on fertilizability of bovine growing oocytes and proliferation of cells surrounding oocytes. Jpn J Vet Res 2014;62:135-41. https://doi.org/10.14943/jjvr.62.3.135.

[37] Chelenga M, Yanagawa Y, Katagiri S, Nagano M. Pre-maturational culture promotes the developmental competence of bovine oocytes derived from an 8-day in vitro growth culture system. J Reprod Dev 2023;69:214-7. https://doi.org/10.1262/jrd.2023-022.

[38] Chelenga M, Sakaguchi K, Kawano K, Furukawa E, Yanagawa Y, Katagiri S, et al. Low oxygen environment and astaxanthin supplementation promote the developmental competence of bovine oocytes derived from early antral follicles during 8 days of in vitro growth in a gas-permeable culture device. Theriogenology 2022;177:116-26. https://doi.org/10.1016/j.theriogenology.2021.10.014.

[39] Chelenga M, Sakaguchi K, Abdel-Ghani MA, Yanagawa Y, Katagiri S, Nagano M. Effect of increased oxygen availability and astaxanthin supplementation on the growth, maturation and developmental competence of bovine oocytes derived from early antral follicles. Theriogenology 2020;157:341-9. https://doi.org/10.1016/j.theriogenology.2020.07.023.

[40] Yang Y, Kanno C, Huang W, Kang SS, Yanagawa Y, Nagano M. Effect of bone morphogenetic protein-4 on in vitro growth, steroidogenesis and subsequent developmental competence of the oocyte-granulosa cell complex derived from bovine early antral follicles. Reprod Biol Endocrinol 2016;14:3. https://doi.org/10.1186/s12958-016-0137-1.

[41] Fukui Y, Ono H. Effects of sera, hormones and granulosa cells added to culture medium for in-vitro maturation, fertilization, cleavage and development of bovine oocytes. J Reprod Fertil 1989;86:501-6. https://doi.org/10.1530/jrf.0.0860501.

[42] Mochizuki H, Fukui Y, Ono H. Effect of the number of granulosa cells added to culture medium for in vitro maturation, fertilization and development of bovine oocytes. Theriogenology 1991;36:973-86. https://doi.org/10.1016/0093-691X(91)90322-5.

[43] Konishi M, Aoyagi Y, Takedomi T, Itakura H, Itoh T, Yazawa S. Presence of granulosa cells during oocyte maturation improved in vitro development of IVM-IVF bovine oocytes that were collected by ultrasound-guided transvaginal aspiration. Theriogenology 1996;45:573-81. https://doi.org/10.1016/0093-691x(95)00404-v.

[44] Hirano K, Nonami Y, Nakamura Y, Sato T, Sato T, Ishiguro KI, et al. Temperature sensitivity of DNA double-strand break repair underpins heat-induced meiotic failure in mouse spermatogenesis. Commun Biol 2022;5:504. https://doi.org/10.1038/s42003-022-03449-y.

