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Progesterone plays a critical role in canine oocyte maturation and fertilization.[1]
Running title. Postovulatory roles of progesterone in dogs.
Summary sentence. In dogs, the ovarian steroid hormone progesterone is essential for resumption of oocyte meiosis, oocyte aging and sperm transit toward the oviducts.

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ABSTRACT

Canine oocyte maturation and fertilization take place within the oviducts under increasing plasma levels of progesterone (P4). In order to investigate the role of P4 in these processes, 51 Beagle bitches were treated with the P4 receptor antagonist aglepristone at the end of proestrus and 32 females were kept untreated. Fifteen treated and 13 control bitches were inseminated at Days +1 and +2 after ovulation (Day 0). Stages of oocyte maturation and embryo development were determined after ovariectomy at different time points after ovulation. Aglepristone did not prevent ovulation but delayed the resumption of oocyte meiosis and inhibited its progression: first metaphase (M) I stages were observed at 173 h post-ovulation and 39% of oocytes reached MII as late as 335 h post-ovulation in treated females whereas first MI occurred at 76 h and 100% of oocytes were in MII at 109 h post-ovulation in controls. Aglepristone extended the stay of morphologically normal oocytes within the oviducts: first signs of oocyte degeneration were observed at 335 h in treated *vs*. 100-110 h post-ovulation in control bitches. In inseminated females, aglepristone prevented sperm progression toward the oviducts and fertilization, although motile spermatozoa were observed in the uterine tip flush and within the cranial uterine glands. A proteomic analysis of the tubal fluid from treated and control non inseminated bitches

at Day +4 evidenced 79 differential proteins potentially involved in the oocyte phenotype. In conclusion, P4 plays key roles in post-ovulatory canine oocyte maturation, aging and in fertilization.

Keywords. Progesterone, antiprogestin, aglepristone, oocyte, canine, maturation, fertilization, spermatozoa

INTRODUCTION

The ovarian steroid hormone progesterone (P4) has been recognized as essential for ovulation, establishment and maintenance of pregnancy, mammary gland development as well as for the expression of sexual behavior in mammals [1, 2]. More recently, several new roles were also suggested for P4 around the time of fertilization, including oocyte maturation and developmental competence [3-6], release of spermatozoa from the sperm reservoir [7, 8], and sperm hyperactivation, chemotaxis and acrosome reaction [9, 10]. In the porcine, the expression of nuclear P4 receptors (PR) was shown to be induced by LH and FSH in cumulus cells and a high level of P4 produced by cumulus cells promoted the resumption of oocyte meiosis [5, 6]. In the bovine, PR were also expressed in cumulus cells during oocyte maturation and inhibition of P4 production by cumulus cells or treatment with a PR antagonist during *in vitro* maturation severely impaired cumulus expansion and reduced blastocyt development [4]. However, to date, the potential roles of P4 in gamete maturation in human and domestic mammals were mostly studied *in vitro* [4-6, 8-10]. Furthermore, despite increasing knowledge on the molecular changes that occur during oocyte aging, very little is known on environmental factors, such as steroids, that influence this process *in vivo* [11].

The domestic bitch offers a useful model to investigate the roles of P4 on oocyte maturation, aging, and sperm progression toward the site of fertilization. In dogs, these events take place in presence of increasing circulating P4 levels over a relatively long time span, allowing sequential *in vivo* examination. Indeed, canine oocytes are ovulated at the germinal vesicle (GV) stage and reach the metaphase (M) II stage after 2-3 days spent in the oviducts, at which time they become fertilizable [12]. In the absence of spermatozoa, canine oocytes may remain fertile for up to 4-5 more days [13]. In this species, the uterine glands and the utero-tubal junction (UTJ) represent the functional sperm reservoir [14, 15]. After mating or artificial insemination during estrus, fresh canine spermatozoa may remain motile and fertile for up to 11 days in the female genital tract [16].

Moreover, canine oocyte maturation and sperm transit take place in the presence of high and increasing concentrations of circulating P4: due to an acute luteinization of preovulatory follicles, the P4 concentration in plasma increases at the time of LH surge, reaches 2 ng/ml concomitant with the LH peak and 6 ng/ml at the time of ovulation, around 60 h later. Circulating P4 levels then increase to a plateau of 15-80 ng/ml between 25 and 30 days post-ovulation (post-ov) [17]. Very little is known, however, on the roles potentially played by P4 in canine gamete maturation, viability and/or transit and in fertilization. Nuclear PR were identified on dog spermatozoa with a demonstrated role in acrosome reaction [18]. Furthermore, the expression of PR was reported on canine cumulus-oocyte complexes (COC) [19, 20], however, no consistent results on oocyte maturation were obtained *in vitro* after addition of P4 [21-24]. Progesterone may also act indirectly on gametes through modulation of their environment. The expression of PR was reported on epithelial, stromal and muscular cells of the canine endometrium [25] and oviduct [26] during the peri-ovulatory period.

In order to investigate *in vivo* the roles of P4 in oocyte maturation and fertilization, aglepristone, a PR antagonist, was administrated to Beagle bitches prior to the endogenous rise in circulating P4 and the oocyte phenotype was analyzed at specific time points after ovulation, with and without insemination. A proteomic analysis of the tubal fluid at the time of divergence between groups in oocyte maturation was also performed to identify some candidate proteins potentially involved in the process.

MATERIALS AND METHODS

Animals, cycle monitoring and insemination

Eighty-three (51 treated and 32 controls) adult Beagle bitches aged 3.1 ± 0.2 years (mean ± SEM; range: 0.6-7.1 years) from our experimental kennel were included. The animals were housed by groups in kennels, fed dry food and given fresh drinking water *ad libitum*. The study was conducted in accordance with the requirements of the ethical committee of the Alfort National Veterinary School. Monitoring of ovarian cycles was carried out as previously described [27]. Briefly, vaginal smears coupled with Harris–Shorr staining were performed weekly and proestrus was considered initiated when the percentage of superficial (cornified) and large intermediate cells started to increase. During proestrus, plasma P4 was assayed every other day (Elecsys enhanced chemiluminescence kit; Roche Diagnostics, Meylan, France; intra- and inter-assay coefficients of variation < 5%) and transabdominal ovarian ultrasonography (MyLab, Biosound Esaote, Saint-Germain-en-Laye, France) was performed daily to monitor follicular growth. When more than 80% of vaginal cells were superficial, plasma P4 was assayed daily until the day of surgery. Once plasma P4 exceeded 2 ng/ml, ovarian ultrasonography was performed twice daily to check ovulation. Time of ovulation (Day 0) was defined as the median time between the first ultrasound when at least one preovulatory follicle disappeared and the previous ultrasound exam.

