Supplementary Material

ZIRC Cryopreservation and Husbandry Protocols

ZIRC E400 Extender

This was a sperm extender solution composed of 130 mM potassium chloride (KCl), 50 mM sodium chloride (NaCl), 2 mM calcium chloride $CaCl_2$, 1 mM magnesium sulfate $MgSO_4$, 10 mM D-(+)-Glucose, and 30 mM HEPES buffer adjusted with potassium hydroxide (pH 7.9). The resulting solution had an osmolality of 400 mmol per kg. One mg per ml gelatin (Sigma-Aldrich Cat#G-7041-100G) from cold-water fish skin was added to this solution.

Sperm Dilution Protocol for Cell Concentration Assessment

Sperm samples were diluted with ZIRC E400 for cell density assessment with a microspectrophotometer (Nanodrop, ThermoFisher, Waltham, MA). For each fish line, immediately after measuring the final volume of combined sperm and extender solution (while still well mixed), 1 µL was transferred to a microcentrifuge tube with 9 µl ZIRC E400 (for a 1 in 10 dilution). A 1.5 – 2 µl aliquot of this dilution was used for each reading.

Raffinose, Milk, Methanol, Bicine (RMMB) Cryoprotectant Solution

This was prepared with 20% weight per volume (w/v) D-(+)-raffinose pentahydrate (Sigma R7630), 2.5% (w/v) Difco™ skim milk (Difco #232100), 6.7% (v/v) methanol (acetone-free, absolute, Certified ACS Reagent Grade, Fisher Scientific A412), and 30 mM bicine-NaOH (pH 8.0).

Sperm-thawing Solution and thawing procedure

1. **To prepare 1L Sperm Thawing Solution with Milk (SS300M)**, 8.2 g NaCl, 5 mL of 1 M KCl, 1 mL of 1 M $CaCl_2$, 1 mL of 1 M $MgSO_4$, 1.8 g D-(+)-Glucose, and 20 mL of 1 M Tris-Cl (pH 8.0) were combined in 800 mL dH_2O and the final volume was adjusted to 1 L. The osmolality was adjusted to 300-310 mOsmol/kg. The resulting SS300 solution will contain 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$2, 1 mM $MgSO_4$, 10 mM D-(þ)-Glucose, and 20 mM Tris-Cl (8.0). 2g Difco Skim Milk was added to 1000 mL SS300 and stirred or vortexed until dissolved (**2 mg/mL Difco Skim Milk**). The Sperm solution was filter sterilized and stored at 4 ºC short term. For long-term storage, the solution was aliquoted into 1.5 ml microcentrifuge tubes and frozen at-20 ºC.
2. For in vitro fertilization an aliqut of SS300M was thawed and used at room temperature. Females were stripped of eggs and once a suitable batch of eggs had been obtained, a cryovial with a sperm sample was removed from the LN_2 Freezer. The cryovial was manually held in a 38 ºC water bath until the frozen pellet was almost entirely thawed and 200 µL room-temperature SS300M was added and gently mixed with the micropipettor. The thawed sperm sample was transferred to the petri dish with the unfertilized eggs and the micropipette tip was gently placed among the eggs. The sperm solution was released among the eggs, and fertilization was allowed to take place undisturbed for 2 minutes. The petri dish was then flooded with a few ml of fish system water and set aside for counting fertilized embryos later.

