

1 **Two transcriptionally distinct pathways drive female development in a
2 reptile with both genetic and temperature dependent sex determination**

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43 **Abstract**

44 How temperature determines sex remains unknown. A recent hypothesis proposes that
45 conserved cellular mechanisms (calcium and redox; 'CaRe' status) sense temperature and
46 identify genes and regulatory pathways likely to be involved in driving sexual development.
47 We take advantage of the unique sex determining system of the model organism, *Pogona*
48 *vitticeps*, to assess predictions of this hypothesis. *P. vitticeps* has ZZ male: ZW female sex
49 chromosomes whose influence can be overridden in genetic males by high temperatures,
50 causing male-to-female sex reversal. We compare a developmental transcriptome series of
51 ZWf females and temperature sex reversed ZZf females. We demonstrate that early
52 developmental cascades differ dramatically between genetically driven and thermally driven
53 females, later converging to produce a common outcome (ovaries). We show that genes
54 proposed as regulators of thermosensitive sex determination play a role in temperature sex
55 reversal. Our study greatly advances the search for the mechanisms by which temperature
56 determines sex.

57 **Author Summary**

58 In many reptiles and fish, environment can determine, or influence, the sex of developing
59 embryos. How this happens at a molecular level that has eluded resolution for half a century
60 of intensive research. We studied the bearded dragon, a lizard that has sex chromosomes
61 (ZZ male and ZW female), but in which that temperature can override ZZ sex chromosomes
62 to cause male to female sex reversal. This provides an unparalleled opportunity to
63 disentangle, in the same species, the biochemical pathways required to make a female by
64 these two different routes. We sequenced the transcriptomes of gonads from developing ZZ
65 reversed and normal ZW dragon embryos and discovered that different sets of genes are
66 active in ovary development driven by genotype or temperature. Females whose sex was
67 initiated by temperature showed a transcriptional profile consistent with the recently-
68 proposed Calcium-Redox hypotheses of cellular temperature sensing. These findings are an
69 important for understanding how the environment influences the development of sex, and
70 more generally how the environment can epigenetically modify the action of genes.

72 **Key words**

73 Sex reversal, gonad differentiation, thermosensitivity, calcium signalling, oxidative stress

74 **Introduction**

75 Sex determination in vertebrates may be genetic or environmental. In genetic sex
76 determination (GSD), offspring sex is determined by sex chromosomes inherited from each
77 parent, which bear either a dominant gene on the heteromorphic sex chromosome (as with
78 *SRY* in humans) (1,2), or a dosage sensitive gene on the homomorphic sex chromosome (as
79 with *DMRT1* in birds) (3). However, some fish and many reptile species exhibit
80 environmental sex determination (ESD), whereby a variety of external stimuli can determine
81 sex, most commonly involving temperature (temperature dependent sex determination,
82 TSD) (4,5). While GSD and ESD are commonly viewed as a dichotomy, the reality is far more
83 complex. Sex determination in vertebrates exists as a continuum of genetic and
84 environmental influences (6) whereby genes and environment can interact to determine sex
85 (7–9).

86 The genetic mechanisms that act in highly conserved pathways that ultimately yield testes
87 or ovaries are quite well characterised (5,10,11). Yet, despite decades of research on ESD
88 systems, and TSD in particular, the upstream mechanisms by which an external signal is
89 transduced to determine sex remains unknown (12). Recent research led to the hypothesis
90 that the cellular sensor initiating ESD is controlled by the balance of redox regulation and
91 calcium (Ca^{2+}) signalling (CaRe) (13). The CaRe hypothesis proposes a link between CaRe
92 sensitive cellular signalling and the highly conserved epigenetic processes that have been
93 implicated in thermolabile sex (TSD and temperature sex reversal) (12,14–17). The CaRe
94 hypothesis posits that in ESD systems a change in intracellular Ca^{2+} (probably mediated by
95 thermosensitive transient receptor potential TRP channels) and increased reactive oxygen
96 species (ROS) levels caused by high temperatures, alter the CaRe status of the cell, triggering
97 cellular signalling cascades that drive differential sex-specific expression of genes to
98 determine sex. The CaRe hypothesis makes several testable predictions for how an
99 environmental signal is captured and transduced by the gonadal cells to deliver a male or a
100 female phenotype.

101 Species in which genes and environment both influence sex determination provide
102 unique opportunities to directly compare the regulatory and developmental processes
103 involved in sex determination. By early gonad differentiation directed by genotype and
104 temperature, it is possible to assess predictions of the CaRe hypothesis. In our model
105 species, the central bearded dragon (*Pogona vitticeps*), we can compare female
106 development via thermal and genetic cues because extreme temperatures ($>32^{\circ}\text{C}$) override
107 the male sex-determining signal from the ZZ sex micro-chromosomes to feminise embryos
108 (8,18). This makes it possible to distinguish between the previously confounded effects of
109 thermal stress and phenotypic sex by comparing gene expression throughout embryonic
110 development in sex reversed ZZf females with genetic ZWf females.

111 We can explore the predictions of the CaRe model, namely that under sex-reversing
112 conditions, we will see differential regulation of: 1) genes involved in responding to Ca^{2+}
113 influx and signalling; 2) genes involved in antioxidant and/or oxidative stress responses; 3)
114 genes with known thermosensitivity, such as heat shock proteins; 4) candidate TSD genes,
115 such as *CIRBP* and Jumonji family genes; 5) signal transduction pathways such as the JAK-
116 STAT and NF- κ B pathways.

117 We compared gene expression profiles in *P. vitticeps* embryonic gonads at three
118 developmental stages (6, 12 and 15; 19,20) for ZWf and ZZf eggs incubated at 28°C and 36°C
119 respectively (Fig. 1). This allowed us to compare drivers of sex determination and
120 differentiation under genetic or thermal influence. We found that very different regulatory
121 processes are involved in temperature-driven regulation compared to gene-driven
122 regulation, although both lead to a conserved outcome (ovaries, Fig. 2). We discovered
123 dramatic changes in cellular calcium homeostasis in the gonads of ZZf individuals incubated
124 at high sex reversing temperatures, which fulfill predictions of the CaRe hypothesis that this
125 is the key driver of temperature induced feminization. We argue that differential expression
126 of calcium channels, and subsequent alterations of the intracellular environment combined
127 with increased ROS production encode, then transduce, the thermal signal into altered gene
128 expression, ultimately triggering male to female sex reversal in *P. vitticeps*.

129 **Results**

130 **Gene-driven female determination in ZWf embryos**

131 Comparisons between stages in ZWf embryos (Fig. 3B, Fig. S1, Additional file S1) showed
132 that many genes were differentially expressed between stages 6 and 12 (210 genes
133 downregulated and 627 genes upregulated at stage 12), but few genes were differentially
134 expressed between stages 12 and 15 (2 genes upregulated at stage 15).

135 *SOX9* and *GADD45G*, genes strongly associated with male development in mammals,
136 were downregulated from stage 6 to stage 12, whereas various female related genes were
137 upregulated, such as *PGR*, *ESR2*, *CYP19A1*, and *CYP17A1*. *BMP7*, a regulator of germ cell
138 proliferation was upregulated at stage 12 (21), as were components of the NOTCH signalling
139 pathway (*JAG2*, *DLL3*, *DLL4*), which are required for the suppression of Leydig cell
140 differentiation (22,23). *SRD5A2*, whose product catalyses the 5- α reduction of steroid
141 hormones such as testosterone and progesterone, was also upregulated (24,25).

142 Notably, there was little differential expression between stages 12 and 15, suggesting
143 that genetically driven ovarian development is complete by stage 12 (Additional file S1).

144 **Temperature-driven female determination in ZZf embryos**

145 Differential expression analysis of temperature-driven female development in ZZf embryos
146 revealed many genes are differentially expressed between stages 6 and 12 (297
147 downregulated and 511 upregulated at stage 12) and no genes are differentially expressed
148 between stage 12 and 15 (Fig. 3, Fig. S1, Additional file S1), suggesting completion of the
149 ovarian development by stage 12 also in ZZf females.

150 Uregulation of *FZD1*, a receptor for *Wnt* family proteins required for female
151 development, suggests the activity of female pathways in ZZf embryos (26). As was seen for
152 ZWf females, canonical NOTCH ligands *DLL3* and *DLL4* were upregulated from stage 6 to
153 stage 12 in ZZf females. However, this did not coincide with upregulation of JAG ligands or
154 NOTCH genes, and the GO term “negative regulation of NOTCH signalling” was enriched
155 within the group of genes upregulated from stage 6 to 12 in ZZf females (Additional file S2).
156 Further, *PDGFB*, which is required for Leydig cell differentiation, was upregulated (27).

157 Together, this suggests that the NOTCH signalling pathway may not be activated, and Leydig
158 cell recruitment is not strongly repressed at stage 12 in ZZf. Alternatively, the absence of
159 NOTCH signalling may indicate an important transition from progenitor cells to
160 differentiated gonadal cell types in the early stages of the developing ovary (28). These
161 apparent differences in NOTCH signalling between ZZf and ZWf embryos suggests that
162 ovarian development has progressed further in ZWf females.

163 Interestingly, genes typically associated with male development show diverse
164 regulation in ZZf embryos, with some being downregulated and some being upregulated
165 from stage 6 to 12. These included *WNT5a* and *SFRP2*, which are both involved in testicular
166 development in mice (29,30), *NCOA4*, which enhances activity of various hormone
167 receptors, and exhibits high expression in testes in mice during development (31), and
168 *HSD17B3*, which catalyses androstenedione to testosterone (32). Unlike what was observed
169 in ZWf embryos, *SOX9* and *GADD45G* were not differentially expressed between stages 6
170 and 12 in ZZf embryos. *TGFB3L*, which is required for Leydig cell function in mouse testis
171 (33), and *NR5A1*, *SOX4*, and *AMHR2* (34–37) were also differentially expressed between
172 stages 6 and 12 (Additional file S1).

173 A suite of genes typically associated with female development were upregulated from
174 stage 6 to 12 (38), for example, *FOXL2*, *CYP17A1*, *RSPO1*, and *ESRRG*. As was also observed
175 in stage 12 ZWfs, *ESR2*, *BMP7*, *CYP19A1*, and *PGR*, were more highly expressed at stage 12
176 in ZZfs. Notably, *CYP19A1* was much more strongly upregulated at stage 12 in ZZfs
177 compared with stage 12 in ZWfs (Additional file S1). The increase in sex specific genes was
178 also reflected in enriched GO terms at stage 12, which included “hormone binding”, “steroid
179 hormone receptor activity”, and “female sex determination” (Additional file S2).

180 **Ovarian maintenance in sex reversed ZZf females**

181 The maintenance of female gene expression and ovarian development at stage 12 in ZZf
182 females may be centrally mediated by *STAT4* (Fig. 4). As a member of the JAK-STAT
183 pathway, *STAT4* is transduced by various signals, including reactive oxygen species, to
184 undergo phosphorylation and translocate from the cytoplasm to nucleus (39–41). At stage
185 12, *STAT4* is upregulated, alongside *PDGFB* compared to stage 6 in ZZf females. *PDGFB* is
186 known to activate *STAT4* (40). Various *STAT4* target genes, notably *AMHR2*, *NR5A1*, *EGR1*,

187 and *KDM6B* (40) are also upregulated at stage 12 (Additional file S1). Consistent with this
188 link is the observation that a member of the same gene family, *STAT3*, is implicated in TSD in
189 *Trachemys scripta* (17).

190 Several targets of *STAT4* are upregulated at stage 12, including *AMHR2* and *NR5A1*.
191 Though typically associated with male development, *AMHR2* and *NR5A1* may also have roles
192 in ovarian development. Although it is the primary receptor for *AMH*, *AMHR2* exhibits
193 considerable evolutionary flexibility is sometimes associated with ovarian development
194 (reviewed by (36)). *NR5A1* is also often associated with male development, as it positively
195 regulates expression of *AMH* and *SOX9* in mammals (42). However, *NR5A1* can also interact
196 with *FOXL2* and bind to *CYP19A1* promoter to promote female development (43,44). The
197 upregulation of *FOXL2* and *CYP19A1*, but not *AMH* or *SOX9*, suggests that *NR5A1* is involved
198 in the establishment of the ovarian pathway in ZZf females.

199 *EGR1* positively regulates *DMRT1* expression through promoter binding in Sertoli cells,
200 but knock-out of this gene can also cause female infertility in mice (45–47). *EGR1* is also
201 associated with female development in birds, likely controlling the production of steroid
202 hormones (34). As was observed for *NR5A1*, *DMRT1* was also lowly expressed, suggesting it
203 is not activated by *EGR1* in ZZf females.

204 One explanation for these expression trends is that male-associated genes are not
205 strongly repressed at this stage during the sex reversal process, and that more prolonged
206 exposure to the sex reversing temperature is required to firmly establish the female
207 phenotype. However, we argue that the results more strongly suggest ROS-induced
208 activation of *STAT4*, and subsequent phosphorylation and translocation, probably mediated
209 by *PDGFB*, allows for the transcriptional activation of *NR5A1*, *AMHR2*, and *EGR1*, which in
210 the temperature driven process of sex reversal in the ZZf embryos serve to maintain the
211 ovarian phenotype.

212 **Differential regulation of female developmental pathways**

213 To better understand differences in ovarian developmental pathways, we compared gene
214 expression of ZZf with ZWf embryos at each developmental stage. There are large gene
215 expression differences between normal ZWf females and ZZf sex reversed females early in

216 development, before the bipotential gonad differentiates into an ovary. These differences
217 are most pronounced early in development and diminish as development progresses. Stage
218 6 had the largest number of differentially expressed genes (DEGs) (281 genes higher
219 expressed in ZWf embryos, 423 genes higher expressed in ZZf), with fewer DEGs at stage 12
220 (51 genes upregulated in ZWf, 63 genes upregulated in ZZf), and fewest at stage 15 (1 gene
221 upregulated in ZWf, 2 genes upregulated in ZZf) (Fig. 3B, Additional file S3, Fig. S1). This
222 suggests that the sex reversed embryos start out on a male developmental trajectory, which
223 they pursue beyond the thermal cue (3 days when the eggs were switched to high
224 incubation temperatures, Fig. 1), but by stage 12 development has been taken over by
225 female genes.

226 Gene ontology (GO) enrichment analysis showed important differences between ZZf and
227 ZWf at stage 6, and provides independent support for the role of calcium and redox
228 regulation in ZZf females as proposed by the CaRe model (Fig. 5 A-D, Additional file S4). GO
229 processes enriched in the gene set higher expressed in ZZf at stage 6 included “oxidation-
230 reduction processes”, “cytosolic calcium ion transport”, and “cellular homeostasis” (Fig. 5A,
231 Additional file S4). GO function enrichment also included several terms related to
232 oxidoreductase activities, as well as “active transmembrane transporter activity” (Fig. 5C,
233 Additional file S4). No such GO terms were enriched in the gene set higher expressed in
234 ZWf. Instead, enriched GO terms included “anatomical structure development”, and
235 “positive regulation of developmental growth” (Fig. 5B, D, Additional file S4).

236 Genes involved in female sex differentiation were higher expressed at stage 6 in normal
237 ZWf embryos compared to sex reversed ZZf embryos (Additional file S3). These included
238 *FOXL2*, *ESR2*, *PGR*, and *GATA6* (48,49). Higher expression of *LHX9*, a gene with a role in
239 bipotential gonad formation in mammals and birds, was more highly expressed in ZWf
240 embryos (42,50–52). Two genes with well described roles in male development, *SOX4* and
241 *ALDH1A2* (53–55), were also higher expressed in ZWf embryos, suggesting they have an as
242 yet unknown function in the early establishment of the ovarian trajectory in *P. vitticeps*.

243 Taken together, these results further suggest that ZWf females are committed to the
244 female pathway earlier than ZZf females. This is not surprising, since ZWf females possess
245 sex chromosomes from fertilisation, whereas ZZf individuals have had only 3 days of
246 exposure to a sex reversal inducing incubation temperature (Fig. 1A). This data is the first to

247 demonstrate a difference in timing of genetic signals between gene and temperature driven
248 development in the same species.

249 Three genes were constantly differentially expressed between ZWf and ZZf embryos at
250 all three developmental stages (Fig. 3A). *GCA* (grancalcin) was upregulated in ZWf embryos,
251 and *KDM6B* and *CIRBP* were upregulated in ZZf embryos at all developmental stages. *GCA* is
252 a calcium binding protein commonly found in neutrophils and is associated with the Nf- κ B
253 pathway (56,57). It has no known roles in sex determination, but its consistent upregulation
254 in ZWf embryos compared to ZZf embryos suggests *GCA* is associated with gene driven
255 ovarian development, at least in *P. vitticeps*.

256 Further analysis of gene expression trends using K-means clustering analysis (58) was
257 used to investigate genes associated with female development, and to determine to what
258 extent these genes are shared between ZZf and ZWf embryos (Fig. 6, Additional file S5).
259 Clusters with upward trends reflect genes likely to be associated with female development,
260 so clusters 1 and 4 in ZWf (ZWC1 and ZWC4), and clusters 1 and 2 in ZZf (ZZC1 and ZZC2),
261 were explored in greater detail (Fig. 6, Additional file S5).

262 ZWC4 and ZZC2 shared 374 genes. Enriched GO terms included “germ cell
263 development” and “reproductive processes” (Additional file S6), consistent with a link with
264 female development. Genes identified included *FIGLA*, a gene known to regulate oocyte-
265 specific genes in the female mammalian sex determination pathway (59), and *STRA8* which
266 controls entry of oocytes into meiosis. Intriguingly, the GO term “spermatid development”
267 was also enriched, encompassing many genes with known roles in testes function, including
268 *ADAD1* and *UBE2J1* (60,61). This suggests that genes involved in male sex determination in
269 mammals may have been co-opted for use in the ovarian pathway in reptiles, so their roles
270 require further investigation in other vertebrate groups, particularly given the complex
271 nature of gene expression in sperm cell types.

272 ZWC1 and ZZC1 shared 998 genes. ZZC1 has about 700 unique genes and ZWC1 about
273 500. GO analysis on shared genes between these clusters (n = 998) revealed enrichment
274 terms such as “kinase binding” and “intracellular signal transduction” (Additional file S5).
275 Genes unique to ZZC1 included members of heat shock protein families (*HSPB11*, *HSPA4*,
276 *HSP90AB1*, *HSPH1*, *HSPB1*, *HSPD1*), heterogenous ribonucleoprotein particles (*HNRNPU1*),

277 mitogen activated proteins (including *MAPK1*, *MAPK9*, *MAP3K8*), and chromatin
278 remodelling genes (*KDM2B*, *KDM1A*, *KDM5B*, *KDM3B*). GO enrichment for genes unique to
279 ZZC1 included “mitochondrion organisation”, “cellular localisation”, and “ion binding”, while
280 GO enrichment for genes unique to ZWC1 included “regulation of hormone levels” and
281 numerous signalling related functions (Additional file S6).

