SUPPLEMENTARY INFORMATION

for

„Postovulatory ageing modifies sperm-induced Ca^{2+} oscillations in mouse oocytes through a conditions-dependent, multi-pathway mechanism”

Marcin Szpila, Agnieszka Walewska, Dorota Sabat-Pośpiech, Patrycja Strączyńska, Takao Ishikawa, Robert Milewski, Katarzyna Szczepańska and Anna Ajduk

Supplementary Table S1. Effect of the cell cycle status on the pattern of Ca^{2+} oscillations in oocytes aged for 25 hrs *in vitro* and *in vivo*

	25 hrs *in vitro*		25 hrs *in vivo*	
	M-phase-arrested	Progressing to interphase	M-phase-arrested	Progressing to interphase
	Median (Q1;Q3)			
No. of Ca^{2+} transients	**9.0** (7.5; 10.5) *n=11*	**7.0** (6.0; 9.0) *n=19*	**14.0** (9.5; 24.3) *n=20*	**9.0** (5.5; 15) *n=23*
Duration of Ca^{2+} oscillations (min)	**199.1** (144.4; 242.9) *n=11*	**130.2** (97.9; 185.0) *n=19*	**246.4**[b] (185.2; 329.1) *n=19*	**125.0**[b] (80.7; 188.5) *n=23*
Mean interval between Ca^{2+} transients during 1st 2 hrs (min)	**24.4** (14.4; 26.3) *n=11*	**18.8** (15.6; 23.9) *n=19*	**13.5** (10.3; 19.2) *n=19*	**14.5** (9.8; 21.6) *n=23*
Amplitude of the 1st Ca^{2+} transient ($\Delta F/F_0$)	**0.7** (0.6; 0.8) *n=11*	**0.7** (0.5; 0.8) *n=19*	**0.5** (0.5; 0;7) *n=20*	**0.6** (0.5; 0.6) *n=23*
Amplitude of the 3rd Ca^{2+} transient ($\Delta F/F_0$)	**0.7** (0.6; 0.7) *n=11*	**0.7** (0.6; 0.7) *n=19*	**0.6** (0.5; 0.6) *n=19*	**0.6** (0.5; 0.7) *n=22*
Duration of the 1st Ca^{2+} transient (min)	**1.5**[a] (1.2; 1.6) *n=11*	**1.7**[a] (1.5; 2.0) *n=19*	**2.2** (2.0; 2.6) *n=20*	**2.3** (1.8; 3.3) *n=23*
Duration of the 3rd Ca^{2+} transient (min)	**1.0** (0.8; 1.0) *n=11*	**1.0** (1.0; 1.2) *n=19*	**1.0** (1.0; 1.2) *n= 19*	**0.8** (0.8; 1.0) *n=22*
Rate of the 1st Ca^{2+} increase (min^{-1})	**3.4** (2.8; 4.2) *n=11*	**3.0** (2.0; 3.2) *n=19*	**2.1** (1.4; 2.2) *n=20*	**2.0** (1.8; 2.4) *n=23*
Rate of the 3rd Ca^{2+} increase (min^{-1})	**3.7** (3.5; 4.0) *n=11*	**3.7** (3.2; 4.0) *n=19*	**3.0** (2.7; 3.4) *n=19*	**3.1** (3.1; 3.3) *n=22*
Rate of the 1st Ca^{2+} decrease (min^{-1})	**-2.7** (-3.1; -2.1) *n=11*	**-2.3** (-3.5; -1.8) *n=19*	**-2.6** (-3.1; -1.8) *n=20*	**-2.8** (-3.1; -2.1) *n=23*
Rate of the 3rd Ca^{2+} decrease (min^{-1})	**-3.6** (-3.7; -3.2) *n=11*	**-3.3** (-3.9; -2.8) *n=19*	**-3.1** (-3.8; -2.8) *n=19*	**-3.3** (-3.7; -3.0) *n=22*

Values marked with the same letter are significantly different: [a,b]$p<0.01$,

Supplementary Table S2. Effect of cumulus cells and Ca^{2+}-independent parthenogenetic activation on the pattern of Ca^{2+} oscillations and the ER Ca^{2+} store in oocytes

	9 hrs *in vitro* + CC	fresh + 6-DMAP + CHX
	Median (Q1;Q3)	
No. of Ca^{2+} transients	**9.0**^{a,ddd} (8.0; 10.0) *n=67*	**6.0**^{bbb,c,eee} (5.0; 7.0) *n=62*
Duration of Ca^{2+} oscillations (min)	**146.1**^{ddd} (135.9; 160.9) *n=67*	**120.0**^{a,b,eee} (63.2; 162.4) *n=61*
Mean interval between Ca^{2+} transients during 1st 2 hrs (min)	**17.0**^{aaa} (15.1; 19.5) *n=67*	**21.1**^{d,eee} (18.3; 24.0) *n=61*
Amplitude of the 1st Ca^{2+} transient ($\Delta F/F_0$)	**0.8**^{ddd,eee} (0.7; 0.9) *n=67*	**1.2**^{bbb,ccc,ddd,eee} (1.0; 1.3) *n=60*
Amplitude of the 3rd Ca^{2+} transient ($\Delta F/F_0$)	**0.8**^{c,eee} (0.7; 0.8) *n=67*	**1.0**^{aa,bbb,ccc,ddd,eee} (1.0; 1.1) *n=56*
Duration of the 1st Ca^{2+} transient (min)	**2.7**^{ccc} (2.2; 3.2) *n=67*	**2.4**^{aaa,c,d} (2.0; 2.7) *n=60*
Duration of the 3rd Ca^{2+} transient (min)	**1.2**^{c,eee} (1.2; 1.3) *n=67*	**1.1** (1.0; 1.3) *n=56*
Rate of the 1st Ca^{2+} increase (min^{-1})	**3.4**^{ddd,eee} (2.8; 4.2) *n=67*	**2.4**^{bbb} (1.5; 3.5) *n=59*
Rate of the 3rd Ca^{2+} increase (min^{-1})	**4.3**^{eee} (3.9; 4.6) *n=67*	**3.4**^{aaa,e} (2.8; 5.5) *n=56*
Rate of the 1st Ca^{2+} decrease (min^{-1})	**-3.3**^{aa} (-3.5; -2.9) *n=67*	**-3.2**^{aa,e} (-4.4; -2.5) *n=60*
Rate of the 3rd Ca^{2+} decrease (min^{-1})	**-4.3**^{cc,eee} (-4.5; -3.8) *n=67*	**-3.5**^{aaa} (-5.8; -3.0) *n-56*
Amplitude of the TG-induced Ca^{2+} release ($\Delta F/F_0$)	**1.2**^{a,b,c,e} (1.2; 1.2) *n=42*	n/a