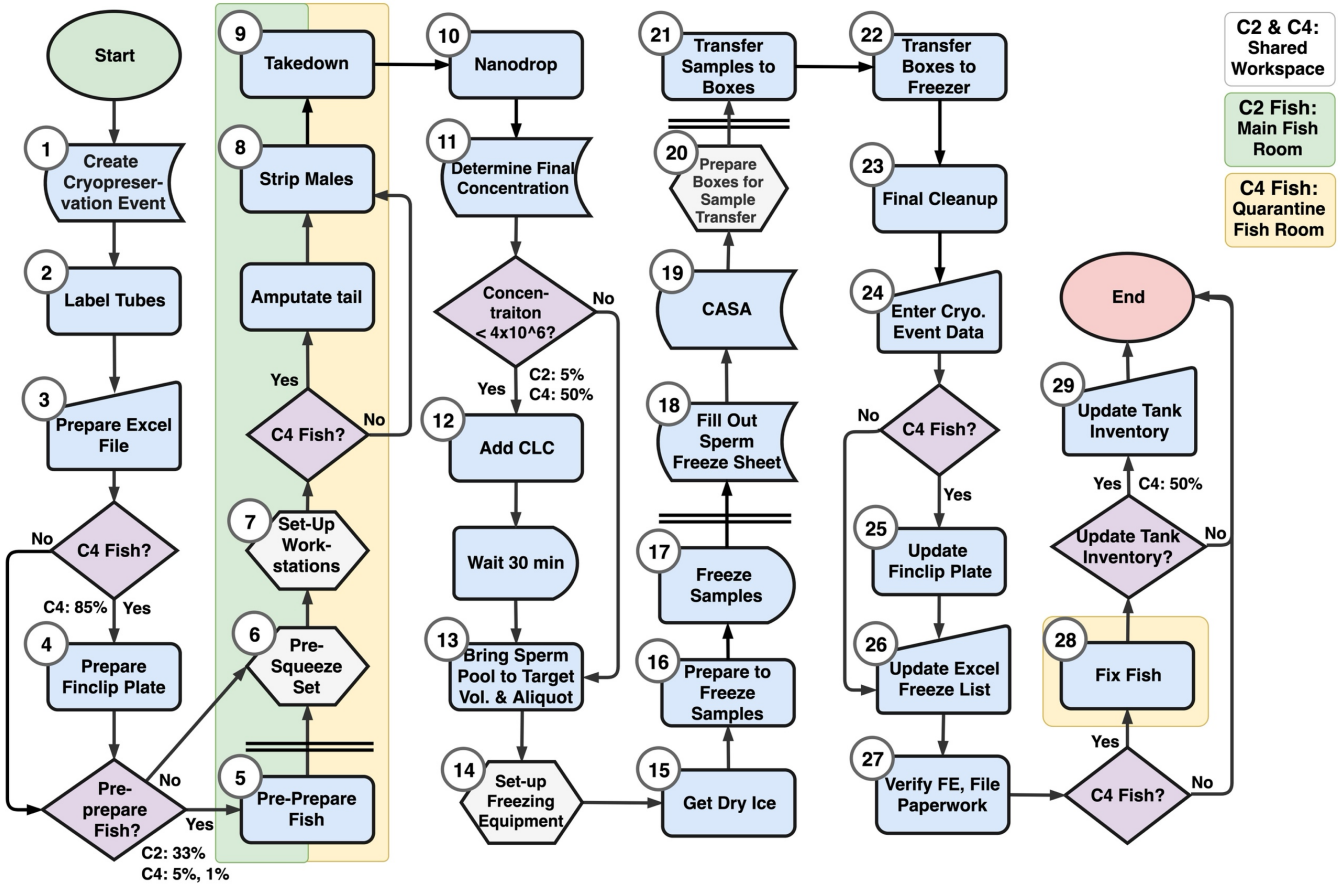
Euthanasia Protocol

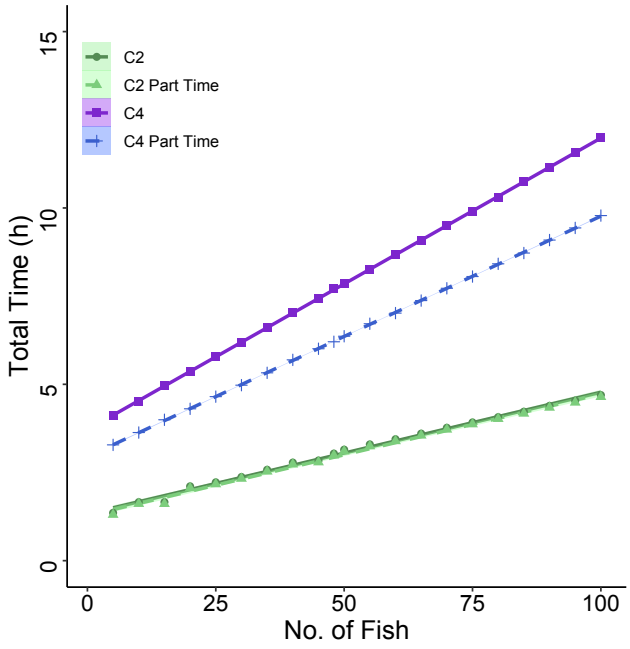
Hypothermic shock has been approved by the American Veterinary Medical Association for euthanasia of zebrafish, and was approved by the University of Oregon Institutional Animal Care and Use Committee (AUP#21-15). An ice water bath was prepared by combining crushed ice and water in a container to produce a slush and submerging a perforated insert. The insert filled with chilled water but not ice crystals, allowed rapid cooling of fish from ambient water temperature (28 °C) to 4 ºC. This induced unconsciousness within 10-15 sec, and death after an additional 10-15 min in the ice water (hypoxia). Carcasses were removed with the perforated insert and placed in a labeled plastic bag for storage in a freezer for later incineration, following local regulations.