282 Taken together, our results show that although the same ovarian phenotype is
283 produced in genetic and temperature induced females, this end is achieved via different
284 gene expression networks. This is most pronounced at stage 6, after which the extent of the
285 differences decreases through development. This reflects canalisation of the gonadal fate to
286 a shared outcome (ovaries, Fig. 2).

287 *Signature of hormonal and cellular stress in ZWf females*

288 Previous work on *P. vitticeps* has shown a more than 50-fold upregulation of a hormonal
289 stress response gene, *POMC*, in sex reversed adult females, leading to the suggestion that
290 induction of sex reversal is in response to temperature stress, or that it is an inherently
291 stressful event, the effects of which persist into adult life (14). We therefore investigated
292 the expression of stress related genes in ZZf and ZWf embryos.

293 We found considerable evidence that ZZf embryos experience oxidative stress, likely
294 resulting from increased ROS production (discussed in detail below). However, contrary to
295 our expectations, we found that ZWf embryos showed higher expression than ZZf of
296 hormonal stress genes and pathways that have been hypothesized to be involved in sex
297 reversal (Fig. 5E-F, Additional file S3). Genes upregulated in ZWf embryos compared to ZZf
298 embryos included *STAT1*, a component of the JAK-STAT pathway, with several roles in stress
299 responses (62), and *MAP3K1* and *MAPK8*, which are typically involved in mediating stress-
300 related signal transduction cascades (63–65). *TERF2IP* is also upregulated; this gene is
301 involved in telomere length maintenance and transcription regulation (66). When
302 cytoplasmic, *TERF2IP* associates with the I-kappa-B-kinase (IKK) complex and promotes IKK-
303 mediated phosphorylation of RELA/p65, activating the NF-κB pathway and increasing
304 expression of its target genes (67). Notably two members of the IKK complex, *IKBKG* (also
305 known as *NEMO*) and *PRKCI*, which are involved in NF-κB induction, were also upregulated
306 in ZWf embryos compared to ZZf embryos (Fig. 4b), implying activation of the NF-κB

307 pathway (68). This pathway is typically associated with transducing external environmental
308 signals to a cellular response (69,70), but also has diverse roles in sex determination in
309 mammals, fish, and invertebrate models (reviewed by Castelli *et al.*, 2019).

310 *CRH*, another gene upregulated at stage 6 in ZWf females compared with ZZf females
311 (Fig. 5b, supplementary file S3), is best known for its role as a neuropeptide synthesised in
312 the brain in response to stresses that trigger the hypothalamic-pituitary-adrenal (HPA) axis
313 (71,72). The role of *CRH* production in the gonads, particularly in ovaries is currently poorly
314 understood (73–76). High *CRH* expression in ZWf gonads is the first observation of this in
315 reptiles. The role of the hormonal stress response during embryonic development, and its
316 apparent discordance with results observed in adults in *P. vitticeps* requires further
317 investigation (14).

318 **Cellular signalling cascades driving sex reversal**

319 Results of this study provide considerable corroborative support for the CaRe model, which
320 proposes a central role for calcium and redox in sensing and transducing environmental
321 signals to determine sex. Many of the genes and pathways predicted by the CaRe model to
322 be involved in sex reversal were shown to be upregulated in ZZf embryos at stage 6
323 compared to ZWf embryos. We use the CaRe model as a framework to understand the roles
324 of each signalling participant in their cellular context during the initiation of sex reversal (Fig.
325 7). This interpretation is also independently supported by GO analysis, showing enrichment
326 of expected terms, such as “cytosolic calcium ion transport” (Additional file S4), as well as k-
327 means clustering analysis (Additional files S4, S5).

328 Cluster 6 in ZZf (Fig. 6A) shows genes whose expression decreases after stage 6, so is likely
329 to include genes responsible for the initial response to temperature and initiation of sex
330 reversal, whose continuing action is not required once the ovarian trajectory has been
331 established. Consistent with this assumption, as well as with predictions from the CaRe
332 model, the 4050 genes in this cluster were enriched for GO terms that included “oxidation-
333 reduction process” and various oxidoreductase activities (Additional files S5, S6).

334 *Calcium transport, signalling, and homeostasis*

335 Our data suggest that exposure to high temperatures may cause a rapid increase in cytosolic
336 Ca^{2+} concentrations, as calcium influx is probably mediated by the thermosensitive calcium
337 channel, *TRPV2* (77,78). *TRPV2* was upregulated in stage 6 ZZf embryos compared to ZWf
338 embryos (Fig. 6). Transient receptor potential (TRP) ion channels, including *TRPV2*, have
339 previously been implicated in TSD in *Alligator sinensis* and *A. mississippiensis*, as well as the
340 turtle *Trachemys scripta* (79–82).

341 *TRPV2* mediated Ca^{2+} influx may trigger a cascade of changes within the gonadal cells
342 of ZZf females, which restore calcium homeostasis, critical to avoid apoptosis (83,84). We
343 observed evidence of such a homeostatic response, with the upregulation of seven genes
344 involved in Ca^{2+} transport and sequestration in ZZf females compared to ZWf females at
345 stage 6 (Fig. 6). Specifically, *MCU*, *ATP2B1*, *ATP2B4*, together regulate calcium homeostasis
346 through active transport of calcium into the mitochondria and into the extracellular space
347 (85–87). *KCNN1* and *CACNB3* encode proteins required for the formation of plasma
348 membrane channels controlling the passage of Ca^{2+} (88–90). *CACNB3* and *KCNN1* have well
349 characterised roles in the nervous system, and excitable cell types in muscle, but their
350 association with TSD in embryonic gonads is novel (88,89). Evidence is also building for a
351 broader role for voltage-gated calcium channels, including *CACNB3*, in orchestrating Ca^{2+}
352 signalling and gene regulation (91). We suggest that *KCNN1* and *CACNB3* in gonads of TSD
353 species play roles in mediating the homeostatic response to elevated cytosolic Ca^{2+}
354 concentrations, and are involved in the subsequent modulation of Ca^{2+} signalling pathways.

355 *TRPC4*, another TRP family gene was, upregulated in stage 12 compared to stage 6 in
356 both ZZf and ZWf embryos. *TRPC4* is expressed in mouse sperm and inhibited by
357 progesterone (92,93) but has no known association with sex determination. *TRPC4* belongs
358 to the TRPC superfamily, which all conduct calcium ions into the cell, typically through
359 phospholipase C and calmodulin signalling pathways, G-protein-coupled receptors, and
360 receptor tyrosine kinases (94,95). Notably, *PLCL2* a phospholipase gene, together with
361 calmodulin genes *CALM1* and *CAMKK1*, were upregulated alongside *TRPC4* from stage 6 to
362 stage 12 in ZZf embryos but not in ZWf embryos (Additional file S3). Given *TRPC4* is
363 upregulated from stage 6 to 12 in both ZZf and ZWf females, it may play a more conserved
364 role in ovarian development in *P. vitticeps*.

365 Several genes with functions in calcium metabolism were upregulated in stage 6 ZZf
366 embryos compared to stage 6 ZWf embryos. *CALR* encodes a multifunctional protein that
367 acts as a calcium binding storage protein in the lumen of the endoplasmic reticulum, so is
368 also important for regulating Ca^{2+} homeostasis (84,96,97). *CALR* is also present in the
369 nucleus, where it may play a role in regulation of transcription factors, notably by
370 interacting with DNA-binding domains of glucocorticoid and hormone receptors, inhibiting
371 the action of androgens and retinoic acid (97–101). *TMEM38B* (commonly known as *TRICB*)
372 is also found on the endoplasmic and sarcoplasmic reticula, where it is responsible for
373 regulating the release of Ca^{2+} stores in response to changes in intracellular conditions (102).

374 *MCU*, *ATP2B1*, *ATP2B4*, *KCNN1*, *CACNB3*, *CALR*, and *TMEM38B* have no known roles in
375 vertebrate sex determination, so their association with sex reversal in *P. vitticeps* is new.
376 This upregulation during the early stage of sex reversal suggests that they are upstream
377 modulators involved in the transduction of environmental cues that trigger sex
378 determination cascades, which is consistent with predictions made by the CaRe hypothesis.

379 We hypothesize that intracellular Ca^{2+} increases in stage 6 ZZf gonads, and further
380 observe that Ca^{2+} signalling related genes are also upregulated in ZZf females compared to
381 stage 6 ZWf females (Fig. 7). *C2CD2* and *C2CD2L* are both thought to be involved in Ca^{2+}
382 signalling, although there is no functional information about these genes. Of note is the
383 significant upregulation of *S100Z*, which is a member of a large group of EF-hand Ca^{2+}
384 binding proteins that play a role in mediating Ca^{2+} signalling (103). The EF-hand domain is
385 responsible for binding Ca^{2+} , allowing proteins like that encoded by *S100Z* to ‘decode’ the
386 Ca^{2+} biochemical signal and translate this to various targets involved in many cellular
387 functions including Ca^{2+} buffering, transport, and enzyme activation (104,105). *PLCB1* also
388 contains an EF-hand binding domain and behaves similarly, being activated by many
389 extracellular stimuli and effecting numerous signalling cascades. It can translocate to the
390 plasma membrane and nucleus, and release Ca^{2+} from intracellular stores (106). Some Ca^{2+}
391 related genes (*GCA* and *CALM1*) are also upregulated in ZWf embryos, but make only a small
392 proportion of the overall response in differential gene expression (Additional file S3).

393 *Oxidative stress in response to high temperatures*

394 The upregulation of antioxidant genes in ZZf compared to ZWf embryos suggests that the
395 gonadal cells in the ZZf embryos are in a state of oxidative stress (Fig. 7). As was proposed
396 by the CaRe model, we see results consistent with the prediction that high incubation
397 temperatures increase metabolism, which increases the production of reactive oxygen
398 species (ROS) by the mitochondria, resulting in oxidative stress (13). ROS are required for
399 proper cellular function, but above an optimal threshold, they can cause cellular damage
400 (107,108). Crossing this threshold launches the antioxidant response, which causes the
401 upregulation of antioxidant genes to produce protein products capable of neutralising ROS
402 (109,110). We observed upregulation of redox related genes, specifically of *TXNDC11*,
403 *PRDX3*, *MGST1* in ZZf embryos compared to ZWf embryos at stage 6. Also upregulated was
404 *FOXO3*, which plays a role in oxidative stress responses, typically by mediating pro-apoptotic
405 cascades (111,112). Importantly, antioxidants play other cellular roles besides neutralisation
406 of ROS. One of these is the alteration of cysteine resides through a process known as S-
407 glutathionylation (113).

408 Various redox related genes were downregulated from stage 6 to 12 in ZZf embryos
409 but not in ZW embryos, including *GLRX* and *PRDX3* (114), as well as numerous genes
410 involved in ROS induced DNA damage repair; *LIG4*, *ENDOD1*, and *HERC2* (115). This indicates
411 a need for expression of these genes specifically in ZZf embryos in early stages that ceases in
412 transition to stage 12. *STAT4*, a member of the ROS-induced JAK-STAT pathway (Simon et al.
413 1998), and *DDIT4*, which is involved stress responses to DNA damage (116), were both
414 upregulated from stage 6 to stage 12 in ZZf embryos.

415 The vertebrate antioxidant response is typically initiated by *NRF2*, but we observed no
416 differential expression of *NRF2*, only upregulation of some of its known targets in ZZf
417 embryos (117). This may mean that the action of *NRF2* is depends more on its translocation
418 from the cytoplasm to the nucleus to modulate transcription of target genes, a process that
419 does not necessarily rely on increased expression of *NRF2* (117). Alternatively, *NRF2*
420 upregulation may have occurred prior to sampling.

421 Oxidative stress has previously been proposed to have a role in TSD, based on the
422 upregulation of genes involved in oxidative stress response. One of these genes, *UCP2*, was

423 upregulated at high male producing temperatures in *A. mississippiensis* (82). *UCP2*, and
424 others genes involved in oxidative stress responses, were also implicated in UV induced
425 masculinisation in larvae of a thermosensitive fish species (*Chiostoma estor*) (118). Notably,
426 we found that *UCP2* was upregulated between stages 6 to 12 in ZZf *P. vitticeps* embryos,
427 suggesting a sustained response to thermal stress in the mitochondria (Additional file S3).

428 *Temperature response and cellular triage*

429 We also observed upregulation of genes involved in response to more generalised
430 environmental stress in ZZf compared to ZWf embryos, as expected since the embryos
431 exposed to high temperature were experiencing a state of thermal stress (Fig. 7). Notably,
432 *CIRBP* a promising candidate for regulation of sex determination under thermal influence, is
433 approximately 10-fold upregulated in ZZf compared to ZWf (Additional file S3). *CIRBP* has a
434 highly conserved role in generalised stress responses (119). It has been suggested to be a
435 putative sex determining gene in the TSD turtle *Chelydra serpentina* (120), and is
436 differentially expressed at different incubation temperatures in *Alligator sinensis* (81). We
437 also observed the upregulation of *CLK4* in ZZf compared to ZWf embryos, a gene that has
438 been recently shown to be inherently thermosensitive, and to regulate splicing of
439 temperature specific *CIRBP* isoforms (121).

440 We found that *ATF5* is upregulated in ZZf embryos compared to ZWf embryos (Fig. 7).
441 *ATF5* has diverse roles in stimulating gene expression or repression through binding of DNA
442 regulatory elements. It is broadly involved in cell specific regulation of proliferation and
443 differentiation, and may also be critical for activating the mitochondrial unfolded protein
444 response (122). This gene is induced in response to various external stressors, and is
445 activated via phosphorylation by eukaryotic translation initiation factors, two of which (*EIF1*
446 and *EIF4A2*; Zhou et al. 2008) are also upregulated in ZZf embryos compared to ZWf
447 embryos.

448 Though not well studied in the context of sex determination, heat shock factors and
449 proteins have been implicated in female sex determination in mammals and fish, and may
450 also play a conserved role in the ovarian pathway in *P. vitticeps* (79,124–127). Surprisingly,
451 only one gene associated with canonical heat shock response (*HSP40*, also known as
452 *DNAJC28*) was differentially expressed following exposure to high temperature in stage 6 ZZf

453 females compared to ZWf embryos (Additional file S3). This could mean either that a heat
454 shock response occurs prior to sampling, or that *P. vitticeps* uses different mechanisms to
455 cope with heat shock.

456 *Chromatin remodelling*

457 We observed upregulation of several components of two major chromatin remodelling
458 complexes, polycomb repressive complexes PRC1 and PRC2, in both the genotype-directed
459 ZWf and the temperature-directed ZZf female pathways in *P. vitticeps* (Fig. 7). Chromatin
460 modifier genes *KDM6B* and *JARID2* are involved in regulation of gene expression during
461 embryonic development and epigenetic modifications in response to environmental
462 stimulus (128,129). *JARID2* and *KDM6B* were both upregulated in ZZf embryos compared to
463 ZWf embryos in stages 6 and 12, and *KDM6B* was also upregulated at stage 15. These genes
464 have recently been implicated in two TSD species (*Alligator mississippiensis*, and *Trachemys*
465 *scripta*) and temperature sex reversed adult *Pogona vitticeps* (14,15,79,82).

466 We also found that two other members of the PRC1 complex, *PCGF6* and *PCGF1*, were
467 upregulated in ZZf embryos at stage 6 compared to ZWf embryos (Fig. 7). *PCGF6* is part of
468 the non-canonical PRC1 complex (ncPRC1) that mediates histone H2A mono-ubiquitination
469 at K119 (H2AK119ub) (130,131). *PCGF6* acts a master regulator for maintaining stem cell
470 identity during embryonic development (132), and is known to bind to promoters of germ
471 cell genes in developing mice (130). *PCGF1* exhibits similar functions by ensuring the proper
472 differentiation of embryonic stem cells (133). The ncPRC1 complex also promotes
473 downstream recruitment of PRC2 and H3K27me3, so that complex synergistic interactions
474 between PRC1 (both canonical and non-canonical) and PRC2 can occur (134,135).

475 We found that other components of both PRC1 and PRC2 complexes were also
476 upregulated in ZWf embryos compared to ZZf embryos (Fig. 5a, supplementary file S3). A
477 member of the canonical PRC1 complex, *PCGF2* (also known as *MEL18*), was upregulated in
478 ZWf embryos compared to ZZf embryos (134). This gene has previously been implicated in
479 temperature induced male development in *Dicentrarchus labrax* (136), and is required for
480 coordinating the timing of sexual differential in female primordial germ cells in mammals
481 (137). *KDM1A*, a histone demethylase that is required for balancing cell differentiation and
482 self-renewal (138), was upregulated in ZWf embryos compared to ZZf embryos. *CHMP1A*

483 was upregulated in ZWf, and is likely to be involved in chromosome condensation, as well as
484 targeting P_cG proteins to regions with condensed chromatin (139).

485 Thus, we conclude that the initiation of sex reversal in ZZf *P. vitticeps* involves a
486 complex cascade of cellular changes initiated by temperature. Our data are consistent with
487 the predictions of the CaRe hypothesis that high temperatures are sensed by the cell via TRP
488 channels, which causes an increase in intracellular increase of Ca²⁺. Coincident with this is an
489 increase of ROS production in the mitochondria that causes a state of oxidative stress.
490 Together, Ca²⁺ and ROS alter the CaRe status of the cell, trigger a suite of alternations in
491 gene expression including chromatin remodelling, which drives sex reversal (Fig. 7).

492 Discussion

493 We used the unique sex characteristics of our model reptile species, *Pogona vitticeps*,
494 which determines sex genetically but sex reverses at high temperature, to assess predictions
495 of the CaRe hypothesis (13). By sequencing isolated embryonic gonads, we provide the first
496 data to represent a suite of key developmental stages with comparable tissue types, and will
497 be a valuable resource for this reptilian model system. There are few transcriptomes of GSD
498 reptiles during embryonic development; the only dataset available prior to this study was a
499 preliminary study of the spiny softshell turtle, *Apalone spinifera* (126), which was
500 inadequate for the inter-stage comparisons required to explore genetic drivers of gonad
501 differentiation.

502 Our analysis of expression data during embryogenesis of normal ZWf females and
503 temperature sex reversed ZZf females revealed for the first time differences in gene-driven
504 and temperature-driven female development in a single species. Early in development, prior
505 to gonad differentiation, the initiation of the sex reversal trajectory differs from the genetic
506 female pathway both in the timing and genes involved. As development proceeds,
507 differences in expression patterns become less until the pathways converge on a conserved
508 developmental outcome (ovaries). Our ability to compare two female types in *P. vitticeps*
509 allowed us to avoid previously intractable confounding factors such as sex or species-
510 specific differences, which provided unprecedented insight into parallel female pathways.
511 We have provided new insight to the conserved evolutionary origins of the labile networks
512 governing environmentally sensitive sex determination pathways. We have identified a suite

513 of candidate genes, which now provide the necessary foundation for functional experiments
514 in the future. This could include pharmacological manipulation of calcium signalling through
515 alteration of intracellular calcium flux and concentration, such as interference with the
516 TRPV4 channel, or via use of calcium chelators and ionophores, in an organ culture system
517 (17,80). Ongoing development of resources for *P. vitticeps* as an emerging model organism
518 may also allow for RNA interference or gene editing experiments, whereby knock-down or
519 knock-out of candidate genes like *CIRBP*, *JARID2*, and *KDM6B*, could be used to demonstrate
520 their roles in sex reversal (15).