^{aaa}p<0.001, ^{aa}p<0.01, ^{a}p<0.05 *vs*. fresh oocytes
^{bbb}p<0.001, ^{bb}p<0.01, ^{b}p<0.05 *vs*. 9h *in vitro*
^{ccc}p<0.001, ^{cc}p<0.01, ^{c}p<0.05 *vs*. 25h *in vitro*
^{ddd}p<0.001, ^{dd}p<0.01, ^{d}p<0.05 *vs*. 9h *in vivo*
^{eee}p<0.001, ^{ee}p<0.01, ^{e}p<0.05 *vs*. 25h *in vivo*

Supplementary figure legends

Supplementary Fig. S1 Fertilization-induced Ca^{2+} oscillations in freshly ovulated and postovulatory aged oocytes

(A) Ca^{2+} trace representative for fresh oocytes. **(B)** Magnification of the 1st Ca^{2+} transient from **(A)** and a schematic representation of how the Ca^{2+} increase rate was calculated (Ca^{2+} decrease rate was calculated analogically). **(C-F)** Ca^{2+} traces representative for oocytes aged for 9 hrs *in vitro* **(C)**, 9 hrs *in vivo* **(D)**, 25 hrs *in vitro* **(E)** and 25 hrs *in vivo* **(F)**. In case of **(E-F),** two of the most frequently occurring types of Ca^{2+} oscillations were presented.

Supplementary Fig. S2 Dynamics of the 3rd fertilization-induced Ca^{2+} transient and IP3R1 and SERCA2 expression in postovulatory aged oocytes

(A-B) Point charts presenting the amplitude and the duration of the 3rd Ca^{2+} transient in freshly ovulated oocytes and oocytes aged for 9 and 25 hrs *in vitro* **(A)** and *in vivo* **(B)**. **(C-D)** Point charts presenting the rates of Ca^{2+} increase and decrease during the 3rd Ca^{2+} transient in freshly ovulated oocytes and oocytes aged for 9 and 25 hrs *in vitro* **(C)** and *in vivo* **(D)**. **(A-D)** Each dot represents one oocyte, the number of analysed oocytes is included in Table 1. **(E)** Original images of the Western blots showing expression of IP3R1 and SERCA2 proteins in brain lysate, freshly ovulated oocytes, oocytes aged for 9 and 25 hrs *in vitro* and *in vivo* and two other samples unrelated to this paper. The red line marks regions showed in Fig. 2 E as blots labelled for IP3R1 and SERCA2, blue line – a region showed in the Ponceau S staining.

Supplementary Fig. S3 Effect of cumulus cells on Ca^{2+} homeostasis in postovulatory aged oocytes.

(A) The mean Ca^{2+} release triggered by thapsigargin (TG), calculated for oocytes aged for 9 hrs *in vivo* and *in vitro* with and without cumulus cells (CC). The number of analysed oocytes is included in Tables 1 and 2. Mean values +/- SD are shown. Time-point "0" was set as a moment when the cytoplasmic Ca^{2+} concentration in oocytes started to rise. **(B)** Ca^{2+} trace representative for oocytes aged for 9 hrs *in vitro* with CC. **(C-G)** Point charts presenting the total duration of the Ca^{2+} oscillations and the mean interval between Ca^{2+} transients **(C)**, the amplitude and the duration of the 1st Ca^{2+} transient **(D)**, the amplitude and the duration of the 3rd Ca^{2+} transient **(E)**, the rates of Ca^{2+} increase and decrease during the 1st Ca^{2+} transient **(F)**, and the rates of Ca^{2+} increase and decrease during the 3rd Ca^{2+} transient **(G)** in oocytes aged for 9 hrs *in vivo* or *in vitro* with and without CC. Each dot represents one oocyte, the number of analysed oocytes is included in Tables 1 and Supplementary Table S2.

Supplementary Fig. S4 Effect of oxidative stress on Ca^{2+} ER store and fertilization-induced Ca^{2+} oscillations in oocytes

(A) The Ca^{2+} release triggered by thapsigargin (TG) and A23187 ionophore in representative freshly ovulated oocytes pre-treated with H_2O_2. Arrow indicates when TG or ionophore was added. **(B)** Ca^{2+} trace representative for fresh oocytes pre-treated with H_2O_2. **(C-D)** Point charts presenting the amplitude and the duration of the 3rd Ca^{2+} transient **(C)** and the rates of Ca^{2+} increase and decrease during the 3rd Ca^{2+} transient **(D)** in fresh oocytes, fresh oocytes pre-treated with H_2O_2 or oocytes aged for 25 hrs *in vitro*. **(E)** The mean Ca^{2+} release triggered by thapsigargin (TG), calculated for freshly ovulated oocytes or oocytes aged for 25 hrs *in vitro* with and without *N*-acetylcysteine (NAC). The number of analysed oocytes is included in Tables 1 and 2. Mean values +/- SD are shown. Time-point "0" was set as a moment when the cytoplasmic Ca^{2+} concentration in oocytes started to rise. **(F)** Ca^{2+} trace representative for oocytes aged for 25 hrs *in vitro* with NAC. **(G-H)** Point charts presenting the amplitude and the duration of the 3rd Ca^{2+} transient **(G)** and the rates of Ca^{2+} increase and decrease during the 3rd Ca^{2+} transient **(H)** in fresh oocytes and oocytes aged for 25 hrs *in vitro* with and without *N*-acetylcysteine (NAC). **(C-D, G-H)** Each dot represents one oocyte, the number of analysed oocytes is included in Tables 1 and 2.

