An *in vitro* ovarian explant culture system to examine sex change in a hermaphroditic fish

Goikoetxea Alexander[1], Damsteegt Erin L[2], Todd Erica V[1], McNaughton Andrew[1], Gemmell Neil J[1], Lokman P Mark[2].

[1]Department of Anatomy, University of Otago, Dunedin, New Zealand
[2]Department of Zoology, University of Otago, Dunedin, New Zealand

Corresponding author:
Alexander Goikoetxea
+34 647 539 500
alexandergoikoetxea@gmail.com

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Abstract

Many teleost fishes undergo natural sex change, and elucidating the physiological and molecular controls of this process offers unique opportunities not only to develop methods of controlling sex in aquaculture settings, but to better understand vertebrate sexual development more broadly. Induction of sex change in some sequentially hermaphroditic or gonochoristic fish can be achieved *in vivo* through social manipulation, inhibition of aromatase activity, and steroid treatment. However, the induction of sex change *in vitro* has been largely unexplored. In this study, we established an *in vitro* culture system for ovarian explants in serum-free medium for a model sequential hermaphrodite, the New Zealand spotty wrasse (*Notolabrus celidotus*). This culture technique enabled evaluating the effect of various treatments with 17ß-estradiol (E_2), 11-ketotestosterone (11KT) or cortisol (CORT) on spotty wrasse ovarian architecture

for 21 days. A quantitative approach to measuring the degree of ovarian atresia within histological images was also developed, using pixel-based machine learning software. Ovarian atresia likely due to culture was observed across all treatments including no-hormone controls, but was minimised with treatment of at least 10 ng/mL E_2. Neither 11KT nor CORT administration induced proliferation of spermatogonia (i.e. sex change) in the cultured ovaries indicating culture beyond 21 days may be needed to induce sex change *in vitro*. The *in vitro* gonadal culture and analysis systems established here enable future studies investigating the paracrine role of sex steroids, glucocorticoids and a variety of other factors during gonadal sex change in fish.

Introduction

In most vertebrates, sex is fixed after birth and remains the same throughout life (1). Some teleost fishes, however, develop as one sex but remain able to change to the other during adulthood (2). *In vitro* culture manipulations enable precise monitoring of the effects of biotic and abiotic factors (e.g. temperature, pH, microplastics, etc.) on gonadal development, but are underutilised in aquaculture and fisheries research. *Ex vivo* approaches show particular potential to study responses of gonadal tissue to sex steroids and other hormones (e.g. glucocorticoids). Testicular and ovarian organ culture has been experimentally achieved with a handful of gonochoristic fish species (3–7). However, with the exception of a conference abstract on the effects of several hormones on the gonadal architecture of sex-changing three-spot wrasse (*Halichoeres trimaculatus* (8)), explant culture systems for the study of sex change in fish remain unreported. Gonadal remodelling in sex-changing fishes occurs in adulthood and, therefore, the study of sex-determining mechanisms is not limited to egg or larval stages as in other vertebrates. Tissue and organoid *in vitro* culture allows evaluating multiple factors in a large number of replicates in well-controlled and standardised experimental conditions, while reducing animal usage and handling compared with *in vivo* manipulations (9–11).

The aim of this study was to develop an organ culture system for a sequential hermaphrodite to establish a new model for investigating the molecular and cellular basis of sex reversal in the gonad. We chose the New Zealand spotty wrasse (*Notolabrus celidotus*), a common and widespread marine species, to evaluate the effects of a suite of hormonal factors on gonadal architecture. In this diandric protogynous hermaphroditic species, sex change is socially regulated, whereby the removal of the dominant male from the social group induces sex change in a resident female (12). Spotty wrasses exhibit sexual dimorphism with alternative male phenotypes. Initial phase (IP) individuals consist of females and primary males which develop directly from a juvenile stage with a bipotential

gonad (13). Both females and IP males can sex or role change, respectively, to become terminal phase (TP) males (13).