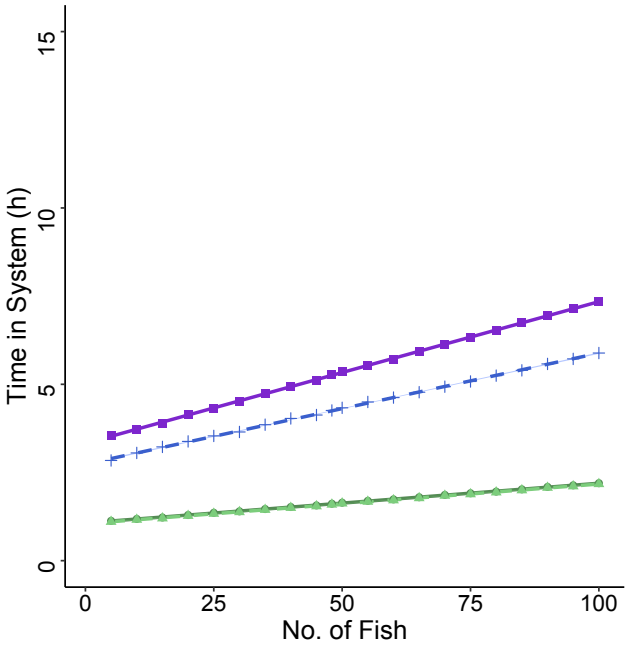
Dietrich's Fixative Solution

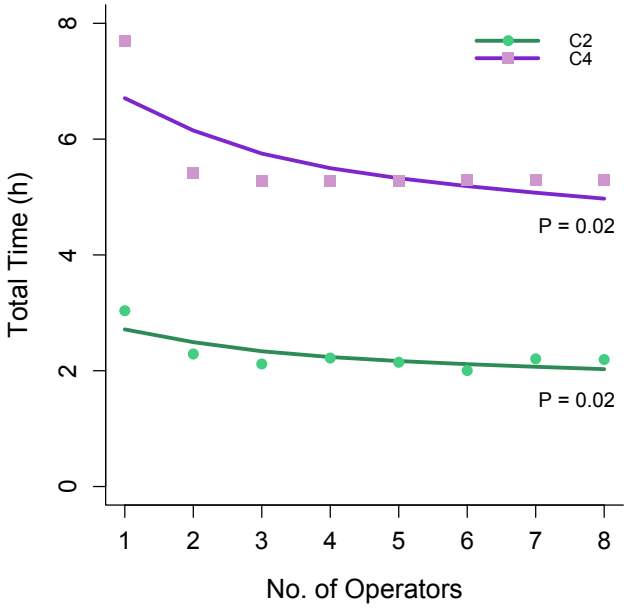
This fixative was prepared with 30% (volume per volume, v/v) ethanol, 10% (v/v) formalin, and 2% (v/v) acetic acid.