Fig. 1 Pictures of OCGC

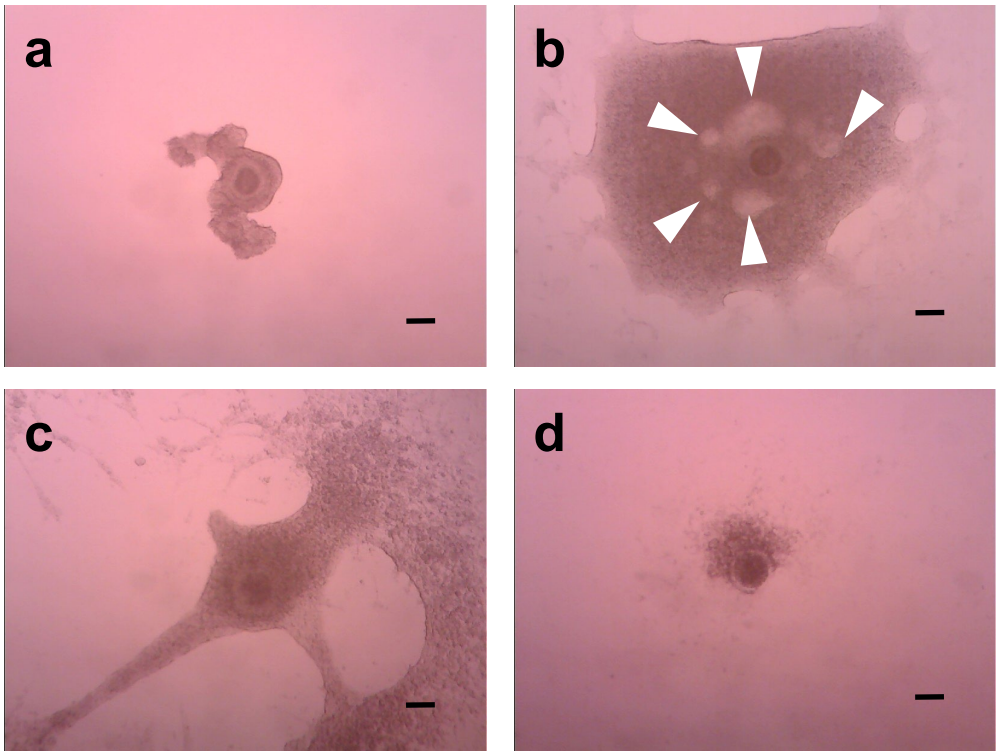


Fig. 2 Schedule of experiment

