## AN ABSTRACT OF THE THESIS OF

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Title: <u>Effects of Synchronous Low Flow and Temperature Maxima on Coastal</u> <u>Cutthroat Trout and Coastal Giant Salamander: An Experimental Approach</u>

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As regional climates warm in the Pacific Northwest, USA, flow minima and temperature maxima may become more synchronous in headwater streams over time. The dual stresses from lower flows and warmer temperatures will be energetically costly for cold-water species such as Coastal Cutthroat Trout and Coastal Giant Salamander. Individual fates will depend on environmental and individual characteristics, as well as the duration of exposures. In this study, I focus on stress responses and behavior of trout and salamanders as response variables to disentangle the impact of changing hydroclimate and its consequences for these sympatric stream-dwelling animals. By testing the synchrony between flow-minima and temperature maxima with mesocosms under a controlled setting, I provide insights about the short-term individual animal responses to the anticipated hydroclimate. Specifically, weight loss may not indicate long-term consequences of environmentalextreme exposure. Other metrics that relate to the animals physiological condition, such as RAMP and glucose may aid in understanding the implications of prolonged drought, but further research is necessary to establish baselines to understand this relationship. Managing for the maintenance of individual variation through population connectivity will increase the likelihood of continued persistence under

anticipated drought conditions. Ultimately, investigating how individuals persist under environmental extremes in headwaters will allow better understanding of population-level responses to climate change. ©Copyright by Francisco Andrew Tinoco Pickens March 16, 2020 All Rights Reserved

## Effects of Synchronous Low Flow and Temperature Maxima on Coastal Cutthroat Trout and Coastal Giant Salamander: An Experimental Approach

by

Francisco Andrew Tinoco Pickens

### A THESIS

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Francisco Andrew Tinoco Pickens, Author

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### 1. Introduction

Headwater streams are dynamic ecosystems that face an array of threats from climate change, along with most freshwaters (Dudgeon et al., 2006). One of these threats is the temporal shift of the hydroclimate (i.e., flow and temperature) (Stewart et al., 2005; Luce & Holden, 2009; Arismendi et al., 2013). For example, the Pacific Northwest (PNW) of North America experiences a Mediterranean climate regime that includes distinct rainy and dry seasons causing high flow and low flow periods, respectively. Historically, high flow periods coincided with cooler winter temperatures, whereas low flow periods occurred after maximum summer temperatures. However, recent evidence suggests that low flow periods are increasing in duration, as well as occurring earlier and temporally closer to maximum stream temperature (Arismendi et al., 2013). Additionally, these summer low flows are reduced relative to historical levels (Luce & Holden, 2009; Safeeq et al., 2013). Consequently, a temporal shift in hydroclimate exposes biota to more synchronous environmental stressors at an unprecedented rate and likely poses ramifications for stream-adapted biota (Lytle & Poff, 2004).

Temperature regulates the metabolic processes of aquatic poikilotherms such as fishes and amphibians (Fry, 1947). Higher temperatures increase metabolic costs, either leaving less energy available for other biological processes (growth and reproduction) or requiring the animal to obtain more energy to balance these costs (Clarke & Johnston, 1999). Concurrently, depth regulates the physical space available as habitat in which ecological interactions occur (Hakala & Hartman, 2004). For smaller streams, low-flow may cause discontinuity in surface flow, creating isolated pool habitats (Hunter et al., 2005; May & Lee, 2011; Hwan & Carlson, 2016). In this scenario, resident animals will be under the influence of extreme flow minima and may become trapped in pool refugia, where the probability of intra- and interspecific interactions increase due to severe reductions in available space. It is reasonable to infer that these hydroclimatic events would be stressful for fish or amphibians alone, but we do not fully understand the implications of their combined effects.

