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A prematuration approach to equine IVM: considering cumulus morphology, seasonality, follicle of origin, gap junction coupling and large-scale chromatin configuration in the germinal vesicle

Valentina Lodde [A] , Silvia Colleoni [B] , Irene Tessaro [A] , Davide Corbani [A] , Giovanna Lazzari [B C] , Alberto M. Luciano [A] , Cesare Galli [B C] and Federica Franciosi [A D]

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In the horse, the array of successful fertility interventions, pivotal to expanding the offspring pool from mares of high genetic merit, is limited. Therefore it is essential that the available treatments are optimized. This study describes an in-depth characterization of functional parameters of equine female gametes that can be used to improve the success of 'test-tube foal' making.

27 **TITLE: A prematuration approach to equine IVM: considering cumulus
28 morphology, seasonality, follicle of origin, gap junction coupling and large-
29 scale chromatin configuration in the germinal vesicle**
30
31 **AUTHORS**: Lodde V[1], Colleoni S[2], Tessaro I[1], Corbani D[1], Lazzari G[2,3], Luciano AM[1],
32 Galli C[2,3], Franciosi F[1]*
33
34 [1]Dipartimento di Scienze Veterinarie per la Salute la Produzione Animale e la
35 Sicurezza Alimentare 'Carlo Cantoni', Reproductive and Developmental Biology
36 Lab, Università degli Studi di Milano, Milano, Italy
37 [2]Laboratory of Reproductive Technologies, Avantea, Cremona, Italy
38 [3]Fondazione Avantea, Cremona, Italy
39
40 *corresponding author:
41 Federica Franciosi
42 Dipartimento di Scienze Veterinarie per la Salute la Produzione Animale e la
43 Sicurezza Alimentare 'Carlo Cantoni'
44 Reproductive and Developmental Biology Lab – ReDBioLab
45 Università degli Studi di Milano
46 via Celoria, 10
47 20133 – Milano
48 ITALIA
49 email: federica.franciosi1@unimi.it
50
51 **RUNNING TITLE**: Horse oocyte prematuration and embryo development
52
53

54 **ABSTRACT**

55 Several studies report that a two-step culture where mammalian oocytes are first
56 kept in meiosis arresting conditions (prematuration) followed by in vitro
57 maturation (IVM) is beneficial to embryo development. The most promising
58 results were obtained by stratifying the oocyte population using morphological
59 criteria and allocating them to different culture conditions to best meet their
60 metabolic needs.

61 In this study horse oocytes are characterized to identify subpopulations that might
62 benefit from prematuration. We investigated gap-junction (GJ) coupling, large-
63 scale chromatin configuration and meiotic competence in compact and expanded
64 cumulus-oocyte complexes (COCs) according to follicle size (<1 cm, 1-2 cm, > 2cm)
65 and season. Then we tested the effect of cilostamide-based prematuration in
66 compact COCs collected from follicles <1 cm and 1-2 cm on embryo development.
67 We observed that meiotic competence was not affected by prematuration, while
68 COCs from follicles 1-2 cm yielded embryos with a higher number of
69 cells/blastocyst than oocytes that underwent direct IVM ($P<0.01$, unpaired Mann-
70 Whitney test), suggesting improved developmental competence. Oocytes collected
71 from follicles <1 cm were apparently not affected.

72 This study represents an extensive characterization of the functional properties of
73 immature horse oocytes and the first report of cilostamide-based prematuration
74 applied to horse oocyte IVM with embryo development as outcome.

75

76 **Additional key words:**

77 assisted reproduction, blastocyst, oocyte maturation, pre-maturation,
78 developmental competence, intracytoplasmic sperm injection (ICSI)

79

80

81

82 **INTRODUCTION**

83 The capacity of mammalian oocytes to resume meiosis once isolated from antral
84 follicles has been widely exploited to obtain fertilizable oocytes without the need
85 for hormonal stimulation through *in vitro* maturation (IVM). This technique is also
86 of great interest for genetic salvage of valuable animals post-mortem. However,
87 while these oocytes undergo nuclear maturation, they do not attain full
88 cytoplasmic maturity, defined as a series of structural and biochemical changes
89 required for successful fertilization and early embryo development (Eppig 1996).
90 Some of these changes occur *in vivo* when follicles are selected for dominance and
91 in the dominant follicle before the LH surge, and have been respectively referred to
92 as 'prematuration' (Hendriksen *et al.* 2000) and 'oocyte capacitation' (Hyttel *et al.*
93 1997). Oocytes collected before undergoing these late differentiation steps are less
94 developmentally competent, even when capable of resuming and completing
95 meiosis I, and undergoing the first rounds of embryonic mitosis (Dieleman *et al.*
96 2002).
97 As a consequence of these observations, starting from the late '90s, several
98 attempts were made to mimic *in vivo* prematuration by culturing COCs in meiotic
99 blocking conditions before IVM. This two-step cultural approach was named *in*
100 *vitro* prematuration (Mermillod *et al.* 1996; Mermillod and Marchal 1999) or pre-
101 IVM.
102
103 Thanks to the elucidation of key molecular mechanisms regulating meiosis
104 inhibition and restart (as reviewed in: (Motlik and Kubelka 1990; Conti *et al.* 1998;
105 Conti *et al.* 2012)), two major prematuration strategies were devised: inhibition of
106 the cyclin-dependent kinase (CDK) responsible for the catalytic activity of the M-
107 phase promoting factor (MPF); or control of phosphodiesterases (PDEs)
108 responsible for maintaining meiosis-inhibiting levels of intra-oocyte cyclic
109 nucleotides, thus targeting mechanisms upstream of MPF activation (Gilchrist *et al.*
110 2016).
111 Most of the reports using butirolactone I or roscovitine as a means of preventing
112 MPF activation gave neutral effects on the subsequent embryo yield, in cows
113 (Mermillod *et al.* 2000; Ponderato *et al.* 2001; Ponderato *et al.* 2002; Adona *et al.*
114 2008b; Adona *et al.* 2008a; Ferreira *et al.* 2009; Sa Barretto *et al.* 2011; Guemra *et*

115 *al.* 2014), horses (Choi *et al.* 2006a; Choi *et al.* 2007) and pigs (Garcia-Rosello *et al.*
116 2006), while positive effects were limited to one study in buffalo (Pandey *et al.*
117 2018) and one in horse oocytes (Franz *et al.* 2003). On the other hand, numerous
118 studies targeting the level of cyclic nucleotides, either through pharmacological
119 compounds or natriuretic peptides naturally present in the follicular fluid, attained
120 higher blastocyst yields compared to direct IVM. For instance increased blastocyst
121 rates were observed in cows (Albuz *et al.* 2010; Huang *et al.* 2013; Huang *et al.*
122 2014; Huang *et al.* 2016; Li *et al.* 2016a; Li *et al.* 2016b; Abdel-Ghani *et al.* 2018;
123 Sugimura *et al.* 2018; Soto-Heras *et al.* 2019), humans (Sanchez *et al.* 2017), mice
124 (Vanhoutte *et al.* 2009a; Vanhoutte *et al.* 2009b; Albuz *et al.* 2010; Zeng *et al.* 2013;
125 Zeng *et al.* 2014; Romero *et al.* 2016; Santiquet *et al.* 2017), pigs (Park *et al.* 2016),
126 sheep (Zhang *et al.* 2015; Azari-Dolatabad *et al.* 2016) and yak (Xiong *et al.* 2017).
127 Notably, in mice higher implantation rate (Albuz *et al.* 2010; Santiquet *et al.* 2017)
128 and fetal yield were also obtained (Albuz *et al.* 2010).
129
130 The above survey of the scientific literature seems to suggest that targeting cyclic
131 nucleotide content is the most promising strategy in prematuration set-up, likely
132 due to the beneficial effects of sustained cAMP levels, as reviewed by (Gilchrist *et*
133 *al.* 2016). However, another emerging consideration when applying prematuration
134 is the heterogeneity of the population of retrieved oocytes. In the absence of
135 treatments that synchronize follicular growth, oocytes retrieved from antral
136 follicles have reached dissimilar stages of differentiation, (Hendriksen *et al.* 2000)
137 with some still accomplishing the final steps of the growth phase, some having
138 reached an optimal differentiation stage, and others undergoing early stages of
139 atresia and degeneration. With this in mind, some research groups proposed that
140 prematuration might be beneficial to a subclass of 'antral' oocytes while
141 detrimental to others, in relation to the differentiation step that they have achieved
142 *in vivo*.
143 Proofs of concept of this hypothesis were independently provided by using
144 customized prematuration approaches in cows (Dieci *et al.* 2016), pigs (Zhang *et*
145 *al.* 2017), and sheep (Azari-Dolatabad *et al.* 2016). Even though different
146 parameters were applied by the three research groups, a stratification based on
147 the metabolic/differentiation stage of 'antral' oocytes was successful in identifying

148 subclasses of gametes that benefited from prematuration. Specifically, gametes at
149 earlier stages of differentiation gained a higher developmental competence when
150 treated with prematuration. Conversely, oocytes that had already reached more
151 advanced stages of differentiation were negatively affected. Essential to this type
152 of approach was an in-depth characterization of the population of oocytes
153 retrieved from the antral follicle pool, in order to obtain information that might be
154 integrated into a decision-making process.
155
156 In the present study we characterized the population of COCs collected from antral
157 follicles of abattoir-derived horse ovaries, taking into consideration season, follicle
158 diameter, morphology of the cumulus cells, gap junction (GJ)-mediated coupling
159 between the cumulus cells and the oocyte, and large-scale configuration of the
160 chromatin in the germinal vesicle (GV). Using these parameters we identified
161 subclasses of COCs that might benefit from prematuration and cultured them in
162 cilostamide, a selective PDE3 inhibitor, before switching to IVM. The
163 developmental competence of oocytes undergoing prematuration was tested by
164 intracytoplasmic sperm injection (ICSI) followed by embryo culture to the
165 blastocyst stage. Oocytes from the same subclass submitted to 'direct IVM' were
166 used as controls.
167
168 Notably, this study represents the first report of customized prematuration
169 treatment in the horse, using a cilostamide-induced meiotic arrest before IVM, and
170 shows that this approach might improve the developmental competence of defined
171 categories of equine oocytes.
172
173