Fifteen treated and 13 control bitches were artificially inseminated at Days +1 and +2 using the Scandinavian catheter and fresh semen collected from two Beagle males with normal semen quality and recorded high fertility.

Treatment

At the end of proestrus and before the endogenous rise in plasma P4 levels (proestrus vaginal smear, mean diameter of largest follicles of 3-4 mm at ultrasonography, and plasma P4 < 1 ng/ml), bitches in the treated group received aglepristone (Alizine; Virbac Laboratories, Carros, France; Marketing Authorization number FR/V/7942089 3/1996) subcutaneously at the dose of 10 mg/kg weight for two subsequent days, according to the recommended use of the product in dogs [28]. If not ovariectomized, the bitches received a third injection of aglepristone at the same dose 7 days after the second injection, based on the 6-day mean residence time of aglepristone [28]. Control animals did not receive any injection.

Collection of tissue samples and oocytes/embryos

Both ovaries, oviducts and uterine tips (3-cm long) were recovered by routine ovariectomy performed at the Alfort veterinary hospital at various time periods after ovulation (Figure 1). The number of corpora lutea on each ovary was recorded, then each oviduct was separated from the ovary and uterus at the UTJ and flushed with 100 µl of phosphate buffer saline (PBS) to collect COC/embryos. The uterine tips were also flushed. Cumulus-oocyte complexes were observed

under light microscopy to check their gross morphology before denudation, if necessary, by pipeting. In inseminated bitches, the presence of spermatozoa attached to the cumulus cells and/or the zona pellucida of oocytes was also checked.

Determination of maturation or developmental stage of oocytes/embryos

Stages of oocyte maturation and embryo development were determined by immunocytochemistry and confocal microscopy, as previously described [29]. Briefly, denuded oocytes and embryos were fixed in 4% paraformaldehyde (PAF) solution (4% PAF + 0.2% Triton + 1mM PMSF in HPEM buffer) for 1h at 39°C. Primary incubation with a mouse, monoclonal anti-α-tubulin antibody (1/1000 dilution in 2% PBS-BSA) was performed overnight at 4°C followed by the incubation with the secondary antibody (rabbit anti-mouse IgG antiblody coupled to Alexa-Fluor 488, 1/1000 dilution; Molecular Probes, Invitrogen, Life Technologies, Saint-Aubin, France) for 2 h at room temperature. Then DNA was stained with ethidium homodimer 2 (Molecular Probes; 1/2000 dilution) for 30 min. Finally, the oocytes/embryos were mounted on slides and observed under confocal microscopy (LSM710; Carl Zeiss, Göttingen, Germany). Oocytes were classified as in germinal vesicle (GV, diffuse or motted chromatin surrounded by a nucleus), MI or MII (chromosomes organized as a plate without and with an extruded polar body, respectively), or as degenerated when chromatin was absent or pyknotic, as previously described [27, 29].

Observation of spermatozoa in the genital tract

The uterine tips (3-cm long) and oviducts of 11 (7 treated and 4 control) inseminated bitches were collected between 6 and 44 h after the second artificial insemination (AI; 54-92 h post-ov) to look for motile spermatozoa. In 3 treated and 2 control bitches, uterine tips and oviducts (2 uterine and 2 oviduct samples/animal) were flushed then opened longitudinally and luminal epithelial cells were collected by scraping, then spread out on a slide. Smears and flushes were observed after centrifugation under light microscopy within 1 h of tissue collection for the presence and motility of spermatozoa. The location and morphology of spermatozoa present in the uterine tips were further studied in 4 treated and 2 control additional bitches (2 samples/animal) by scanning electron microscopy. After longitudinal opening of uterine tips, 1-cm long pieces of tissue were fixed in 2% glutaraldehyde solution in 0.1M sodium cacodylate for 12 h at room temperature and stored at 4°C until use. Dehydration and drying of samples was performed using the critical point method. Briefly, the fixed samples were dehydrated in a series of ethanol solutions of ascending concentrations, then ethanol was replaced with liquid CO_2, which was subsequently evaporated under 120-bar pressure at 38°C. The samples were coated with platinium in a vacuum vessel with 1.65 kV for 1-3 min, and observed under a field emission scanning electron microscopy (Hitachi S4500, Elexience, Verrières-le-Buisson, France).

Proteomic analysis of the tubal fluid

In order to identify proteins potentially involved in the observed oocyte phenotype, tubal fluids were collected from both oviducts of 8 control and 7 treated non inseminated bitches at Day +4 (90-110 h post-ov) by flushing. At that time point, oocytes from control bitches had resumed meiosis in the oviducts whereas oocytes from treated bitches were all still at the GV stage. After flushing with 100 µl of PBS, the COC were collected and the remaining fluids stored at −80°C until used. The oviductal fluids of control and treated groups were then pooled and 50 µg of proteins per pool were fractionated by SDS-PAGE in three bands and in-gel digested with

trypsin. Each peptide mixture was analyzed by nanoflow liquid chromatography tandem mass spectrometry (nanoLC-MS/MS) using a LTQ Orbitrap Velos Mass Spectrometer coupled with an Ultimate 3000 RSLC chromatographer (Thermo Fisher Scientific, Bremen, Germany) as previously described [30]. MS/MS ion searches were performed using Mascot search engine v 2.2 (Matrix Science, London, UK) via Proteome Discoverer 1.4 software (Thermo Fisher Scientific, Bremen, Germany) against a local database targeting mammalian taxons (292862 entries) extracted from NCBInr (10/22/14). The search parameters included trypsin as a protease with allowed two missed cleavages, carbamidomethylcysteine, methionine oxidation and acetylation of N-term protein as variable modifications. The tolerance of the ions was set at 5 ppm for parent and 0.8 Da for fragment ion matches. Mascot results were subjected to Scaffold 4 software (v 4.4, Proteome Software, Portland, USA). Peptide and protein identification was done by the Peptide and Protein Prophet algorithms [31] with a 95.0% probability. All proteins with more than two peptides identified were considered for protein quantification. For quantitative analysis, spectral count and Extracted Ion Chromatogram (XIC) extracted with Proteome Discoverer 1.4 software were performed. Scaffold 4 Q+ software (version 4.4, Proteome Software, Portland, USA) using spectral count and average precursor intensity quantitative modules were used. To eliminate quantitative ambiguity into protein groups, we ignored all the spectra matching any peptide which was shared between proteins. Thereby, quantification performed with normalized spectral counts or normalized XIC was carried out on distinct proteins. For differential proteins, only proteins common to the two quantitative methods presenting a P-value < 0.05 and a fold-change between treated and control groups > 2 were retained. The top 10 up- and down-regulated proteins were selected among differential proteins with more than five peptides identified and based on the highest XIC fold-changes.