Although temperature and flow tend to be the primary environmental regulators of streams (Magnuson et al., 1979; Lytle & Poff, 2004), most research effort has focused on these regimes individually, rather than their interactive effects. Some studies have taken advantage of naturally occurring drought to understand low flow effects on the physical and/or community structure in streams (May & Lee, 2011; VerWey et al., 2018; Kaylor et al., 2019). May & Lee (2011) investigated the importance of pool refuge for salmonids during summer drought and found that the geomorphic setting determines a pool's persistence through summer. In this study, bedrock and bedrock-mixed substrate pools were more resilient to complete drying as opposed to gravel bed pools. Similarly, substrate type and the dominant source of water inputs (surface or subsurface) influenced the magnitude of diel temperature fluctuations. This influence posed important ramifications for resident fishes, such as mortality of fish not able to find stable pool refugia, increased densities within isolated pools (due to decrease in physical space), and decreased food availability caused by reduced flow (Chapman, 1966; Harvey et al., 2011).

VerWey et al. (2018) and Kaylor et al. (2019) observed decreased density and growth rates of adult Coastal Cutthroat Trout (*Oncorhynchus clarkii clarkii*) during a summer drought in PNW headwater streams. Coastal Giant Salamander (*Dicamptodon tenebrosus*) also exhibited decreases in condition factor, but abundances and biomass were not different during drought. Each of these studies found common responses in abundance, growth, and condition of stream animals due to drought, and show patterns identified in similar studies investigating drought impacts on trout in Virginia and California (Hakala & Hartman, 2004; Harvey et al., 2011). These studies have provided insight into the changing stream hydroclimate and its potential effects on stream animals; however, they do not directly investigate effects of temporal synchrony between flow minima and temperature maxima on individual animals (Arismendi et al., 2013).

Research focusing on the ecological impacts of the stream hydroclimate tends to focus on population-level processes. Because populations are comprised of individuals, individual fitness, behavior, and stress are the fundamental components that generate population-level processes (Fefferman & Romero, 2013). At the individual level, an animal must continually respond to its environmental scenario and does so to maintain homeostasis; internal ion balances, temperature, energy (glucose), and pH are regulatory systems that have been selected for over evolutionary time with respect to the environment (McEwen & Wingfield, 2003). Because the environment changes continually, an animal must similarly respond (behaviorally and/or physiologically) to maintain homeostasis, achieved through allostasis. The energy required to maintain homeostasis, referred to as the allostatic load, balances energy input (consumption) with metabolic costs associated to life stressors, and can become overwhelmed (allostatic overload). While maintaining homeostasis, the animal undergoes stress when allostasis achieves homeostasis (eustress). However, in the face of acute or prolonged stress, the energy required to maintain homeostasis exceeds that of net intake, which results in allostatic overload (distress). The distinction between eustress and distress is that eustress may be adaptive whereas distress operates outside the animals physiological tolerance (Schreck, 2000; McEwen & Wingfield, 2003). Additionally, a population of a single species will have a range of individual variation in physiological tolerances to environmental stimuli (Cockrem, 2013). Because of this variation, natural selection can act at the population level by selecting for physiological tolerances

(grounded in allelic differences) best suited to environmental conditions (Calow & Forbes, 1998).

Linking physiological measures to environmental conditions, determining what stressors are distressing opposed to eustressing, and making population-level inferences from this information is a major component in our understanding of individual physiology (Calow & Forbes, 1998; Wikelski & Cooke, 2006; Fefferman & Romero, 2013). Hematological (blood) measurements provide insight into an animals' homeostatic condition; however, physiological systems are intricate and responses can be idiosyncratic among individuals (Mommsen et al., 1999; Cockrem, 2013). Because of the natural variation in physiological systems, individuals will have different sensitivities and/or capacities to respond to a short- or long-term environmental stressor (Cockrem, 2013). Plasma cortisol and glucose concentrations are used often to assess fish stress responses because they relate to mobilization of energy, and several studies have been conducted within the genus Oncorhynchus (Morgan & Iwama, 1996; Martínez-Porchas et al., 2009). In general, cortisol increases immediately after exposure to a stressor, leading to the increase of glucose, which can occur rapidly by means of catecholamines (glycogenolysis) or gradually over time by means of glucocorticoids (gluconeogenesis) in response to a chronic stressor (Morgan & Iwama, 1996; Vijayan et al., 2010; Faught et al., 2016).