174 **MATERIALS AND METHODS**

175 Unless otherwise stated, all the consumables were purchased from Sigma-Aldrich,
176 Milan, Italy.

177

178 **Collection of cumulus-oocyte complexes (COCs)**

179 Ovaries were collected at the local slaughterhouse during the breeding season and
180 the transitional periods that precede and follow it, leaving 1-2 months of interval
181 between the different periods considered. Specifically, collections were carried out
182 in the Winter (January-February) for the first annual transition, in the Spring-
183 Summer (April to July) for the reproductive season, and in the Fall (October-
184 November) for the second annual transition (Dell'Aquila *et al.* 2008).
185 Ovaries were transported in 26°C 0.9% saline to the lab within 3 hrs. Upon
186 removal of the tunica albuginea, follicles were measured with a caliper,
187 individually opened with a razor blade and the inner follicular wall was washed
188 several times with phosphate buffer saline (PBS) supplemented with Pen/Strep
189 solution and carefully scraped, as recommended in (Alm *et al.* 1997). The follicular
190 fluid and scraped cells were collected in dishes according to the follicle size: <1 cm,
191 1-2 cm, >2 cm.
192 Dishes were examined under a dissection microscope for COC retrieval. Only COCs
193 with complete cumulus investment, intact zona pellucida and oolemma were used
194 in downstream procedures. COCs were classified according to the morphology of
195 the outer layer of cumulus cells in compact (Cp) when showing no signs of
196 expansion, or expanded (Ex) if the outermost layers of cumulus cells were affected
197 by any degree of expansion, as previously described (Hinrichs and Schmidt 2000).

198

199 **Lucifer Yellow dye injection and assessment of the GJ-mediated coupling**
200 **between the cumulus cells and the oocyte**

201 GJ-mediated coupling between the cumulus cells and the oocyte was assessed by
202 injecting the GJ-permeable fluorescent dye Lucifer Yellow (LY - 3% in 5 mM
203 solution of lithium chloride) into the oocytes still enclosed within the cumulus
204 cells, as previously described (Colleoni *et al.* 2004).
205 COCs were incubated 15 mins in HEPES supplemented synthetic oviductal fluid (H-
206 SOF) at 38.5°C, before monitoring LY diffusion. GJ were classified as open if LY

207 signal was detectable in basically all the cells surrounding the oocytes, partially
208 open if LY reached approx. half of the cumulus cells, or closed if LY remained
209 confined to the ooplasm or reached only a few cumulus cells.
210 For the purpose of injecting and classifying Ex COCs, part of the expanded mass
211 was mechanically removed to allow the injection, and only diffusion to the
212 innermost layers of cumulus cells was considered.
213
214 **Nuclear staining and classification of chromatin configuration**
215 Cumulus cells were removed by pipetting and the oocytes were fixed in 4%
216 paraformaldehyde (PFA), washed in PBS plus 0.1% polyvinyl-alcohol (PVA),
217 permeabilized with Triton-X 100 0.1% and mounted in Vectashield containing 4',6-
218 Diamidine-2-phenylindole dihydrochloride (DAPI) (Vector Laboratories, Inc.,
219 Burlingame, CA, USA).
220 As previously described by (Luciano *et al.* 2006), oocytes were classified as being
221 in Prophase I (or GV) when the chromatin was still enclosed within the GV; Pro-
222 metaphase I (pro-MI) when the GV envelope was dissolved and the chromatin
223 started to compact into chromosomes; Metaphase I (MI) when chromosomes were
224 well defined and aligned but a polar body was not yet visible; Anaphase I (AI)
225 when the two sets of aligned chromosomes started to separate; Telophase I (TI)
226 when two sets of chromosomes were well segregated, usually more condensed
227 than the meiotic plate configuration; Metaphase II (MII) when a set of
228 chromosomes was well defined and aligned, while the other set was segregated
229 into a polar body (PB); degenerated (deg) when none of the above configuration
230 was recognizable, chromatin was randomly dispersed in the cytoplasm, or not
231 visible at all, or the cytoplasm was fragmented or shrunken.
232 Prophase I oocytes were further classified according to the large-scale
233 configuration of the chromatin within the GV into Fibrillar, Intermediate and
234 Condensed, according to the description given by (Franciosi *et al.* 2012).
235
236 **Oocyte and embryo culture**
237 After retrieval COCs were washed in H-SOF and either cultured in prematuration
238 or IVM conditions.

239 For prematuration, COCs were cultured in DMEM-F12 based medium
240 supplemented with 10% Serum Replacement, insulin, transferrin and sodium
241 selenite (ITS), sodium pyruvate, $1x10^{-4}$ IU/ml follicle stimulating hormone (FSH,
242 Serono, Merck Inc., Taguig City, Philippines) and 10 μM cilostamide at 38.5°C in
243 5% CO2 for 6 hrs. The ideal window of cilostamide exposure was preliminarily
244 investigated by monitoring its effect at different time points between 0-16 hrs.
245 For IVM, COCs were cultured in DMEM-F12 based medium supplemented with
246 10% Serum Replacement, insulin, transferrin and sodium selenite (ITS), sodium
247 pyruvate, 0.1 IU/ml follicle stimulating hormone (FSH) and 50 ng/ml epidermal
248 growth factor (EGF) at 38.5°C in 5% CO2 for 24 hrs.
249
250 At the end of IVM, cumulus cells were removed by a brief incubation in H-SOF
251 supplemented with 25 μg/ml hyaluronidase followed by 2 mins incubation in H-
252 SOF supplemented with 2.5 mg/ml trypsin. Oocytes were then transferred in H-
253 SOF supplemented with serum to inactivate trypsin and examined for PB
254 extrusion. Oocytes that had extruded the PB were returned to the IVM medium
255 until ICSI was performed, as described by (Galli *et al.* 2014).
256 Briefly, frozen-thawed semen of a stallion of proven fertility was centrifuged at
257 750 g for 40 mins on a 90-45% Redigrad (Amersham Bioscience Inc, Piscataway,
258 NJ, USA) gradient. The pellet was recovered and washed by centrifugation 400g for
259 10 mins in TALP Ca^{2+} free. The washed pellet was suspended in SOF medium
260 supplemented with BSA, MEM amino acids and sperm motility factors (heparin,
261 epinephrine, penicillamine and hypotaurine) (SOF-IVF medium) (Lazzari *et al.*
262 2002). Before ICSI, the sperm suspension was diluted 1:1 with a
263 polyvinylpyrrolidone (PVP) solution (12% in H-SOF).
264 ICSI was performed using a Piezo-driven micropipette (Prime Tech, Japan). Briefly,
265 motile spermatozoa were immobilized by Piezo pulses and injected into oocytes
266 held by a holding pipette with the polar body oriented either to the 6 or 12 o'clock
267 position.
268 Injected oocytes were cultured in SOF medium supplemented with MEM amino
269 acids and 4 mg/ml of BSA (SOF-BSA-AA) (Lazzari *et al.* 2002) for 9 days.
270 Cleavage rate was evaluated at day 2, where day 0 was the day of ICSI. Half of the
271 culture medium was replaced on day 4 and day 6. From day 6 to day 9 the embryos

272 were evaluated for blastocyst formation, with embryos showing an organized
273 outer layer of aligned trophoblast cells considered to be blastocysts.
274
275 **Blastocyst cell count (total, apoptotic and mitotic cell)**
276 Differential staining of vital cells was conducted as previously described
277 (Oberstein *et al.* 2001) with minor modifications. Briefly, blastocysts were stained
278 with Propidium Iodide 10 μg/ml washed and fixed in PFA 4% in PBS. Blastocysts
279 were then mounted on coverslips in Vectashield containing DAPI and imaged
280 under an epifluorescent microscope using TRITC and DAPI filters.
281 Digital images were captured and the merged blue and red images were used to
282 count vital nuclei in blue as opposed to severely apoptotic nuclei in purple (blue
283 plus red fluorescence). Additionally, mitotic figures, also in blue, were separately
284 counted (Leroy *et al.* 2010). Cell count was carried out using the 'Cell Count' tool of
285 NIH Image J (Schneider *et al.* 2012). Total nuclei were the sum of vital, apoptotic
286 and mitotic cells.
287
288 **Statistical analysis**
289 GraphPad Prism 8.1.1 (GraphPad Software, San Diego, CA) was used for graphical
290 representation and statistical analysis.
291 Data representing the distribution in discrete classes (GJ-mediated
292 communication, chromatin configuration in the GV, meiotic stages, cleavage and
293 blastocyst rate) are expressed as percentage along with the actual number of
294 observations (n). These experiments were repeated 2-6 times and analyzed by
295 two-tailed Fisher's Exact test.
296 Numerical values (blastocyst cell number, as well as apoptotic and mitotic cells
297 number) are represented as the mean±S.E.M. of at least 3 independent
298 experiments. After determining that the assumption of normal distribution of the
299 sample population could not be made using D'Agostino & Pearson's normality test,
300 data were analyzed by two-tailed unpaired Mann-Whitney test (non parametric).
301 $P<0.05$ was considered statistically significant (*). Whenever possible, keeping in
302 mind the overall clarity of the graph, further differences ($P<0.01$, $P<0.0001$) are
303 also indicated as ** and ****, respectively.