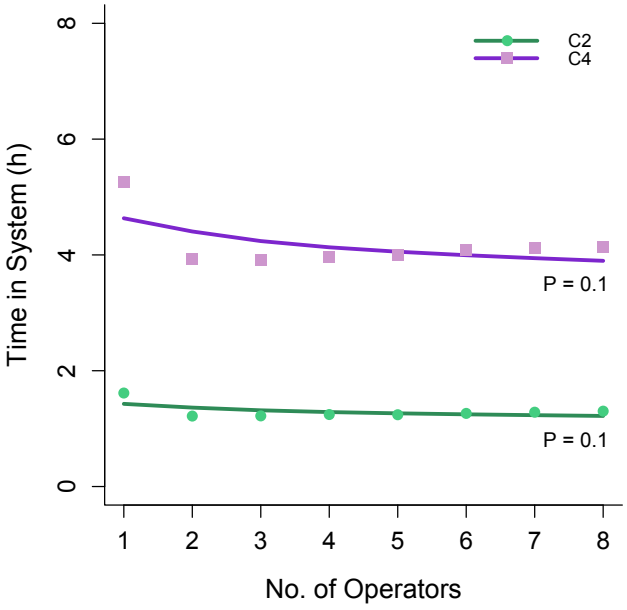
Figures


Start
1 Create Cryopreser-vation Event
2 Label Tubes
3 Prepare Excel File
C4 Fish?
No
C4: 85%
Yes
4 Prepare Finclip Plate
Pre-prepare Fish?
No
Yes
C2: 33%
C4: 5%, 1%
5 Pre-Prepare Fish
6 Pre-Squeeze Set
7 Set-Up Work-stations
C4 Fish?
Yes
No
8 Amputate tail
8 Strip Males
9 Takedown
10 Nanodrop
11 Determine Final Concentration
Concen-traiton < 4x10^6?
No
Yes
C2: 5%
C4: 50%
12 Add CLC
Wait 30 min
13 Bring Sperm Pool to Target Vol. & Aliquot
14 Set-up Freezing Equipment
15 Get Dry Ice
16 Prepare to Freeze Samples
17 Freeze Samples
18 Fill Out Sperm Freeze Sheet
19 CASA
20 Prepare Boxes for Sample Transfer
21 Transfer Samples to Boxes
22 Transfer Boxes to Freezer
23 Final Cleanup
24 Enter Cryo. Event Data
C4 Fish?
No
Yes
25 Update Finclip Plate
26 Update Excel Freeze List
27 Verify FE, File Paperwork
C4 Fish?
Yes
No
28 Fix Fish
Update Tank Inventory?
Yes
C4: 50%
No
29 Update Tank Inventory
End
C2 & C4: Shared Workspace
C2 Fish: Main Fish Room
C4 Fish: Quarantine Fish Room


C2
C2 Part Time
C4
C4 Part Time
Total Time (h)
No. of Fish
0
5
10
15
0
25
50
75
100


Time in System (h)
No. of Fish
0
5
10
15
0
25
50
75
100


C2
C4
P = 0.02
P = 0.02
Total Time (h)
No. of Operators
0
2
4
6
8
1
2
3
4
5
6
7
8


C2
C4
P = 0.1
P = 0.1
Time in System (h)
No. of Operators
0
2
4
6
8
1
2
3
4
5
6
7
8