While natural sex change involves modifications at the behavioural, physiological, molecular and anatomical levels (1), herein, the term 'sex change' is used to refer to the development of testicular tissue in female gonads. The effect of different doses of steroid hormones 17ß-estradiol (E_2), 11-ketestosterone (11KT) or cortisol (CORT) on spotty wrasse ovary explants was evaluated *in vitro* to elucidate their effect on gonadal sex change in this species. Controls (C, no steroid) were used to determine the viability of the culture system and the potential triggering of gonadal sex change in the absence of exogenous factors. Treatment with E_2 was used to evaluate its role in the successful maintenance of spotty wrasse ovaries *in vitro*. 11KT, the most potent androgen in fish (14), was employed to explore its potential to induce sex change (i.e. proliferation of spermatogonia) in cultured spotty wrasse ovaries. In addition, treatment with the stress hormone CORT was predicted to induce oocyte degeneration through the inhibition of aromatase (15), and it was hypothesised that this effect alone may trigger the female-to-male gonadal transformation. A strong effect of CORT, the main glucocorticoid in fish, has been linked to fish sex determination and sex change in several studies (16–20). This is the first study to evaluate the effects of CORT in an *in vitro* gonadal culture system of a sex-changing fish.

Materials and Methods

Sample collection

Five IP spotty wrasse individuals ranging from 160 – 230 mm total length were captured by hook and line at the Portobello Marine Laboratory, Department of Marine Science, University of Otago, New Zealand during May 2019. Live fish were immediately transported to cell culture facilities at the Department of Zoology, University of Otago, where fish were euthanised by overdose in benzocaine (0.3 g/L) and gonads dissected within 1.5 h of capture. At this time,

105 gonadal fragments from each fish were preserved for histological analysis as day
106 0 reference tissues (D0). Gonads fixed in 4% PFA were processed for routine
107 embedding in paraffin (Otago Histology Services Unit, Department of Pathology,
108 University of Otago). Fish were captured and manipulated with approval from the
109 University of Otago Animal Ethics Committee, and in accordance with New
110 Zealand National Animal Ethics Advisory Committee guidelines.
111

112 Ovarian organ culture technique

113

114 *In vitro* culture of spotty wrasse ovary was carried out using the floating tissue
115 culture method originally described by (6) with minor modifications. For each of 5
116 IP fish, freshly removed ovaries were cut into pieces of approximately 2 – 3 mm
117 and ovarian fragments were placed on floats of 1.5% agarose covered with a
118 nitrocellulose membrane in a 24-well plastic tissue culture dish. The basal
119 medium consisted of Leibovitz L-15 medium supplemented with 0.5% bovine
120 serum albumin, 10,000 U/L penicillin, 10 mg/L streptomycin and 1 μg/mL porcine
121 insulin adjusted to pH 7.4. Ovarian fragments were exposed to E_2, 11KT or CORT
122 for 21 days at 16 °C. The effect of various concentrations of each hormone (1, 10 or
123 100 ng/mL) was examined. Steroids were first dissolved in EtOH and then diluted
124 with the medium (0.1% EtOH), which was changed every 7 days. Controls (no
125 steroid, 0.1% EtOH) were included. At the end of the experiment, tissues were
126 fixed in 4% PFA and processed for routine paraffin embedding.
127

128 Image analysis

129

130 An open source pixel-based machine learning trainable classifier, Ilastik (v. 1.3.2)
131 (https://www.ilastik.org) (21), was employed to discriminate cell types in the
132 gonadal images captured using brightfield light microscopy. Approximately 85
133 previtellogenic oocytes (PVO), from a subset of 5 images, were used to train Ilastik.
134 Atretic oocytes, stromal tissue and background were labelled separately with a
135 paintbrush-style tool using the Pixel Classification module. The training process
136 was refined iteratively, updating pixel classifications until object types were

accurately discriminated in the segmentation maps, as described in (22). The Ilastik classifier files were extracted in HDF5 (Hierarchical Data Format 5) format and loaded into open source image processing software ImageJ (v. 2.0.0-rc-69/1.52p), where the different object types were each assigned a different threshold. ImageJ 'Analyse particles' function was used to calculate the total relative surface area occupied by non-atretic PVO and measure the total surface of the tissue section in each image. 'Remove outliers' function was used to remove any outlier < 10 pixels from analysis and 'Fill holes' function was used to mark PVO nuclei in the exceptional occasions when Ilastik failed to correctly recognise these.