304 **RESULTS**

305 **Compact COCs collected during the spring-summer and the fall show a high**
306 **rate of open GJs**

307 We investigated the permeability of GJs in 301 Cp and 178 Ex COCs retrieved
308 during different seasons and from follicles of different size to assess whether
309 cumulus morphology can be used as a predictor of GJ-mediated coupling (Fig.1).
310 We observed that, during the spring-summer, Cp COCs had significantly higher
311 rates of open GJs (69-75%) compared to Ex COCs (17-42%) for all follicle sizes
312 considered. A similar relationship was found during the fall, but was limited to Cp
313 and Ex COCs collected from <1 cm and 1-2 cm follicles, while no difference in GJ
314 permeability was seen in COCs from >2 cm follicles (58% and 67% in Cp and Ex,
315 respectively).
316 In the winter, Cp and Ex COCs had a similar and relatively low rate of GJ opening,
317 ranging from a minimum of 19% to a maximum of 58%. By comparing the
318 frequency of open GJs only in Cp COCs according to season and follicle size (Suppl.
319 Fig.1), we indeed observed that Cp COCs collected from <1 cm and 1-2 cm follicles
320 during the winter had significantly lower rates of open GJs compared to the same
321 follicle classes in the spring-summer and fall.
322
323 Since COCs collected from <1 cm and 1-2 cm follicles during spring-summer and
324 fall showed a similar rate of open GJs according to the cumulus morphology, these
325 periods were considered together in the following experiments, i.e. as spring-fall
326 class.
327

328 **Compact COCs collected from <1 cm and 1-2 cm follicles have low meiotic**
329 **competence**

330 In a second series of experiments we investigated whether cumulus morphology,
331 season and follicle size can predict the meiotic competence of the oocyte (Fig. 2).
332 To this end we in vitro matured 639 Cp and 307 Ex COCs.
333 In agreement with previous reports (Alm and Hinrichs 1996; Love *et al.* 2003; Choi
334 *et al.* 2004; Hinrichs 2010), a lower percentage of Cp COCs collected from <1 cm
335 and 1-2 cm follicles reached the MII stage compared to Ex COCs, regardless of the
336 season considered (overall 27-42% MII for Cp and 58-100% for Ex).

337 On the other hand, approx. 60% of Cp COCs collected from larger follicles (>2 cm)
338 matured in vitro up to the MII stage, a significantly higher rate than Cp from <1 cm
339 and 1-2 cm follicles, and comparable to Ex COCs.
340 Data analysis on the distribution of the 'not MII' stages (Suppl. Fig.2) revealed that
341 at the end of the IVM period most of the not matured oocytes derived from Cp
342 COCs from <1 cm and 1-2 cm were arrested at the GV stage or degenerated, rather
343 than being at an intermediate stage of meiotic progression.
344
345 We note that the absence of a significant difference between Cp and Ex COCs from
346 >2 cm is likely due to the low number of COCs retrieved from this class.
347
348 We also observed that, if comparing the meiotic competence of Cp COCs retrieved
349 from <1 cm follicles in the different reproductive periods, the MII rate achieved
350 during winter is significantly higher compared to the same class in spring-fall (42
351 and 27%, respectively, P=0.008). This increase is accompanied by a lower rate of
352 oocyte degeneration in the winter (24 %) as opposed to spring-fall (41%,
353 P=0.0127).
354 We did not observe differences in the MII rate in the other COC classes according
355 to seasons.
356
357 **Characterization of the large-scale chromatin configuration in the GV of Cp**
358 **COCs collected from <1 cm and 1-2 cm follicles**
359 The high rate of oocytes unable to resume meiosis led us to examine the oocyte
360 chromatin status in 325 Cp COCs right after retrieval from <1 cm and 1-2 cm
361 follicles (Fig.3), in order to assess if they have very early stages of chromatin
362 organization, which are usually linked to the growing phases of oocyte
363 development (Luciano *et al.* 2012), and whether degeneration had already
364 occurred or arose during IVM.
365 Even though the Fibrillar GV type (less condensed chromatin) was apparently
366 more frequent in oocytes from <1 cm follicles than 1-2 cm follicles (ranging from
367 18-27% and 11-12%, respectively), significant differences were not observed.
368 Conversely the frequency of Condensed GV type was higher in oocytes from 1-2 cm
369 follicles (approx. 60%), together with a lower incidence of degeneration. The

370 degeneration rate was considerably higher (approx. 30%) in oocytes from <1 cm,
371 albeit statistical differences were only noticeable during the spring-fall.
372
373 **Effect of cilostamide-induced prematuration on the developmental**
374 **competence of Cp COCs collected from <1 cm and 1-2 cm follicles**
375 Persistence of open GJs between the oocyte and companion cumulus cells is
376 considered essential for successful prematuration (Luciano *et al.* 2014; Dieci *et al.*
377 2016) and the data gathered in the 'population characterization' phase of this
378 study showed that this parameter was satisfied by Cp COCs during spring-fall.
379 Hence the following phase of 'setting up of a prematuration treatment' was
380 conducted in this subpopulation of gametes. Specifically, we focused on the <1 cm
381 and 1-2 cm Cp COCs, and excluded COCs from follicles >2 cm mainly due to
382 difficulties in retrieving sufficient samples to carefully evaluate responses to the
383 prematuration treatment in this subpopulation.
384
385 Prematuration treatment with cilostamide and low FSH was effective in other
386 mammalian species to maintain the meiotic arrest, while stimulating the
387 functionality of the GJs in the COCs (Dieci *et al.* 2013; Franciosi *et al.* 2014).
388 Also, in horse oocytes treated with cilostamide GJs stayed open for up to 10 hrs, at
389 levels comparable to those observed at the time of retrieval (Fig.4). Further
390 prolonging the culture up to 16 hrs resulted in a sharp decrease of the GJ-mediated
391 coupling, irrespective of the cilostamide treatment. Overall 465 COCs were used to
392 conduct this set of experiments.
393
394 After having confirmed on 440 COCs that the cilostamide-induced meiotic arrest
395 for 6-10 hrs was reversible (Suppl. Fig.3), we performed IVM-ICSI and embryo
396 culture with or without the prematuration step on 492 COCs. Despite not
397 increasing the embryo yield (Fig.5), 6 hrs prematuration significantly improved
398 the cell number per blastocyst derived from oocytes collected from 1-2 cm follicles,
399 from 243±40.11 in the control to 401.1±18.21 in prematured group (Fig.6B). In an
400 attempt to shed light on the mechanisms responsible for improving the total cell
401 number, we also measured apoptotic and mitotic nuclei (Fig.6C-D). However, at
402 the blastocyst stage, no differences were observed.

403 No differences in blastocyst cell number were observed for the oocytes collected

404 from <1 cm follicles.

405 Overall 48 of the 85 blastocysts produced were stained for cell counting.

406

407 **DISCUSSION**
408 Due to the unreliable responsiveness of mares to superovulation treatments, IVM-
409 ICSI and embryo culture is currently the only means to increase the number of
410 foals per treatment for individual mares of high genetic merit. Hence improving
411 the developmental competence of *in vitro* matured oocytes has considerable
412 interest in horse breeding.
413 Given the successful attempt described by the Hinrichs group, where Cp equine
414 COCs treated with roscovitine and matured for 30 hrs achieved higher cleavage
415 rates and cell number per embryo at day 4 (Franz *et al.* 2003), it is surprising that
416 the development of prematuration protocols, similar to those successful in
417 improving embryo quality and yield in other species, have not been extensively
418 pursued for horse embryo production. Studies in the horse instead, focused on
419 keeping ovaries (Love *et al*. 2003; Ribeiro *et al*. 2008; Hinrichs *et al*. 2012) or
420 isolated COCs (Choi *et al*. 2006b; Choi *et al*. 2007; Foss *et al.* 2013; Galli *et al.* 2014;
421 Martino *et al.* 2014; Dini *et al.* 2016; Diaw *et al.* 2018) at room temperature for a
422 prolonged time before performing IVM, which led to the development of user-
423 friendly procedures for oocyte transportation and scheduling of the manipulations
424 (Hinrichs 2018). This system shares some similarities with prematuration, as the
425 oocytes remain at the GV stage (Pedersen *et al*. 2004; Choi *et al*. 2006b, Galli *et al.*
426 2014), hence it might be supposedly considered an '*ex vivo* prematuration'.
427 However, besides the demonstration of no adverse effects of holding at room
428 temperature (HRT) on oocyte competence, and the consequent practical relevance,
429 improvements in the developmental competence of such treated oocytes have not
430 been reported thus far (as reviewed in: Hinrichs 2018). Moreover the mechanisms
431 that function in keeping meiotic arrest in absence of inhibitors have not been
432 elucidated.
433
434 Here we present a series of experiments aimed at characterizing thoroughly the
435 heterogeneous population of equine COCs retrieved from antral follicles, taking
436 into account GJ functionality, diameter of the follicle of origin, status of the
437 chromatin in the GV at the time of retrieval and intrinsic meiotic competence.
438 These parameters have been used previously in other species to develop
439 customized prematuration strategies according to the differentiation step that the