Statistical analysis

Data are presented as means ± SEM. The F-test was used to examine the equality of variance and Student *t*-tests were used for comparison between groups of time intervals, numbers of corpora lutea, of oocytes, and for plasma P4 concentrations. Differences were considered significant when $P < 0.05$.

RESULTS

Ovulation and plasma steroid hormone concentrations

Treated animals ovulated on average 5.5 ± 0.3 days (range: 3-10 days) after the first administration of aglepristone. The time of ovulation relative to the time at which the treatment would have started was not different in the control group (5.1 ± 0.2 days; range: 3-8 days). The mean number of corpora lutea at the time of oocyte/embryo collection did not differ between groups (6.9 ± 0.4 and 6.8 ± 0.5 in treated and control groups, respectively). Plasma P4 concentrations were lower in treated than in control females at Days +3 and +4 (Figure 2). At Day+10, plasma P4 tended to be higher in treated animals compared with controls (P=0.054).

Kinetics of oocyte maturation in non inseminated females

A total of 197 and 91 oocytes were recovered and analyzed for determination of maturation stage between 53 and 344 h post-ov from non inseminated treated (n=35) and control (n=17) animals, respectively. The mean number of oocytes collected from treated (5.6 ± 0.5) and control (5.4 ± 0.7) females were similar. All oocytes were recovered from the oviducts and none from the uterine tips. Aglepristone treatment severely delayed the resumption of oocyte meiosis: the

first MI stages were observed at 76 h post-ov in control bitches *vs.* 173 h post-ov, i.e. 97 h later, in the treated group (Figure 3). Furthermore, aglepristone inhibited the progression of oocyte meiosis up to the MII stage: the proportion of oocytes at the MII stage reached 100% at 109 h post-ov (i.e. 33 h after observation of the first MI) in the control group whereas in treated animals, only 39% (29/75) of the collected oocytes reached the MII stage between the time point at which oocytes resumed meiosis (173 h post-ov) and the last time point when oocytes were observed (335 h post-ov). Some parthenogenetic embryos were also observed, alone or together with non activated oocytes, between 312 h and 344 h post-ov (Figure 3).

Morphology of post-ovulatory COC

Aglepristone treatment altered the morphology of ovulated COC and extended the post-ovulatory stay of oocytes within the oviducts. Until 100 h post-ov, oocytes from both groups were of similar morphology, surrounded by 2-3 layers of cumulus cells (Figure 4A,B). After 100-110 h (4.5 days) post-ov, empty zona pellucida and partially or totally denuded oocytes were recovered from control bitches, whereas oocytes retrieved from treated females were still surrounded by 2-3 compact layers of cumulus cells (Figure 4C,D). In treated bitches, cumulus cells started to be less numerous around oocytes after 220 h post-ov and only one empty zona pellucida was recovered at 335 h post-ov. After chromatin/microtubule staining and observation under confocal microscopy, oocytes harvested from control bitches showed clear signs of degeneration after 100-110 h post-ov (abnormal or absent chromatin, abnormal meiotic spindle; Figure 4E) whereas, until the end of the observation period, oocytes from treated bitches showed normal chromatin and microtubule morphology, in accordance with their stage of maturation (Figure 3F).

Presence of embryos and kinetics of oocyte maturation in inseminated females

A total of 64 oocytes and 58 embryos were recovered and analyzed for determination of maturation/devepmental stage between 90 and 178 h post-ov from the oviducts of inseminated treated (n=10) and control (n=9) bitches, respectively. No embryo was recovered from treated bitches, whatever the collection time point, whereas embryos at the expected stage of development [29] were collected from control bitches (Figure 3). Nevertheless, insemination seemed to accelerate the kinetics of oocyte maturation in treated bitches: first oocytes at the MI stage were observed at 147 h post-ov, that is 26 h earlier than in non inseminated treated bitches (Figure 3).

Location and motility of spermatozoa in inseminated females

In inseminated control females, tens of spermatozoa were found attached to the cumulus cells and zona pellucida of retrieved embryos, whereas no spermatozoa were observed around COC in the treated group. No spermatozoa were found in tubal flushes and smears from both groups, however, in flushes and smears from the uterine tips, tens of motile spermatozoa were observed in both treated and control inseminated animals. Observations of uterine tips under scanning electron micrsocopy confirmed the presence of spermatozoa with intact acrosome at the surface of uterine epithelial cells and within the uterine glands in both treated and control inseminated bitches (Figure 5).

Identification of changes induced by aglepristone in the proteome of tubal fluid

In order to identify proteins potentially involved in the delayed resumption of oocyte meiosis and in the prolonged lifespan of oocytes observed in treated animals, tubal fluids from both groups were subjected to mass spectrometry analysis at Day +4, i.e. at the time when all oocytes in control females had resumed meiosis whereas all oocytes in treated females were still at the GV stage. A total of 342 proteins were identified (Supplemental Table S1; Supplemental Data are available online at www.biolreprod.org), among which 79 proteins were differentially concentrated in the tubal fluid of treated *vs*. control bitches (fold-change > 2; $P < 0.05$;): 19 (24%) were upregulated and 60 (76%) downregulated by the aglepristone treatment (Supplemental Table S2). The list of the top up-regulated and down-regulated proteins is shown in Table 1.

DISCUSSION

The high P4 concentrations in circulating blood during the periovulatory period in the bitch raises questions regarding the roles of this hormone on both gamete maturation and fertilization. In this study on Beagle bitches, *in vivo* inhibition of P4 actions via administration of aglepristone severely delayed oocyte meiotic resumption, inhibited oocyte degeneration and prevented sperm transit across the UTJ. These results assign crucial roles to P4 in gamete maturation, viability and transport in dogs.