Statistical analysis

Generalised linear mixed models were used to assess the effect of steroid (i.e. E_2, 11KT or CORT) treatments on the proportion of total tissue surface occupied by non-atretic PVO ('PVO_area') in comparison to the total area of each ovarian section ('total_area'), using individual fish as a random effect. Models followed a negative binomial distribution and were performed using the following command line in R (v. 3.6.1): 'glmmPQL (PVO_area/total_area ~ Treatment, random = ~1 | Fish, weights = total_area, family = negative.binomial (theta = 1), data = dat)'. If significant differences between treatments were found, Tukey's multiple comparison tests were performed to determine where the significance lay between treatments. All statistical analyses were performed using R (23). Results are expressed as means ± SEM of 5 biological replicates, except for the control (n = 15) or unless stated otherwise.

Results and Discussion

Histological analysis of D0 reference tissues confirmed that all 5 IP spotty wrasse captured were female. Visual inspection of gonadal sections under light microscopy revealed that oocyte degeneration was present in all experimental groups, including controls, but not the D0 reference tissues. However, initiation of

169 spermatogenesis was not observed in any group, and no male structures were
170 identified.
171
172 Atresia of PVO appeared to be a background effect of the *in vitro* culture (8). This
173 was reflected in reduced oocyte surface area in controls compared to D0 reference
174 tissues, with the proportion of total tissue area filled with non-atretic PVO being
175 significantly higher in D0 (73.6 ± 2.4 %) versus control ovaries (31.8 ± 3.7 %) (p-
176 value < 0.001) (Figure 2A). Estrogen treatment reduced the incidence of oocyte
177 degeneration. A greater surface area covered by non-atretic PVO was observed in
178 ovaries treated with the two highest E_2 doses (10 ng/mL, 51.1 ± 5.1 %; 100 ng/mL,
179 50.7 ± 9.8 %) (Figure 2A). Differences between the control ovaries and those
180 treated with 100 ng/mL of E_2 were significant (p-value < 0.05). Therefore,
181 exogenous E_2 administration to cultured ovaries can effectively, albeit partially,
182 prevent oocyte degeneration (i.e. increased surface area occupied by non-atretic
183 PVO) associated with explant culture. This reinforces observations in female coho
184 salmon (*Oncorhynchus kisutch*), in which *in vivo* fadrozole treatment (an
185 aromatase inhibitor) induced reductions in E_2 and a higher incidence of associated
186 atresia (24). In future culture systems, the addition of growth factors or the use of
187 an artificial extracellular matrix (25) could be investigated to further mitigate
188 oocyte degeneration as a consequence of culture.
189
190 Unexpectedly, there was no histological evidence that treatment with 11KT
191 induced sex change in spotty wrasse ovaries *in vitro*. The proportion of non-atretic
192 PVO found in the ovarian tissues was not dose-dependent and did not significantly
193 vary between any of the 11KT-treated ovaries. Although all 11KT-treated ovary
194 explants showed a much lower percentage of PVO compared to total tissue area
195 than D0 ovaries, values were comparable to the control (Figure 2B). The
196 proportion of non-atretic PVO in 11KT-treated ovarian tissues was also unaffected
197 by dose (1 ng/mL, 24.0 ± 10.7 %; 10 ng/mL, 25.6 ± 9.1 %; 100 ng/mL 21.6 ± 9.7 %).
198
199 Successful induction of spermatogenesis via *in vitro* androgen treatment in
200 cultured testes has been demonstrated in Japanese eel (*Anguilla japonica*), in