521 The maintenance of ovarian differentiation seems to require the operation of
522 different pathways in gene and temperature driven female development. This may involve a
523 pathway centrally mediated by *STAT4* in sex reversed *P. vitticeps*, which has not been
524 previously described, so requires additional confirmation with functional experiments. It will
525 be interesting to determine if a role for these genes occurs in other species. Another STAT
526 family gene, *STAT3*, has recently been demonstrated to play a critical role in the
527 phosphorylation of *KDM6B* and subsequent demethylation of the *DMRT1* promoter
528 required for male development in *T. scripta* (17). The involvement of different genes in the
529 same family is intriguing in its implications; while different genes may be co-opted, natural
530 selection may favour gene families with conserved functions even between evolutionarily
531 disparate lineages.

532 Our data provided insight into the molecular landscape of the cell required to initiate
533 temperature induced sex reversal. This is the first dataset to capture temperature-induced
534 sex reversal in a reptile, and remarkably we have simultaneously implicated all functional
535 candidates that have previously been identified to be involved in TSD across a range of
536 other species (Table 1). Our results also identified novel genes involved with
537 thermosensitive sex determination, and provide corroborative evidence for the CaRe
538 hypothesis (13). Importantly, our work highlights avenues for future studies to conduct
539 functional experiments to definitively identify the genes and pathways implicated here in
540 sex reversal. Observation and manipulation of intracellular calcium concentrations, as has
541 been conducted in *T. scripta* (17), will also be crucial for fully understanding the role of
542 calcium signalling in sex reversal.

543 Our results highlight the complexity of initiating thermolabile systems. Indeed, it has
544 been suggested that thermolabile sex determination involves system-wide displacement of
545 gene regulation with multiple genes and gene products responding to temperature leading
546 to the production of one sex or the other – a parliamentary system of sex determination
547 (151). We take an intermediate position, arguing for a central role for Calcium-Redox
548 balance as the proximal cellular sensor for temperature, but interacting with other required
549 thermosensitive genes or gene products (e.g. *CLK4*) to influence ubiquitous signalling
550 pathways and downstream splicing regulation, epigenetic modification and sex gene
551 expression. The level of interaction between each thermosensitive element remains to be
552 explored. For example, if temperature can be sensed by both *TRPV2* and *CLK4*, are both
553 required to initiate sex reversal, or is the signal from only one sufficient? This raises the
554 possibility that no single proximal sensor of the environmental exists, but that several
555 thermosensitive elements early in development must come together to orchestrate
556 alterations in gene expression.

557 It has been suggested that the products of TRP family genes act as mediators between
558 the temperature signal and a cellular response through Ca^{2+} signalling and subsequent
559 modulation of downstream gene targets (80–82). Notably, different TRP channels are
560 implicated in two alligator species; *TRPV4* in *A. mississippiensis*, but *TRPV2*, *TRPC6*, and
561 *TRPM6* in *A. sinsensis*. In *T. scripta*, *TRPC3* and *TRPV6* are upregulated at male producing
562 temperatures (26°C), while *TRPM4* and *TRPV2* are upregulated at female producing
563 temperatures (31°C), as is the case for *TRPV2* in *P. vitticeps* (79). The diversity of TRP
564 channels recruited for roles in environmental sex determination hints at considerable
565 evolutionary flexibility, perhaps the result of repeated and independent co-option of these
566 channels in TSD species. As may be the case for STAT family genes, the evolution of
567 environmentally sensitive sex determination pathways may involve the use of different
568 genes within gene families that have conserved functions.

569 Our data also highlights the importance of chromatin remodelling genes in sex
570 reversal in *P. vitticeps*. *KDM6B* and *JARID2* have been previously implicated sex
571 differentiation in adult *P. vitticeps* (14), embryonic *T. scripta* (15,17) and embryonic *A.*
572 *mississippiensis* (14). Sex-specific intron retention was observed in TSD alligators and turtle,
573 and was exclusively associated with sex reversal in adult *P. vitticeps* (14). Subsequently,

574 knockdown of *KDM6B* in *T. scripta* caused male to female sex reversal by removing
575 methylation marks on the promoter of *DMRT1*, a gene critical in the male sex
576 determination pathway (15). *KDM6B* and *JARID2* have also been associated with TSD in
577 another turtle species (*Chrysemys picta*) (126), female to male sex change in the bluehead
578 wrasse, *Thalassoma bifasciatum* (140), and thermal responses in the European bass,
579 *Dicentrarchus labrax* (141).

580 It is currently unknown if the unique splicing events in *KDM6B* and *JARID2* in adult sex
581 reversed *P. vitticeps* that cause intron retention and presumed gene inactivation, also occur
582 in embryos. Given the high expression of these genes during embryonic development at sex
583 reversing temperatures, it would be surprising if this pattern was observed. We also show a
584 significant role for *CIRBP* as the only other gene, alongside *KDM6B*, to be consistently
585 upregulated during sex reversal in all developmental stages assessed. *CIRBP* is a mRNA
586 chaperone, which could be required to stabilise transcripts of crucial sex specific genes
587 during oxidative, cellular and/or thermal stress. It has been proposed as a novel TSD
588 candidate gene in the turtle, *Chelydra serpentina* (120). This gene remains a promising
589 candidate for mediating thermosensitive responses in TSD more broadly, and its role needs
590 to be explored in more detail.

591 **Conclusions**

592 The alternative female pathways in *P. vitticeps* demonstrates that there is inherent flexibility
593 in sex determination cascades even within the same species. This is consistent with the idea
594 that, provided a functional gonad is produced, considerable variation in sex determining and
595 differentiation processes at the early stages of development is tolerated under natural
596 selection (151). Perhaps this makes the astonishing variability in sex determination between
597 diverse species less surprising. Our findings provide novel insights, and are a critical
598 foundation for future studies of the mechanisms by which temperature determines sex.

599 **Materials and Methods**

600 *Animal breeding and egg incubations*

601 Eggs were obtained during the 2017-18 breeding season from the research breeding colony
602 at the University of Canberra. Breeding groups comprised three sex reversed females (ZZf)

603 to one male (ZZ), and three concordant females (ZWf) to two males (Fig. 1). Paternity was
604 confirmed by SNP genotyping (Fig. S2). Females were allowed to lay naturally, and eggs
605 were collected at lay or within two hours of lay. Eggs were inspected for viability as
606 indicated by presence of vasculature in the egg, and viable eggs were incubated in
607 temperature-controlled incubators ($\pm 1^\circ\text{C}$) on damp vermiculite (4 parts water to 5 parts
608 vermiculite by weight). Clutches from sex reversed females (that is, ZZf x ZZm crosses)
609 comprised eggs with only ZZ genotypes. These were initially incubated at 28°C (male
610 producing temperature, MPT) to entrain and synchronise development. After 10 d of
611 incubation, half of the eggs selected at random from each clutch was shifted to 36°C (female
612 producing temperature, FPT). Clutches from ZWf x ZZm crosses were incubated at 28°C
613 throughout the incubation period (Fig. 1A). Sample sizes are given in Fig. 1 and Additional
614 file S7.

615 *Embryo sampling and genotyping*

616 Eggs from both temperatures were sampled at times corresponding to three developmental
617 stages (6, 12 and 15) (20), taking into account the differing developmental rates between
618 28°C and 36°C. These stages equate to the bipotential gonad, recently differentiated gonad,
619 and differentiated gonad respectively (19). Embryos were euthanized by intracranial
620 injection of 0.1 ml sodium pentobarbitone (60mg/ml in isotonic saline). Individual gonads
621 were dissected from the mesonephros under a dissection microscope and snap frozen in
622 liquid nitrogen. Isolation of the gonad from the surrounding mesonephros was considered
623 essential for studying transcriptional profiles within the gonad. Embryos from three
624 different ZZf x ZZm clutches from each treatment class (temperature x stage) were selected
625 for sequencing, and randomized across sequence runs to avoid batch effects. Embryos from
626 concordant ZWf x ZZm crosses potentially yield both ZW and ZZ eggs, so these were
627 genotyped using previously established protocols (8,20). Briefly, this involved obtaining a
628 blood sample from the vasculature on the inside of the eggshell on a FTA Elute micro card
629 (Whatman). DNA was extracted from the card following the manufacturer protocols, and
630 PCR was used to amplify a W specific region (8) so allowing the identification of ZW and ZZ
631 samples.

632 *RNA extraction and sequencing*

633 RNA from isolated gonad samples was extracted in randomized batches using the Qiagen
634 RNeasy Micro Kit (Cat. No. 74004) according to the manufacturer protocols. RNA was eluted
635 in 14 µl of RNAase free water and frozen at -80°C prior to sequencing. Sequencing libraries
636 were prepared in randomized batches using 50 ng RNA input and the Roche NimbleGen
637 KAPA Stranded mRNA-Seq Kit (Cat. No. KK8420). Nine randomly selected samples were
638 sequenced per lane using the Illumina HiSeq 2500 system, and 25 million read-pairs per
639 sample were obtained on average. Read lengths of 2 x 150 bp were used. All samples were
640 sequenced at the Kinghorn Centre for Clinical Genomics (Garvan Institute of Medical
641 Research, Sydney). All sample RNA and library DNA was quantified using a Qubit Instrument
642 (ThermoFisher Scientific, Scoresby, Australia), with fragment size and quality assessed using
643 a Bioanalyzer (Agilent Technologies, Mulgrave, Australia).

644 *Gene expression profiling*

645 Paired-end RNA-seq libraries (.fastq format) were trimmed using trim_galore with default
646 parameters (v0.4.1; https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/, last
647 access 21-Apr-2020). Trimmed reads were aligned to the *Pogona vitticeps* NCBI reference
648 genome (pvi1.1, GenBank GCA_900067755.1; (142)) using STAR (v2.5.3; (143)), with splice-
649 aware alignment guided by the accompanying NCBI gene (*Pvi1.1*) annotation (.gtf format).
650 Likely PCR duplicates and non-unique alignments were removed using samtools (v1.5 ;
651 (144)). Gene expression counts and normalised expression values (reported in TPM) were
652 determined using RSEM (rsem-calculate-expression; v1.3.1; (145)).

653 *Identification of non-sex reversed specimens*

654 Normalised transcripts per million (TPM) for a panel of sex-specific genes (*SOX9*, *AMH*,
655 *DMRT1*, *FOXL2*, *CYP19A1*, *CYP17A1*) were inspected across the three stages to identify if any
656 samples showed aberrant expression patterns. This approach was also used to determine if
657 any of the stage 12 and 15 samples from the 36°C treatment had not undergone sex
658 reversal by comparing expression levels between ZWf and ZZf embryos; the rate of sex
659 reversal is 96% at 36°C (8) (Fig. S3, Additional file S8). The five samples from clutch 9
660 exhibited significantly higher expression values for *SOX9*, *AMH*, and *DMRT1* and represented
661 clear outliers. This was also supported by multidimensional scaling (MDS) plots, so the

662 decision was made to regard the five samples from clutch 9 as aberrant and exclude them
663 from subsequent analyses (Figs S3, S4, Additional file S9). Any ZZf samples with male-like
664 gene expression patterns (high expression for male-specific genes, and low expression for
665 female-specific genes) were considered to have not been reversed (sex reversal is not 100%
666 at 36°C) and were removed (two stage 15 samples).

667 *Differential expression analysis*

668 Differential expression analysis of ZZf and ZWf transcripts was conducted on raw counts
669 using the EdgeR package (Bioconductor v 3.9 (146)) in R (v 1.2.1335, (147)), following
670 standard procedures outlined in the EdgeR users guide (146,148). Lowly expressed genes,
671 which was applied to genes with fewer than ten counts across three samples, were removed
672 from the raw counts (19,285 genes) so that the total number of genes retained was 17,075.
673 Following conversion to a DGElist object in EdgeR, raw counts were normalised using the
674 upper-quartile method (calcNormFactors function) (149). Estimates for common negative
675 binomial dispersion parameters were generated (estimateGLMCommonDisp function) (148),
676 followed by generation of empirical Bayes dispersion estimates for each gene
677 (estimateGLMTagwiseDisp function) (148,150). A quasi-likelihood binomial generalised log-
678 linear model was fitted (glmQLFit function) and the glmQFTest function was used to
679 compare contrasts within the design matrix (151–155). A P-value cut-off of 0.01 and a log₂-
680 fold change threshold of 1 or -1 was applied to all contrasts (topTags function) (151).
681 Contrasts were used to assess differential expression between ZZf and ZWf samples across
682 each developmental stage. Raw count (Additional file S10) and expression files (Additional
683 file S11) from this analysis are supplied.

684 Gene ontology (GO) analysis was conducted for each set of differentially expressed
685 genes using GOrilla (156,157). The filtered count data file (17,075 genes) was used for the
686 background gene set at a P-value threshold of 10⁻³.

687 *K-means clustering analysis*

688 K-means clustering analysis was performed on normalised counts per million extracted from
689 the DGElist object produced by the initial process of the DGE analysis using edgeR (see
690 above). Counts for each gene were averaged for each treatment group, and the number of
691 clusters was selected using the sum of squared error approach, which was further validated

692 by checking that each cluster centroid was poorly correlated with all other cluster centroids
693 (maximum correlation 0.703 in ZWf clusters, and 0.65 in ZZf clusters). A total of 6 clusters
694 was chosen, and clustering analysis was conducted using the kmeans function in R package
695 stats v3.6.2. Resultant gene lists were sorted by unique and shared genes between clusters
696 with similar trends between ZWf and ZZf (cluster 1 in ZWf, cluster 3 in ZZf, and cluster 3 in
697 ZWf and cluster 5 in ZZf). Both unique and shared genes from each cluster and pairs of
698 clusters (cluster 1 and 3, and clusters 3 and 5) were then analysed for gene ontology (GO)
699 enrichment using GOrilla (156,157). The filtered count data file (17,075 genes) was used for
700 the background gene set at a P-value threshold of 10^{-3} .

701 **Declarations**

702 *Ethics Approval*

703 All procedures were conducted in accordance with approved animal ethics protocols from
704 the University of Canberra Animal Ethics Committee (AEC 17-17).

705 *Consent for publication*

706 Not applicable

707 *Availability of data and materials*

708 The raw input files (counts and transcripts per million) that were analysed for this study are
709 available as supplementary files. Raw sequencing data is available under NCBI BioProject
710 PRJNA699086 (Biosample accession IDs SAMN17765903 to SAMN17765941).

711 *Competing interests*

712 The authors declare that they have no competing interests.

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718 *Author Contributions*

719 AG and CEH led and designed the experiment, AG and JMG built the “Sex in Dragons”
720 program of which this is a part. SLW carried out all experimental procedures and differential
721 gene expression analysis. JB created all RNA libraries and carried out all sequencing. IWD
722 generated all data files from sequencing outputs. SW assisted with data analysis, particularly
723 K-means clustering. SLW lead the preparation of the manuscript, with AG, CEH, and JMG. All
724 authors provided feedback on the manuscript and approved the final draft.

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1091 visualization of enriched GO terms in ranked gene lists. BMC Bioinformatics. 2009;10:48.

1092 157. Eden E, Lipson D, Yoge S, Yakhini Z. Discovering motifs in ranked lists of DNA sequences.
1093 PLoS Comput Biol. 2007;3:0508–22.

1094

1095 **Table 1:** All genes, full gene names, functional categories and associations with either gene
1096 (ZWf) or temperature driven (ZZf) female development mentioned in the paper. NA denotes
1097 a gene that was mentioned, but was not differentially expressed. Genes with an asterisk are
1098 those that have previously been implicated in thermosensitive sex determination cascades,
1099 either in *Pogona vitticeps*, or in another reptile species.

Gene ID	Gene Name	Functional Category	Association
ADAD1	Adenosine deaminase domain containing 1 [testis-specific]	Sex determination and differentiation (Male-specific)	ZWf/Zf
ALDH1A2	Retinal dehydrogenase 2	Sex determination and differentiation (Male-specific)	ZWf
AMH	Anti-Müllerian hormone	Sex determination and differentiation	NA
AMHR2	Anti-Müllerian hormone receptor 2	Sex determination and differentiation	ZZf
ATF5	Activating transcription factor 5	Stress response	ZZf
ATP2B1	ATPase plasma membrane Ca ²⁺ transporting 1	Calcium signalling	ZZf
ATP2B4	ATPase plasma membrane Ca ²⁺ transporting 4	Calcium signalling	ZZf
BMP7	Bone morphogenetic protein 7	Sex determination and differentiation	ZZf
C2CD2	C2 calcium-dependent domain containing 2	Calcium signalling	ZZf
C2CD2L	C2 calcium-dependent domain containing 2 like	Calcium signalling	ZZf
CACNB3	Calcium voltage-gated channel auxiliary subunit beta 3	Calcium signalling	ZZf
CALM1	Calmodulin 1	Calcium signalling	ZWf/Zf
CALR	Calreticulin	Calcium signalling	ZZf
CAMKK1	Calcium/calmodulin dependent protein kinase kinase 1	Calcium signalling	ZZf
CHMP1A	Chromatin modifying protein 1A	Chromatin remodelling	ZWf
CIRBP*	Cold-inducible binding protein	Temperature-sensing	ZZf
CLK4*	CDC like kinase 4	Temperature-sensing	ZZf
CRH	Corticotropin releasing hormone/factor	Stress response	ZWf
CYP17A1	Cytochrome P450 17A1	Sex determination and differentiation (Female-Specific)	ZWf/Zf
CYP19A1	Aromatase	Sex determination and differentiation (Female-)	ZWf/Zf