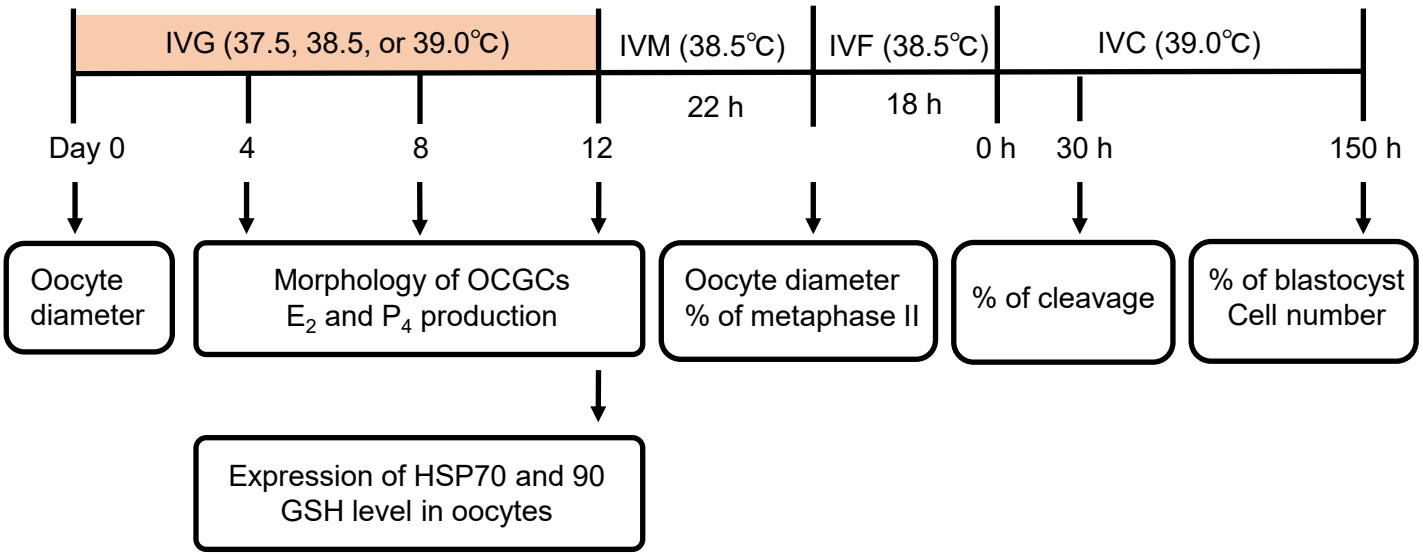
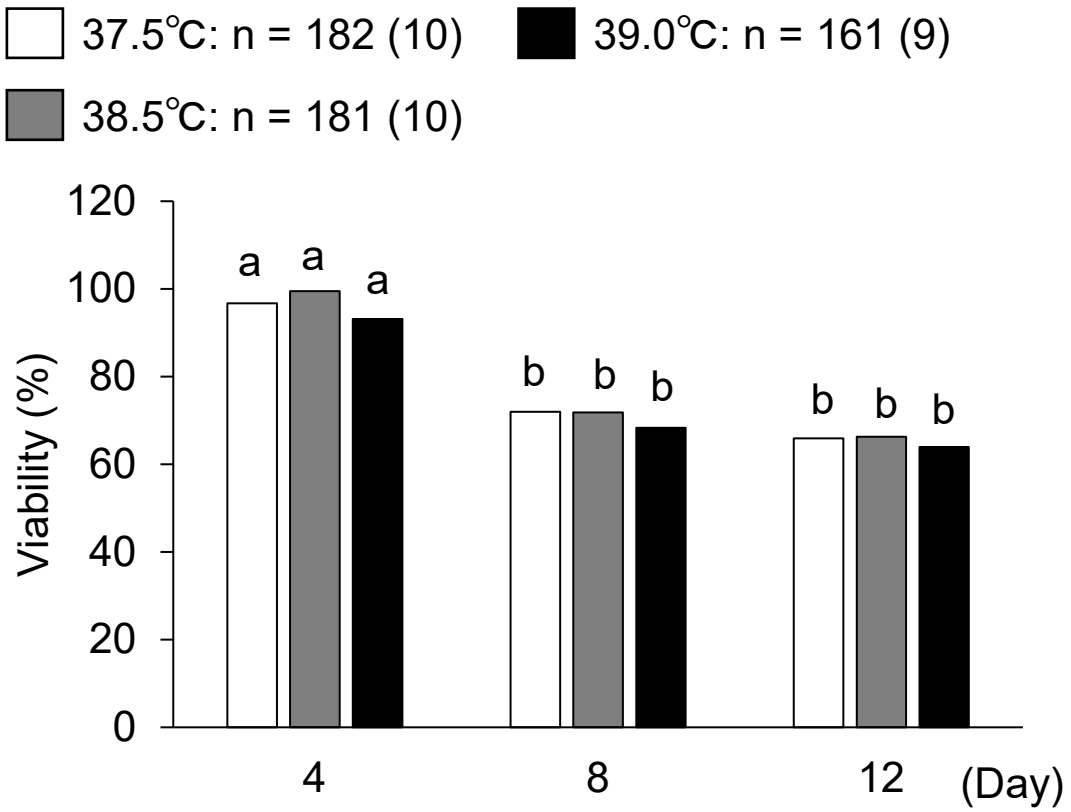
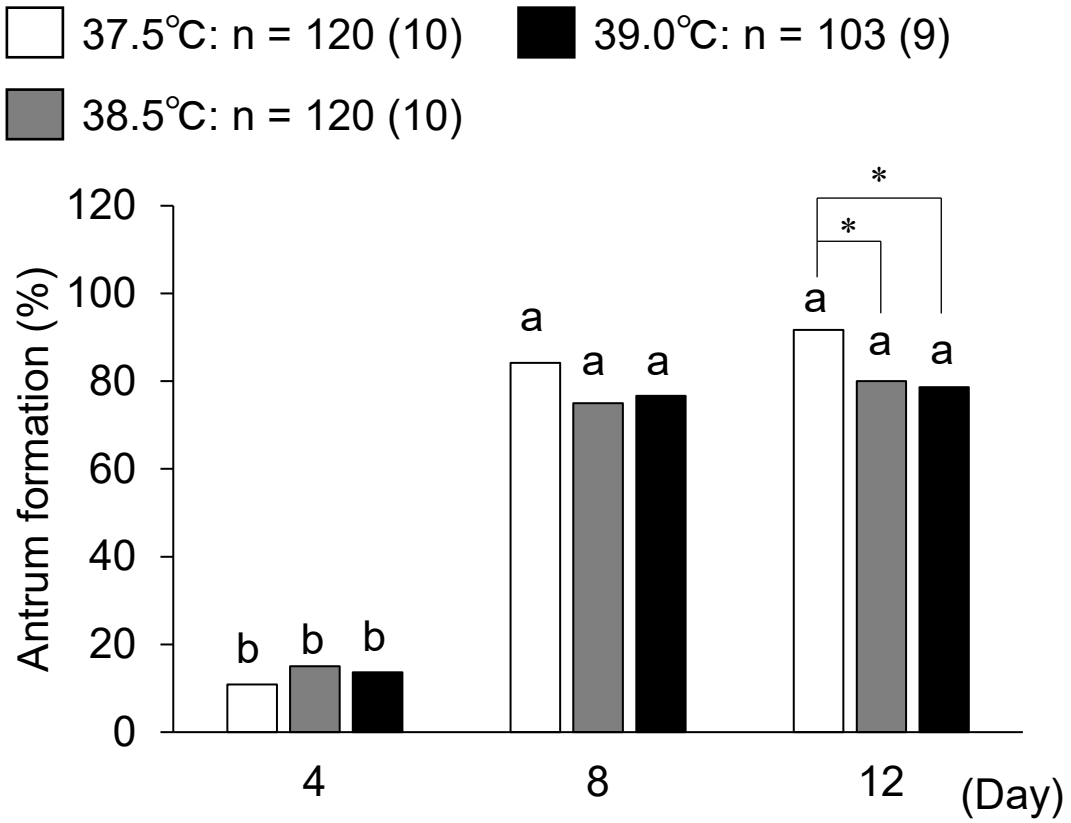