Although aglepristone treatment started on average 5.5 ± 0.3 days before ovulation, it did not prevent ovulation. In contrast, *Pr*-null mice [1] and mice treated with the PR antagonist mifepristone (RU 486) at the time of hCG-induced ovulation [32] totally failed to ovulate. Differences in ovarian responses to PR antagonists may be due to differences in the compounds and doses used as well as to the onset of the treatment relative to ovulation: mice treated with mifepristone one day after PMSG administration, i.e. one day before hCG treatment, showed only a decrease in the ovulation rate and those treated 2 days before hCG treatment ovulated normally [32]. Furthermore, the role of P4 in triggering ovulation appears to be species specific. In accordance, the *in vivo* administration of the PR antagonist onapristone before ovulation in ewes [33] or the inhibition of steroid synthesis in preovulatory follicles by trilostane in cows [34] did not affect ovulation.

Aglepristone as used in the present study had globally limited effects on circulating P4 levels, as reported earlier in Beagle bitches treated at the same dose during the early luteal phase [35]. Temporary lower circulating P4 concentrations were observed at Days +3 and +4 in treated compared to control bitches, suggesting a slight anti-luteotropic action of aglepristone. Administration of aglepristone at the same regiment but starting in the mid-luteal phase (29 and 30 days after the LH peak) was reported to induce early luteal regression in non pregnant bitches [36]. Thus, a lack of autocrine supportive activity of P4 in the corpus luteum seem less critical in the early than mid-luteal phase in the bitch.

Oocytes and parthenogenetic embryos collected in this study were all recovered from the oviducts. This oviductal stay was particularly long in treated animals, in which oocytes were collected up to 344 h (14.3 days) post-ov. In inseminated bitches, the entry of embryos into the uterine horns has been well described: it starts at 204-216 h (8.5-9 days) post-ov, at the morula or early blastocyst stage [37]. The hormone P4 is one major regulator of the contractions in the oviductal muscular layer and of ciliary beating in the oviductal epithelium, both implicated in oocyte and embryo transport [3]. The first possible hypothesis to explain why oocytes in treated females stayed in oviducts for more than 14 days is that aglepristone prevented or at least markedly slowed their tubal transport toward the uterus. In contrast, a treatment with

mifepristone was reported to accelerate the flow of oocytes through the oviducts in cycling rats [38]. The second hypothesis is that some unknown secreted factor(s) originating from canine embryos are needed to cross the UTJ toward the uterine horn, as described for the horse embryo [39]. Oocytes and non viable parthenogenetic embryos would thus remain trapped in the caudal oviduct because of the absence of these embryonic factors.

Aglepristone treatment also delayed the resumption of meiosis in ovulated oocytes and inhibited their progression toward the MII stage. The role of P4 in promoting oocyte maturation is still under debate in mammals. The pre-ovulatory *in vivo* administration of mifepristone in mice [40] and human [41] or of onapristone in ewes [33] did not affect oocyte maturation. Furthermore, *in vitro* addition of the PR agonist R5020 did not stimulate the resumption of meiosis in mouse and rat follicle-enclosed oocytes or COC, and accordingly, addition of the PR antagonists mifepristone and Org31710 did not prevent oocyte meiosis resumption in these models [42]. However, other studies in mice demonstrated that P4 promoted oocyte maturation, whether in preovulatory follicles or in isolated COC *in vitro* [43, 44]. This promoting role of P4 was also suggested by *in vitro* studies in other mammals. Treatment of COC with mifepristone impaired oocyte maturation and cumulus cell expansion in the bovine [4] and porcine [6]. In porcine, P4 produced by cumulus cells has been shown to play a positive role in oocyte meiosis resumption [5]. In dogs, the expression of PR is upregulated in the COC just before ovulation [20], suggesting important local actions of P4 in the post-ovulatory period. However, *in vitro* studies that tested the effect of P4 on canine oocyte maturation gave contradictory results [21-24]. This may be due to inappropriate *in vitro* conditions; these conditions remain to be optimized in this species [12]. Noteworthy, most of these studies used ovaries from anestrous or metestrous bitches, in which oocytes are known to be of low competence for meiosis resumption [45]. Nevertheless, the present results suggest at least three possible non exclusive mechanisms of actions of P4 on oocyte maturation: a direct promoting action of P4 on the oocyte, an action of P4 on cumulus cells that in turn modulates oocyte meiosis resumption, and/or the P4-induced secretion of oviductal factors that promote oocyte maturation.

In the treated group, a delayed loss of attached cumulus cells was observed in parallel with the delayed resumption of meiosis in oocytes. Cumulus cells in dogs, as in other mammals, were reported to respond to the ovulatory LH surge with cumulus expansion, this ability being acquired at a follicular diameter of minimum 4–4.5 mm [27]. It is however a peculiarity of the dog that the 2-3 innermost layers of cumulus cells remain in close contact with the post-ovulatory oocyte and start to be shed concomitantly with oocyte meiosis resumption, around 72 h after ovulation [12]. The aglepristone-induced delay in oocyte meiosis resumption may thus be linked to the delayed loss of the cumulus cells around oocytes. The exact mechanisms of action of P4 in promoting cumulus expansion and meiosis resumption in the dog remain to be elucidated.

The post-ovulatory viability of oocytes is particularly long in dogs compared to other mammals: oocytes become fertilizable once they have reached the MII stage, 2-3 days after ovulation [12]. However, if fertilization does not occur within that time and based on conception rates, oocytes may remain fertile up to 8 days post-ovulation, although with decreased litter size [13]. In the present study, the absence of degeneration of oocytes was assessed based on chromatin and microtubule morphology, two well-established criteria for classification of degenerated oocytes in this species [27, 45, 46]. The time period over which morphologically normal oocytes were observed in aglepristone-treated bitches was very long, lasting at least 14 days (335 h), which was the last time point at which oocytes were retrieved. It is however not

known whether oocytes at the MII stage among those collected up to 14 days post-ov were fertilizable and able to sustain embryo development. Since no medium has yet been developed to obtain *in vitro* fertilization and embryo development in the dog [12], this question remains unanswered. The factors responsible for the long viability of canine oocytes after ovulation are largely unknown. It is not clear whether the delayed loss of cumulus cells in the treated group was linked to the delayed oocyte degeneration, and if so, whether it was the cause or a consequence of it. The proportion of degenerated canine oocytes was either increased [46] or decreased [47] by spontaneous denudation *in vitro*, while transferred oocytes within the oviducts of recipient bitches showed a high rate of degeneration in parallel with denudation [45]. In the mouse, cumulus cells were shown to accelerate oocyte aging of both *in vivo* and *in vitro* matured oocytes [48]. To our knowledge, this is the first report showing a possible negative role of P4 in postovulatory oocyte lifespan in mammals. In contrast, in rhesus monkeys, steroid depletion (by an *in vivo* treatment with trilostane) increased the percentage of degenerating oocytes recovered 12 h post-hCG, while progestin replacement prevented oocyte degeneration [49]. Nevertheless, the follicular environment in which primate oocytes aged in that study was barely comparable to the oviductal fluid in which canine oocytes and those from other mammals age under physiological conditions.