201 which Sertoli cells were activated by 11KT to stimulate the spermatogenic cascade
202 (3,6). The role of 11KT as a promoter of spermatogenesis in ovaries of sex-changing
203 fish has also been demonstrated (8,26). In the protogynous honeycomb grouper
204 (*Epinephelus merra*), *in vivo* implantation of 11KT into pre-spawning females
205 caused masculinisation in all fish (26). Likewise, *in vitro* 11KT treatment of
206 ovarian explants from protogynous three-spot wrasse induced the formation of
207 presumed spermatogenic crypts and sperm within 2 – 3 weeks (8). However, three-
208 spot wrasses live in tropical waters, and sex change in captive populations has
209 been observed in under 40 days (27), around half that required in spotty wrasse
210 (60 – 70 days (12)). In the current study, it may be that spotty wrasse ovaries were
211 not cultured long enough for 11KT to promote male germ cell proliferation, or that
212 11KT alone is insufficient to induce gonadal transformation in this species. It has
213 been hypothesised that endogenous E_2 levels need to decrease below a
214 physiological threshold for oocyte maintenance to become jeopardised and
215 androgen levels to effectively induce proliferation of bipotential gonial stem cells
216 in the ovary (26). Co-treatment of 11KT with an aromatase inhibitor (e.g.
217 fadrozole) should be performed in the future to further characterise the triggering
218 of *in vitro* gonadal restructuring, potentially establishing this technique as a
219 model for sex change research.
220
221 It was hypothesised that CORT treatment would promote oocyte degeneration,
222 following evidence that CORT may mediate sex change in fish by inhibiting
223 aromatase transcription and promoting androgen production (14,15). However, no
224 significant differences were observed in the proportion of non-atretic PVO between
225 the controls and ovaries treated with 1 and 10 ng/mL of CORT (43.3 ± 7.7 % and
226 42.9 ± 6.2 %, respectively) (Figure 2C). While a significant increase in the
227 percentage area of PVO was detected between the controls and 100 ng/mL CORT
228 treatment group (39.5 ± 18.0 %) (p-value < 0.005), this may reflect variability
229 between fish and the effect of smaller sample size (n=4, as one biological replicate
230 was lost during histological processing due to technical issues) on the statistical
231 analysis. Therefore, these differences were not deemed a direct effect of CORT
232 administration to the ovaries. Cortisol has been observed to negatively impact

233 ovarian development by suppressing E_2 secretion in cultured rainbow trout
234 (*Oncorhynchus mykiss*) follicles (7). However, in that same study, Carragher &
235 Sumpter (7) argued that there may be a time lag for CORT suppressive effects to
236 become evident during *in vitro* ovarian culture. Longer-term culture of spotty
237 wrasse ovaries may be needed to observe any effects of CORT on ovarian structure,
238 a finding in keeping with our findings for 11KT.
239
240 Cortisol also failed to initiate obvious sex change in cultured spotty wrasse ovaries,
241 with no evidence of male tissues observed in our ovarian sections. It may be that
242 CORT alone is ineffective in inducing spotty wrasse sex change, or that the
243 experiment needs technical adjustments (e.g. longer duration) for the successful
244 induction of sex change in spotty wrasse. Low doses of CORT (i.e. 0.01 – 10 ng/mL)
245 were reported to promote testicular development in Japanese eel and pejerrey
246 (*Odontesthes bonariensis*) by increasing the production of 11KT, which, in turn,
247 induced spermatogonial proliferation (3,5). Interestingly, higher doses of CORT
248 (i.e. 100 ng/mL) had the opposite effect and inhibited 11KT synthesis in these
249 species (3,5). Although measurement of 11KT concentration in the cultured media
250 should be performed to evaluate such effects on spotty wrasse ovaries, absence of
251 spermatogonia in the cultured ovaries suggests 11KT levels were not significantly
252 affected. The possible metabolic conversion of CORT into 11KT and other 11-
253 oxygenated androgens has also been suggested in several teleosts (3,28), as there
254 is cross-talk between the glucocorticoid and androgen synthesis pathways (14,29).
255 However, the mechanisms underlying 11KT synthesis are still not fully
256 understood and a potential CORT-to-11KT direct conversion remains to be
257 clarified (14,30). This work constitutes the first-ever *in vitro* organ culture system
258 in a temperate sex-changing teleost, and opens doors for future work in a wide
259 variety of hermaphroditic fish.
260
261 Although the current experiment could not induce any observable female-to-male
262 restructuring of spotty wrasse gonads in culture, ovarian tissues were successfully
263 maintained in culture for 21 days. Such long-term culture of fish organ explants
264 has only been accomplished in a few instances (3–7). Moreover, this work validates

265 the use of open-source machine learning software Ilastik to reliably recognise cell
266 types in fish gonads, with enormous potential in future studies. The image
267 analysis strategy presented here can be adapted and applied to other studies
268 where accurate cell segmentation is required.
269