Fig. 3 Viability and antrum formation





• ab: P < 0.05, ＊: P < 0.05

Fig.4 Steroidogenesis

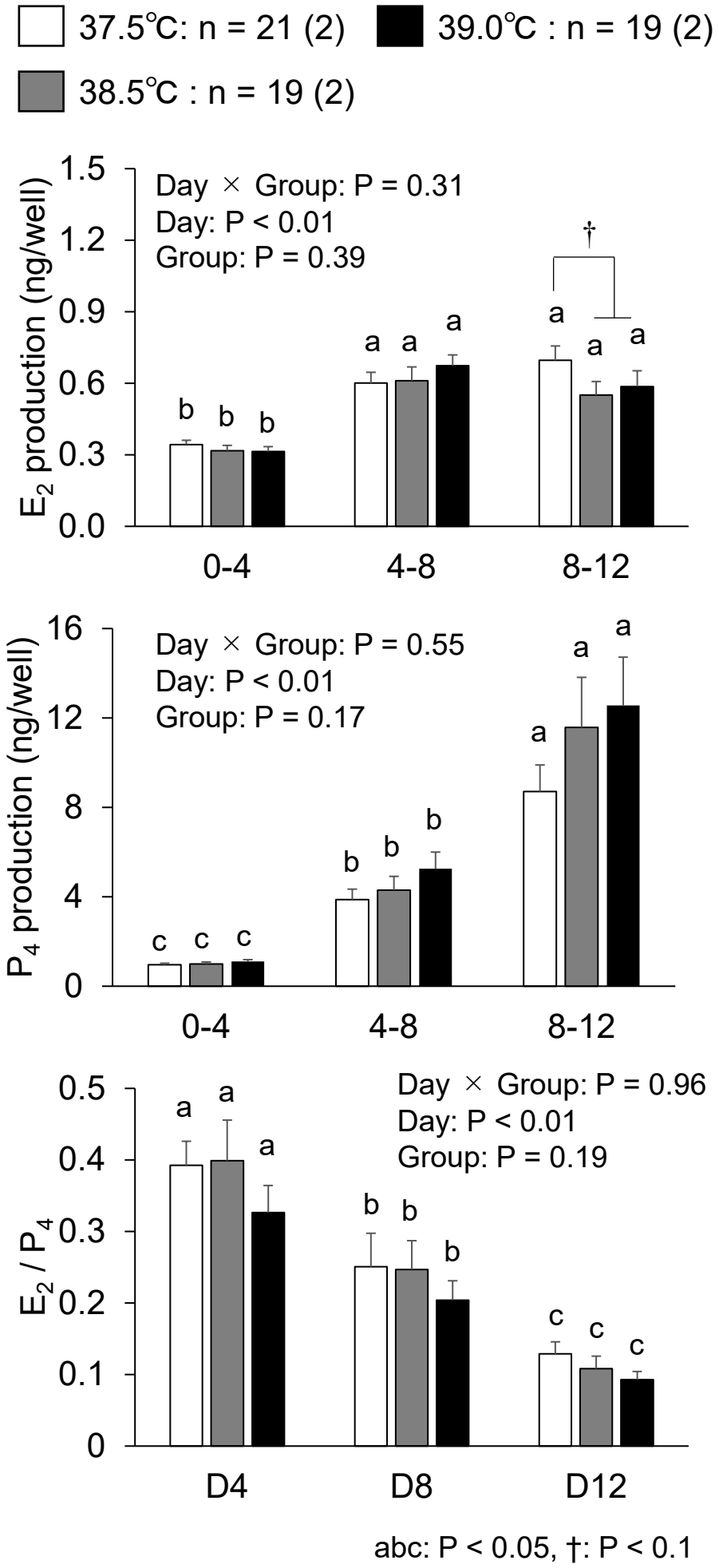


Fig. 5 Steroidogenesis

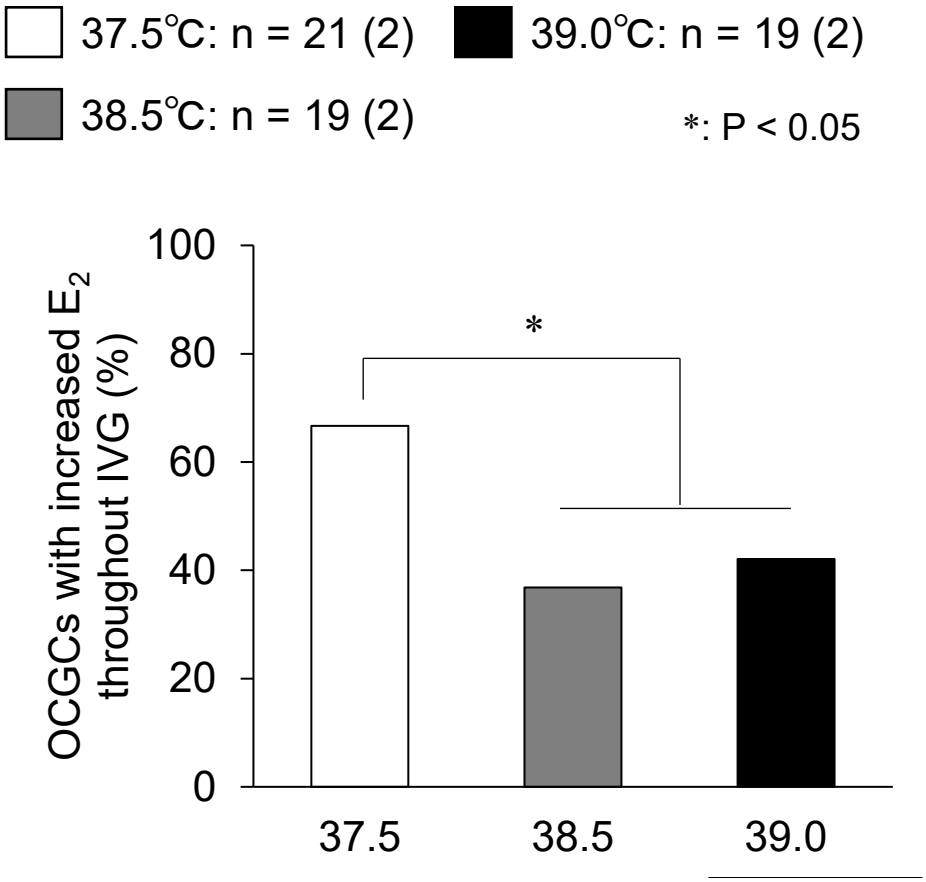


Fig. 6 GSH level