Aglepristone in the present study also prevented fertilization, probably due a sperm arrest before the UTJ. Indeed, numerous spermatozoa were observed attached to the cumulus cells and/or zona pellucida of embryos in control inseminated bitches, which demonstrates that spermatozoa were able to reach the oviducts in that group. In accordance with previous reports [14, 15], numerous spermatozoa were also observed in the cranial uterine glands, which represent the uterine sperm reservoir in this species. In contrast, the absence of spermatozoa around oocytes and their presence in large numbers in uterine tips in inseminated treated bitches suggest that aglepristone prevented spermatozoa to detach from the uterine sperm reservoir and/or to cross the UTJ toward the oviducts. In a recent study, follicular fluids collected from large antral follicles (in which the P4 concentration was high) and from post-ov but not pre-ov oviductal flushes were shown to induce the detachment of canine spermatozoa from uterine epithelial cells *in vitro* [50]. Thus, in dogs, P4 seems to play a crucial role in the release of spermatozoa from the sperm reservoir, as previously reported in pigs [7] and birds [51]. Alternatively, it could be that some spermatozoa were able to detach from the uterine reservoir but unable to cross the UTJ, possibly due to a reduced motility and/or to an inability to interact with the UTJ epithelium. Indeed, the mammalian UTJ was reported to be a functional barrier, that only spermatozoa with normal motility and surface molecular integrity are able to pass through [52]. Furthermore, the systemic level of P4 has been shown to modulate the availability of ions, amino acids and energy substrates in the uterine lumen in mammals [53-55]. Aglepristone may thus have modified the uterine environment in a way that decreased sperm ability to cross the UTJ. Similarly, onapristone administrated before ovulation in inseminated ewes severely impaired fertilization due to sperm inability to progress across the cervix [33]. In contrast, mice treated with mifepristone concomitantly with hCG and the day after coitus had unchanged numbers of two-cell embryos compared with untreated controls [40], suggesting that P4 has species-specific effects on sperm progression and fertilization. Nevertheless, our results suggest a potential application in contraception for dogs, although such an application would require a delivery system avoiding repeated administrations.

Aglepristone altered the level of numerous proteins in the oviductal fluid of treated bitches, which is not surprising since systemic P4 was reported to modulate the composition of bovine

tubal fluid in energy substrates, ions and amino acids [53]. In dogs, the oviduct epithelium was shown to express PR at high levels in epithelial secretory cells from the Pre-LH stage to Day 7 post-ov [26]. The proteomic analysis in this study brings new insights into the regulatory role of P4 on oviductal functions. The vast majority (76%) of the differential proteins at Day +4 were down-regulated by aglepristone; this is in accordance with a recent transcriptomic study comparing *Pr*–/– with *Pr*+/– mouse oviductal cells, in which 83% of the differentially expressed genes were down-regulated [56]. Furthermore, 9 down-regulated proteins in the present study were among the down-regulated genes in *Pr*-null mice (adenosylhomocysteinase-like, aldehyde dehydrogenase 1 family member L1, transgelin, sulfotransferase 1A1, alpha-N-acetylglucosaminidase, chaperonin containing Tcp1 subunit 5, metalloproteinase inhibitor 2, myosin-11-like isoform 1 and ribosomal protein S8) [56]. However, 2 up-regulated proteins in the present study belonged to the down-regulated genes in *Pr*-null mice (transforming growth factor-beta-induced protein, SPARC-like protein 1) and the majority of down-regulated proteins in aglepristone-treated bitches were not among down-regulated genes in *Pr*-null mice, pointing out probable species-specific PR-targets in the oviduct and/or post-transcriptional modifications of these target genes.

The differential proteins evidenced in the present study are assumed to play a role in the delayed meiosis resumption and absence of degeneration of oocytes observed after ovulation in treated bitches. Consistent with this hypothesis, lactoferrin, up-regulated here by aglepristone, was recently reported to interact with post-ovulatory oocytes in humans [57] and was shown to be an effective antioxidant for porcine oocytes [58]. However, in the latter study, lactoferrin increased the maturation rates of porcine oocytes [58], which is not consistent with our results. Clusterin, another protein among the top-ten up-regulated in our treated bitches, was reported to have an anti-apoptotic action on granulosa cells [59] and may be involved in the prolonged lifespan of oocytes in the treated group. Clusterin was also reported to play a role in oocyte meiosis resumption in the bovine [60]. Further investigations on the differential secreted proteins appear desirable to determine which interact with post-ovulatory canine oocytes and may regulate oocyte maturation and aging. In addition, the different stages of maturation reached by oocytes at Day+4 in control and treated groups may have contributed to the modification of the tubal fluid composition. Indeed, gametes have been reported to modulate the oviductal environment in porcine [61].

In conclusion, the results of this study highlight some key roles played by P4 in canine oocyte meiosis resumption and aging, as well as in sperm transit toward the site of fertilization. Further studies are now needed to determine the exact mechanisms of action of P4, by direct actions on gametes and by changes in the uterine and tubal environments.

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FIGURE LEGENDS

Figure 1. Time period of ovaries/oviducts collection, numbers of animals and types of analysis made in each group of animals after ovulation (OV). AI, artificial insemination.

Figure 2. Plasma P4 concentrations in treated and control bitches from Day −10 to Day +10 relative to ovulation. The number of animals per day is indicated in brackets (treated/controls). *$P < 0.05$.