270 **Conclusion**

271

272 This study successfully developed an organ culture system for gonadal tissue
273 explants in New Zealand spotty wrasse, providing an *in vitro* system for
274 investigating control and perturbation of gonadal sex change in this and other
275 species. Although culture was associated with a degree of PVO degeneration, it
276 was shown that administration of at least 10 ng/mL E_2 can partially mitigate
277 tissue degeneration during culture. However, neither 11KT nor CORT treatment
278 successfully accelerated ovarian atresia or induced spermatogenic proliferation.
279 Future *in vitro* manipulations should explore longer cultures and/or co-treatment
280 of multiple steroids to analyse potential antagonistic or synergistic effects. This
281 culture system will facilitate future investigations of ovarian and testicular trans-
282 differentiation in sex-changing fish. For example, an *in vitro* system would be
283 especially amenable to the use of small interfering RNAs to knock down expression
284 of specific candidate genes or application of methylation inhibitors to further
285 investigate the genetic and epigenetic cascade underlying sex change. *In vitro*
286 approaches also offer valuable ethical benefits over experiments with live fish,
287 enabling the application of the 3Rs guiding principles: reduce, replace and refine
288 (31).
289

290 **Abbreviations**

291

292 11KT: 11-ketestosterone; C: control (no steroid); D0: day 0 reference tissues;
293 CORT: cortisol; E_2: 17β-estradiol; HDF5: hierarchical data format 5; IP: initial
294 phase; PVO: previtellogenic oocytes; TP: terminal phase.
295
296

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Competing Interests

The authors have declared that no competing interest exists.

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404 **Figure legends**

405

406 Figure 1. Pixel-based trainable classifier Ilastik workflow. Example of spotty
407 wrasse (*Notolabrus celidotus*) ovarian tissue treated with 100 ng/mL of 17ß-
408 estradiol processed for histology and visualised under light microscopy (A). Ilastik
409 was manually trained to discriminate previtellogenic oocytes (yellow), atretic
410 oocytes (turquoise), stromal/connective tissue (red) and background (blue) (B).
411 Segmentation map with different object types created with Ilastik (C). Scale bar:
412 100 µm.

413

414 Figure 2. Histological measurement of area covered by non-atretic previtellogenic
415 oocytes (PVO) in ovaries of spotty wrasse (*Notolabrus celidotus*) treated with E_2
416 (A), 11KT (B) or CORT (C) (day 21). Bars show the surface area of all non-atretic
417 PVO over total tissue area (expressed as %). Same day 0 reference tissues and
418 controls (day 21) were used for all treatments. Results are shown as the mean ±
419 SEM. Letters denote a significant difference between groups. Samples sizes: D0 n
420 = 5; C (no steroid) n = 15; E_2, 1 ng/mL n = 5, 10 ng/mL n = 5, 100 ng/mL n = 5;
421 11KT, 1 ng/mL n = 5, 10 ng/mL n = 5, 100 ng/mL n = 5; CORT, 1 ng/mL n = 5, 10
422 ng/mL n = 5, 100 ng/mL n = 4. Abbreviations: 11-ketotestosterone (11KT), control
423 (C), cortisol (CORT), day 0 reference tissues (D0), 17ß-estradiol (E_2).

424

425 **Authors' biography**

426

427 Alexander Goikoetxea obtained his BSc in Biotechnology and MSc in Aquaculture
428 from the *Universitat of Barcelona* and completed his PhD at the University of
429 Otago. His PhD thesis focuses on the elucidation of the genetic basis underlying
430 sex change in the protogynous New Zealand spotty wrasse (*Notolabrus celidotus*),
431 in particular, on the role of stress during this astonishing transformation.

432

433 Erin Damsteegt completed a BSc (Hons) in Zoology and a PhD at the University
434 of Otago. After completing a Post-Doctoral research position combined between
435 King's College London and The University of Roehampton (United Kingdom), she

is currently working as a Research Fellow in the Department of Zoology, University of Otago, New Zealand.