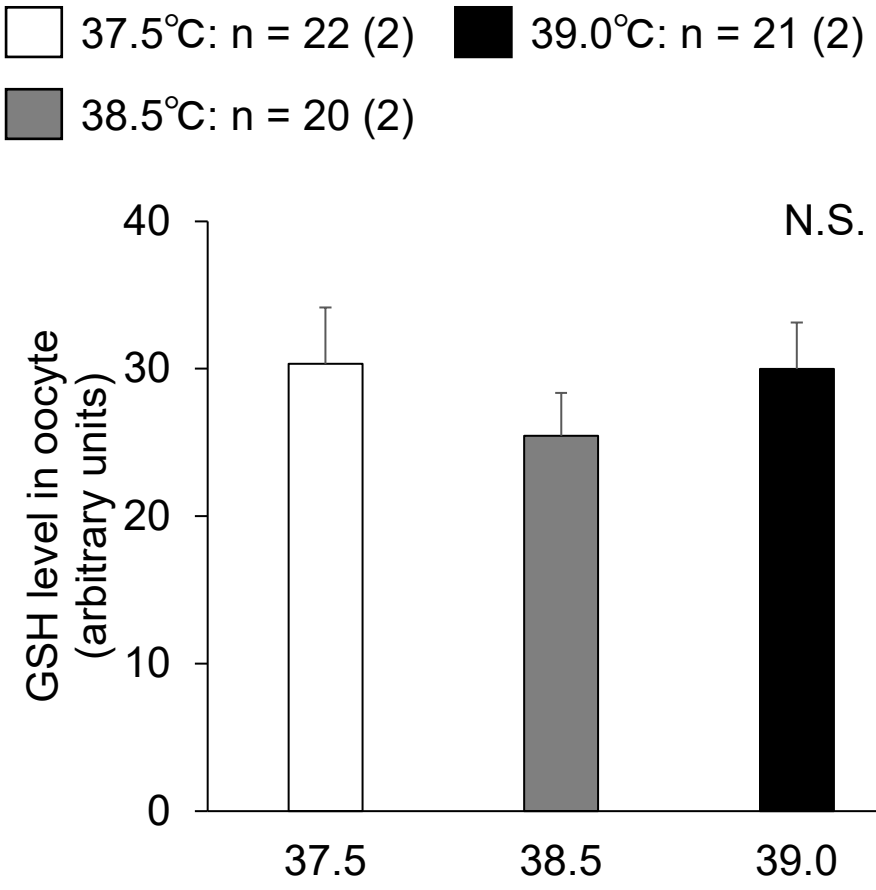


Fig. 7 Expression of HSP70 and 90

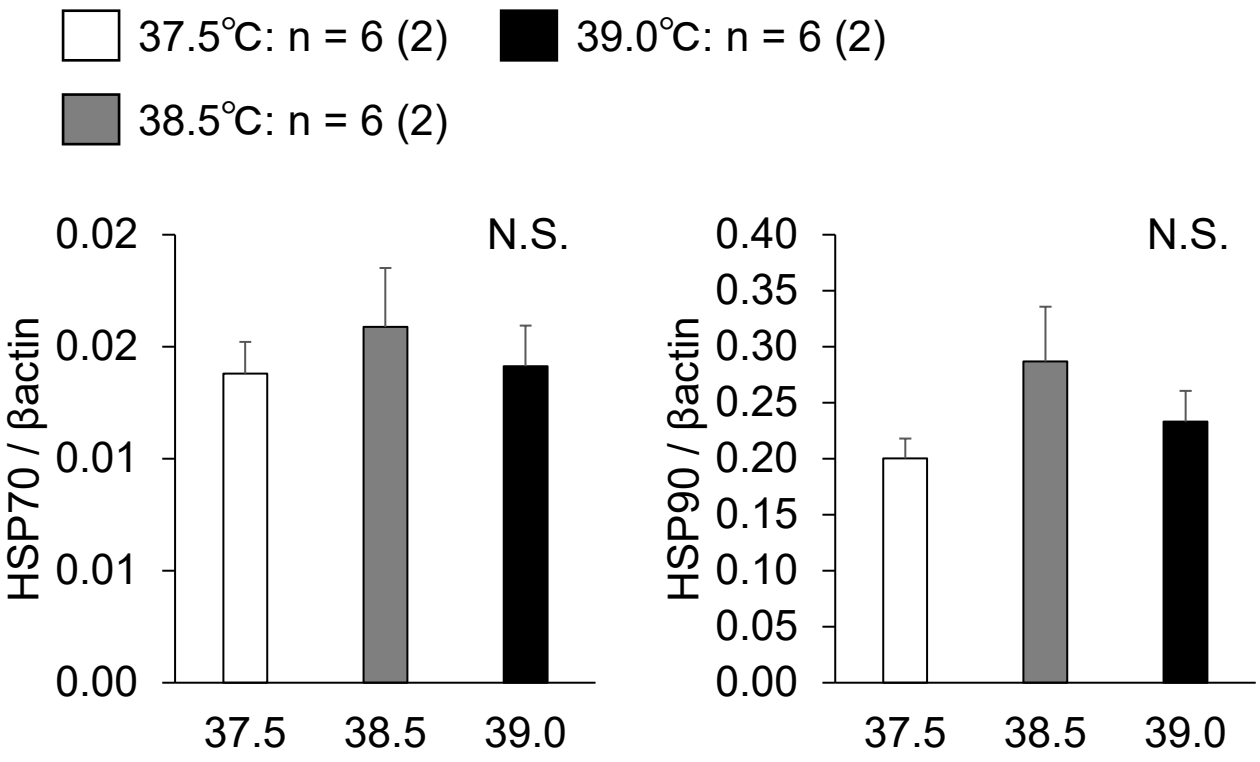