Supplementary Fig. S5 Specificity of TMRE and MgGreen stainings

(A-B) Mg^{2+} (MgGreen in blue) and Ca^{2+} (Rhod-2 in orange) traces recorded for oocytes subjected to FCCP **(A)** or thapsigargin (TG) **(B)**. Arrows indicate when FCCP or TG was added. **(C)** Confocal image of a representative oocyte stained with MgGreen, displaying predominantly cytoplasmic localization of the dye. Although there are some signs of potential internalization of the dye (arrowheads), it does not form a pattern typical for ER cistern distribution (see **Fig. 4E**). Scale bar 50 µm.

Supplementary Fig. S6 Effect of postovulatory ageing on the readjustment of ATP production during Ca^{2+} oscillations.

(A-D) Mg^{2+} (MgGreen in blue) and Ca^{2+} (Rhod-2 in orange) oscillations in representative oocytes aged for 9 hrs *in vitro* **(A)** or *in vivo* **(B)** and for 25 hrs *in vitro* **(C)** or *in vivo* **(D).** The cytoplasmic concentration of free Mg^{2+} ions is inversely proportional to the ATP concentration. **(E)** A decrease in the Mg^{2+} concentration, indicative of an increase in the ATP concentration, during the 1st Ca^{2+} transient (value 'a' – value 'b' in **Fig. 4 C**) calculated for freshly ovulated oocytes (n=31), oocytes aged *in vitro* for 9 and 25 hrs (n=25 and 11, respectively) and oocytes aged *in vivo* for 9 and 25 hrs (n=18 and 32). $^{a}p<0.05$, $^{b}p<0.001$ *vs*. fresh oocytes. **(F)** A decrease in the Mg^{2+} concentration, indicative of an increase in the ATP concentration, during the 3rd Ca^{2+} transient (value 'c' – value 'd' in **Fig. 4 C**) calculated for freshly ovulated oocytes (n=31), oocytes aged *in vitro* for 9 and 25 hrs (n=25 and 10,

respectively) and oocytes aged *in vivo* for 9 and 25 hrs (n=15 and 29). [a]p<0.001, [b]p<0.01 *vs*. fresh oocytes, [c]p<0.001 *vs*. 9 hrs *in vitro*. **(A-B)** Graphs present medians and the 1st and the 3rd quartile values. The ends of the whiskers are set at 1.5*IQR above the third quartile and 1.5*IQR below the first quartile. Dots show the minimum and maximum values if they are outside the range (outliers).

Supplementary Fig. S7 Effect of actin depolymerisation on fertilization-induced Ca^{2+} oscillations in oocytes

(A) Ca^{2+} trace for a representative fresh oocyte treated with cytochalasin D (CCD). **(B-C)** Point charts presenting the amplitude and the duration of the 3rd Ca^{2+} transient **(B)** and the rates of Ca^{2+} increase and decrease during the 3rd Ca^{2+} transient **(C)** in freshly ovulated oocytes treated with CCD, and oocytes aged for 25 hrs *in vitro* and *in vivo*. Blue ovals indicate regions, where dots representing freshly ovulated oocytes would have been located. Each dot represents one oocyte, the number of analysed oocytes is included in Tables 1 and 2.