		Specific)	
<i>DDIT4</i>	DNA damage inducible transcript 4	DNA damage repair	Zwf
<i>DLL3</i>	Delta like canonical Notch ligand 3	Sex determination and differentiation (Male-specific)	Zwf/Zf
<i>DLL4</i>	Delta like canonical Notch ligand 4	Sex determination and differentiation (Male-specific)	Zwf/Zf
<i>DMRT1</i>	Doublesex and mab-3 related transcription factor 1	Sex determination and differentiation (Male-specific)	NA
<i>EGR1</i>	Early growth response 1	Sex determination and differentiation	Zwf
<i>EIF1</i>	Eukaryotic translation initiation factor 1	Translation initiation	Zwf
<i>EIF4A2</i>	Eukaryotic translation initiation factor 4A2	Translation initiation	Zwf
<i>ENDOD1</i>	Endonuclease domain containing 1	DNA damage repair	Zwf
<i>ESR2</i>	Estrogen receptor 2	Sex determination and differentiation (Female-Specific)	Zwf
<i>ESRRG</i>	Estrogen related receptor gamma	Sex determination and differentiation (Female-Specific)	Zwf
<i>FIGLA</i>	Folliculogenesis specific basic helix-loop-helix	Sex determination and differentiation (Female-Specific)	Zwf/Zf
<i>FOXL2</i>	Forkhead box L2	Sex determination and differentiation (Female-Specific)	Zwf/Zf
<i>FOXO3</i>	Forkhead box O3	Redox regulation	Zwf
<i>FZD1</i>	Frizzled class receptor 1	Sex determination and differentiation	Zwf
<i>GADD45G</i>	Growth arrest and DNA damage inducible gamma	Sex determination and differentiation	Zwf
<i>GATA6</i>	GATA binding factor 6	Sex determination and differentiation	Zwf
<i>GCA</i>	Grancalcin	Calcium signalling	Zwf
<i>GLRX</i>	Glutaredoxin	Redox regulation	Zwf
<i>GPX1</i>	Glutathione peroxidase	Redox regulation	Zwf
<i>HERC2</i>	HECT and RLD domain containing E3 ubiquitin protein ligase 2	DNA damage repair	Zwf
<i>HNRNPU</i> <i>L1</i>	Heterogeneous nuclear ribonucleoprotein U like 1	Splicing	Zwf/Zf
<i>HSD17B3</i>	Hydroxysteroid 17-beta dehydrogenase 3	Sex determination and differentiation	Zwf
<i>HSP40</i>	DnaJ heat shock protein family (hsp40) member B1	Temperature-sensing	Zwf
<i>HSP90AB1</i>	Heat shock protein 90 alpha family class B member 1	Temperature-sensing	Zwf/Zf
<i>HSPA4</i>	Heat shock protein family A (Hsp70) member 4	Temperature-sensing	Zwf/Zf
<i>HSPB1</i>	Heat shock protein family B (Small) member 1	Temperature-sensing	Zwf/Zf

<i>HSPB11</i>	Heat shock protein family B (Small) member 11	Temperature-sensing	ZWf/Zf
<i>HSPD1</i>	Heat shock protein family D (Hsp60) member 1	Temperature-sensing	ZWf/Zf
<i>HSPH1</i>	Heat shock protein family H (Hsp110) member 1	Temperature-sensing	ZWf/Zf
<i>IKBKG/NEMO</i>	NF-κB essential modulator	NF-κB pathway	ZWf
<i>JAG2</i>	Jagged 2	Sex determination and differentiation	ZWf
<i>JARID2*</i>	Jumonji and AT-rich interaction domain containing 2	Chromatin remodelling	ZZf
<i>KCNN1</i>	Small conductance calcium-activated potassium channel protein 1	Calcium signalling	ZZf
<i>KCTD1</i>	Potassium channel tetramerization domain containing 1	Sex determination and differentiation (Male-specific)	ZZf
<i>KDM1A</i>	Lysine demethylase 1A	Chromatin remodelling	ZWf/Zf
<i>KDM2B</i>	Lysine demethylase 2B	Chromatin remodelling	ZWf/Zf
<i>KDM3B</i>	Lysine demethylase 3B	Chromatin remodelling	ZWf/Zf
<i>KDM5B</i>	Lysine demethylase 5B	Chromatin remodelling	ZWf/Zf
<i>KDM6B*</i>	Lysine demethylase 6B	Chromatin remodelling	ZZf
<i>LHX9</i>	LIM homeobox 9	DNA damage repair	ZWf
<i>LIG4</i>	DNA ligase 4	DNA damage repair	ZZf
<i>MAP3K8</i>	Mitogen-activated protein kinase kinase kinase 8	Stress response	ZWf/Zf
<i>MAPK1</i>	Mitogen-activated protein kinase 1	Stress response	ZWf/Zf
<i>MAPK9</i>	Mitogen-activated protein kinase 9	Stress response	ZWf/Zf
<i>MCU</i>	Mitochondrial calcium uniporter	Calcium signalling	ZZf
<i>MGST1</i>	Microsomal glutathione S-transferase 1	Redox regulation	ZZf
<i>NANOS1</i>	Nanos C2HC-type zinc finger 1	Sex determination and differentiation (Female-Specific)	ZWf
<i>NCOA4</i>	Nuclear receptor coactivator 4	Sex determination and differentiation	ZZf
<i>NEIL3</i>	Nei like DNA glycosylase 3	DNA damage repair	ZZf
<i>NR5A1</i>	Nuclear receptor subfamily 5 group A member 1	Sex determination and differentiation	ZZf
<i>NRF2</i>	Nuclear factor, erythroid 2 like 2	Redox regulation	NA
<i>PCGF1</i>	Polycomb group ring finger 1	Chromatin remodelling	ZZf
<i>PCGF2/Mel18</i>	Polycomb group ring finger 2	Chromatin remodelling	ZWf
<i>PCGF6</i>	Polycomb group ring finger 6	Chromatin remodelling	ZZf
<i>PCYOX1L</i>	Prenylcysteine oxidate 1 like	Redox regulation	ZZf

PDGFB	Platelet derived growth factor subunit B, paralog of mammalian <i>PDGFA</i>	Sex determination and differentiation	ZZf
PGR	Progesterone receptor	Sex determination and differentiation (Female-Specific)	ZWf
PLCB1	Phospholipase C Beta 1	Calcium signalling	ZZf
PLCL2	Phospholipase C like 2	Calcium signalling	ZZf
POMC	Proopiomelanocortin	Stress response	NA
PRDX3	Peroiredoxin 3	Redox regulation	ZZf
PRKCI	Protein kinase C iota	NF-kB pathway	ZWf
RSPO1	R-spondin 1	Sex determination and differentiation (Female-Specific)	ZWf/Zf
S100Z	S100 calcium binding protein Z	Calcium signalling	ZZf
SFRP2	Secreted frizzled related protein 2	Sex determination and differentiation (Male-specific)	ZZf
SOX4	SRY-box transcription factor 4	Sex determination and differentiation (Male-specific)	ZWf
SOX9	Sry-box 9	Sex determination and differentiation (Male-specific)	NA
SQOR	Sulfide quinone oxidoreductase	Redox regulation	ZZf
SRD5A2	Steroid 5 alpha reductase 2	Sex determination and differentiation	ZWf
STAT1	Signal transducer and activator of transcription 1	Stress response	ZWf
STAT4	Signal transducer and activator of transcription 4	Stress response	ZZf
STRA8	Stimulated by retinoic acid 8	Sex determination and differentiation	ZWf/Zf
TERF2IP	Telomeric repeat-binding factor 2-interacting protein 1	NF-kB pathway	ZWf
TGFB3L	Transforming growth factor beta receptor 3-like, paralog of mammalian <i>TGFB3</i>	Sex determination and differentiation	ZZf
TMEM38B/TRICB	Trimeric intracellular cation channel type B	Calcium signalling	ZZf
TRPC4	Transient receptor potential cation channel subfamily C member 4	Temperature-sensing	ZZf
TRPV2*	Transient receptor potential cation channel subfamily V member 2	Temperature-sensing	ZZf
TXND11	Thioredoxin domain containing 11	Redox regulation	ZZf
UBE2J1	Ubiquitin-conjugating enzyme E2 J1	Sex determination and differentiation	ZWf/Zf
UCP2	Oxidative stress responsive-gene uncoupling protein-2	Redox regulation	ZZf
WNT5a	Wnt family member 5a	Sex determination and differentiation	ZZf

1101 **Figure Captions**

1102 **Fig. 1:** Schematic representation of experimental design used in this study to compare the
1103 differences between genetic sex determination and temperature dependent sex
1104 determination. **(A)** Summary of experiment showing how the parental crosses were
1105 designed, and how eggs were allocated and incubated. Eggs from sex reversed females (ZZf)
1106 were initially incubated at 28°C for 10 days, then were switched to 36°C. Eggs were sampled
1107 at the same three developmental stages (6, 12, and 15) based on (19,20). At stage 6 the
1108 gonad is bipotential, at stage 12 the gonad is in the early stages of differentiation, and it
1109 completely differentiated by stage 15. Eggs from concordant females (ZWf) were incubated
1110 at 28°C and sampled at the same three developmental stages as the ZZf eggs. **(B)** PCA plots
1111 showing the first and second principal components of read count per gene between ZZf
1112 (red) and ZWf (blue) at each stage of development.

1113 **Fig. 2:** Schematic overview of gene-driven (blue) and temperature-driven (red) female
1114 developmental pathways in *Pogona vitticeps*. The pathways are initially different (from
1115 stages 6 to 12), but they ultimately converge on highly similar expression profiles when
1116 ovarian differentiation has occurred by stage 15. Both pathways are characterised by
1117 repression of a male signal, however this signal is stronger in temperature-driven females
1118 and appears to require ongoing repression when compared with the gene-driven females.

1119 **Fig. 3:** **(A)** Expression (transcripts per million, TPM) \pm SE of three genes differentially
1120 expressed at all three developmental stages between ZZf and ZWf, with *KDM6B* and *CIRPB*
1121 (outlined in red) having consistently higher expression in ZZf embryos, and *GCA* having
1122 higher expression in ZWf. **(B)** Bar graphs representing the number of differentially expressed
1123 genes in all comparisons between ZZf and ZWf, and between developmental stages. MA
1124 plots of this data are available in Fig. S1. Differentially expressed genes were determined as
1125 having *P* values ≤ 0.01 and log₂-fold changes of 1, -1.

1126 **Fig. 4:** Hypothesized pathway for the maintenance of the ovarian phenotype in stage 12 sex
1127 reversed ZZf *Pogona vitticeps*. Given the upregulation of these genes, it is likely that reactive
1128 oxygen species induce the phosphorylation, and subsequent activation and nuclear
1129 translocation of STAT4, likely mediated by PDGFB. Once in the nucleus, STAT4 is able to bind

1130 to promoter regions of known target genes, *NR5A1*, *AMHR2* and *EGR2* to regulate their
1131 expression and promote ovarian development.

1132 **Fig. 5:** (A) A subset of GO processes and (C) GO functions enriched in stage 6 ZZf embryos
1133 compared with ZWf. (B) A subset of GO processes and (D) GO functions enriched in stage 6
1134 ZWf embryos compared with ZZf. Complete results of GO analysis for all developmental
1135 stages in ZZf and ZWf for enriched GO processes and functions is provided in Additional file
1136 S2. Differentially expressed chromatin modifier (E) and cellular stress (F) genes in *Pogona*
1137 *vitticeps* at stage 6 comparing ZWf and ZZf females.

1138 **Fig. 6:** K-means clustering analysis on normalized counts per million for ZZf (A) and ZWf (B)
1139 across all developmental stages. The colour depicts the correlation score of each gene in the
1140 cluster, where numbers approaching one (red) have the strongest correlation. All gene lists
1141 produced for each cluster are provided in Additional file S5.

1142 **Fig. 7:** Hypothesised cellular environment (A) of a ZZf gonad at stage 6 in *Pogona vitticeps*
1143 based on differential expression analysis (B) using the CaRe model as a framework (13). We
1144 used this approach to understand the cellular context responsible for driving sex reversal in
1145 ZZf samples. This reveals that calcium signalling likely plays a very important role in
1146 mediating the temperature signal to determine sex. Influx of intracellular calcium is likely
1147 mediated primarily by TRPV2, and may also be influenced by KCNN1 and CACNB3. This influx
1148 appears to trigger significant changes in the cell to maintain calcium homeostasis. MCU,
1149 ATP2B1, CALR and TRICB all play a role in this process by sequestering calcium and pumping
1150 it back out of the cell, in which KCNN1 and CACNB3 may have a role. Calcium signalling
1151 molecules C2CD2, C2CDL2, and S100Z are likely responsible for encoding and translating the
1152 calcium signal leading to changes in gene transcription. Changes in gene expression are
1153 likely mediated primarily by the two major Polycomb Repressive Complexes, PRC1 and
1154 PRC2. Members of these two complexes (PCGF1, PCGF6, KDM6B, and JARID2)
1155 transcriptionally regulate genes by controlling methylation dynamics of their targets, the
1156 latter two of which have been previously implicated in sex reversal (14,15). ATF5 may also
1157 play a role in gene regulation, and alternative splicing, which has been implicated in sex
1158 reversal (14) may be mediated by CLK4. High temperatures necessarily increase cellular
1159 metabolism, which in turn increases the amount of reactive oxygen species (ROS) produced
1160 by the mitochondria. ROS can cause cellular damage at high levels, so trigger an antioxidant

1161 response, which is observed here in the upregulation of MGST1, PRDX3, TXNDC11 and
1162 FOXO3. Also of note is the upregulation of CIRBP, which has numerous functions in response
1163 to diverse cellular stresses, and has been implicated in TSD.

1164 **Supplementary Materials**

1165 **Additional file S1:** Differentially expressed genes between developmental stages 6 and 12,
1166 and 12 and 15 for ZZf and ZWf females generated from EdgeR's "topTags" function. Results
1167 are sorted by log-fold change, with a cut-off of 1 or -1 applied, and a P-value threshold of
1168 0.01. For the stage 6 and 12 comparison, genes with positive log-fold changes are
1169 upregulated at stage 12, while for the stage 12 and 15 comparison, genes with a positive
1170 log-fold change are upregulated at stage 15. No genes were differentially expressed
1171 between stages 12 and 15 in ZZf females.

1172 **Additional file S2:** Gene ontology (GO) enrichment for process and function for the stage 6
1173 and 12 comparison for ZZf and ZWf (Additional file S1). GO enrichment was not possible for
1174 the stage 12 and 15 comparison because differentially expressed genes were lacking. GO
1175 enrichment was generated from GOrilla (156,157) at a significance threshold of $P \leq 0.05$.

1176 **Additional file S3:** Differentially expressed genes between ZZf and ZWf for each
1177 developmental stage generated from EdgeR's "topTags" function. Results are sorted by log-
1178 fold change, with a cut-off of 1 or -1 applied, and a P-value threshold of 0.01. Genes with
1179 positive log-fold changes are upregulated in ZZf embryos, genes with negative log-fold
1180 changes are upregulated in ZWf.

1181 **Additional file S4:** Gene ontology (GO) enrichment for process and function generated from
1182 GOrilla (156,157) at a significance threshold of $P \leq 0.05$ for differentially expressed genes at
1183 stages 6 and 12 for ZZf and ZWf samples (Additional file S3).

1184 **Additional file S5:** Gene list outputs for K-means clustering analysis ($n = 6$), and comparative
1185 information for matched clusters between ZZf and ZWf (ZZC1 and ZWC1, and ZZC2 and
1186 ZWC4) including genes that are unique and shared between each cluster.

1187 **Additional file S6:** Gene ontology (GO) process and function enrichment for genes in ZZf C1,
1188 and genes shared between ZZC5 and ZWC3 generated from GOrilla (156,157) at a
1189 significance threshold of $P \leq 0.05$.

1190 **Additional file S7:** Summary of all embryonic gonad samples sequenced for this study,
1191 including incubation temperature, genotype, parental cross, developmental stage, and
1192 clutch for each sample. Unique sample identifiers are matched to those used in raw data
1193 inputs (Additional file S10, S11).

1194 **Additional file S8:** Outputs from pairwise T-tests conducted between stage 15 ($n = 7$) and
1195 stage 15 ZZf samples suspected not to have undergone sex reversal ($n = 2$). The normalised
1196 transcripts per million (TPM) for six genes, three male (*AMH*, *DMRT1*, *SOX9*) and three
1197 female genes (*FOXL2*, *CYP19A1*, *CYP17A1*) were used. The two samples suspected of not
1198 undergoing sex reversal show significantly different expression levels for four of these
1199 genes, and differences just above the significance threshold of ≤ 0.05 for the other two
1200 genes. On this basis, these two samples were removed from further analysis.

1201 **Additional file S9:** Outputs from one way analysis of variance (ANOVAs) between all
1202 clutches (clutch 1, 2, 3, 6, and 9) across each developmental stage (6, 12 and 15) for a panel
1203 of sex specific genes (*AMH*, *SOX9* and *DMRT1*) to determine whether clutch 9 exhibits
1204 aberrant expression levels. The normalised transcripts per million (TPM) generates from the
1205 EdgeR pipeline described in the materials and methods was used. Based on the results from
1206 this analysis, five samples from clutch 9 were excluded from further analysis.

1207 **Additional file S10:** Raw counts for all samples ($n = 39$) for all genes ($n = 19,284$) prior to any
1208 filtering or sample removal. Sample ID labels correspond to incubation temperature (36 or
1209 28), maternal genotype/maternal homozygosity/maternal heterozygosity (ZZf or ZWf),
1210 sample stage (s1 = stage 6, s2 = stage 12, s3 = stage 15), clutch number (c1, c2, etc.), and
1211 replicate ID (e.g., “a” denotes the sample was the first replicate for that sampling point).
1212 Sample data is also available in Additional file S7.

1213 **Additional file S11:** Raw expression values (TPM, transcripts per million) all samples ($n = 39$)
1214 for all genes ($n = 19,284$) prior to any filtering or sample removal. Sample ID labels
1215 correspond to incubation temperature (36 or 28), maternal genotype/maternal

1216 homozygosity/maternal heterozygosity (ZZf or ZWf), sample stage (s1 = stage 6, s2 = stage
1217 12, s3 = stage 15), clutch number (c1, c2, etc.), and replicate ID (e.g., “a” denotes the sample
1218 was the first replicate for that sampling point). Sample data is also available in Additional file
1219 S7.