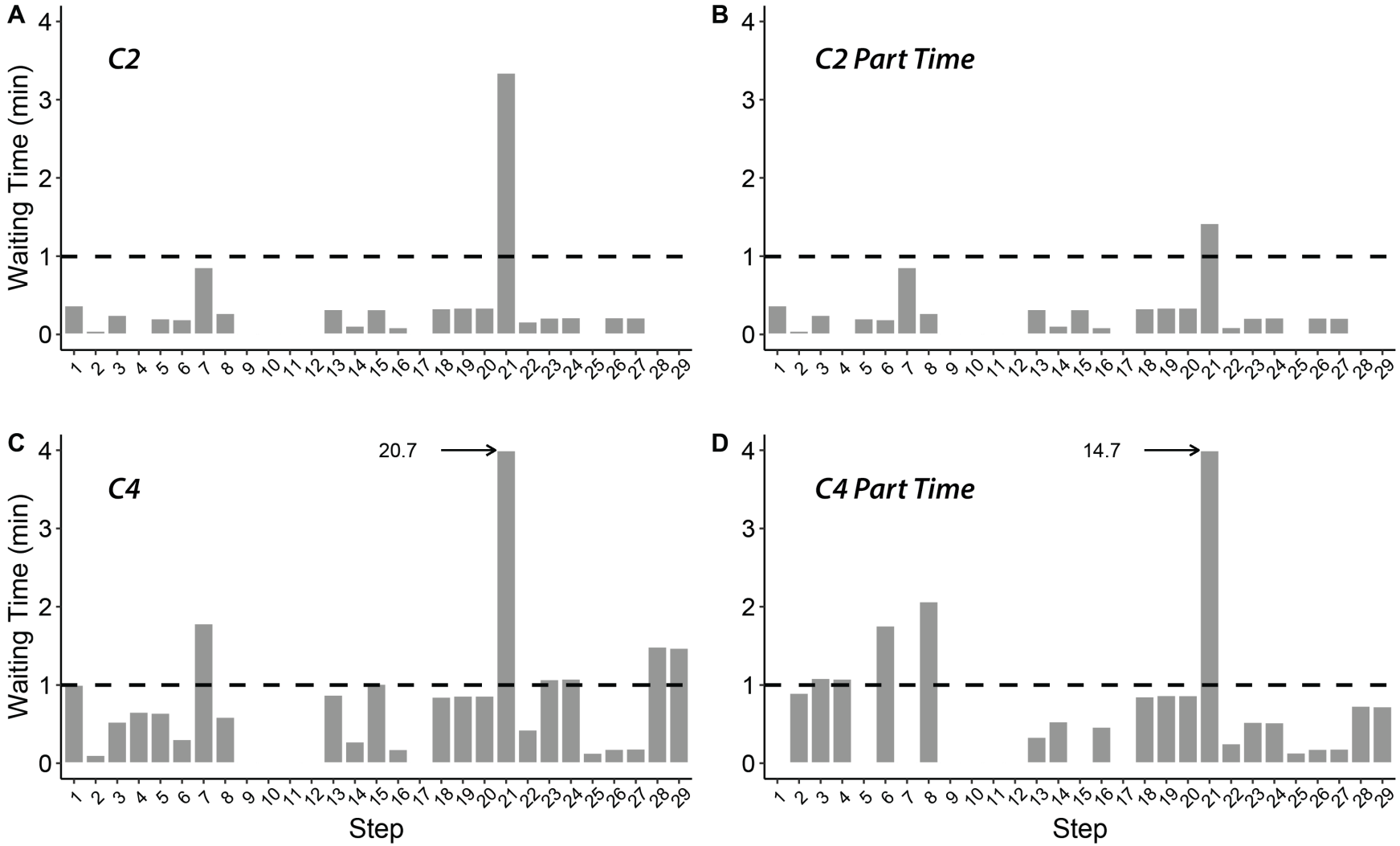

A
C2
B
C2 Part Time
C
C4
20.7
D
C4 Part Time
14.7
Waiting Time (min)
Step

Figure Legends

Fig. 1. Process flow diagram of the C2 and C4 zebrafish cryopreservation pathways used in this study. Ovals represent the start and end points; rectangles represent steps in the pathway; rectangles with a curved side represent steps where data were recorded; trapezoids represent steps where operators manually entered data; diamonds represent a decision point (a Quality Assurance checkpoint); hexagons represent set-up steps, and rectangles with a rounded side represent a delay in the protocol. Arrows indicate the flow of materials. Short parallel lines represent transition points between protocol stages. The text near Steps 4, 5, 12 and 29 represent the decision weights in the models that show their occurrence rate (Logic Rule 7). Colored backgrounds indicate where each step took place (white: Shared Workspace, green: Main Fish Room, and yellow: Quarantine Fish Room).

Fig. 2. Linear regressions of Number of Fish on the Total Time (h) and Time in System (h). All four models (C2, C4, C2 Part Time, and C4 Part Time) are represented. Symbols represent data points generated in Simio.

Fig. 3. Power models. Analyses of the effects of Number of Operators on Total Time (h) and Time in System (h) for C2 and C4 models. Shapes represent averages generated in Simio.

Fig. 4. Waiting times for individual steps. Steps 1 – 29 in the C2 and C4 models before and after an addition of an operator to steps with Waiting Times averaging above the 1-min threshold. Plot A represents the original C2 model; Plot B represents the C2 model with an additional operator in Step 21. Plots C represents the original C4 model; Plot D represents the C4 model with an additional operator in Steps 1, 7, 15, 21, 23,24, 28, and 29.