440 gametes have achieved *in vivo* (Dieci *et al.* 2016; Zhang *et al.* 2017). In addition, we
441 considered peculiar features of horse gametes such as cumulus morphology and
442 seasonality. In a second experimental phase, we tested the effect of a cilostamide-
443 based, two-step culture on the developmental competence of selected sub-
444 populations of equine oocytes, compared to direct IVM.
445
446 Due to the inability of the oocyte to efficiently metabolize glucose and uptake some
447 amino acids, GJ-mediated coupling with the cumulus cells is indispensable for
448 sustaining the metabolism of the oocyte (Gilula *et al.* 1978; Colonna and Mangia
449 1983). Furthermore the GJ-mediated passage of cyclic nucleotides, by imposing
450 Prophase-arrest on meiotically competent gametes, allows for continued
451 differentiation of the oocyte and supports the acquisition of developmental
452 competence (Gharibi *et al.* 2013; Franciosi *et al.* 2014; Li *et al.* 2016a; Romero *et al.*
453 2016; Santiquet *et al.* 2017; Sugimura *et al.* 2018; Soto-Heras *et al.* 2019).
454 Therefore, investigation of GJ coupling, both at the time of retrieval and, during a
455 second experimental phase, in response to the cilostamide-induced prematuration,
456 played an essential role in the set-up of a customized cultural approach.
457 Indeed, the first set of experiments showed that GJ functionality is compromised in
458 Ex COCs during spring-summer and fall and, independent of cumulus morphology,
459 during winter, making these gametes less suitable for sustaining GJ coupling
460 during a prolonged culture in meiotic arrest.
461 Ex COCs were also characterized by higher maturation rates compared to Cp COCs
462 retrieved from <1 cm and 1-2 cm follicles, indicating that the latter gamete
463 subclasses are still developing to some extent and have not attained full meiotic
464 competence yet. This observation is in line with the results on coupling, as growing
465 gametes would still require the GJ-mediated support in order to continue their
466 growth and differentiation program. Furthermore, a higher meiotic competence of
467 Ex COCs was previously reported both in horses (Alm and Hinrichs 1996; Love *et*
468 *al.* 2003; Choi *et al.* 2004; Hinrichs 2010) and in humans (Nogueira *et al.* 2006).
469 Indeed, when using human oocytes from expanded COCs, prematuration did not
470 improve further the meiotic competence, while an increase was shown for the
471 compact class (Nogueira *et al.* 2006). These findings suggest that, also in humans,
472 impairment of GJ functionality might arise from expansion, as demonstrated in the

473 present study for the horse. Most importantly, they also indicate that it is not ideal
474 to expose expanded COCs to prematuration.
475 That differences in developmental competence also exist in equine Ex vs Cp COCs
476 is controversial, with studies from the same group either showing differences
477 according to the IVM time and medium composition (Choi *et al.* 2004), or
478 concluding that cumulus morphology does not affect embryo development
479 (Hinrichs 2010). While these results might have to be confirmed on a larger
480 population, it is intriguing to ask whether equine Ex COCs derive from atretic
481 follicles. In cows, it has been hypothesized that morphological changes occurring
482 during prematuration in dominant follicles are similar to those arising during early
483 atresia in follicles 3-8 mm (Hyttel *et al.* 1997), giving a possible explanation for the
484 higher developmental competence of oocytes coming from early atretic follicles.
485 Whether Ex equine COCs are comparable with mild atretic stages in bovine is an
486 open question, but if that was the case, their higher competence might arise from
487 an *in vivo*-occurred prematuration.
488
489 The above-discussed experiments indicated that Cp COCs collected from <1 cm and
490 1-2 cm from spring to fall likely represent subpopulations of equine oocytes that
491 might benefit from a prematuration culture step. In line with this conclusion, it was
492 previously reported, using a roscovitine-based system, that prematuration of Ex
493 COCs is probably unnecessary, if not harmful (Franz *et al*. 2003; Choi *et al*. 2006b).
494 We therefore focused the following part of the study on defined subclasses of Cp
495 COCs.
496 As a first step, we investigated the organization of the chromatin within the GV,
497 since it can provide additional information on the differentiation and metabolic
498 state of the oocyte (Luciano *et al.* 2014). Indeed, starting from the first reports
499 demonstrating increased meiotic and developmental competence in mice oocytes
500 according to the compaction of chromatin structure in the GV (Zuccotti *et al.* 1998;
501 Zuccotti *et al.* 2002), it became clear that, in most mammals, oocytes from more
502 advanced follicular stages and with higher developmental potential show a more
503 condensed chromatin (Tan *et al.* 2009; Luciano and Lodde 2013). In horses we
504 classified the GV chromatin of oocytes collected from the 'antral pool' in 3
505 subtypes: Fibrillar, Intermediate and Condensed going from a more dispersed

506 organization to a compact clump completely enclosing the nucleolar body
507 remnants (Franciosi *et al.* 2012).
508 Based on other animal models, it was expected that oocytes coming from smaller
509 follicles, still functionally coupled with the somatic environment and with limited
510 meiotic competence, would mainly have Fibrillar or Intermediate chromatin
511 configuration. This prediction was instead contradicted by the experimental
512 observations, as the Condensed GV type was predominant. These findings may
513 suggest that equine chromatin compaction temporally precedes full competence
514 acquisition, in contrast to other mammalian species. Alternatively, Cp COCs
515 collected from <1 cm and 1-2 cm are already at advanced stages of growth, and
516 only the late differentiation steps are pending.
517
518 The latter hypothesis fits well with the 'shut down' of GJs during the first 10-16 hrs
519 of cilostamide-induced meiotic arrest. In the cow, for instance, growing oocytes
520 cultured *in vitro* tend to prolong the functional coupling for at least 24 hrs (Luciano
521 *et al.* 2011), while GJs in cultured fully-grown oocytes close rather earlier
522 (Franciosi *et al.* 2014).
523 Such limited lifespan of the GJs in culture and the prevalence of the Condensed GV
524 type narrowed down to a few hours the temporal frame of intervention of the
525 prematuration, especially for the COCs derived from 1-2 cm follicles. It was indeed
526 in this class of gametes that the short prematuration tested in our experiments was
527 associated with an increase in the total number of cells in the blastocyst, usually
528 considered a parameter of higher embryo quality, also in the horse (Pomar *et al*.
529 2015; Salgado *et al*. 2018).
530 Even though the overall maturation and blastocyst rates were not affected, these
531 results might represent a promising starting point, especially if considering that an
532 improvement limited to embryo quality is consistent with results obtained in cows
533 with *in vivo* prematuration (Dieleman *et al.* 2002). We must nevertheless
534 acknowledge, as a word of caution, that the ultimate proof of effective acquisition
535 of higher developmental competence in response to prematuration would be the
536 delivery of more foals after transfer to recipient mares, which remains to be tested.
537

538 Furthermore, the lack of an increase in overall embryo yield might be due to the
539 characteristics of the starting oocyte population, namely enrichment in the
540 Condensed GV type. Since prematuration promotes a late folliculogenesis and
541 dominance stage-like differentiation, it would not markedly affect oocytes with
542 Fibrillar and Intermediate GV type. Similarly, it would not rescue oocytes that are
543 already degenerated at the time of retrieval. We cannot currently exclude the
544 possibility that extending the prematuration time past the 10 hrs might be
545 beneficial for oocytes in the <1 cm class. However, given the overall abundance of
546 Condensed GV, a major concern would be 'aging' most of the oocytes in order to
547 rescue few. Ideally, one would have to develop non-invasive biomarkers allowing
548 further stratification of the oocytes according to their GV type, without removing
549 the cumulus cells.
550
551 Another consideration in respect to Cp COCs retrieved from follicles <1 cm is that
552 the maturation rate was higher in the winter than in the spring-fall. Since this
553 increase in meiotic competence was also accompanied by a decrease in the
554 degeneration rate, we are tempted to speculate that it might be linked to the
555 resumption of ovarian activity in the spring transitional period (winter). At this
556 time, deviation of the dominant follicles has not occurred yet, or it has occurred to
557 a limited extent, hence subordinate follicles undergoing atresia should be less
558 compared to the breeding season and the fall transition, when subordinate follicles
559 from the growing cohorts have accumulated from the previous cycles. Of course
560 this hypothesis remains to be tested and other factors may instead be responsible
561 for the observed difference.
562
563 Overall the present findings suggest that a cilostamide-based prematuration might
564 support the cytoplasmic maturation of a subset of equine oocytes, in agreement
565 with studies conducted in other mammalian species. As expected, and in
566 agreement with (Franz *et al.* 2003), the improvement was confined to a subclass of
567 gametes, but opens the possibility of being modified to meet the needs of other
568 subclass(es). In this respect the initial characterization of the 'antral' pool of
569 oocytes might represent a valuable source of information when devising new
570 culture approaches in a 'decision making process' more sub-divided than the one

571 currently in use (i.e. direct IVM for all), and even of the one we are proposing here
572 (i.e. short prematuration plus IVM of Cp COCs collected from 1-2 cm follicles from
573 spring to fall, and direct IVM for all others).
574 Clearly, many questions remained unresolved. However, we note that a deeper
575 knowledge of horse oocyte physiology and folliculogenesis will be pivotal to
576 improving the success of equine IVM and IVEP.
577
578

579 **CONFLICT OF INTEREST**

580 The Authors declare no conflicts of interest

581

582

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974

FIGURE LEGENDS

Figure 1: Gap junction-mediated cumulus cell-oocyte coupling according to cumulus morphology, follicle size and season. LY was injected in the ooplasm of compact or expanded COCs collected either during winter, spring-summer or fall from follicles <1 cm, 1-2 cm and >2 cm in diameter (<1, 1-2 and>2, respectively). The diffusion of LY to the cumulus cells was monitored as an indication of functionally open GJs. The bar graph represents the proportion of COCs with open GJs of the total COCs injected in each group, across 2-6 independent experiments. Actual number of COCs analyzed (n) is given on top of each bar. Data were analyzed by two-tailed Fisher's exact test. * represents significant differences (P<0.05) between compact and expanded class within the same follicle diameter and season.

Figure 2: Meiotic competence according to cumulus morphology, follicle size and seasonality. Compact and expanded COCs collected either during winter or spring-summer-fall (spring-fall) from follicles <1 cm, 1-2 cm and >2 cm in diameter (<1, 1-2 and>2, respectively) were in vitro matured and the stage of meiosis was evaluated by DNA staining. The bar graph represents the rate of oocytes that at the end of IVM were at the MII stage of the total samples cultured in each group, across 2-6 independent experiments. Actual number of oocytes analyzed (n) is given on top of each bar. Data were analyzed by two-tailed Fisher's exact test. * represents significant differences (P<0.05) within the season.