1220 **Fig. S1:** MA plots of read counts per gene from differential expression analysis conducted
1221 between ZZf and ZWf (**A**) and comparisons between stages for both ZZf and ZW (**B**).
1222 Differentially expressed genes (P values ≤ 0.01 , \log_2 -fold change of 1, -1) are coloured
1223 (colour indicative of significant fold change), and the total number of genes are indicated in
1224 each plot. Grey indicates no differential expression, horizontal lines indicate \log_2 -fold
1225 changes of 1, -1. CPM: normalised counts per million

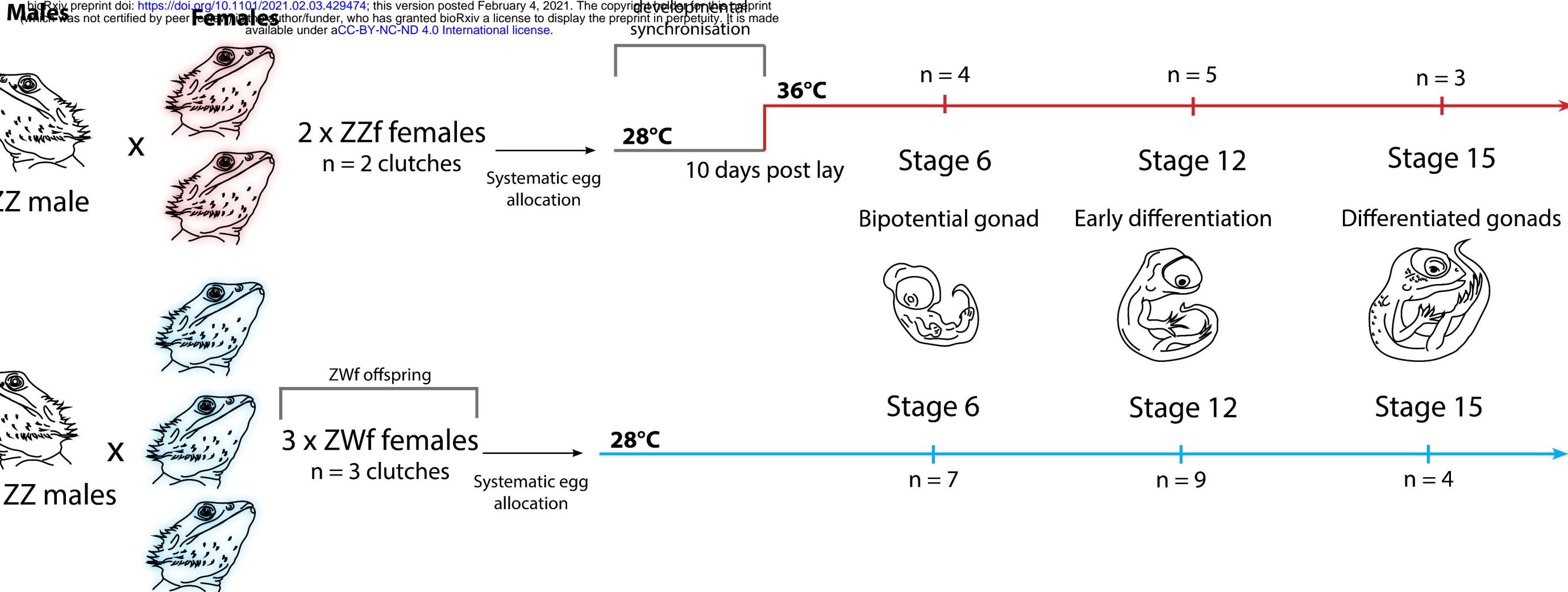
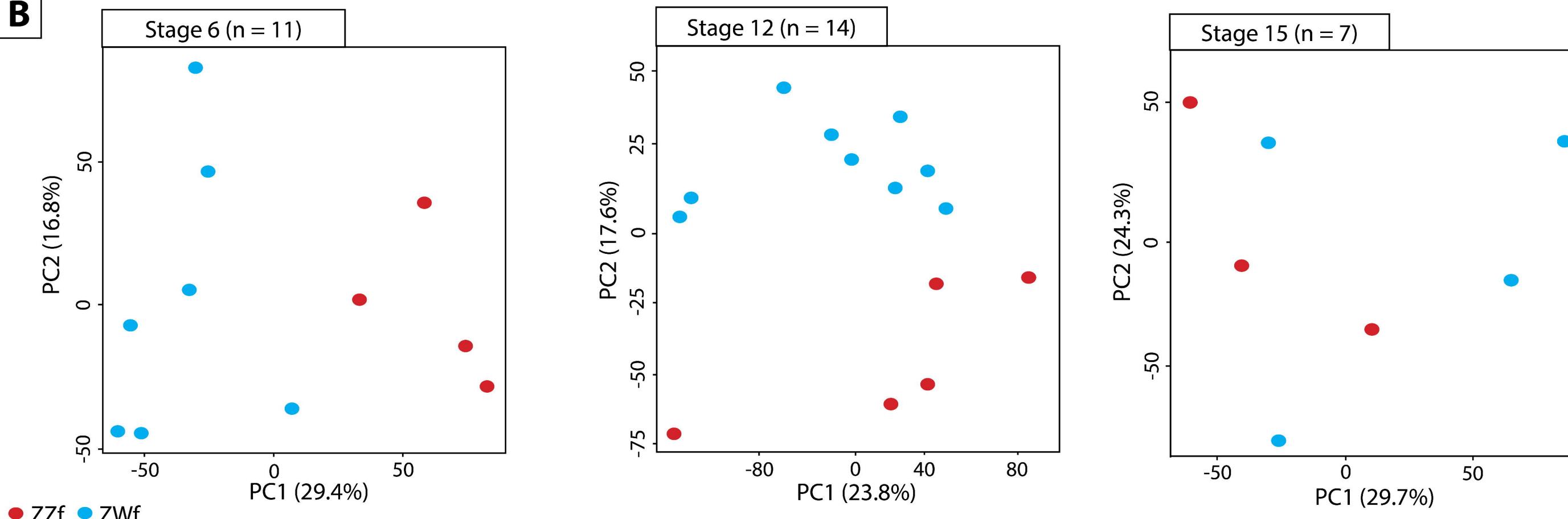
1226 **Fig. S2:** Network analysis of parental and offspring SNPs to confirm paternity of clutches
1227 used in this experiment. SNP data was generated by Dart sequencing, a reduced genome
1228 representation sequencing method at Diversity Arrays Technology, University of Canberra.

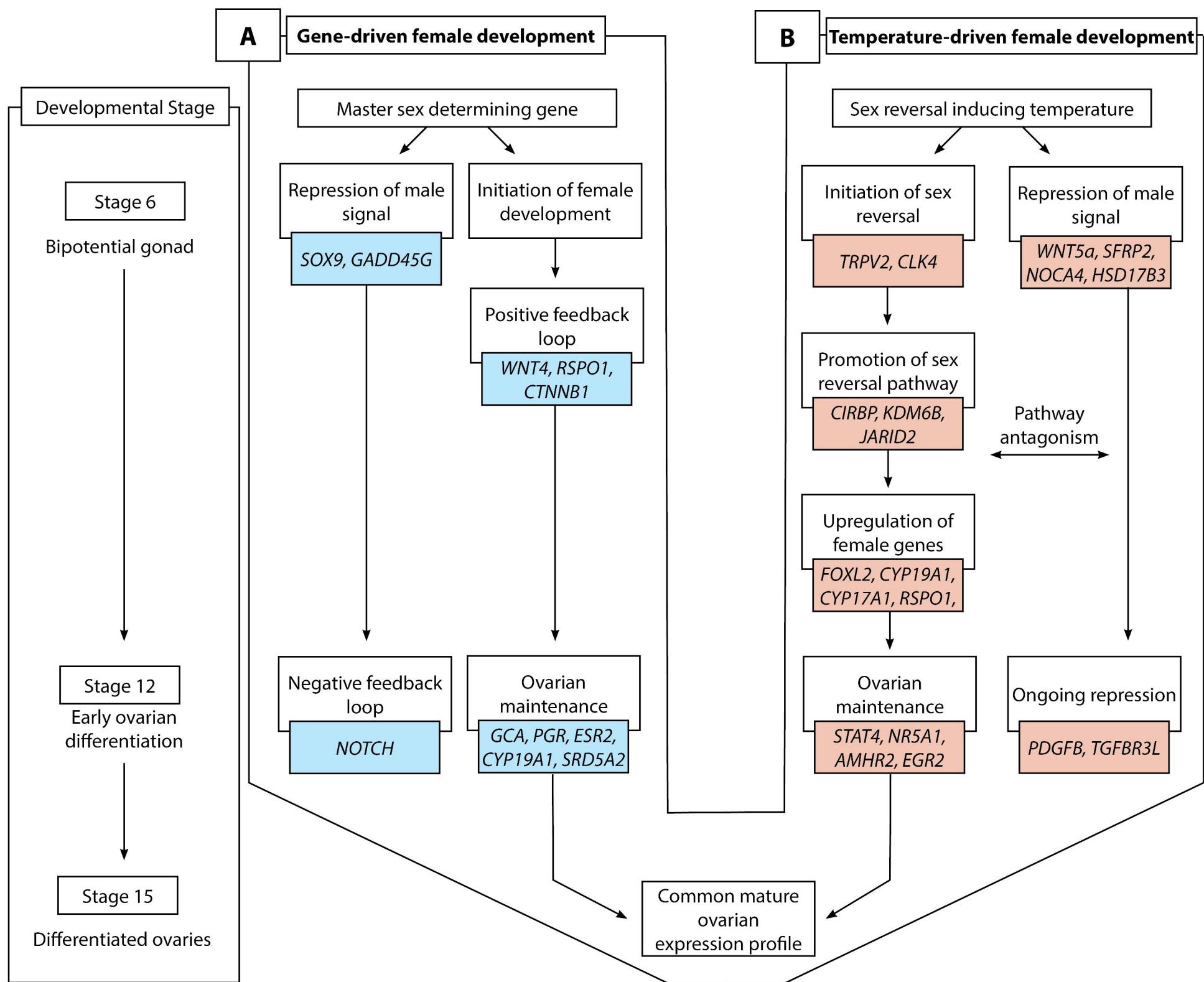
1229 **Fig. S3:** Expression (TPM, transcripts per million) of female-specific genes (*CYP17A1*, *FOXL2*,
1230 *CYP19A1*; panel A) and male-specific genes (*DMRT1*, *SOX9*, *AMH*; panel B) across three
1231 developmental stages (6, 12, 15) (19,20) for all samples to aid in the identification of
1232 samples with aberrant expression patterns. Samples from later developmental stages that
1233 exhibit low expression of female-specific genes are likely to have not undergone sex
1234 reversal. Sample ID labels correspond to incubation temperature (36°C or 28°C in red or
1235 blue respectively), maternal genotype/maternal homozygosity/maternal heterozygosity (ZZf
1236 or ZWf), sample stage (s1 = stage 6, s2 = stage 12, s3 = stage 15), clutch number (c1, c2,
1237 etc.), and replicate ID (e.g., “a” denotes the sample was the first replicate for that sampling
1238 point).

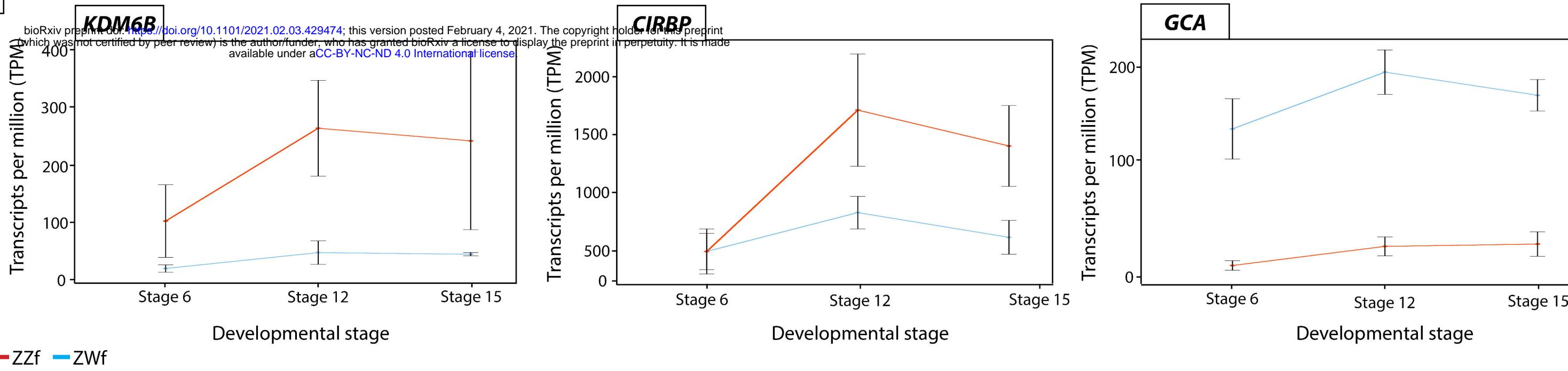
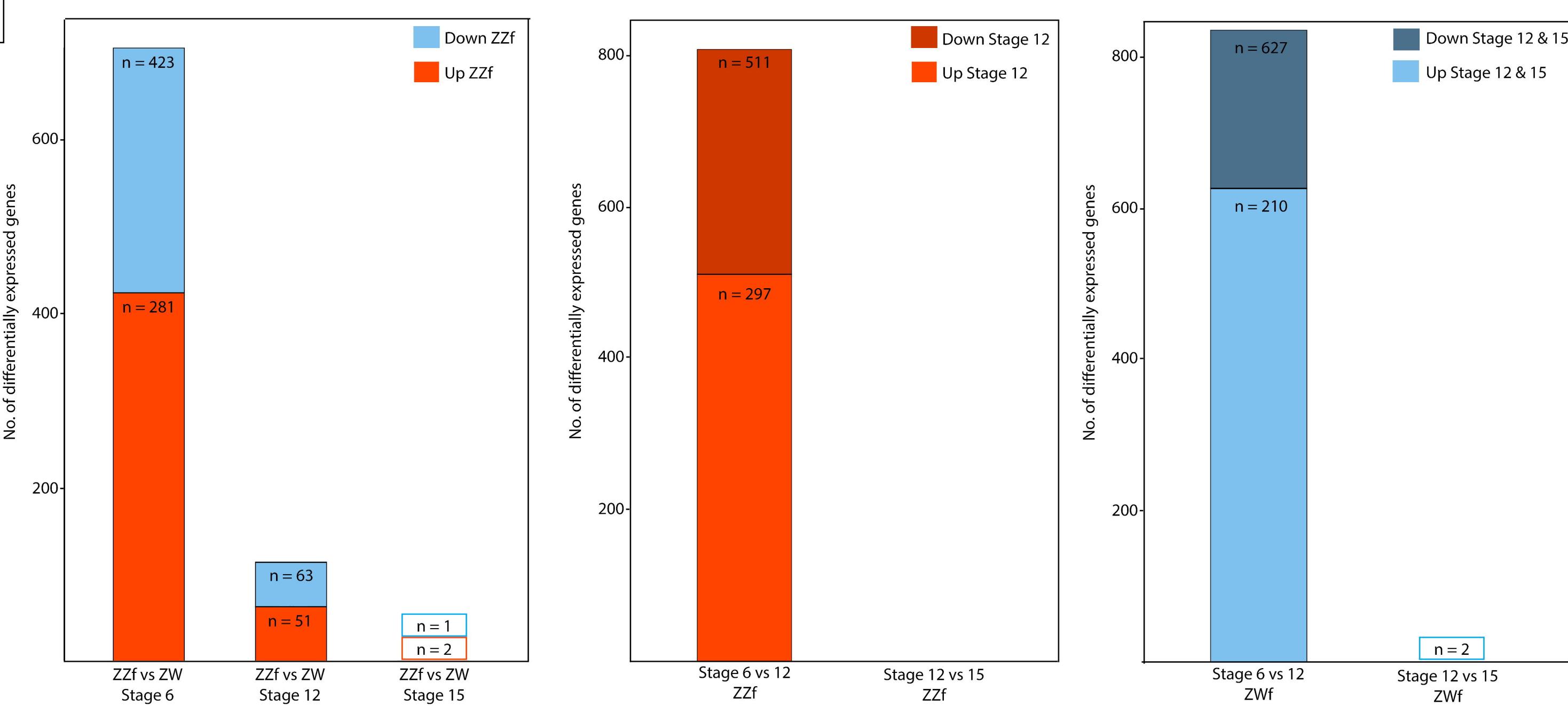
1239 **Fig. S4:** Principal components analysis (PCA) plots performed on normalised counts per
1240 million for filtered genes following the EdgeR pipeline described in the materials and
1241 methods section. (A) PCA of all samples ($n = 39$) (B) PCA of samples with clutch 9 removed (n
1242 = 32) (C) PCA of samples with clutch 9 samples removed and two samples that had not
1243 undergone sex reversal. This is the final dataset upon which all analysis was performed ($n =$
1244 30).