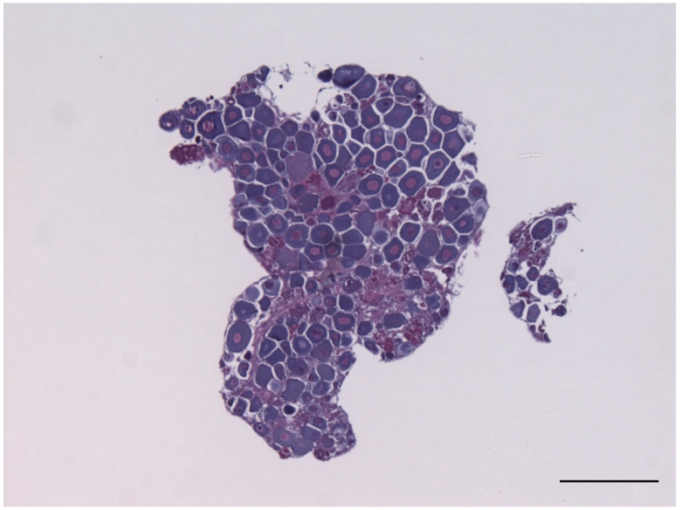
Erica Todd received her PhD from James Cook University, Australia. Her Post-Doctoral research at the University of Otago, New Zealand, focused on the molecular basis of female-to-male sex change in wrasses using transcriptomic, epigenomic and comparative genomic approaches. Currently, she is a lecturer in Marine Genomics at Deakin University, Australia.

Neil Gemmell is a Professor at the University of Otago. His research broadly spans reproduction and genomics, with the genetic basis of sex determination and differentiation a long-standing topic of interest.

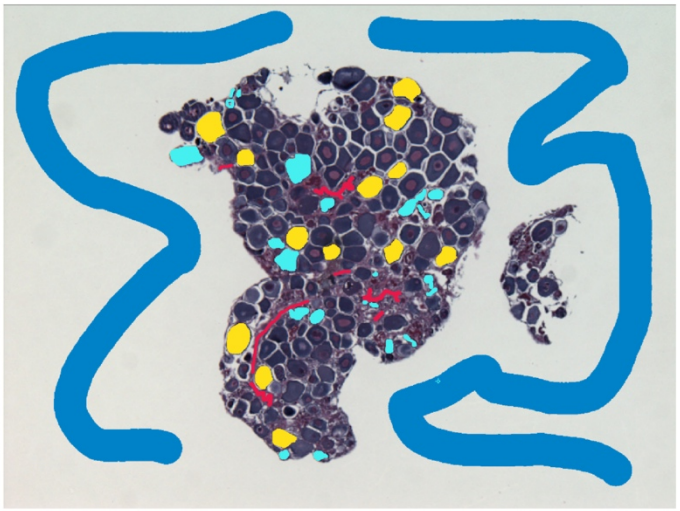
Mark Lokman is an academic at the University of Otago. His research interests centre around effects of androgens on ovarian physiology and around endocrine control of oogenesis in fish, chiefly using anguillid eels as research models.