Figure 3: Large-scale chromatin configuration in the GV of oocytes from compact COCs. Compact COCs collected either during winter or spring-summer-fall (spring-fall) from follicles <1 cm and 1-2 cm (<1 and 1-2, respectively) were fixed and the DNA was stained. The bar graph represents the percentage of oocytes in the various configurations of large-scale chromatin organization within the GV: Fibrillar, Intermediate and Condensed (Fibr, Int, Cond respectively) or degenerated (Deg) of the total samples across 2-6 independent experiments. Actual number of oocytes analyzed (n) is given on top of each bar. Data were analyzed by two-tailed Fisher's exact test. *, ** and **** represent significant

differences ($P<0.05$, $P<0.01$ and $P<0.0001$, respectively) in the GV type between follicle diameter in a given season.

Figure 4: Effect of cilostamide treatment on GJ-mediated coupling in compact COCs. Compact COCs collected from follicles <1 cm and 1-2 cm (<1 and 1-2, respectively) were cultured in prematuration conditions for up to 16 hrs and the permeability of GJs was investigated by LY injection. The bar graph represents the percentage of COCs with open GJs of the total COCs injected in each group, across 3 independent experiments. Actual number of COCs analyzed (n) is given on top of each bar. Data were analyzed by two-tailed Fisher's exact test. * represents significant differences ($P<0.05$) between time/treatment within the same follicle diameter.

Figure 5: Effect of prematuration on embryo yield. Compact COCs collected from follicles <1 cm and 1-2 cm (<1 and 1-2, respectively) were cultured in prematuration conditions before undergoing IVM, ICSI and embryo culture. Control COCs underwent IVM directly. Cleavage and blastocyst rate were evaluated at day 2 and 9 post-ICSI, respectively. The bar graph represents the percentage of cleaved oocytes of total oocytes (Cleav/TOT), cleaved oocytes of oocytes that were ICSI-ed (Cleav/ICSI), blastocysts of total oocytes (Blast/TOT), and blastocyst of oocytes that were ICSI-ed (Blast/ICSI) for each treatment/follicle diameter, across 3-6 independent experiments. Actual number of oocytes and embryos analyzed (n) is given on top of each bar. Data were analyzed by two-tailed Fisher's exact test. No differences were observed.

Figure 6: Effect of prematuration on blastocyst quality. 18 blastocysts obtained from COCs collected from follicles <1 cm (9 Control and 9 Prematuration, respectively) and 30 blastocysts obtained from COCs collected from follicles 1-2 cm (16 Control and 14 Prematuration, respectively) were stained with propidium iodide before fixing, then fixed and stained with DAPI. A) Representative images of day 9 post-ICSI equine blastocysts where the nuclei were stained with DAPI post-fixation (blue), propidium iodide before being fixed (red) and the merge of the two channels. Arrows point at mitotic figures, visible in blue. Bar represents 100 μm. B)

1040 The bar graph represents the mean±S.E.M. of DAPI stained nuclei per blastocyst.
1041 Data distribution was analyzed by D'Agostino & Pearson normality test and
1042 statistical differences were evaluated by two-tailed unpaired Mann-Whitney test.
1043 ** represent significant differences ($P<0.01$) in total cells/blastocyst between
1044 prematuration and control group for the 1-2 cm follicle class. C) The bar graph
1045 represents the mean±S.E.M. percentage of propidium iodide stained nuclei per
1046 blastocyst, as a measure of advanced apoptosis/cell death. No differences were
1047 observed. D) The bar graph represents the mean±S.E.M. percentage of mitotic
1048 figures per blastocyst, as a measure of proliferation. No differences were observed.
1049

1050 **Supplemental Figure 1: Gap junction-mediated cumulus cell-oocyte coupling**
1051 **in compact COCs according to follicle size and season.** Data presented in Fig.1
1052 were re-analyzed considering only the compact class of cumulus morphology. The
1053 bar graph represents the rate of COCs with open GJs of the total COCs injected for
1054 each follicle diameter/season, across 2-6 independent experiments. Actual number
1055 of COCs analyzed (n) is given on top of each bar. Data were analyzed by two-tailed
1056 Fisher's exact test. * represents significant differences ($P<0.05$) between COCs
1057 collected in winter compared to spring-summer and fall, in the follicles <1 cm and
1058 1-2 cm.
1059

1060 **Supplemental Figure 2: Meiotic stage at the end of IVM in not MII oocytes**
1061 **according to cumulus morphology, follicle size and seasonality.** Compact and
1062 expanded COCs collected either during winter or spring-summer-fall (spring-fall)
1063 from follicles >1 cm, 1-2 cm and >2 cm in diameter (<1, 1-2 and>2, respectively)
1064 were in vitro matured and the stage of meiosis was evaluated by DNA staining. The
1065 bar graph represents the percentage of oocytes that at the end of IVM were at the
1066 GV stage (A), proMI-MI-AI-TI stages (B) or degenerated (C), across 2-6
1067 independent experiments. Actual number of oocytes analyzed (n) is given on top of
1068 each bar. Data were analyzed by two-tailed Fisher's exact test. * and ** represent
1069 significant differences ($P<0.05$ and $P<0.01$) in the rate of GV and Deg.
1070

1071 **Supplemental Figure 3: Effect of prematuration on maturation rate.** Compact
1072 COCs collected from follicles <1 cm and 1-2 cm (<1 and 1-2, respectively) were

1073 cultured in prematuration conditions before undergoing IVM. Control COCs
1074 underwent IVM directly. The bar graph represents the percentage of GV, ProMI-MI-
1075 AI-TI, MII, and deg oocytes at the end of IVM. Actual number of oocytes analyzed
1076 (n) is given on top of each bar. Data were analyzed by two-tailed Fisher's exact test.
1077 No differences were observed.
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Fig 1