Figure 3. Kinetics of oocyte maturation and embryo development relative to ovulation. Each vertical line represents one time point of oocyte/embryo collection. Aglepristone delayed oocyte meiosis resumption: first metaphase I (MI) stages were observed at 76 h post-ov (left arrow) in non inseminated control bitches (white circles, n=91 oocytes) *vs.* at 173 h post-ov (right arrow) in non inseminated treated bitches (grey circles, n=197 oocytes). The treatment prevented

fertilization: embryos at various developmental stages were observed in inseminated control bitches (white triangles, n=58 embryos) whereas only oocytes were retrieved from inseminated treated bitches (grey triangles, n=64 oocytes). GV, germinal vesicle stage; M I/II, metaphase I/II stage; 2 PN, embryos at the 2-pronuclei stage; 2-4 C, embryos at the 2- or 4-cell stages; 8C-Mo, embryos at the 8-cell or morula stages.

Figure 4. Morphology of COC and oocytes. Up to 100 h post-ov, compact COC with 2-3 layers of cumulus cells were recovered from both control (**A**, at 94 h post-ov) and treated (**B**, at 97 h post-ov) bitches. After 100 h, partially denuded and empty zona pellucida were observed in control bitches (**C**, at 168 h post-ov) but not in treated ones (**D**, at 188 h post-ov). Under confocal microscopy, chromatin (in red) and microtubule (in green) staining revealed signs of oocyte degeneration in control bitches after 100-110 h post-ov (**E**, abnormal meiotic spindle at 168 h post-ov) whereas oocytes from treated bitches displayed normal chromatin and microtubule patterns (**F**, metaphase I at 273 h post-ov). Inset in **F** is a focus on the metaphase plate. Bars = 100 µm (**A, B, C, D**) and 50 µm (**E, F**).

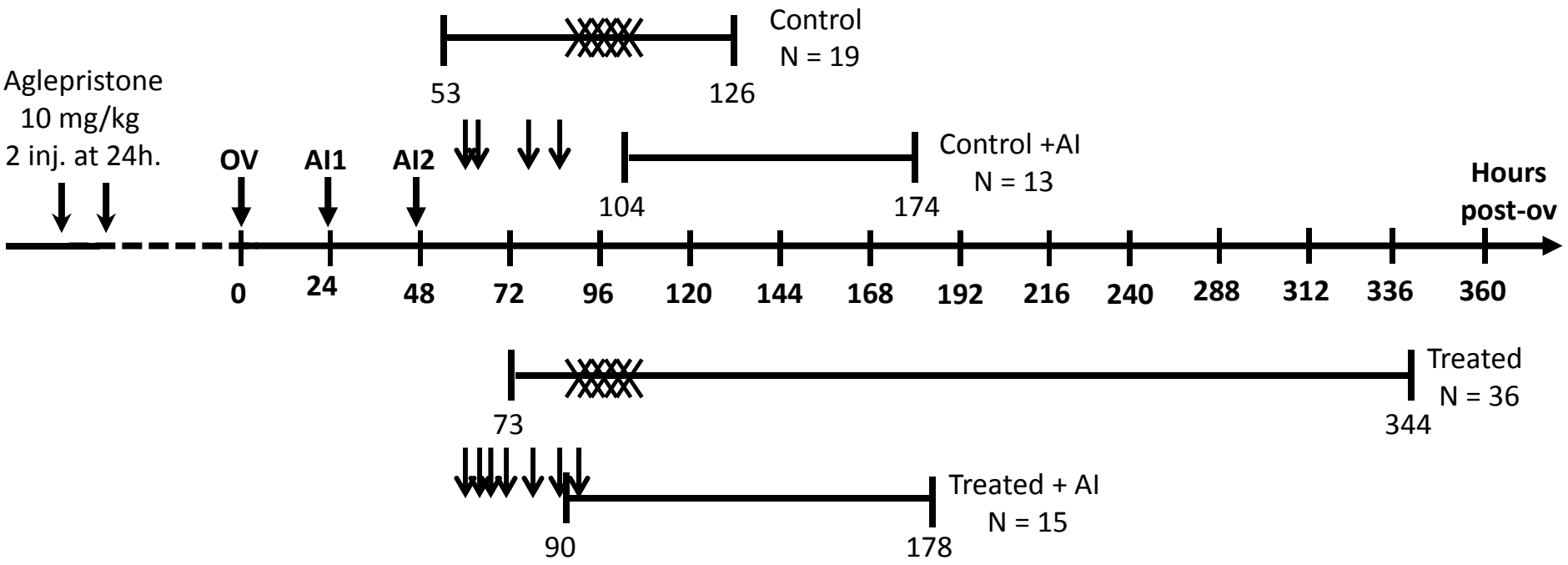
Figure 5. Location and morphology of spermatozoa under scanning electron microscopy. Numerous spermatozoa with intact acrosome were observed in uterine tips, some of them being nestled within the uterine glands (**A**, treated female at 42 h post-AI). Spermatozoon with intact acrosome (**B**). Bar = 30 µm.

TABLE 1. Top 10 up- and down-regulated proteins in the tubal fluid in treated bitches as compared with control animals at Day +4 (see all differential proteins in Supplemental Table S2).

Upregulated proteins		Downregulated proteins	
Name of the protein (gene symbol)	Fold-change	Name of the protein (gene symbol)	Fold-change
Lactoferrin (*LTF*)	*	Carbonic anhydrase 1 (*CA1*)	**
Folate receptor beta isoform 1 (*FOLR2*)	18	Tryptophan-tRNA ligase, cytoplasmic isoform 1 (*WARS*)	**
Mucin-1 precursor (*MUC1*)	7.2	Vimentin (*VIM*)	10
Pantetheinase (*VNN1*)	5.8	Filamin-B isoform 1 (*FLNB*)	10
Clusterin (*CLU*)	4.9	Hemoglobin subunit beta-like (*HBB*)	5
Transforming growth factor-beta-induced protein (*TGFBI*)	3.2	Aldehyde dehydrogenase 1 family, member L1 (*ALDH1L1*)	5
CD109 antigen isoform 3 (*CD109*)	2.9	Rab GDP dissociation inhibitor alpha isoform 1 (*GDI1*)	5
Plastin-3 (*PLS3*)	2.4	Apolipoprotein A-IV (*APOA4*)	5
Ceruloplasmin (*CP*)	2.2	Protein-arginine deiminase type-2 (*PADI2*)	5
SPARC-like protein 1 (*SPARCL1*)	2.2	Glutathione S-transferase Mu 3 isoform 1 (*GSTM3*)	3.3

*Proteins detected only in the treated group.
**Proteins detected only in the control group.