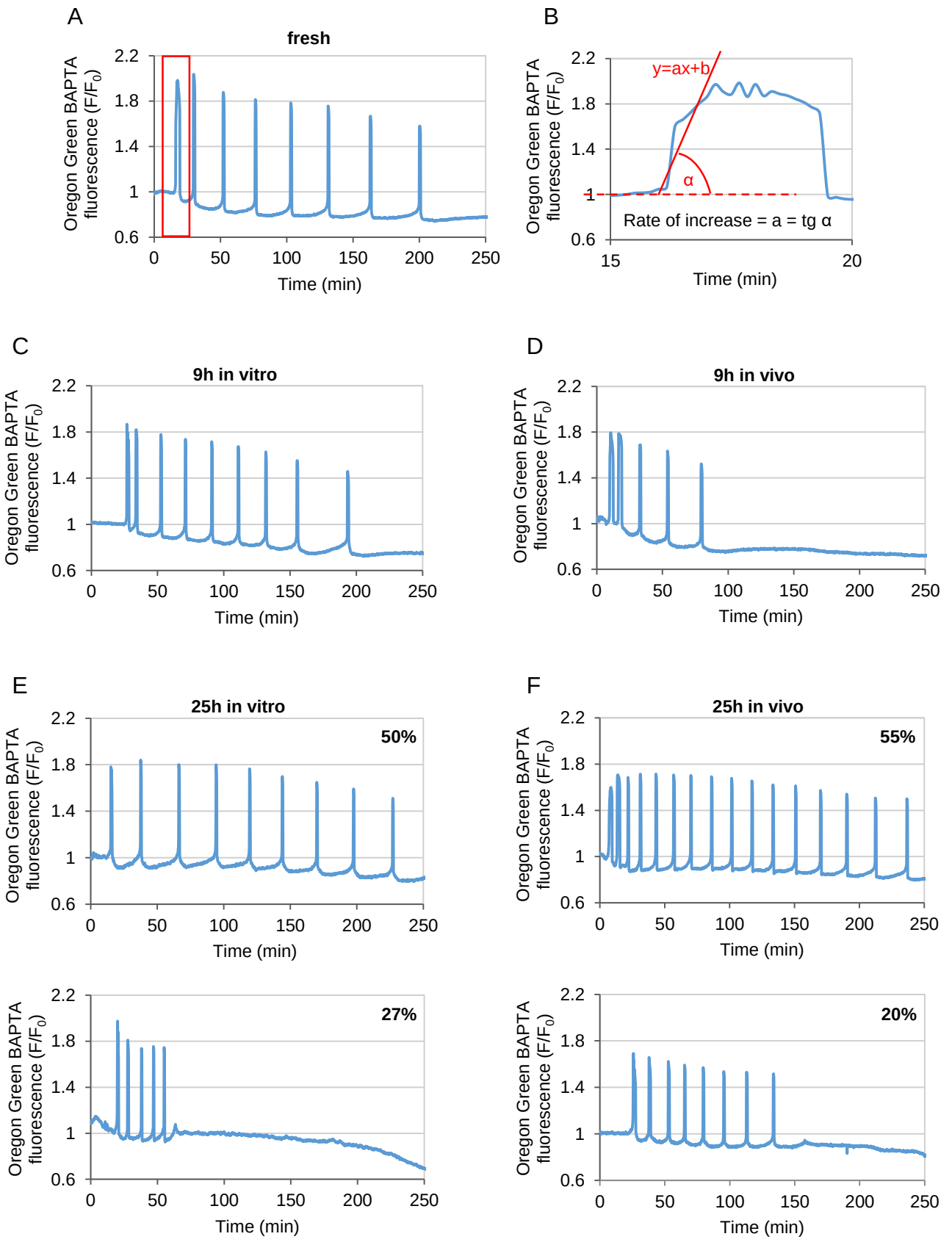
Supplementary Fig. S8 Effect of parthenogenetic activation with EtOH on fertilization-induced Ca^{2+} oscillations in oocytes

(A) Images of fresh oocytes, oocytes aged for 9 hrs *in vivo*, and fresh oocytes activated with ethanol (EtOH) or 6-DMAP and cycloheximide (CHX) used for the recording of Ca^{2+} oscillations. Images were taken just before fertilization. Asterisks indicate forming 2nd polar bodies and arrowheads – anaphase bulges. Scale bar 100 μm. **(B)** Ca^{2+} trace for a representative fresh oocyte pre-activated with EtOH. **(C-D)** Point chart presenting the amplitude and the duration of the 3rd Ca^{2+} transient **(C)** and the rates of Ca^{2+} increase and decrease during the 3rd Ca^{2+} transient **(D)** in freshly ovulated oocytes, oocytes pre-activated with EtOH and oocytes aged for 9 hrs *in vivo*. **(A-D)** Each dot represents one oocyte, numbers of analysed oocytes are included in Tables 1 and 2.

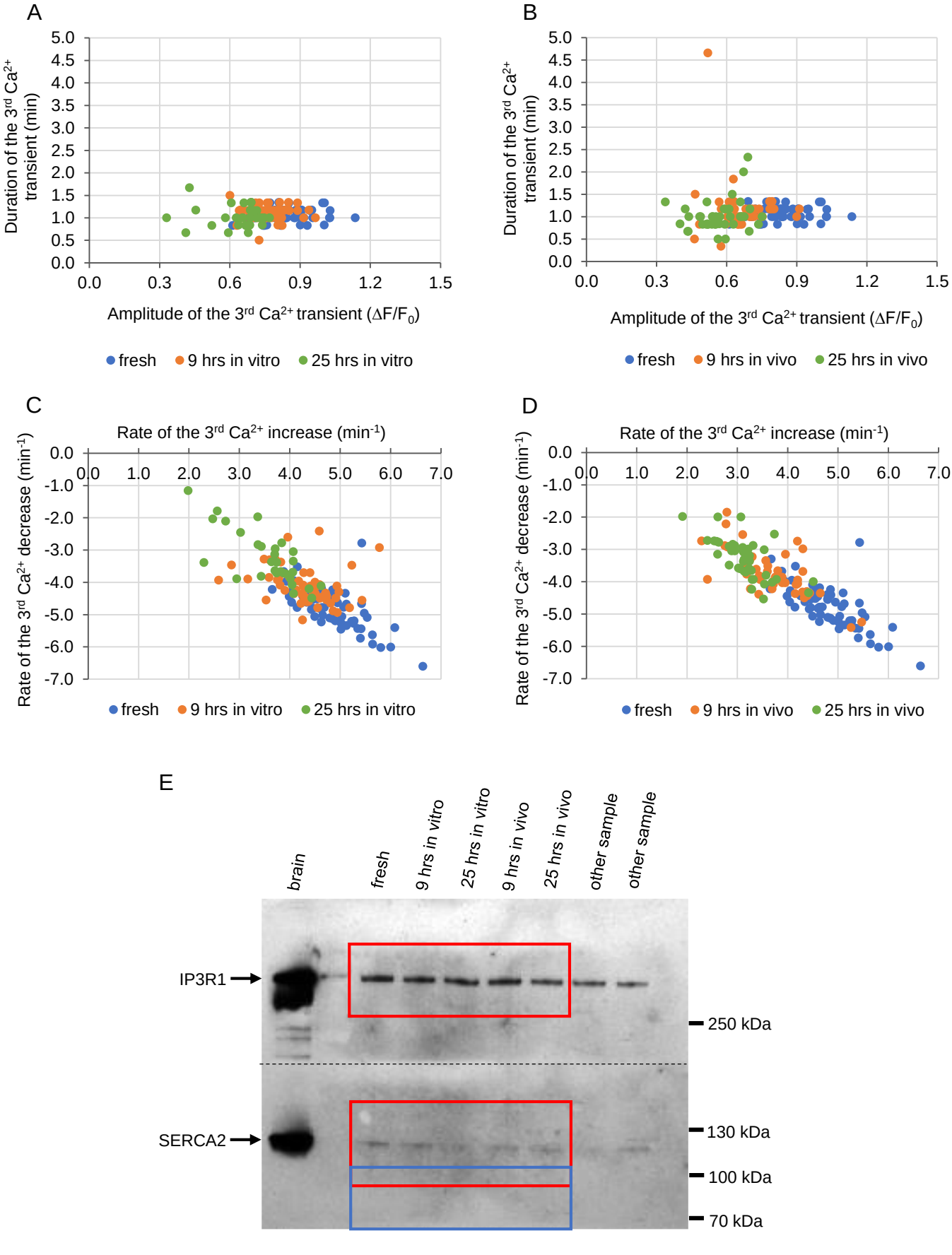
Supplementary Fig. S9 Effect of parthenogenetic activation with 6-DMAP and cycloheximide on fertilization-induced Ca^{2+} oscillations in oocytes