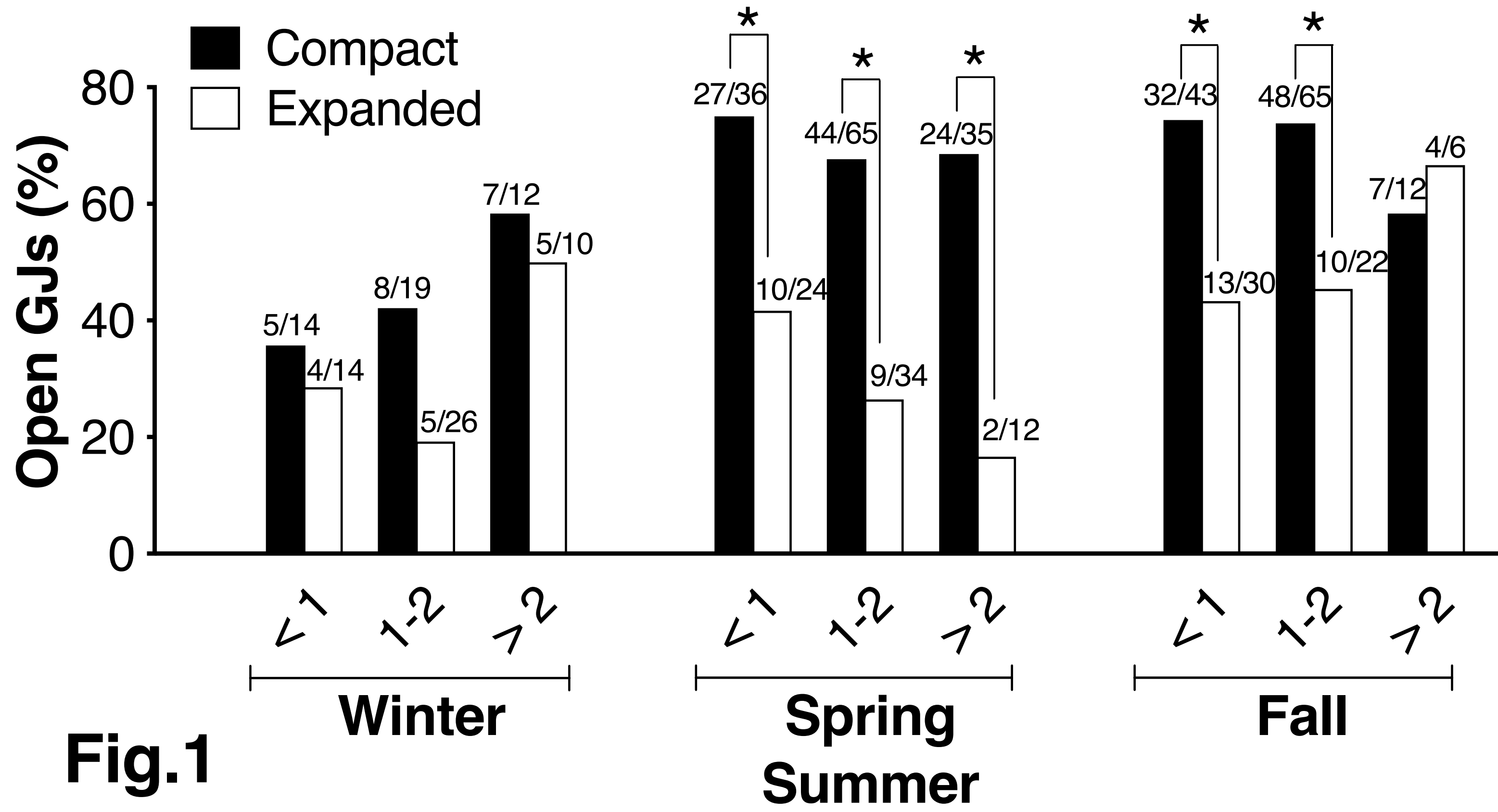


Fig 2

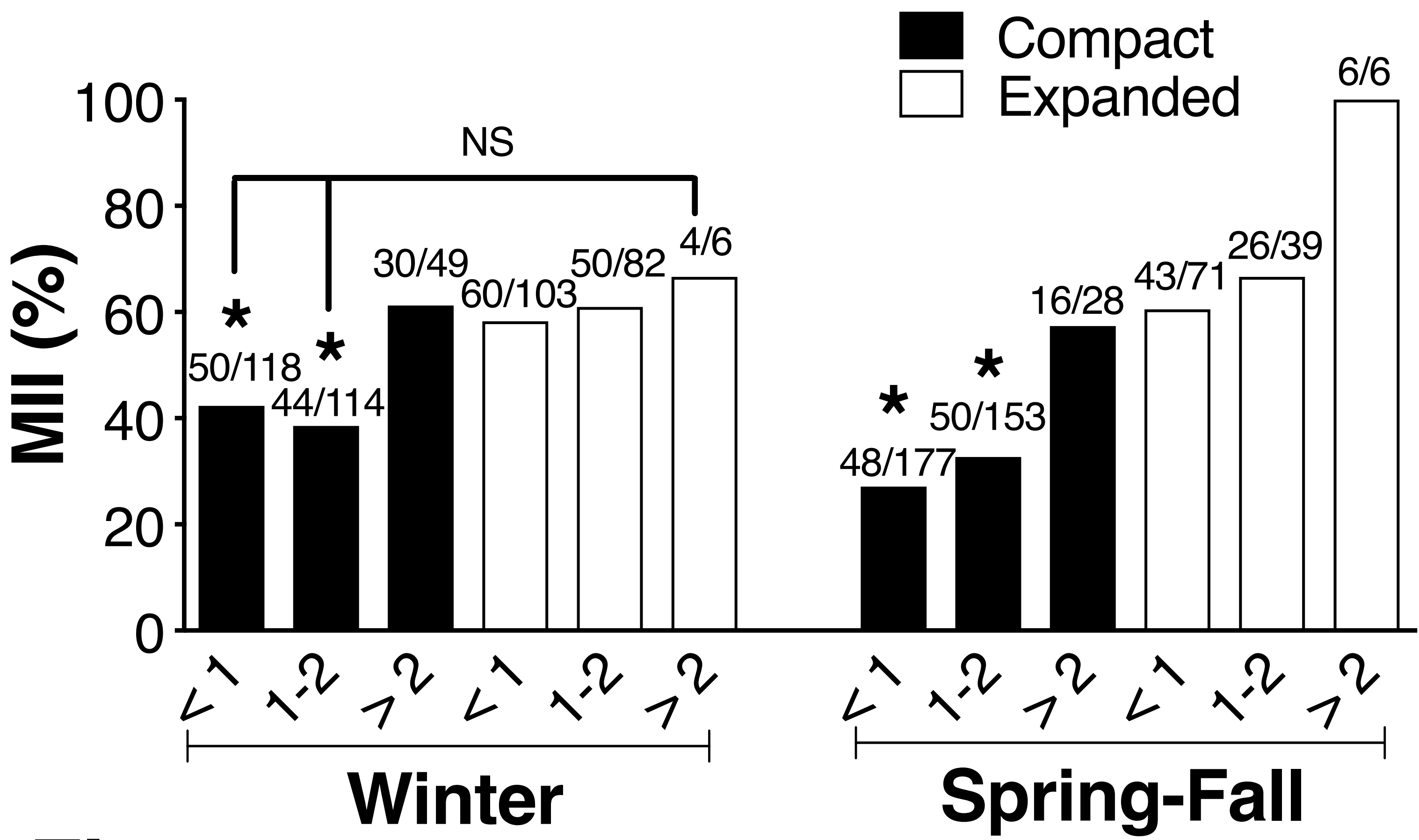
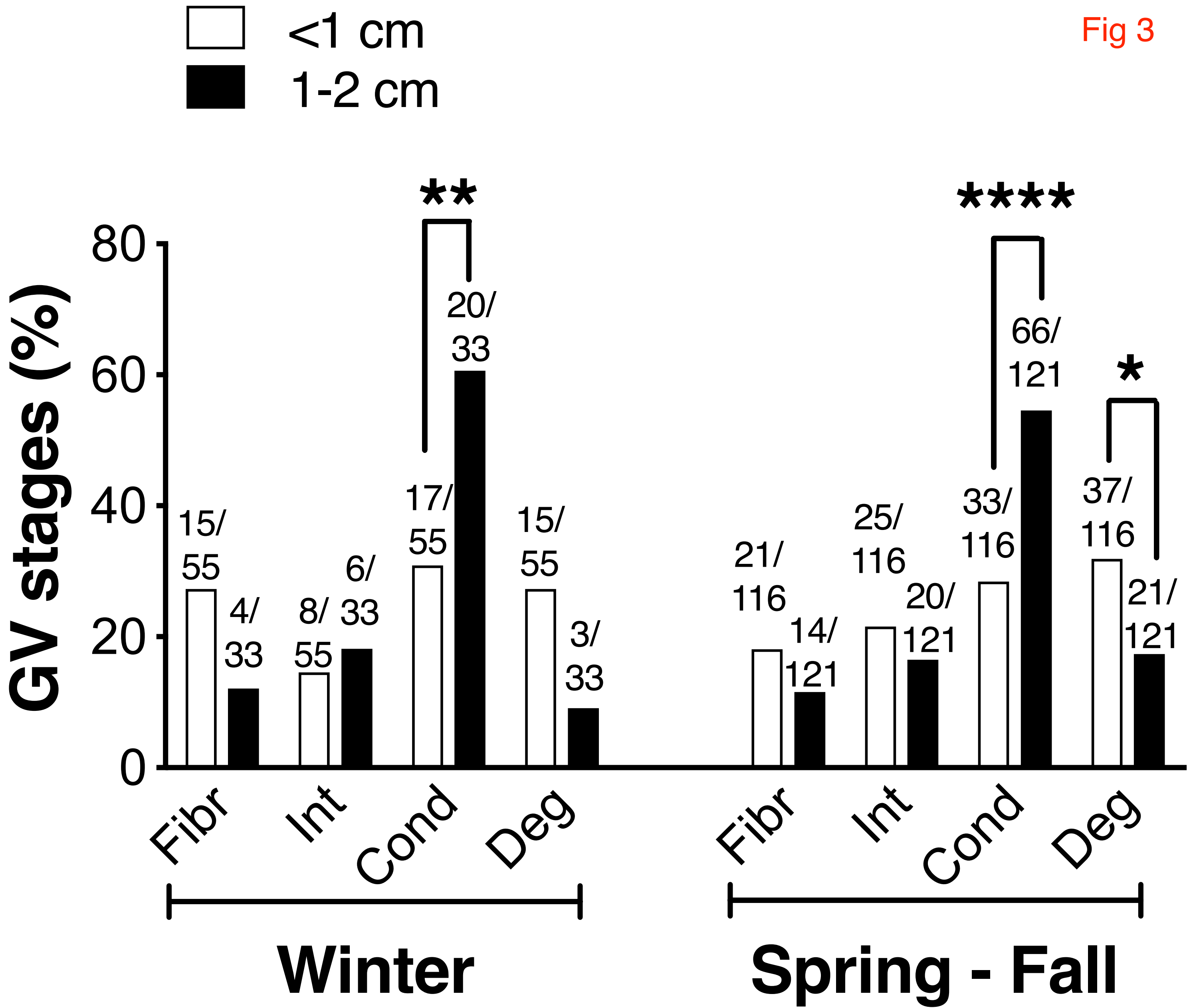


Fig.2



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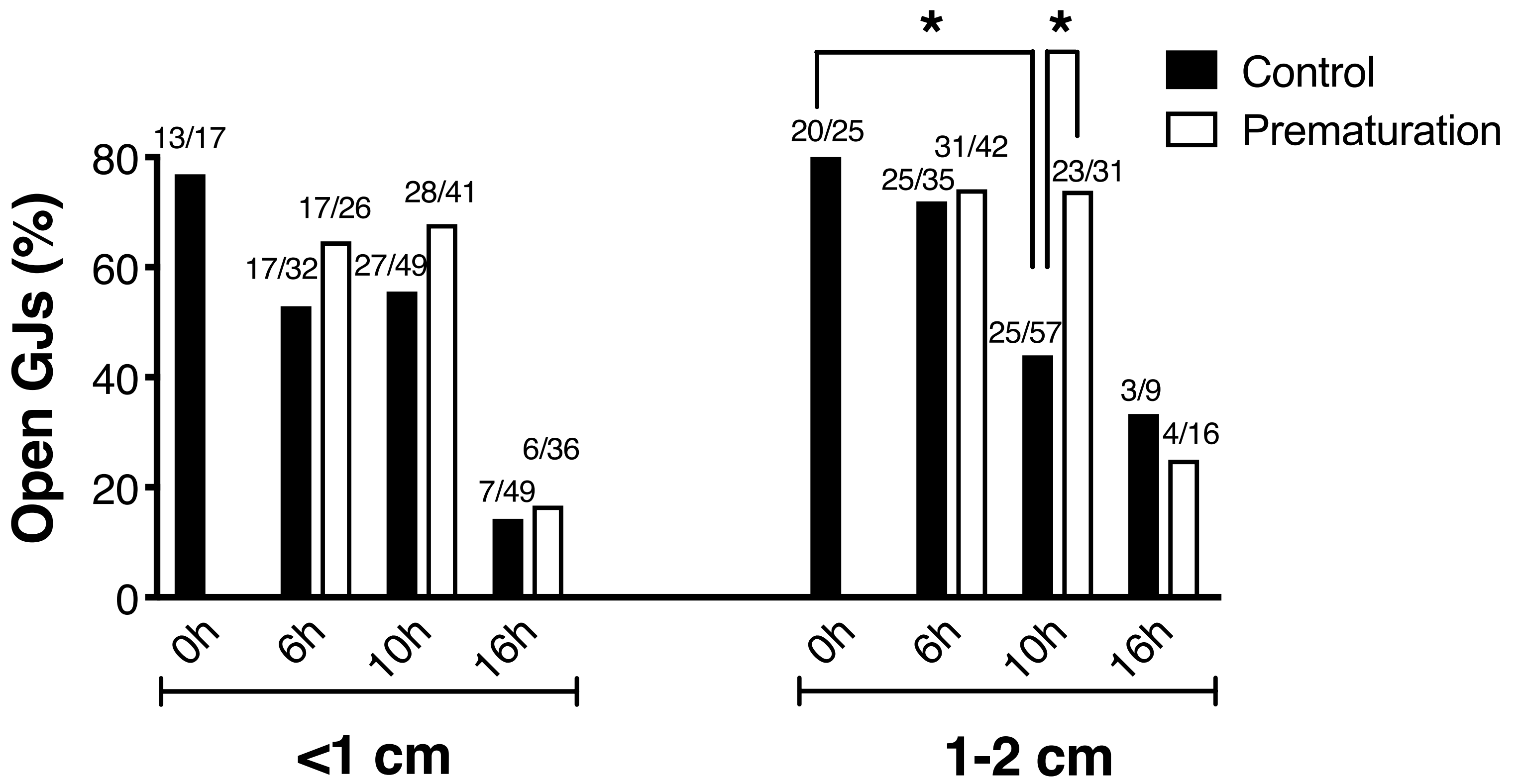