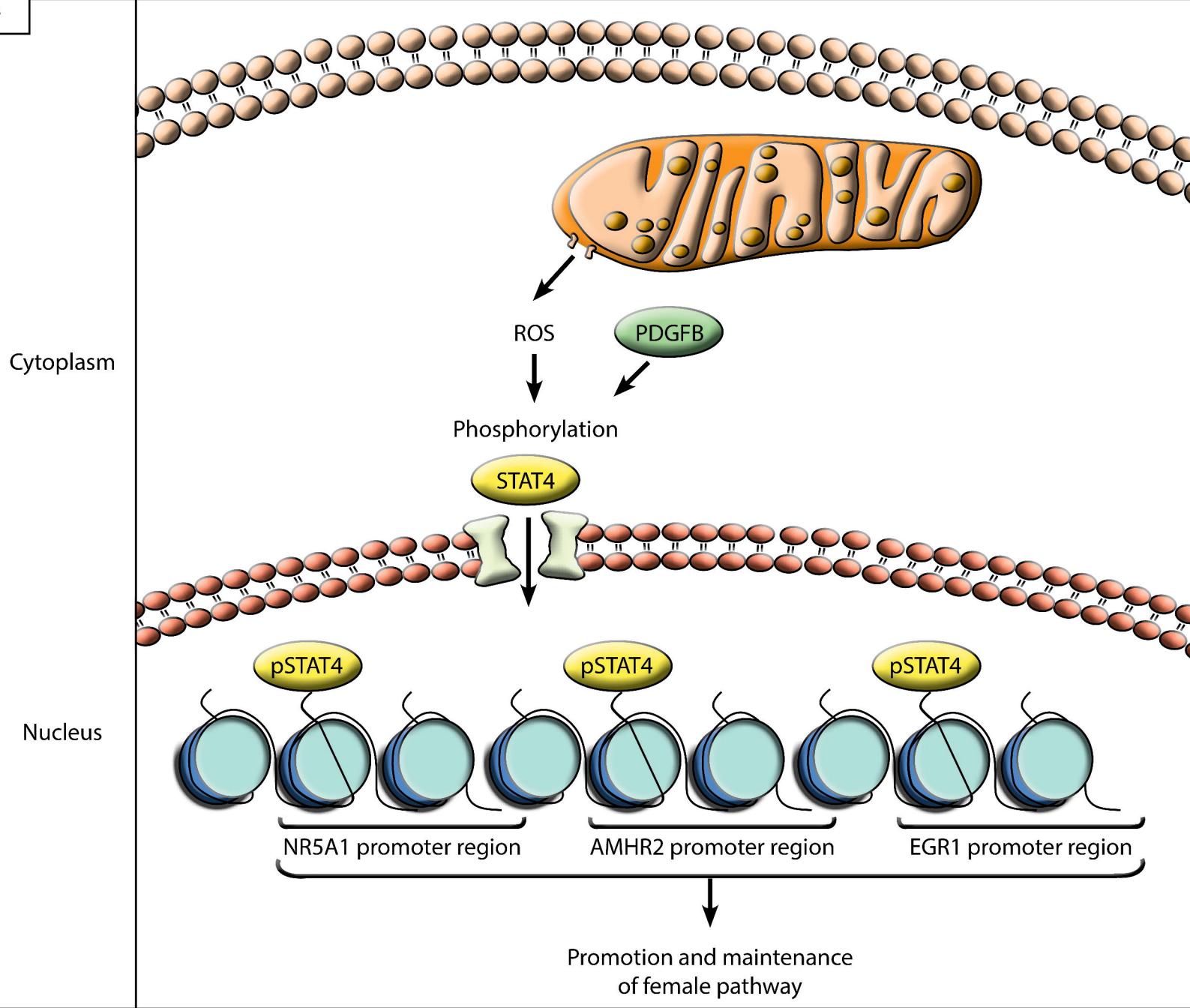
A**Breeding Design**

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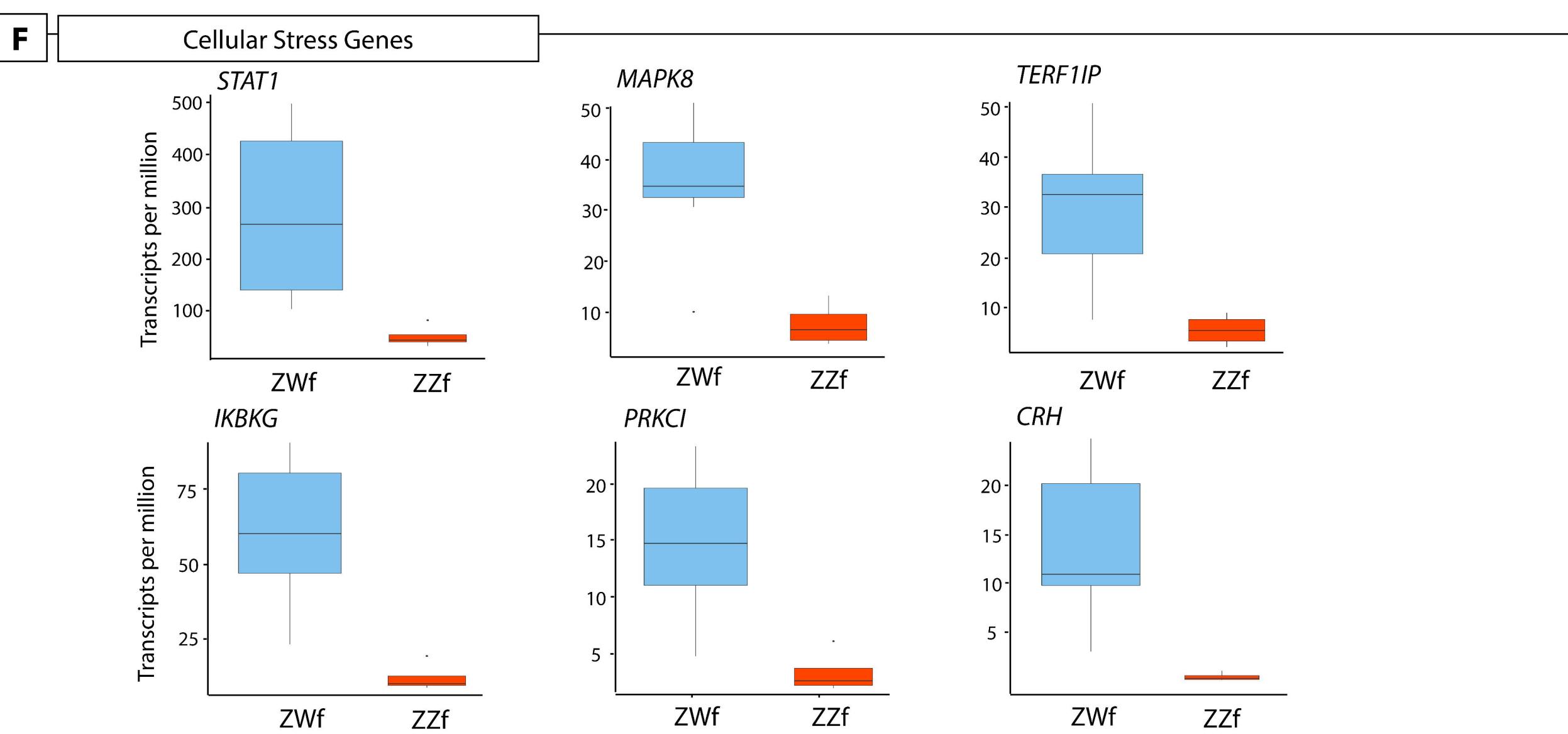
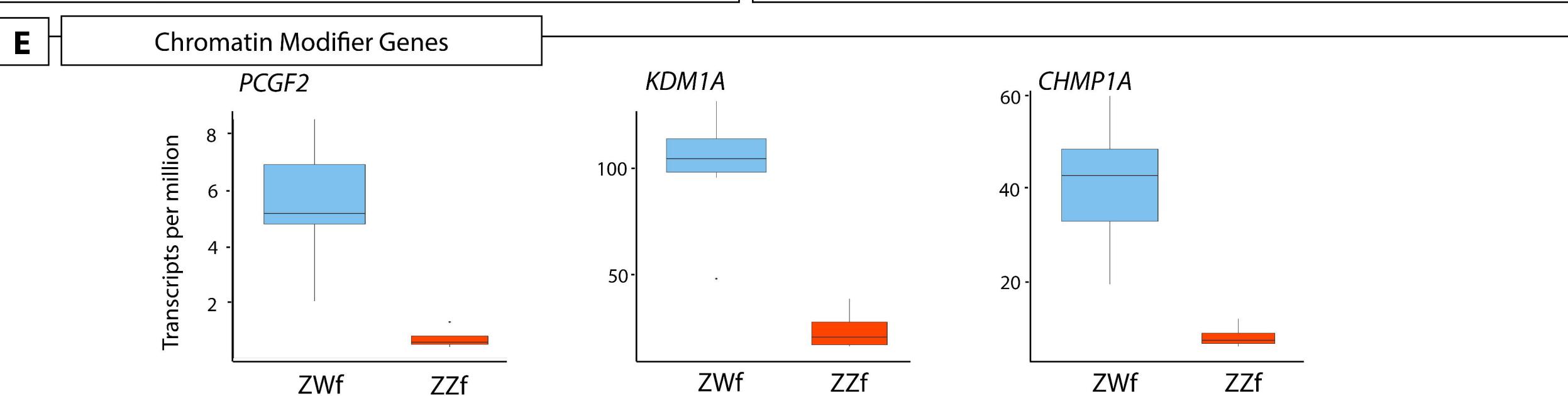
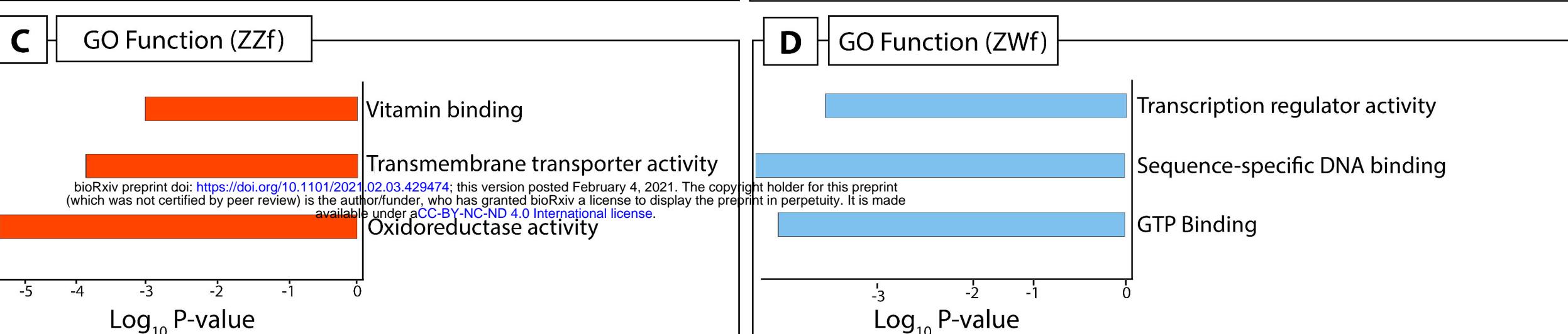
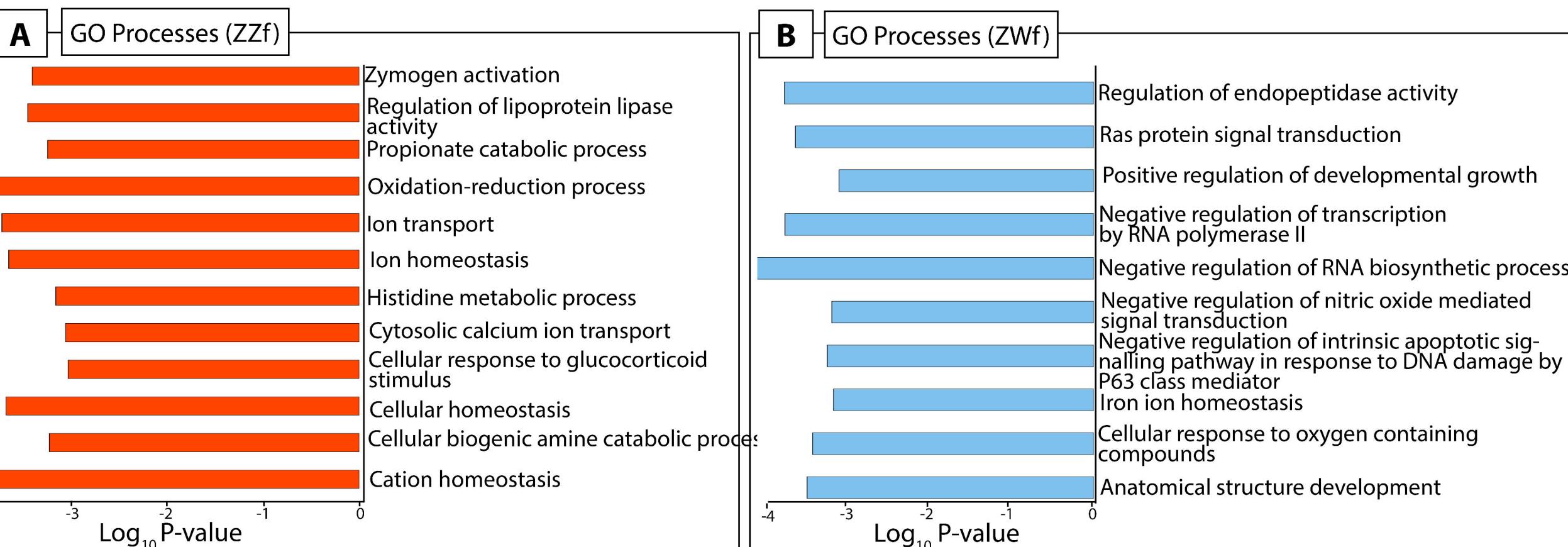
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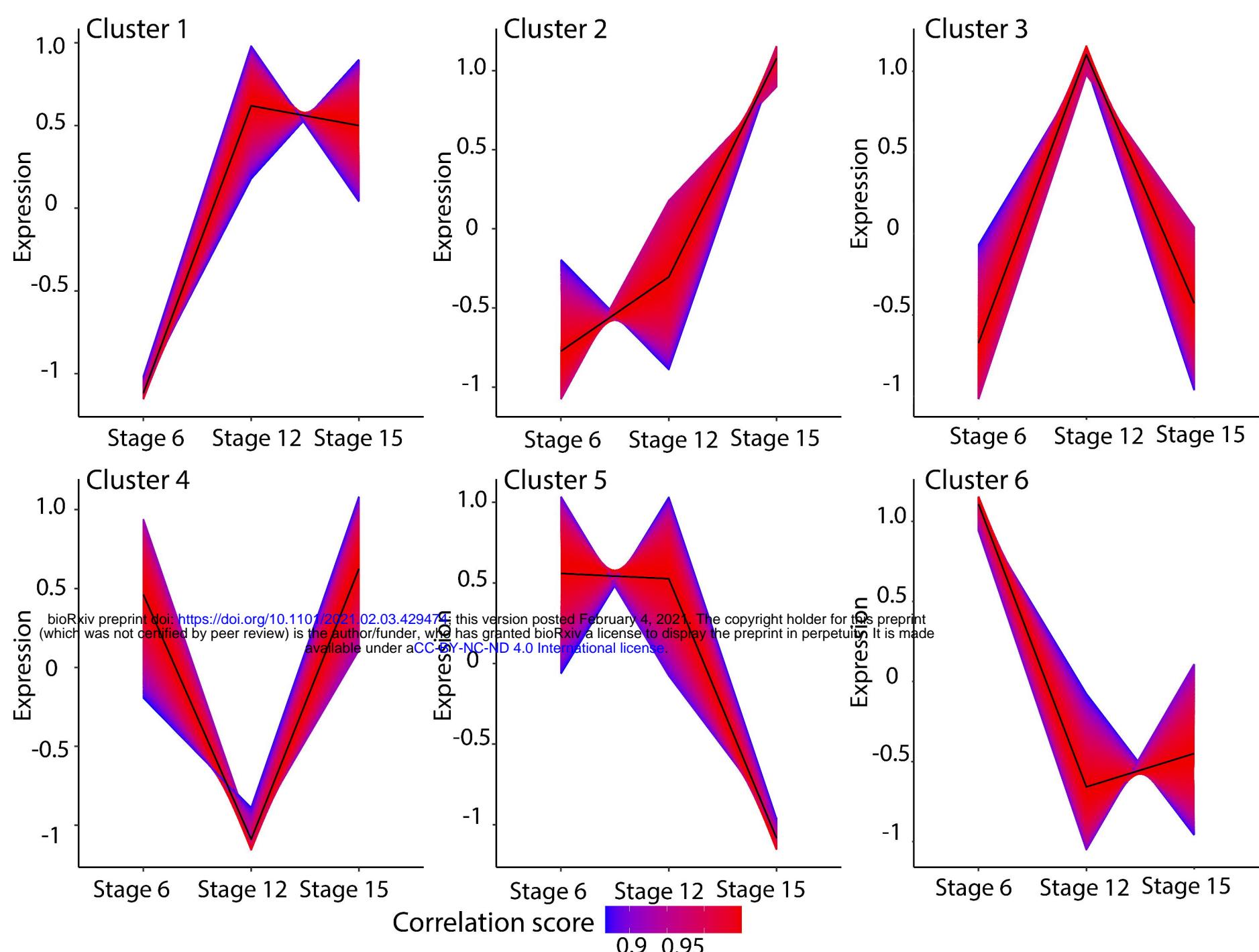
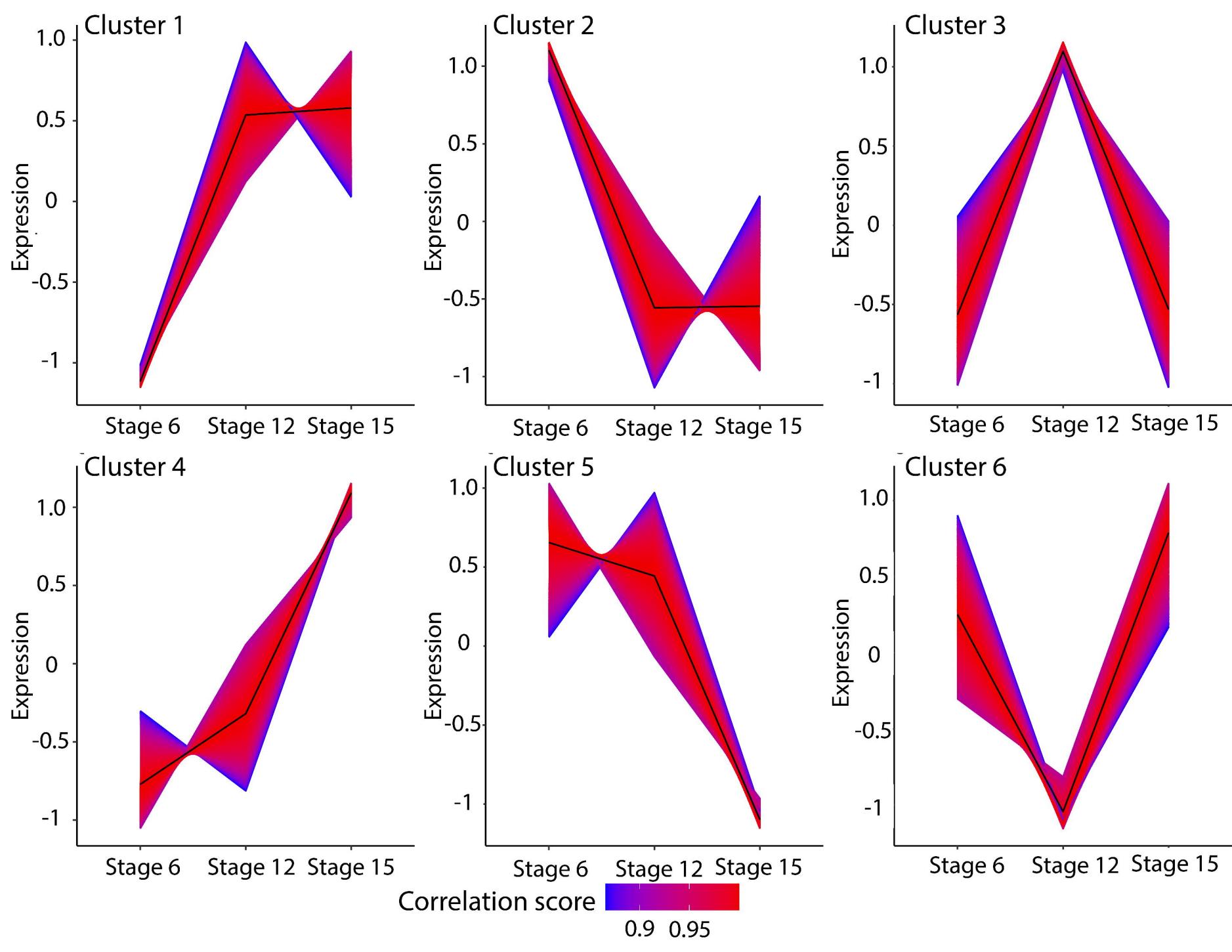