Tables

Table 1. *The time distributions (created from 16 time-study trials) for each step in the zebrafish cryopreservation protocol for the C2 and C4 models. Different time distributions were selected for use in the individual servers (steps) in each model from the following statistics: normal (mean, standard deviation), lognormal (normal mean, normal standard deviation), poisson (mean) and gamma (shape, scale), Exponential(mean).*

Step No.	Step Names	C2 Time Distributions (sec)	C4 Time Distributions (sec)
1	Create New Cryopreservation Event (CE)	Normal (4.57,1.03)	Lognormal (2.37,0.27)
2	Label Tubes	Normal (12.48,3.19)	Normal (39.65,9.12)
3	Prepare Excel CE Sheet	Lognormal (1.00,0.43)	Poisson (6.18)
4	Prepare Finclip Plate	-	Lognormal (3.17,0.13)
5	Pre-Prepare Fish	Poisson (35.73)	Poisson (29.24)
6	Pre-Strip Setup	Lognormal (2.76,0.36)	Lognormal (3.57,0.20)
7	Setup Workstation	Poisson (11.92)	Gamma (7.81,2.46)
8	Strip Males	Gamma (33.04,1.60)	Poisson (110.42)
9	Takedown	Gamma (13.45,1.33)	Poisson (19.24)
10	Nanodrop	Gamma (6.03,3.11)	Exponential (64.83)
11	Determine Final Concentration	Poisson (6.36)	Gamma (6.77,1.57)
12	Add CLC	30 min	30 min
13	Bring Sperm to Target Vol. & Aliquot	Lognormal (1.58,0.37)	Poisson (18.84)
14	Setup Freezing Equipment	Gamma (10.33,0.57)	Poisson (11.24)
15	Get Dry Ice	Poisson (6.77)	Lognormal (2.79,0.31)
16	Prepare to Freeze Samples	Normal (14.31,2.37)	Lognormal (3.74,0.22)
17	Freeze Samples	30 min	30 min
18	Fill Out Sperm Freeze Sheet	Gamma (9.63,0.37)	Lognormal (2.50,0.37)
19	CASA	Lognormal (2.49,0.24)	Poisson (29.10)
20	Prepare Boxes for Sample Transfer	Normal (5.14,0.44)	Lognormal (2.42,0.26)
21	Transfer Samples to Boxes	Normal (6.29,1.00)	Lognormal (2.63,0.16)
22	Transfer Boxes to Freezer	Gamma (3.29,1.17)	Exponential (11.52)

Step No.	Step Names	C2 Time Distributions (sec)	C4 Time Distributions (sec)
23	Final Cleanup	Exponential (3.87)	Poisson (50.25)
24	Enter CE Data	Gamma (6.53,0.82)	Lognormal (2.73,0.26)
25	Update Finclip Plate	-	Lognormal (1.96,0.55)
26	Update Excel File	Gamma (14.01,0.07)	Lognormal (1.09,0.49)
27	Verify CE & File Paperwork	Normal (3.05,0.55)	Poisson (8.04)
28	Fix Fish	-	Poisson (90.03)
29	Update Tank Inventory	-	Normal(121,33)/num of fish

Table 2. *Output statistics (Average Time and Time is System (TIS)) and identified bottlenecks (steps with the longest waiting and processing time) for all four models (C2, C4, C2 Part Time, and C4 Part Time) under baseline conditions (48 zebrafish and one operator). Half-widths (distance from the confidence limits to the mean for two-sided intervals) are displayed next to average values.*

Model	Avg. Time[1] (h)	TIS (h)	Longest Waiting Time (step name and min)		Longest Processing Time (step name and min)	
C2	3.0 ± 0.12	1.6 ± 0.03	Step 21	3.3 ± 0.12	Step 8	0.9 ± 0.02
C4	7.7 ± 0.05	5.3 ± 0.07	Step 21	20.7 ± 0.86	Step 8	1.8 ± 0.02
C2 Part Time	3.0 ± 0.13	1.6 ± 0.03	Step 21	1.4 ± 0.04	Step 8	0.9 ± 0.02
C4 Part Time	6.2 ± 0.04	4.2 ± 0.07	Step 21	14.7 ± 0.68	Step 8	1.8 ± 0.02

[1]*(Average* ± half-width)