Figure 1



Collection of oocytes/embryos for the determination of maturation stages

Collection of oviductal fluids for proteomics

Collection of oviducts and uterine tips for the localization of spermatozoa

Figure 2

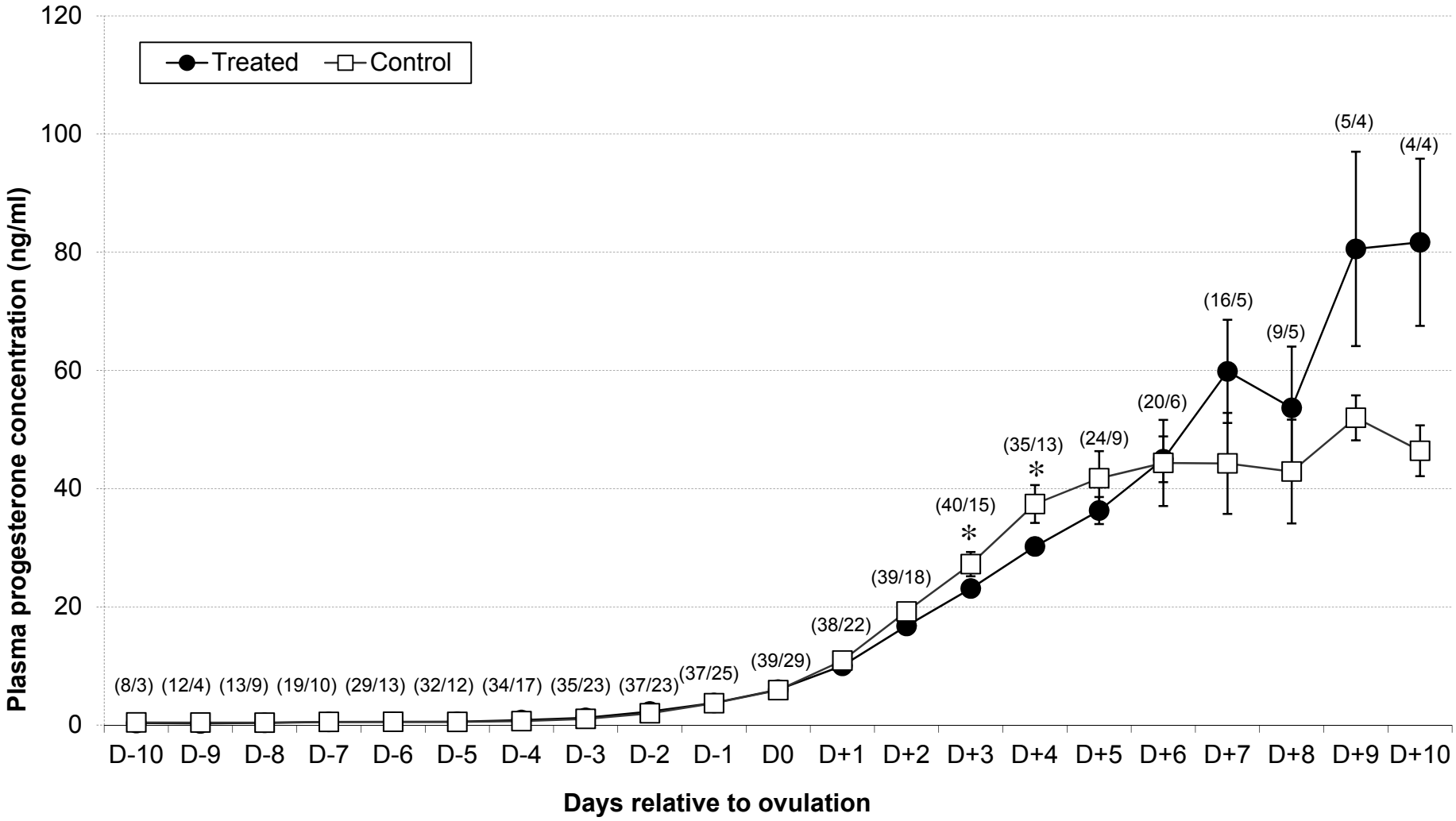

Treated
Control
Plasma progesterone concentration (ng/ml)
120
100
80
60
40
20
0
(8/3)
(12/4)
(13/9)
(19/10)
(29/13)
(32/12)
(34/17)
(35/23)
(37/23)
(37/25)
(39/29)
(38/22)
(39/18)
(40/15)
(35/13)
(24/9)
(20/6)
(16/5)
(9/5)
(5/4)
(4/4)
*
*
D-10
D-9
D-8
D-7
D-6
D-5
D-4
D-3
D-2
D-1
D0
D+1
D+2
D+3
D+4
D+5
D+6
D+7
D+8
D+9
D+10
Days relative to ovulation