(A) Ca^{2+} trace for a representative fresh oocyte pre-activated with 6-DMAP and cycloheximide (CHX). **(B-F)** Point charts presenting the total duration of Ca^{2+} oscillations and the mean interval between Ca^{2+} transients **(B)**, the amplitude and the duration of the 1st Ca^{2+} transient **(C)**, the amplitude and the duration of the 3rd Ca^{2+} transient **(D)**, the rates of Ca^{2+} increase and decrease during the 1st Ca^{2+} transient **(E)**, and the rates of Ca^{2+} increase and decrease during the 3rd Ca^{2+} transient **(F)** in freshly ovulated oocytes, oocytes pre-activated with 6-DMAP and CHX and oocytes aged for 9 hrs *in vivo*. Each dot represents one oocyte, numbers of analysed oocytes are included in Tables 1 and Supplementary Table S2.

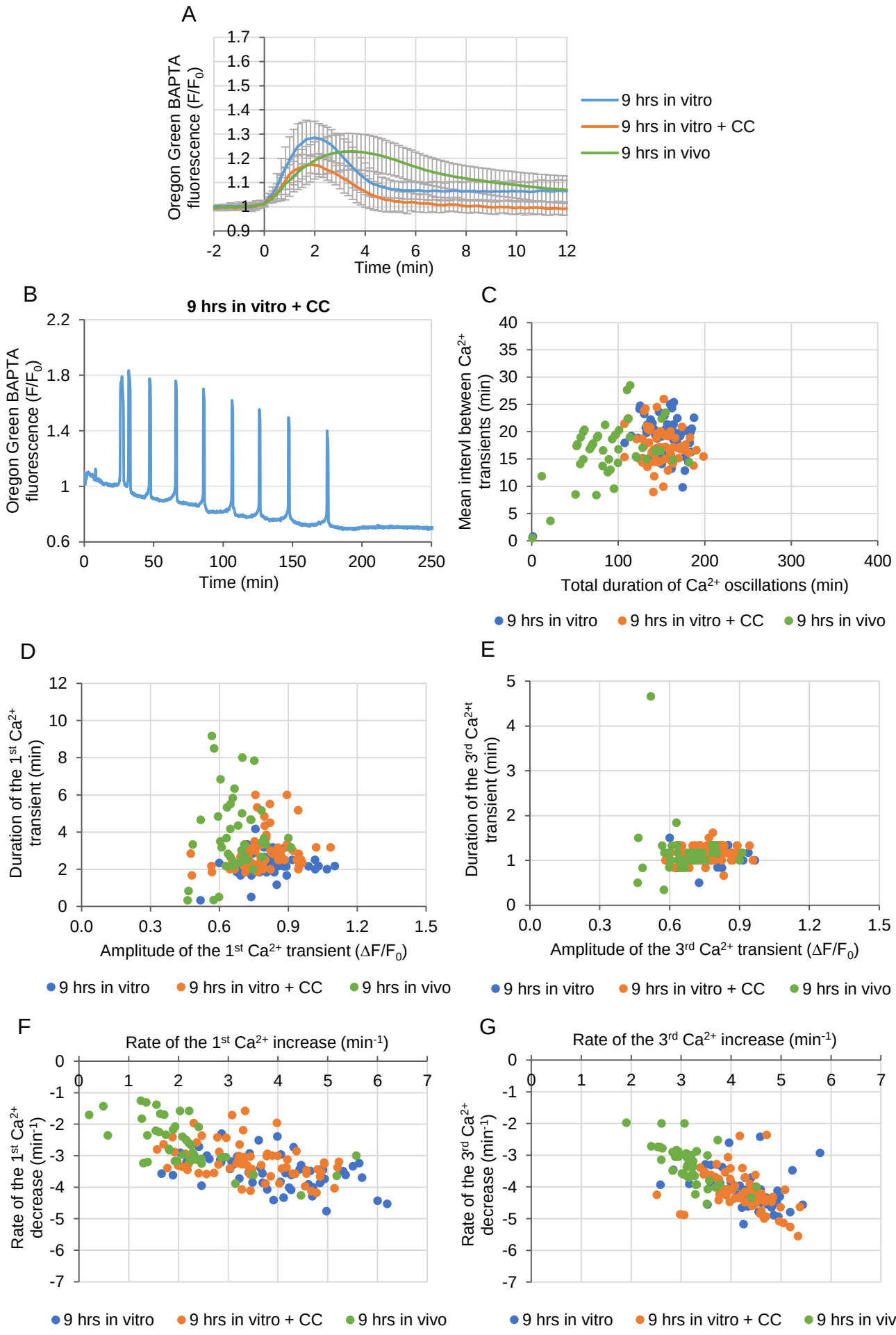
Supplementary Figure S1



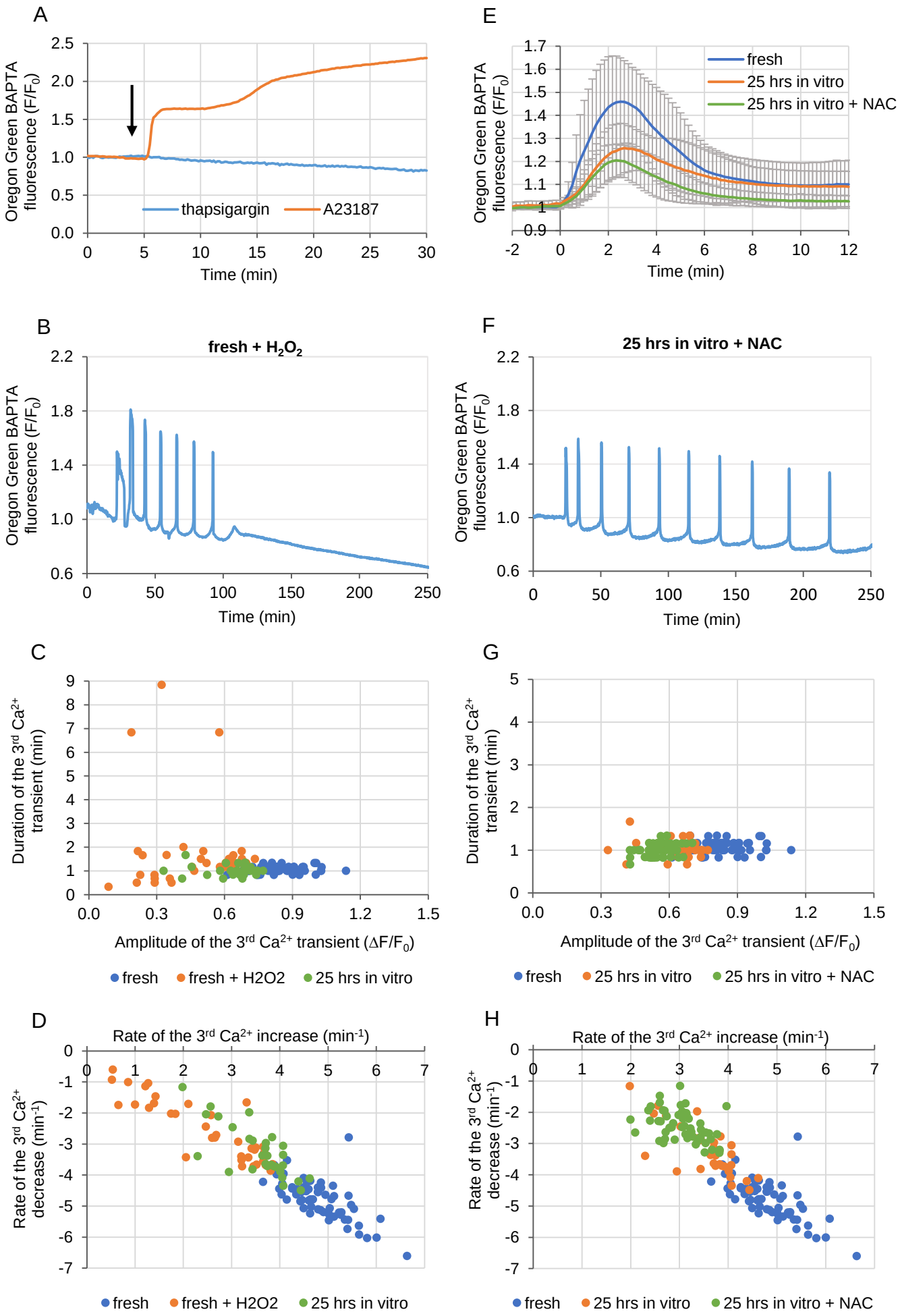
Supplementary Figure S2



Supplementary Figure S3



Supplementary Figure S4



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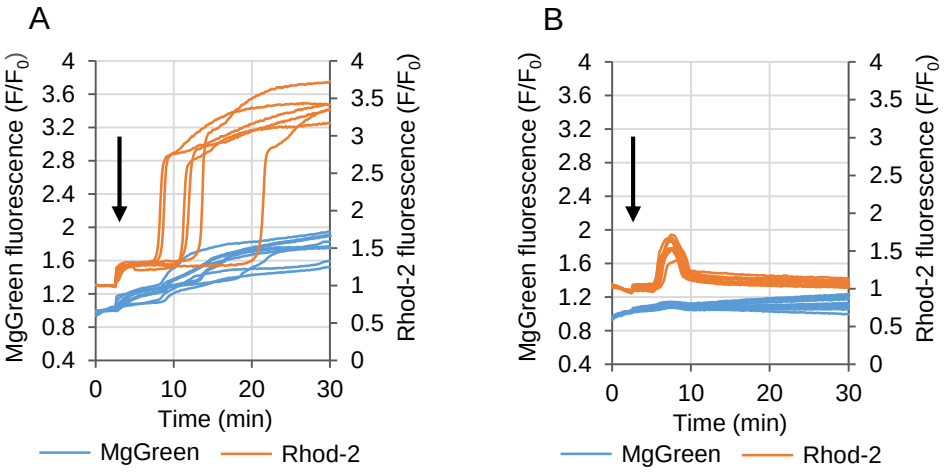