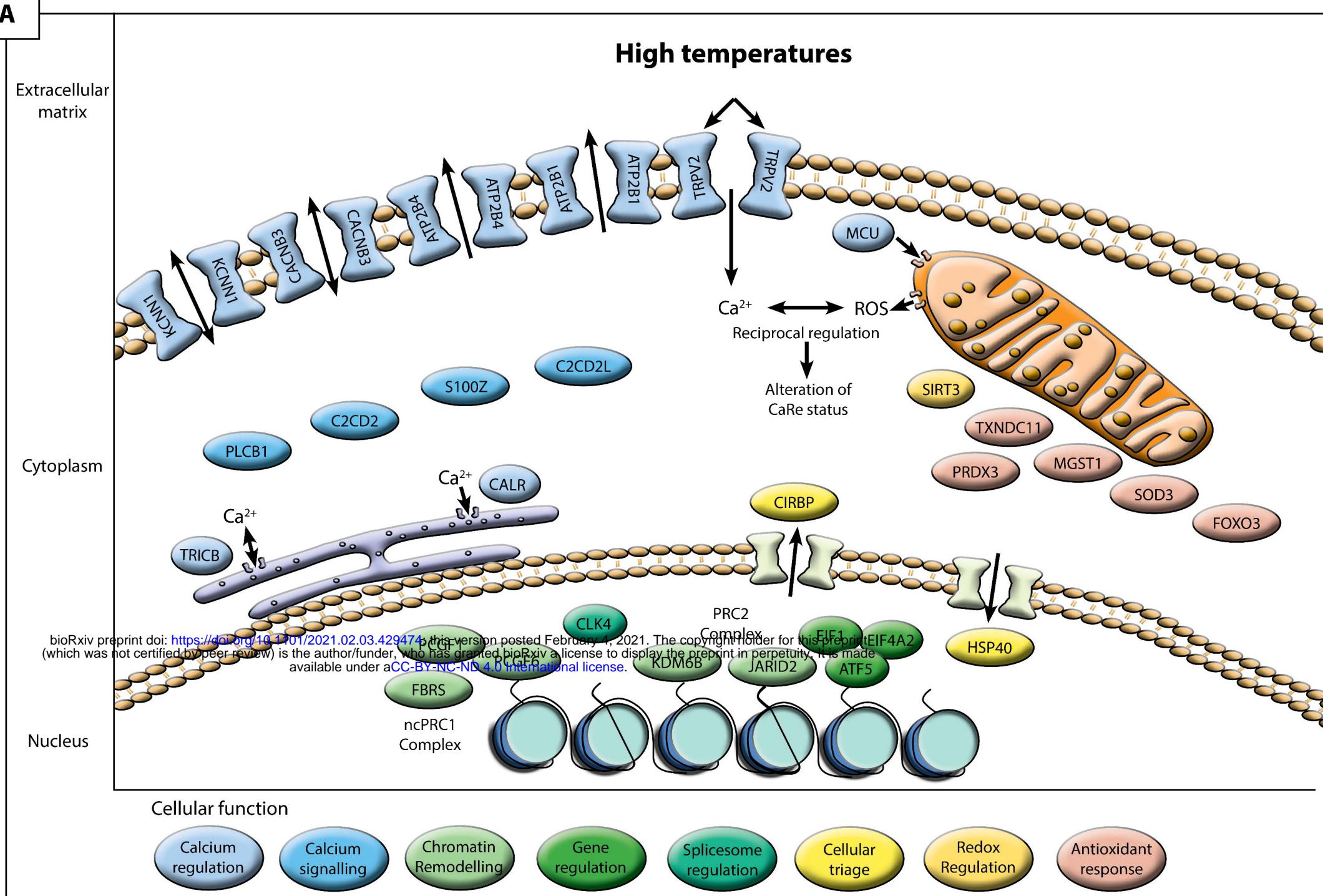
A**B**

A**B**

Gene ID	Gene Name	Log FC	Log CPM	F	P-value	FDR
AMHR2	Anti-Müllerian hormone receptor type 2	-1.96	7.85	23.49	3.17E-5	1.56E-3
EGR1	Early growth response 1	-2.58	5.74	30.80	4.14E-6	4.05E-4
NR5A1	Nuclear receptor subfamily 5 group A member 1	-1.30	7.87	15.21	4.69E-4	9.66E-3
PDGFB	Platelet derived growth factor subunit B	-2.83	4.58	72.33	1.10E-9	2.35E-6
STAT4	Signal transducer and activator of transcription 4	-1.36	3.57	16.94	2.56E-4	6.47E-3



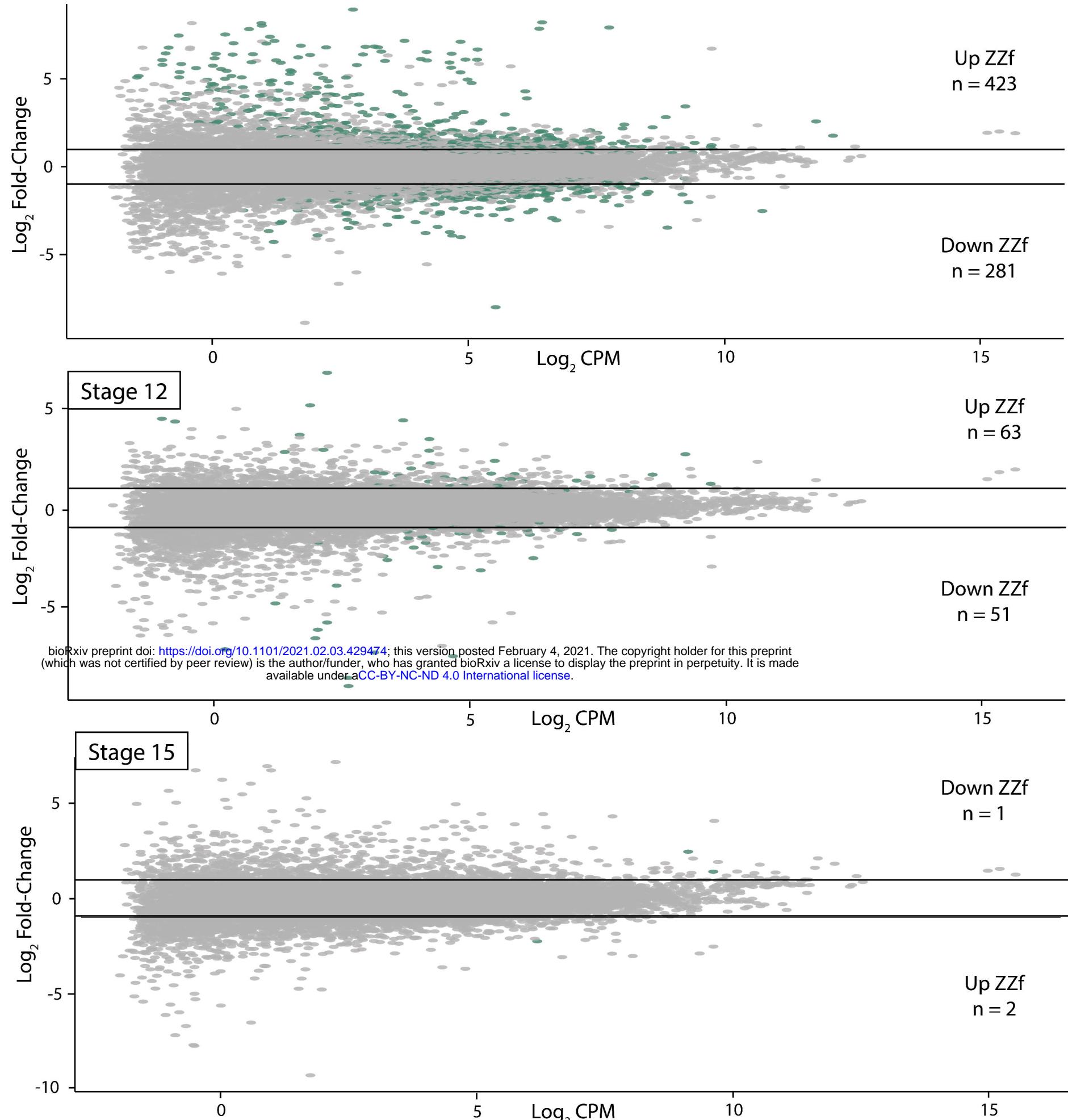
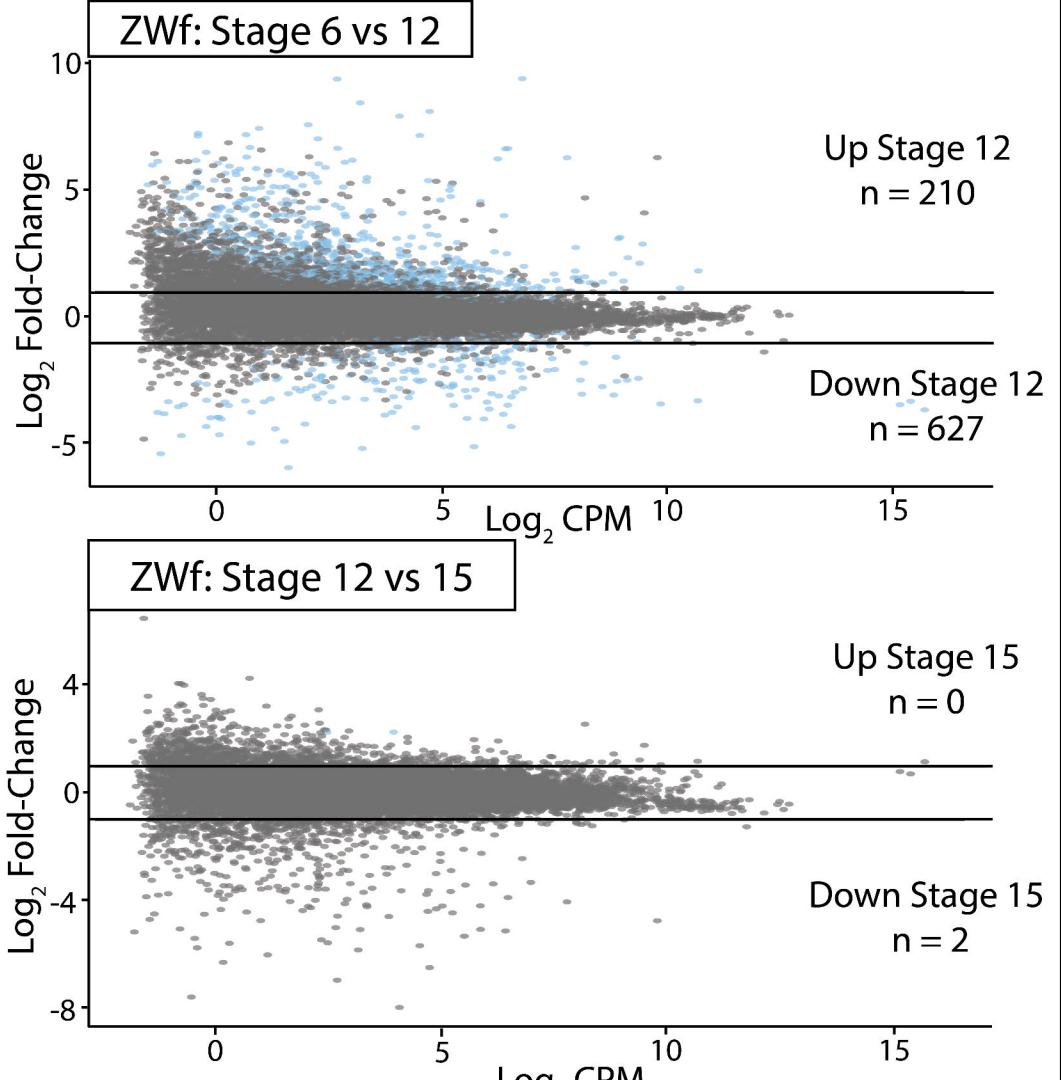
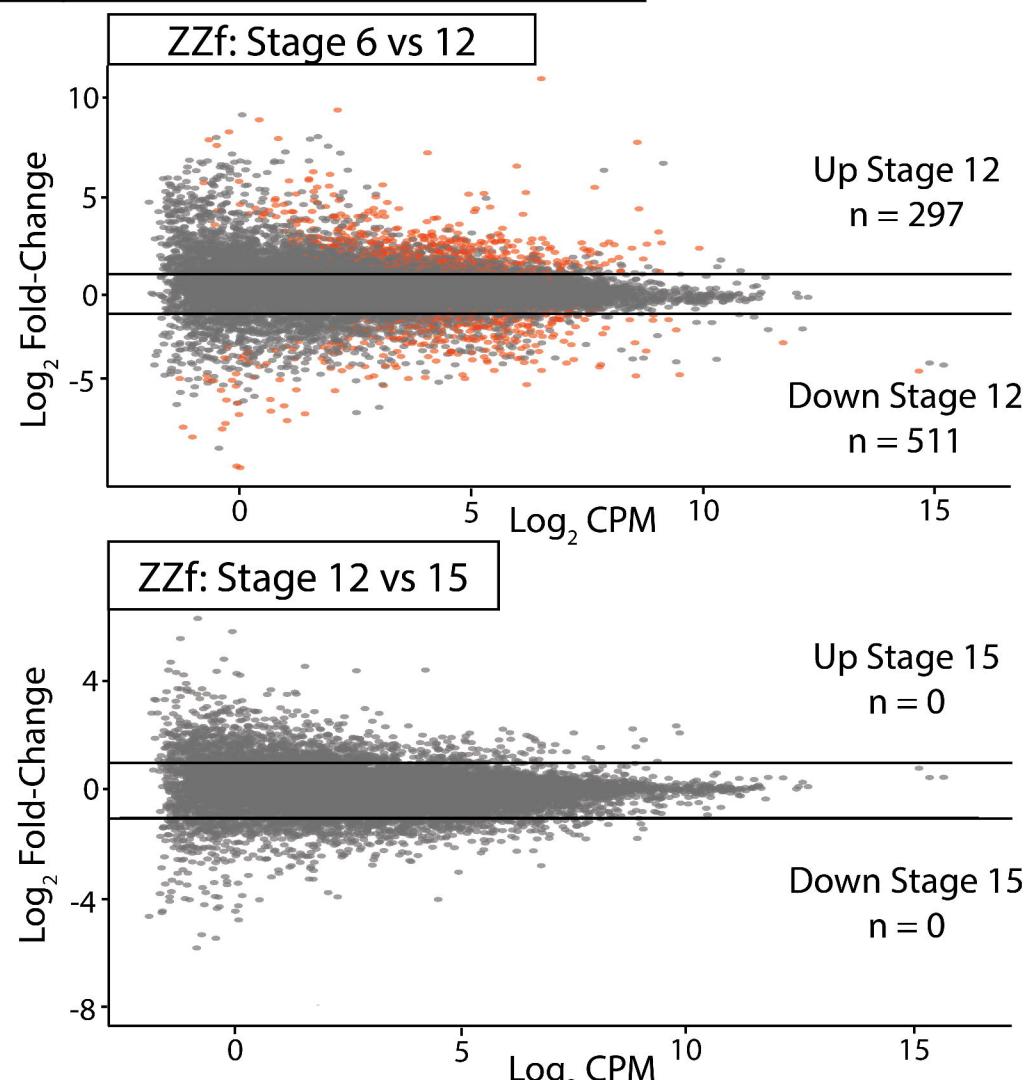
A ZZf clusters**B** ZWf clusters