Figure 3

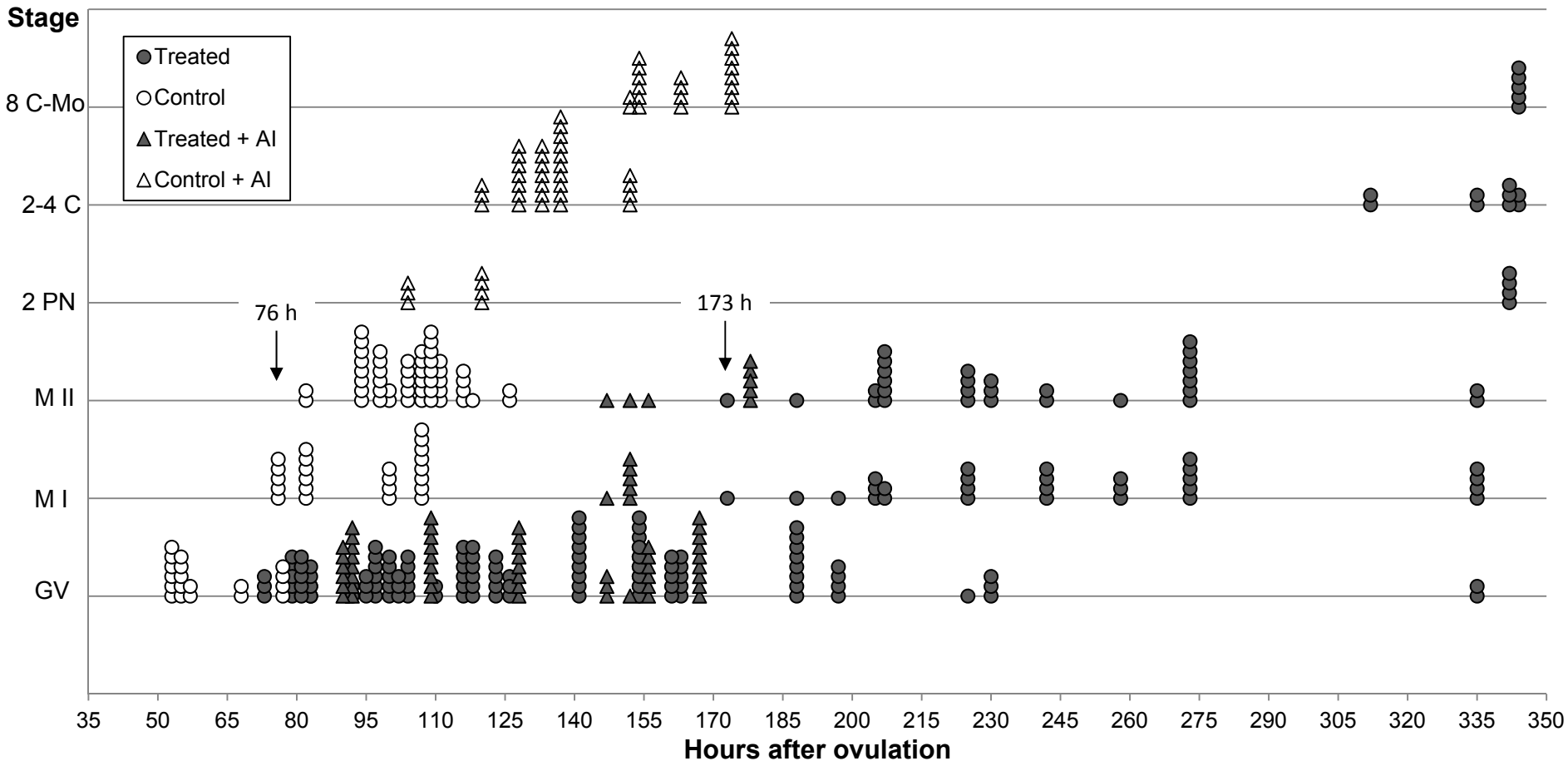

Stage
Treated
Control
Treated + AI
Control + AI
8 C-Mo
2-4 C
2 PN
M II
M I
GV
76 h
173 h
35
50
65
80
95
110
125
140
155
170
185
200
215
230
245
260
275
290
305
320
335
350
Hours after ovulation

Figure 4

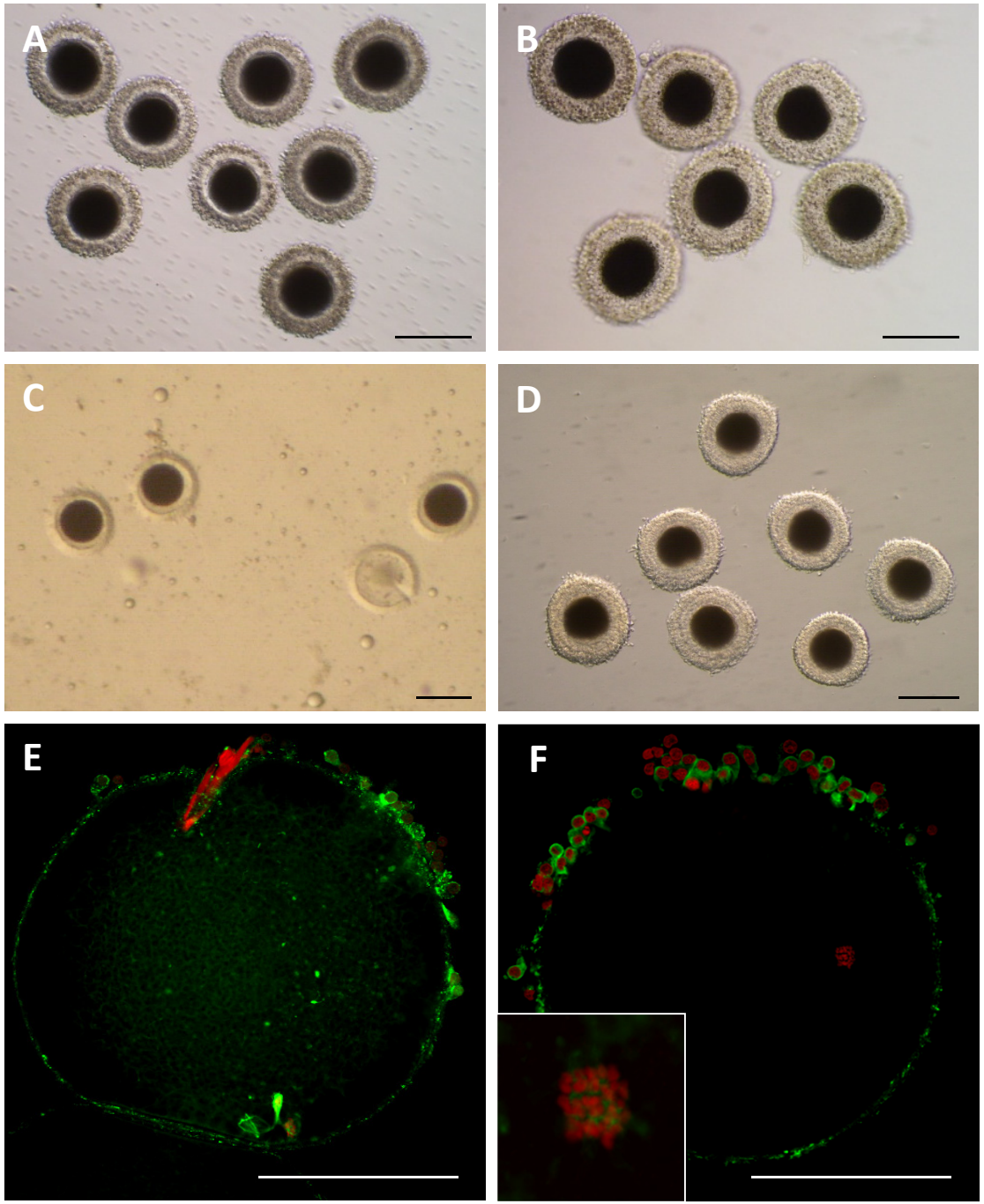


Figure 5