Fig. 8 Oocyte diameter

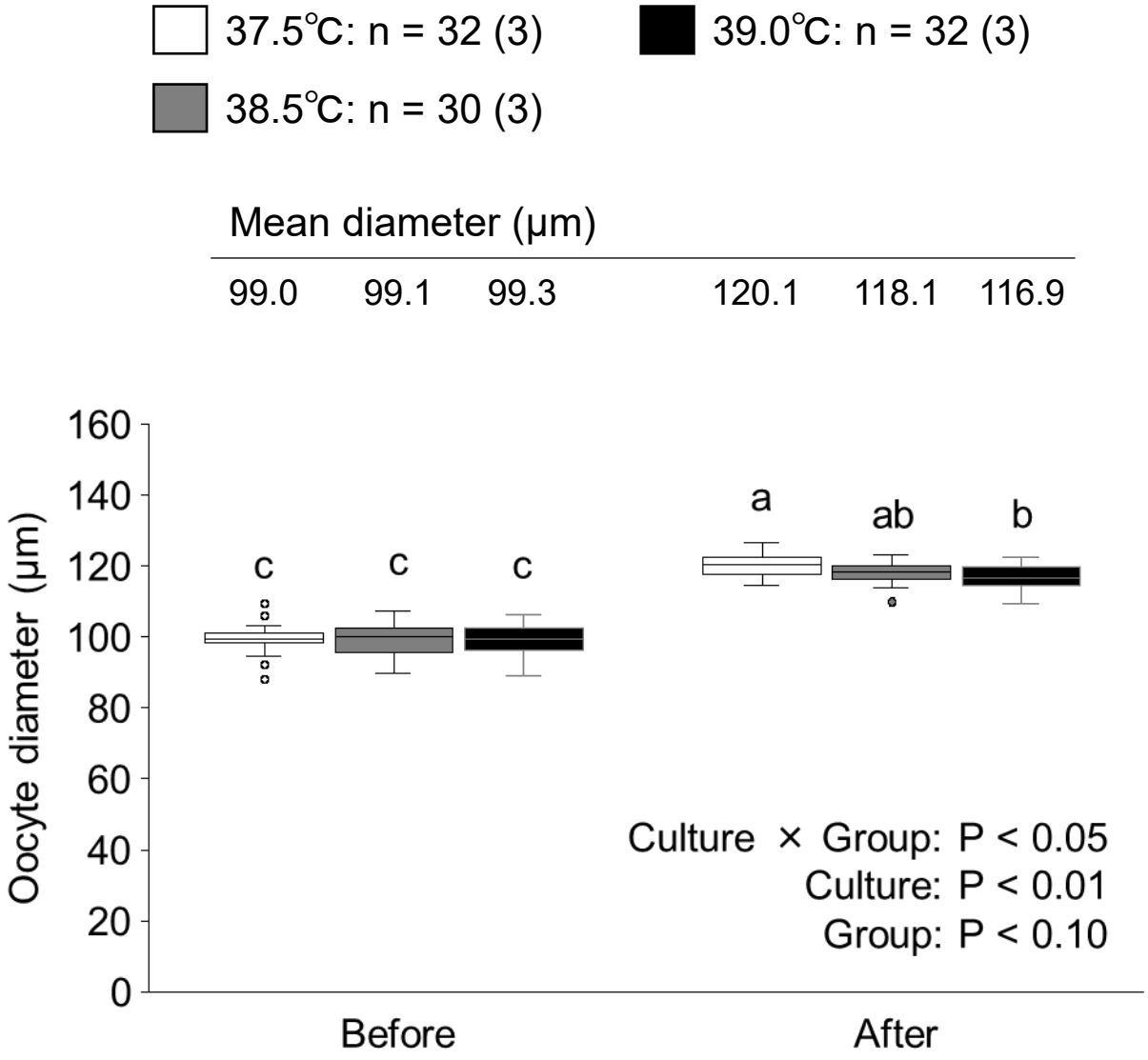
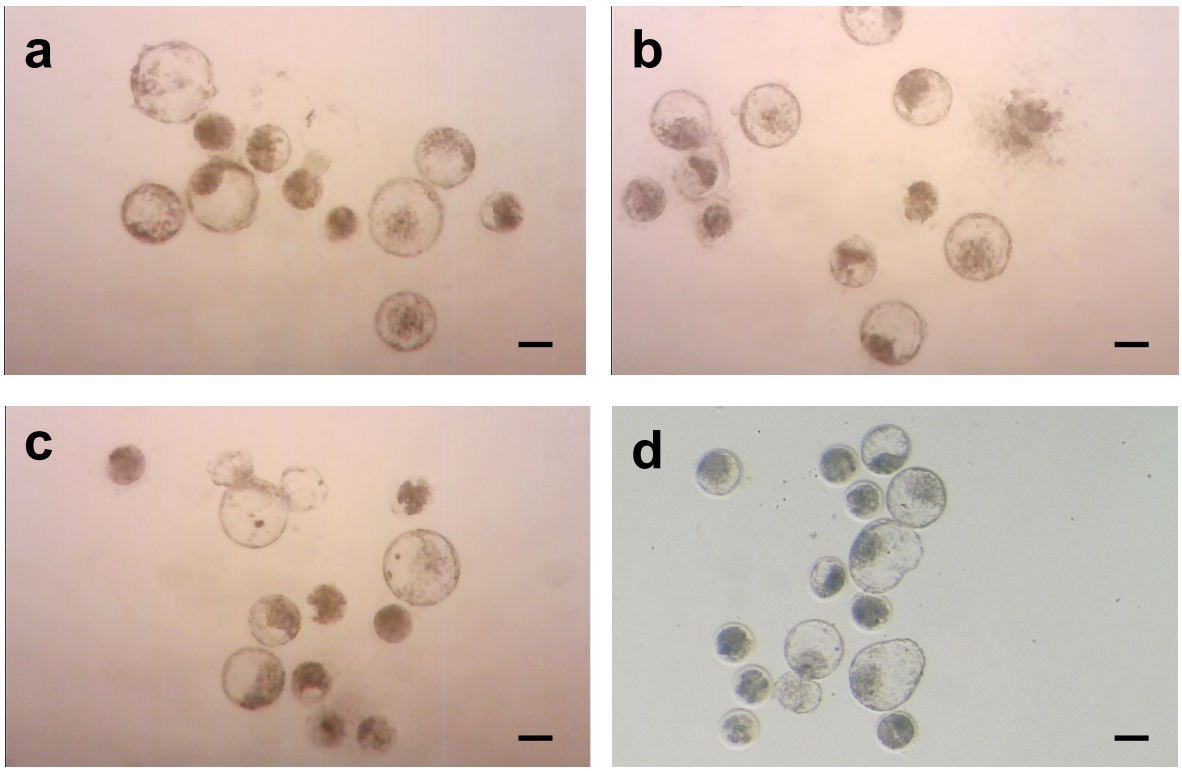