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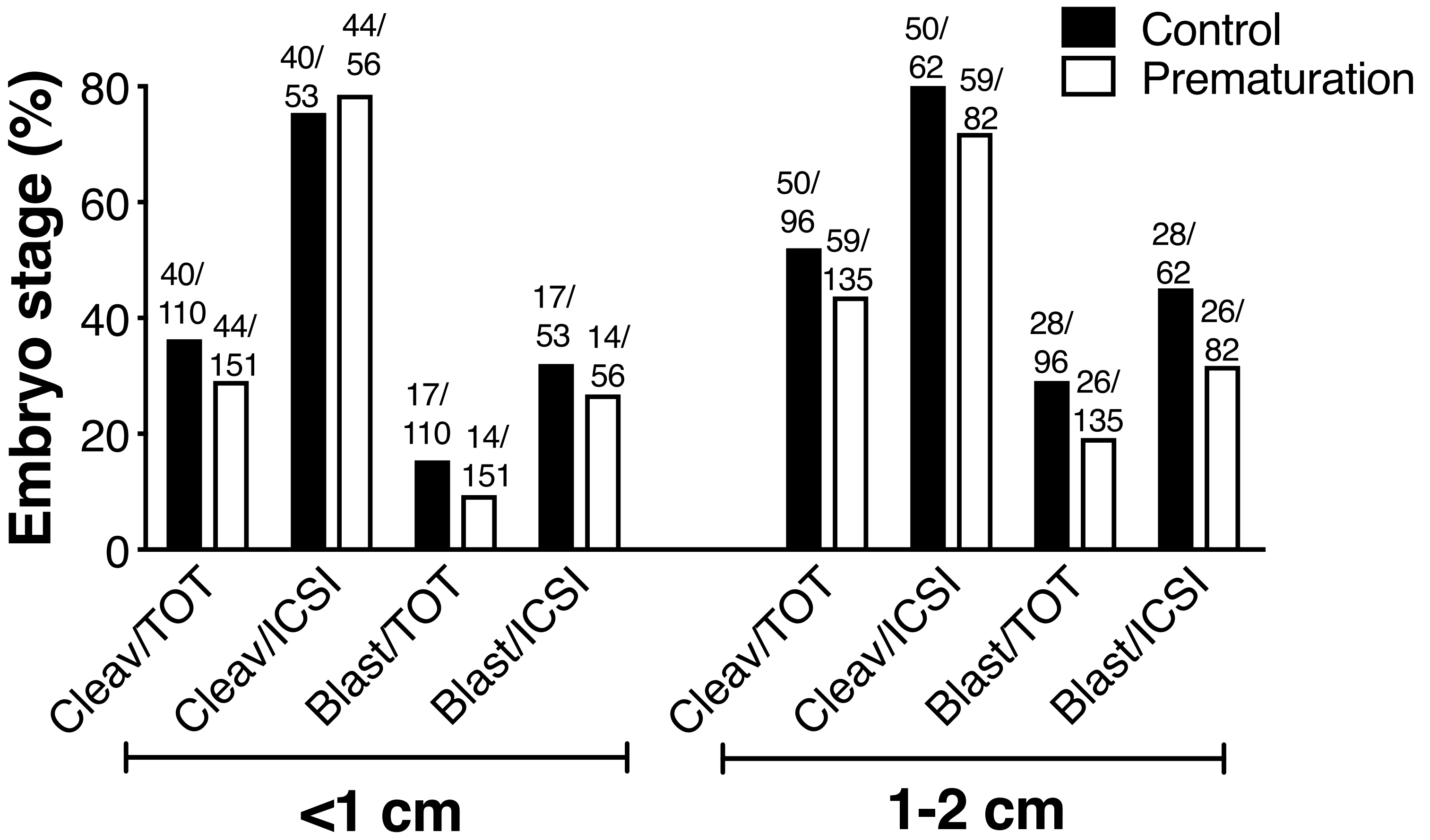

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Fig 6

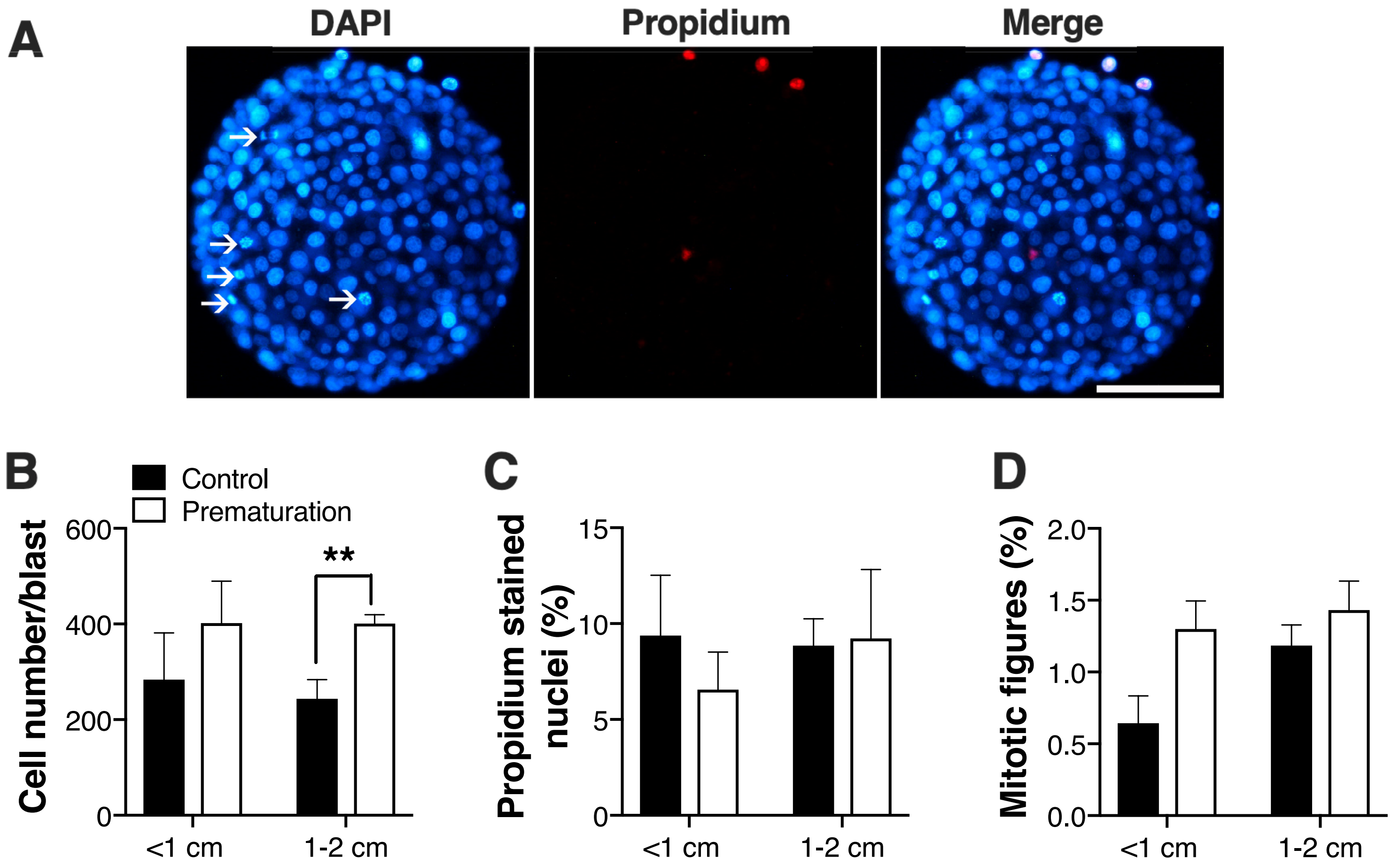
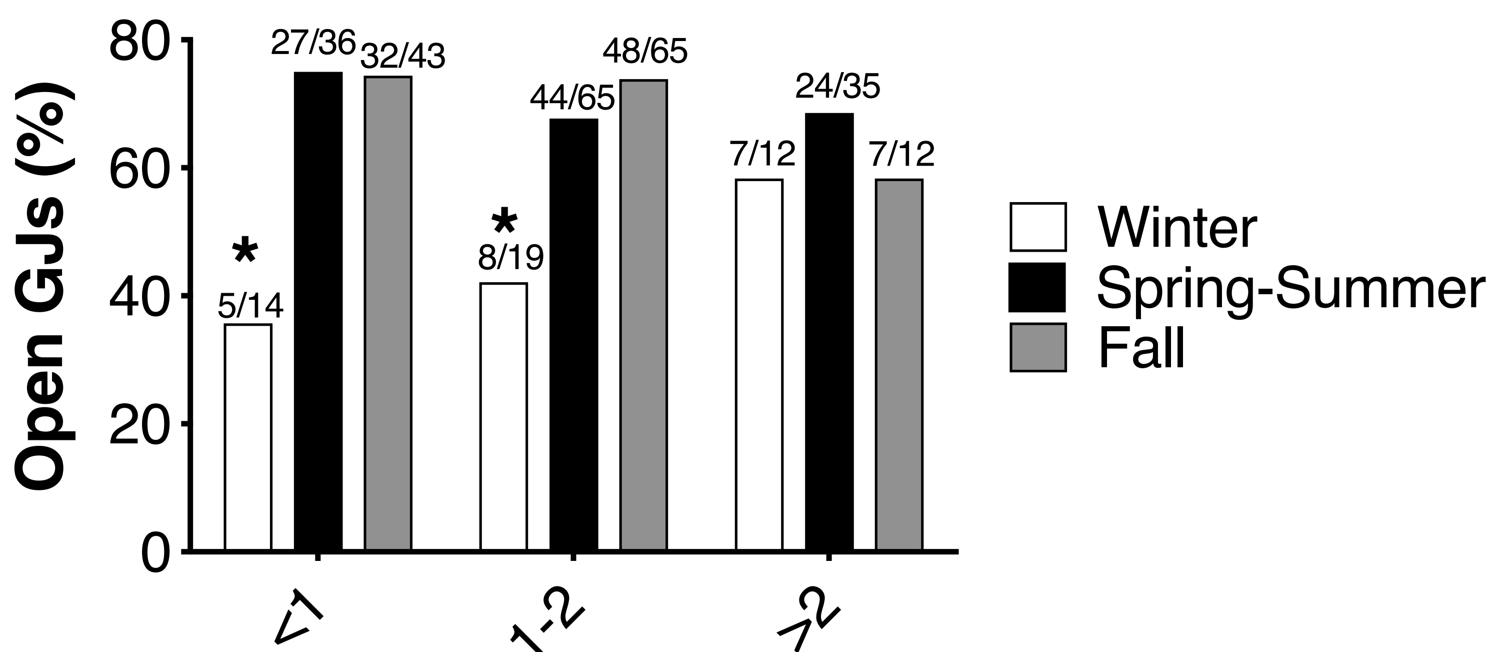