Figure 1

A



B



C

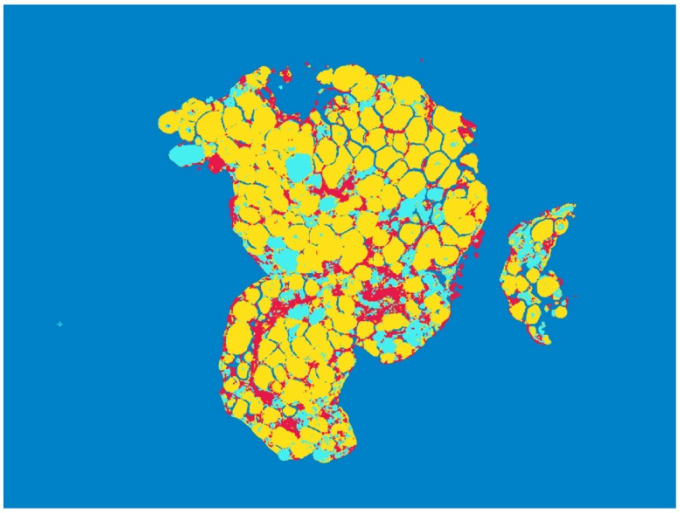
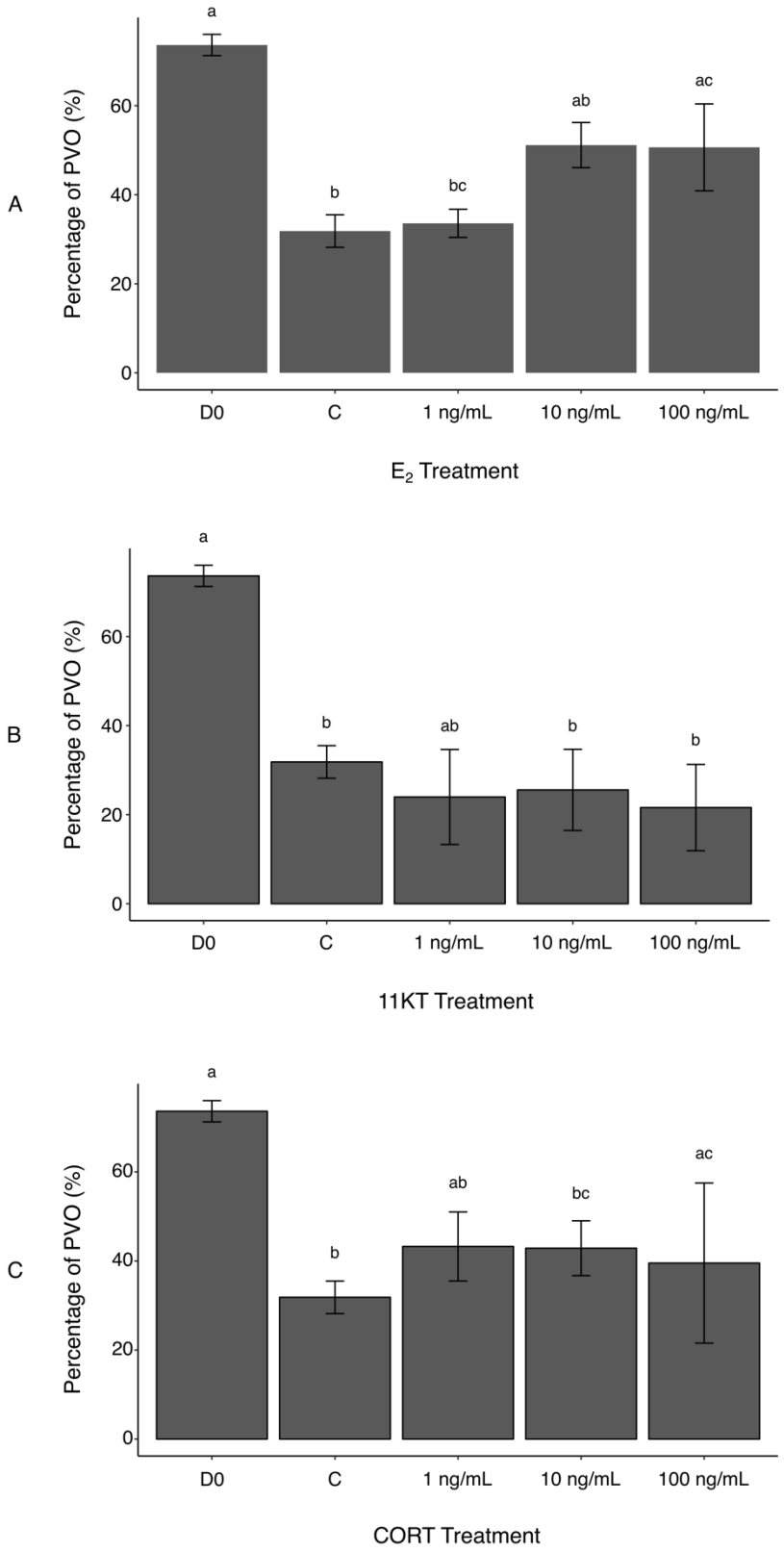


Figure 2


A
Percentage of PVO (%)
0
20
40
60
a
b
bc
ab
ac
D0
C
1 ng/mL
10 ng/mL
100 ng/mL
E2 Treatment
B
Percentage of PVO (%)
0
20
40
60
a
b
ab
b
b
D0
C
1 ng/mL
10 ng/mL
100 ng/mL
11KT Treatment
C
Percentage of PVO (%)
0
20
40
60
a
b
ab
bc
ac
D0
C
1 ng/mL
10 ng/mL
100 ng/mL
CORT Treatment