Fig. 9 Picture of blastocysts



Tables

Table 1. Primers used in real-time PCR

Gene	Sequence (5'-3')	Product Length (bp)	Accession number	References
HSP70	F: TCATCAACGACGGAGACAAGCCTA R: TTCATCTTGGTCAGCACCATCGAC	103	NM_203322.3	Singh et al. 2014 [22]
HSP90	F: TTGGAACTCTTCACTGAACTGG R: CCATCTCATCACCAGAAGCAG	163	NM_001012670.2	-
βactin	F: TCCCTGGAGAAGAGCTACGA R: GGTAGTTTCGTGAATGCCGC	132	NM_173979.3	-

Table 2. Effects of temperature conditions on the nuclear maturation of oocytes derived from early antral follicles

Group	No. of oocytes (replicates)	% of nuclear status				
		GVBD	M I	A I/T I	M II	Deg
37.5℃	32 (3)	0	25.0	3.1	71.9	0
38.5℃	30 (3)	3.3	23.3	0	73.3	0
39.0℃	32 (3)	0	15.6	9.4	71.9	3.1

No significant differences between the groups.

GVBD: germinal vesicle breakdown, M I: metaphase I, A I/T I: anaphase I/telophase I, MII: metaphase II (Nuclear maturation), Deg: degenerated.

Table 3. Effects of temperature conditions on the developmental competence of oocytes derived from early antral follicles

Group		No. of oocytes (replicates)	% of cleaved zygotes	% of blastocyst based on		Cell no. in blastocysts (n)
				Inseminated oocytes	Cleaved zygotes	
In vitro-grown	37.5℃	63 (5)	82.5	61.9	75.0	149.5 ± 10.5 (37)
	38.5℃	67 (5)	82.1	61.2	74.5	121.8 ± 7.8 (38)**
	39.0℃	48 (4)	81.3	41.7*	51.3*	147.5 ± 10.7 (19)
In vivo-grown	-	94 (4)	85.1	62.8	73.8	156.2 ± 7.8 (41)

*An asterisk indicates significant difference compared to the other groups ($P < 0.05$).

** Asterisks indicate significant difference compared to the *in vivo*-grown oocytes ($P < 0.05$).

Data of cell number of blastocysts in the *in vivo*-grown oocytes was derived from the three experimental sessions.

Data are presented as means ± SEM.