Fig.6

Suppl
Fig 1



Suppl. Fig.1

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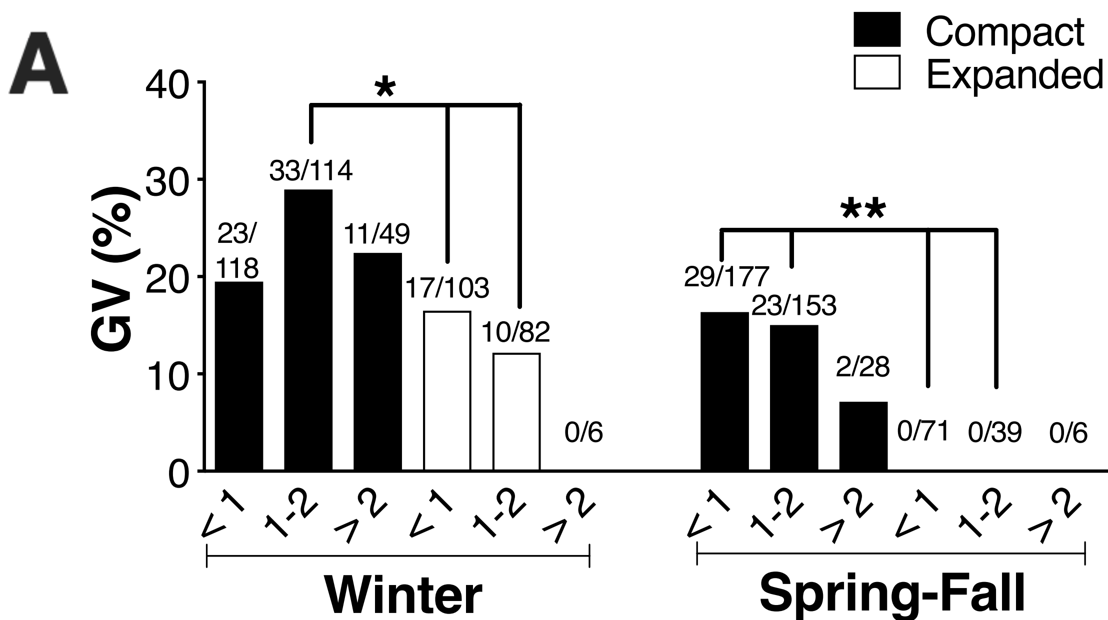
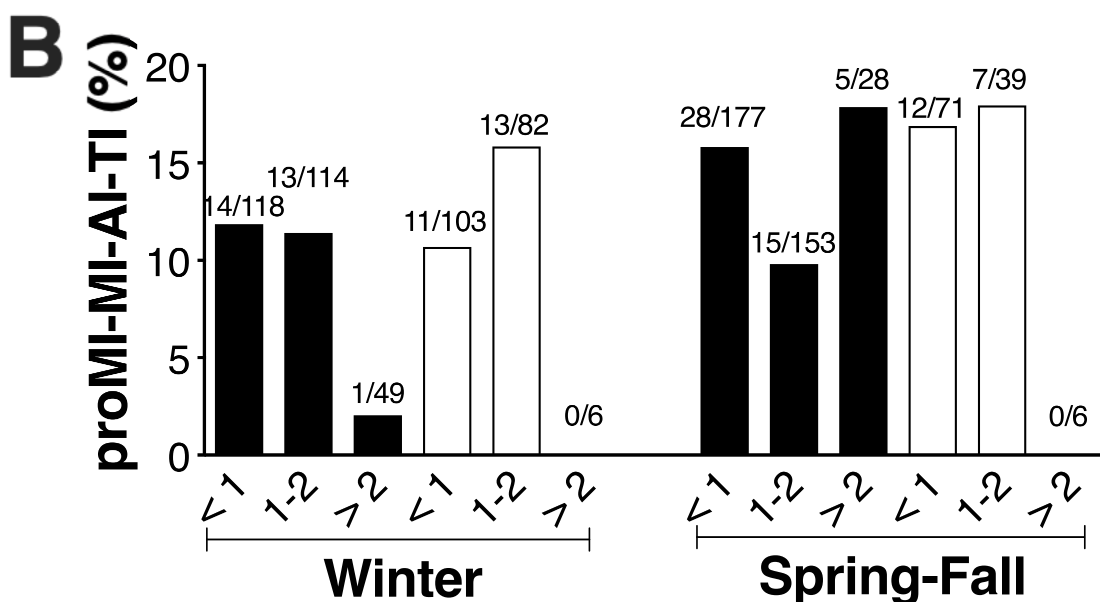
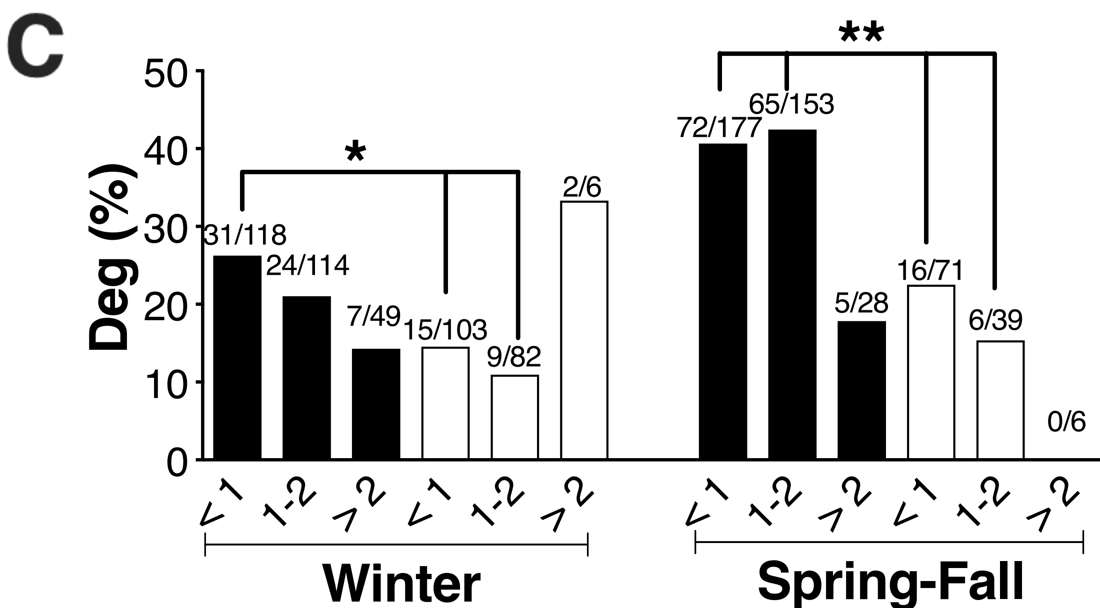
Fig 2







Suppl. Fig.2