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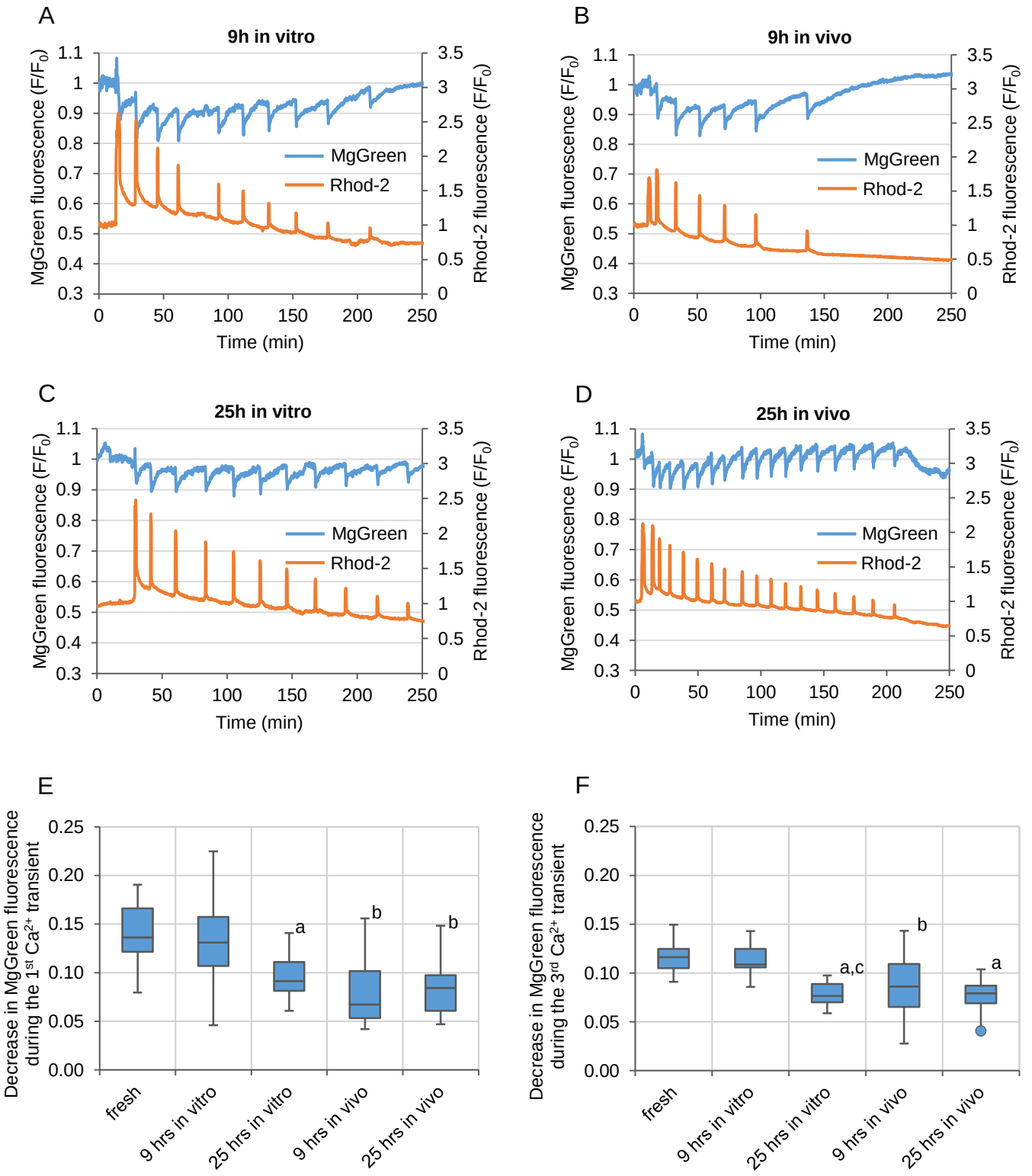