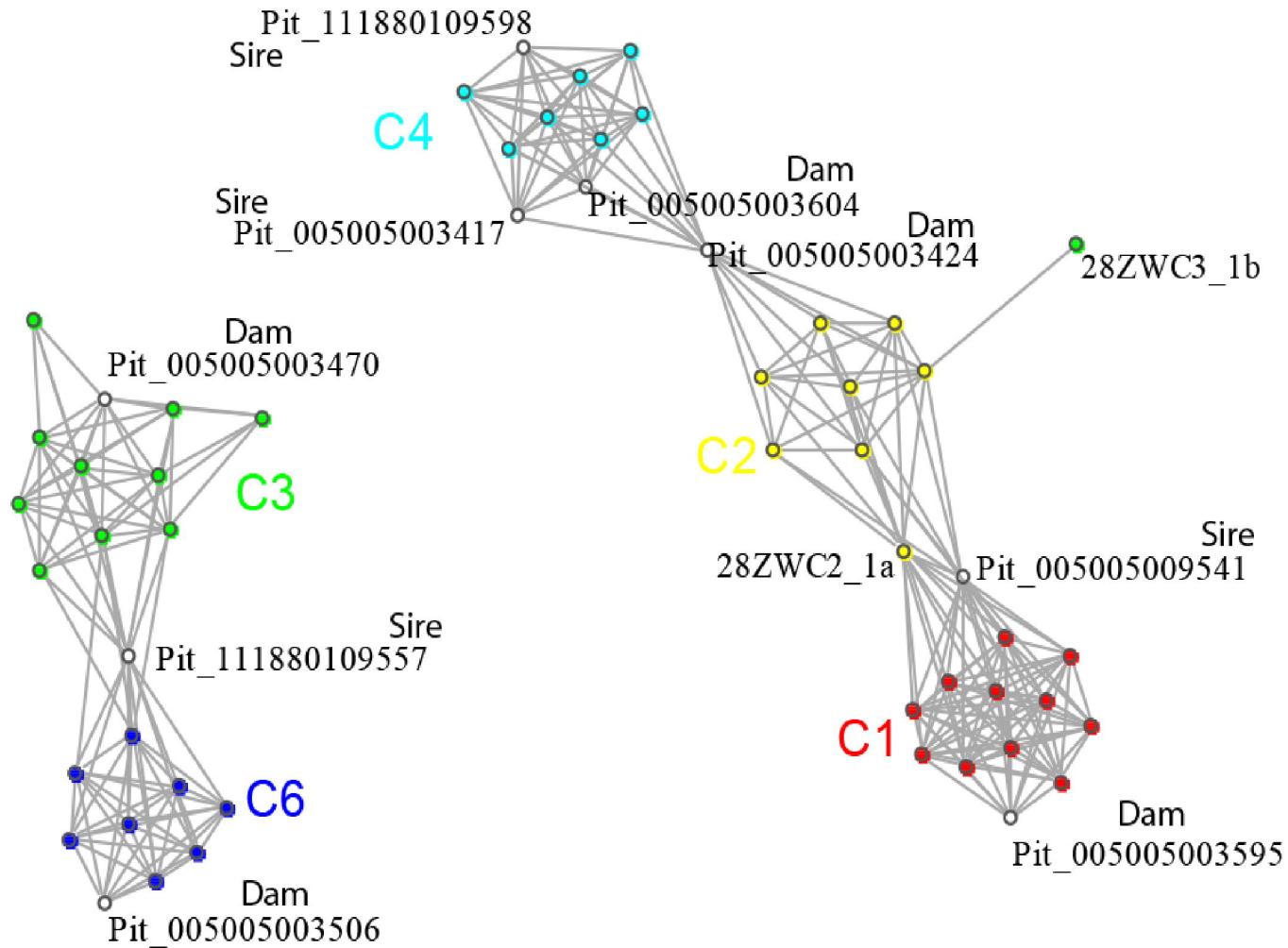
A**B**

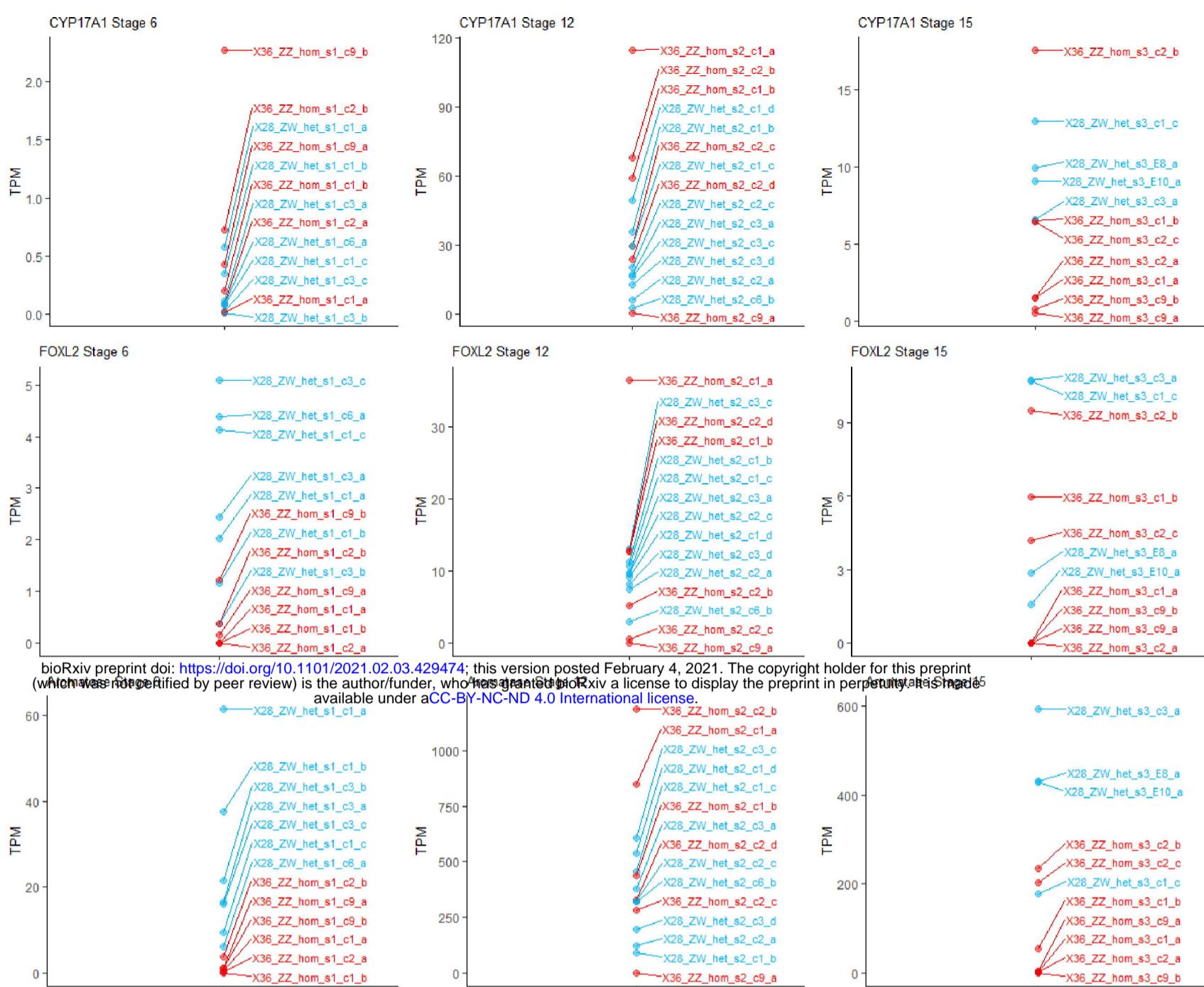
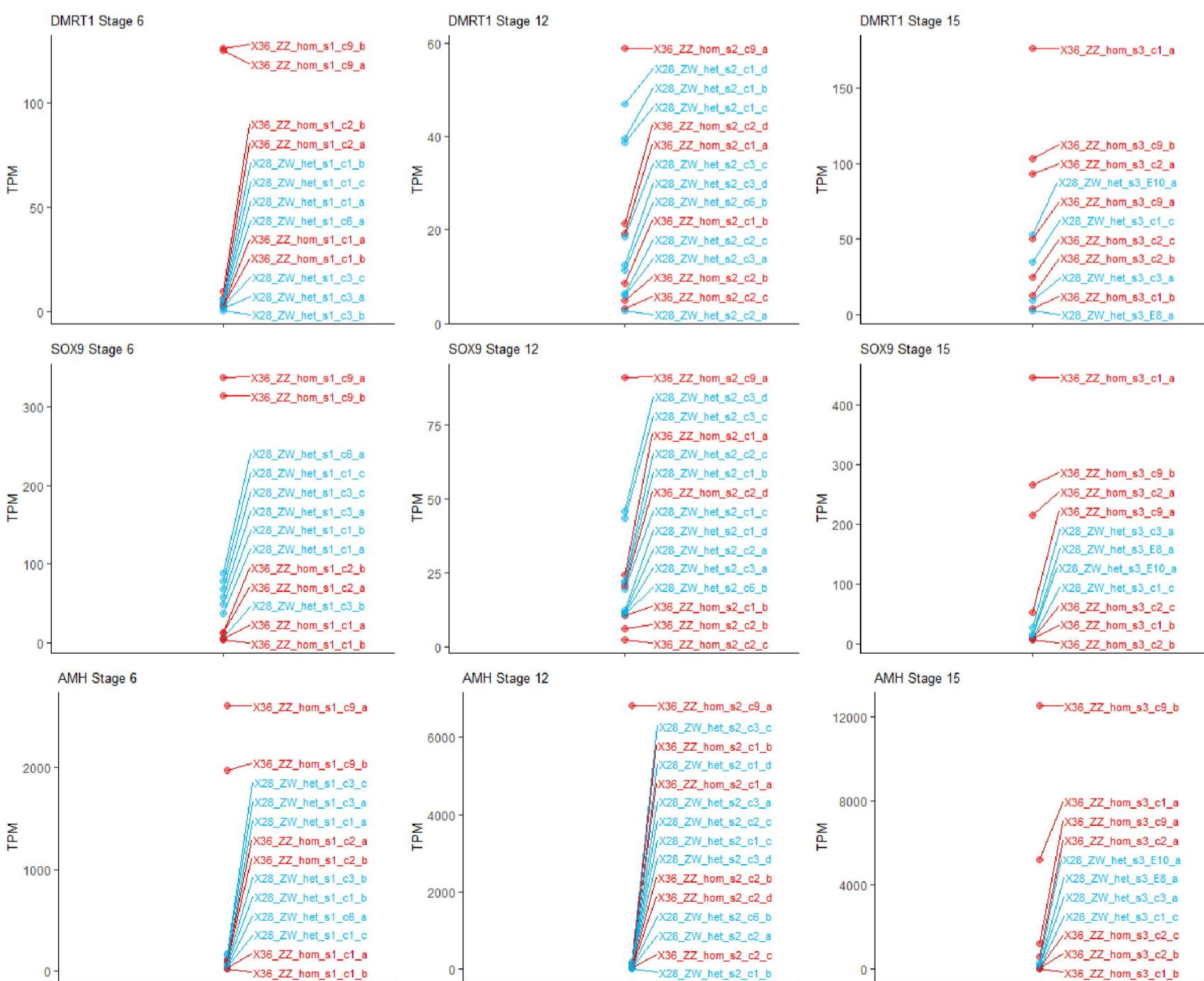
Gene ID	Gene Name	logFC	logCPM	F	P-Value	FDR
ATF5	Activating transcription factor 5	2.075	6.445	20.868	8.20E-5	3.42E-3
ATP2B1	ATPase plasma membrane Ca^{2+} transporting 1	1.162	5.455	24.183	3.09E-5	2.13E-3
ATP2B4	ATPase plasma membrane Ca^{2+} transporting 4	1.561	6.369	35.685	1.63E-6	4.31E-4
C2CD2	C2 calcium dependent domain containing 2	1.320	4.706	26.474	1.64E-5	1.60E-3
C2CD2L	C2CD2 like	1.330	3.288	17.857	2.12E-4	5.71E-3
CACNB3	Calcium voltage-gated channel auxiliary subunit beta 3	1.541	7.079	44.339	2.50E-7	1.69E-4
CALR	Calreticulin	1.397	9.378	23.407	3.86E-5	2.37E-3
CIRBP	Cold inducible RNA binding protein	1.166	9.763	41.691	4.32E-7	2.29E-4
CLK4	CDC like kinase 4	1.330	5.681	19.118	1.41E-4	4.47E-3
DNAJC28	DnaJ heat shock protein family (Hsp40) member C28	1.252	3.039	15.643	4.44E-4	8.72E-3
EIF1	Eukaryotic translation initiation factor 1	1.176	8.435	17.443	2.43E-4	6.11E-3
EIF4A2	Eukaryotic translation initiation factor 4A2	1.168	8.158	59.491	1.53E-8	2.87E-5
FBRs	Fibrosin	1.153	8.243	30.247	6.08E-6	8.95E-4
FOXO3	Forkhead box O3	1.129	5.798	18.598	1.67E-4	4.88E-3
JARID2	Jumonji and AT-rich interaction domain containing 2	2.296	8.620	40.274	5.85E-7	2.57E-4
KCNN1	Potassium calcium-activated channel subfamily N member 1	1.164	5.594	15.939	4.01E-4	8.21E-3
KDM6B	Lysine demethylase 6B	3.503	9.291	120.703	6.40E-12	1.08E-7
MCU	Mitochondrial calcium uniporter	1.018	5.466	26.624	1.57E-5	1.56E-3
MGST1	Microsomal glutathione S-transferase 1	1.351	7.830	29.858	6.71E-6	9.47E-4
PCGF1	Polycomb group ring finger 1	1.408	5.170	19.275	1.34E-4	4.42E-3
PCGF6	Polycomb group ring finger 6	1.072	4.330	19.318	1.33E-4	4.39E-3
PLCB1	Phospholipase C beta 1	1.763	3.649	24.040	3.22E-5	2.17E-3
PRDX3	Peroxiredoxin 3	1.336	7.212	29.894	6.65E-6	9.46E-4
S100Z	S100 calcium binding protein Z	4.202	3.813	23.471	3.79E-5	2.37E-3
SIRT3	Sirtuin 3	1.264	2.359	18.039	2.00E-4	5.52E-3
SOD3	Superoxide dismutase 3	2.317	1.806	17.239	2.59E-4	6.33E-3
TMEM38B	Transmembrane protein 38B	1.437	3.311	26.252	1.74E-5	1.62E-3
TRPV2	Transient receptor potential cation channel subfamily V member 2	3.239	3.951	45.162	2.12E-7	1.49E-4
TXNDC11	Thioredoxin domain containing 11	1.024	6.160	23.816	3.43E-5	2.26E-3

A ZZf vs ZWf at each stage

Stage 6

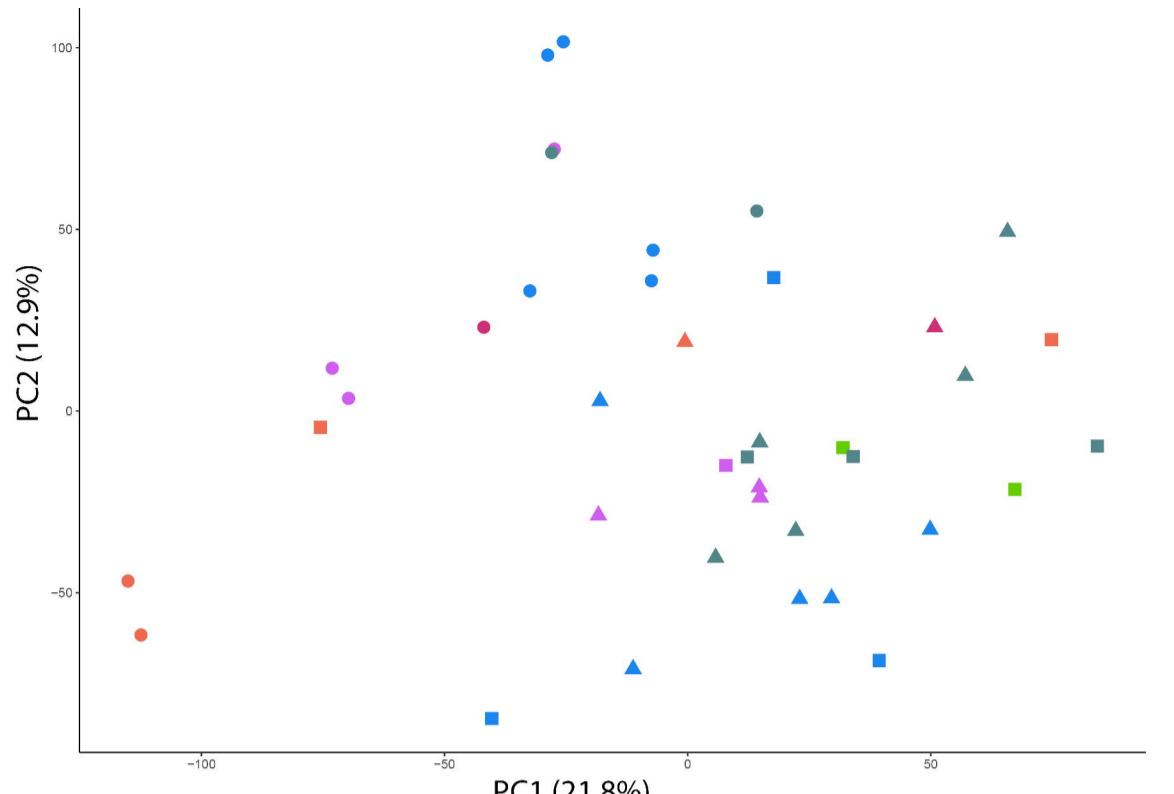
**B** Between stage comparisons



A**Female Gene Panel****B****Male Gene Panel**

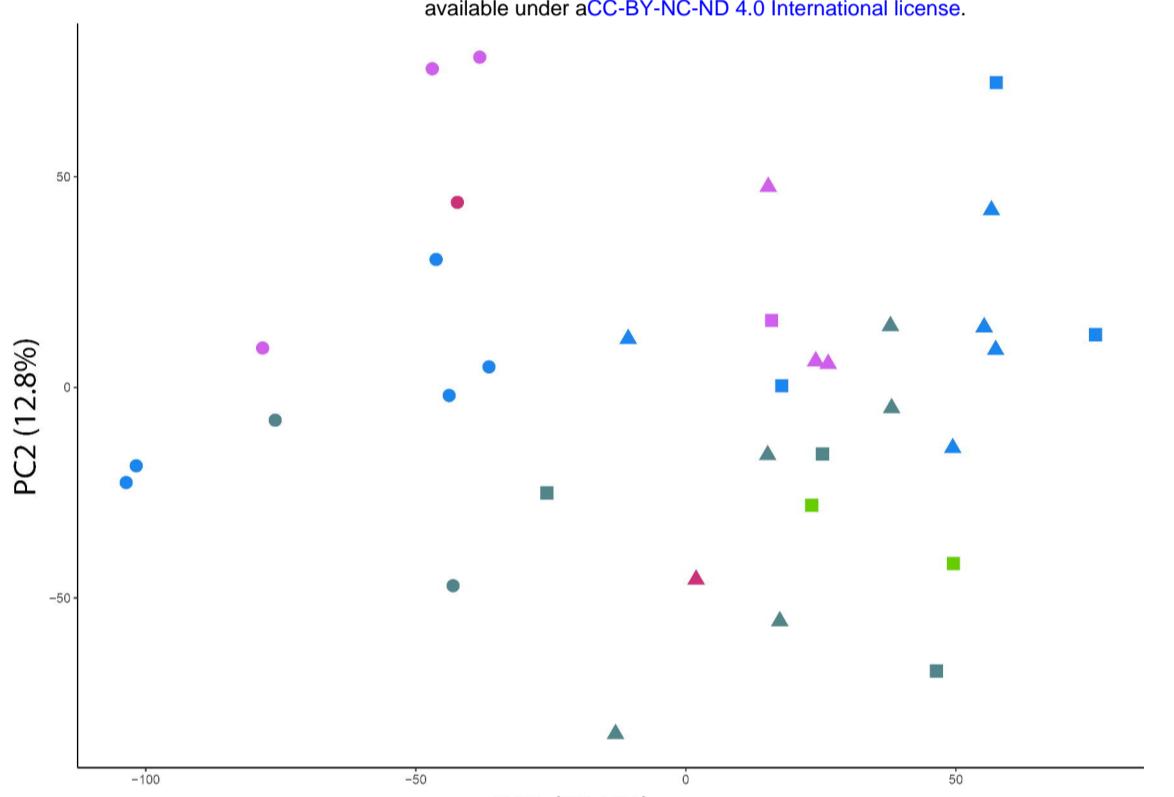
A

All Samples

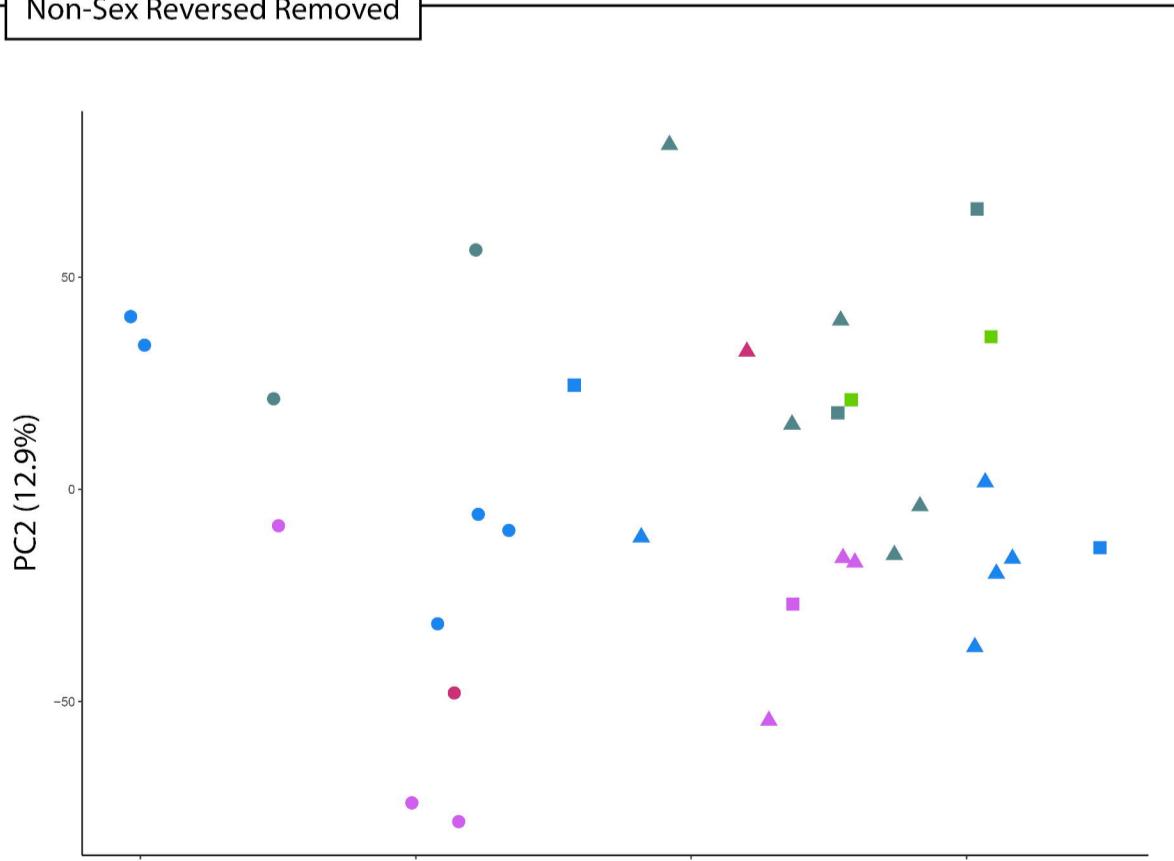
**B**

Clutch 9 Removed

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**C**

Non-Sex Reversed Removed



Legend

○	Stage 6	△	Stage 12	□	Stage 15						
●	Clutch 1	●	Clutch 2	●	Clutch 3	●	Clutch 6	●	Clutch 9	●	Clutch 10