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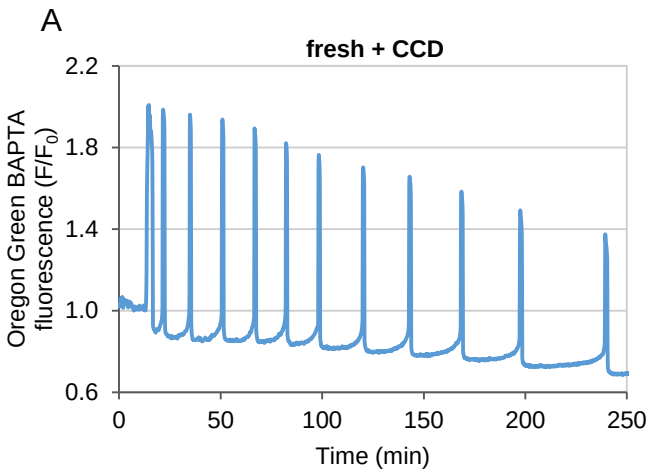
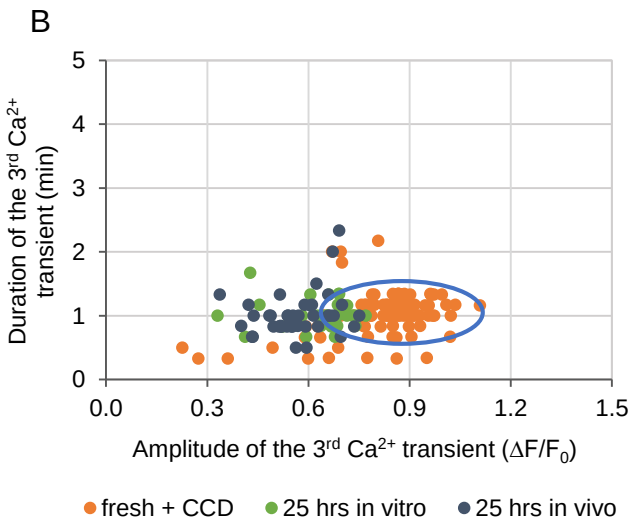
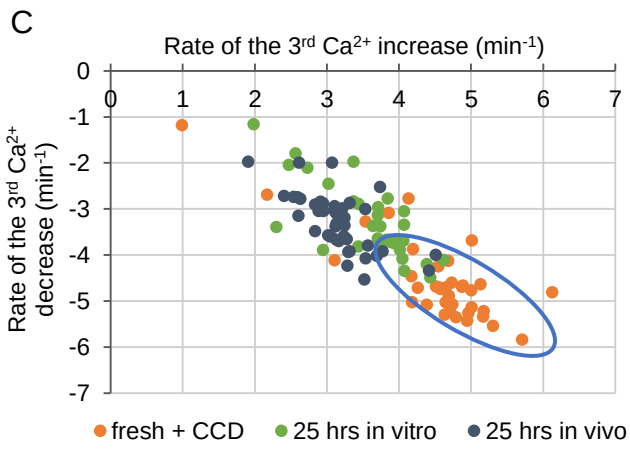
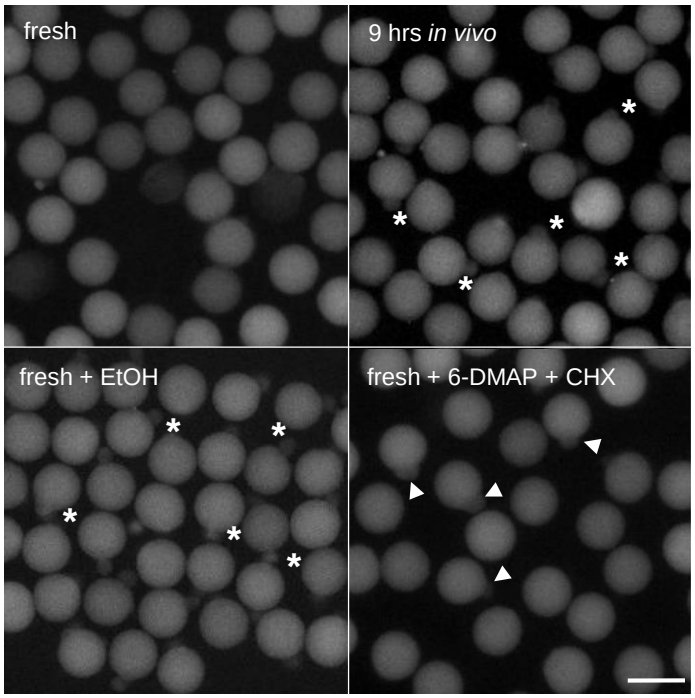
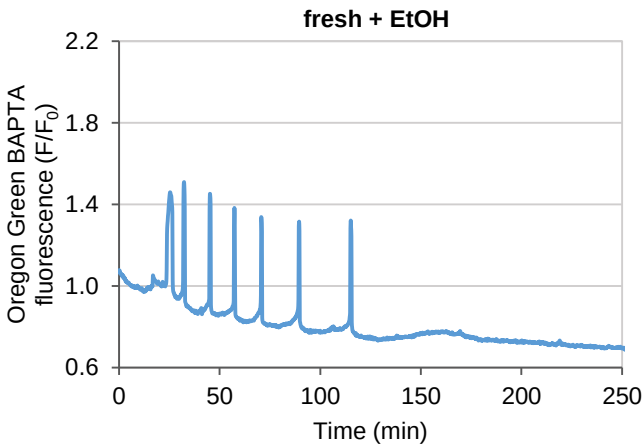


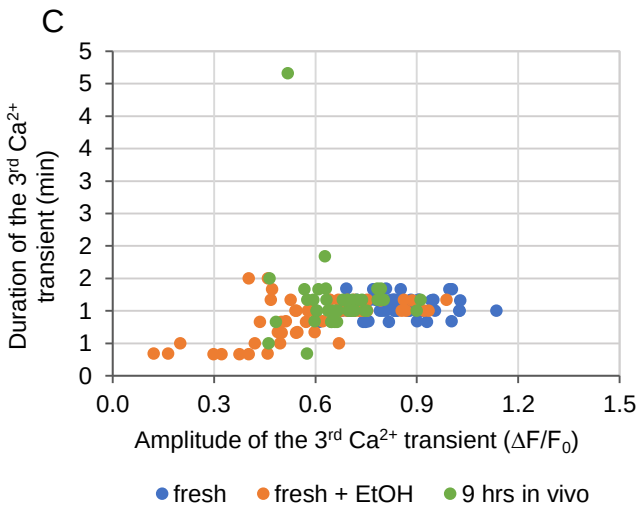


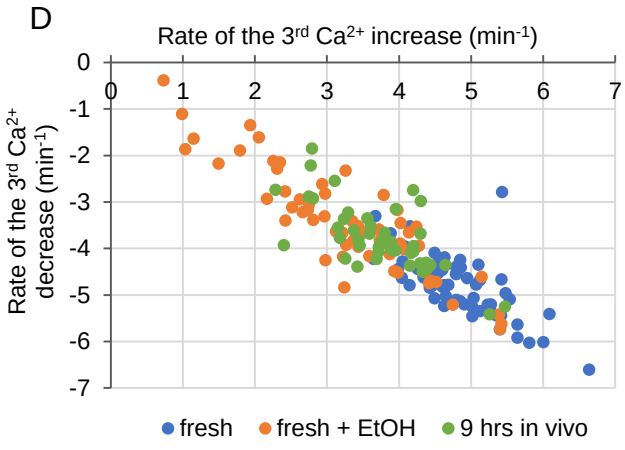


Supplementary Figure S8









Supplementary Figure S9