Similar to fishes, amphibians exhibit elevated corticosterone, a stress hormone similar to cortisol, upon exposure to an acute, chronic, or life-cycle stressor (Denver, 1997; Moore & Jessop, 2003; Woodley, 2017). Some work has described glucose responses for frog and salamander species (Hutchison & Turney, 1975; Hervant et al., 2001; Xia & Li, 2010). Similar to fish, corticosterone increases after exposure to stress followed by elevated levels of glucose. However, information regarding Coastal Giant Salamander physiology is relatively sparse

(Wagner, 2014). We have a general understanding that higher levels of corticosterone relate to stress in Coastal Giant Salamander (Wagner, 2014), but we do not know the nature of the relationship between stress and glucose for this species. Furthermore, caution is advised towards solely using glucocorticoids to determine stress in any animal, due to the complexity of hormone pathways and variability among individual responses (Mommsen et al., 1999; Martínez-Porchas et al., 2009; Cockrem, 2013).

Behavioral responses can also reflect underlying physiological conditions of an animal and can help us understand stress. Similar to physiological responses, behaviors can vary considerably at the individual level within a species (Carere & Eens, 2005). Extensive effort has focused on environmental factors and their influence on behavior. Temperature preference is a well-known concept for fish and amphibians, in which an animal actively explores the thermal niche within its respective lethal minimum and maximum temperature tolerance (Fry, 1947; Brett, 1952; Coutant, 1977; Wagner, 2014). However, in complex environments such as headwaters, there is a tradeoff between multiple interacting factors based on an animal's tolerance and preference for each environmental factor (Noakes & Jones, 2016). Ultimately, gaining insight into how individuals respond both physiologically and behaviorally to synchronous environmental extremes can allow greater understanding of the underlying mechanisms governing population-level responses.

In this study, I experimentally investigated the physiological and behavioral responses of two stream animals, Coastal Cutthroat Trout (*Oncorhynchus clarkii clarkii*) and Coastal Giant Salamander (*Dicamptodon tenebrosus*), using mesocosms under different levels of synchrony between flow minima and temperature maxima. Both species are widely distributed across headwater streams in Oregon (Behnke, 1979; Trotter, 1989) and represent a considerable fraction of stream vertebrate biomass (Murphy et al., 1981; Hawkins et al., 1983; Davic & Welsh, 2004).

Equally important, these animals live in sympatry (Roni, 2002) and have persisted in these headwater ecosystems across millennia, potentially forging a unique evolutionary relationship with this hydroclimate (Behnke, 1979; Steele & Storfer, 2006).

I focus on stress and behavior of Coastal Cutthroat Trout as response variables due to the larger body of knowledge for this species compared to Coastal Giant Salamander. I hypothesize that the synchrony of flow minima and temperature maxima would be more unfavorable relative to an asynchronous scenario due to the combined effects of reduced physical space and temperature maxima. Similarly, I expected that animals in the least favorable scenario (synchronous) would exhibit less activity in order to conserve energy, and that individual measures such as weight change would indicate a more energetically stressful condition. This experiment aims to disentangle the impact of changing hydroclimate and its consequences for these sympatric stream dwelling animals at the individual level. Testing the synchrony between flow minima and temperature maxima under a controlled setting provides insights about the short-term individual animal responses that may be encountered in future hydroclimate scenarios.

#### 2. Methods

#### 2.1 Field Collection

123.3317W). I used minnow traps and a backpack electrofisher (Smith-Root model LR-18) to collect the animals along a selected stream section. I transported collected animals to the John L. Fryer Aquatic Animal Health Lab (AAHL), Corvallis OR, and placed them in sterilized 1.2m-diameter outdoor tanks with approximately 1000-L of aerated flow-through well-water that

I collected wild animals from Oak Creek on 7-9th July 2017, near Corvallis, OR (44.6111N,

maintained temperatures between 10-12 °C. Trout and salamanders were acclimated in separate tanks to reduce the potential for agonistic encounters for 8-10 days.

#### 2.2 Feeding

Red-wiggler worms (*Eisenia fetida*) (purchased from Three Trees Farm, Cottage Grove, OR) were given to the animals during the experiment. Dietary content of the worm food source was previously determined through dry-mass bomb calorimetry (61.38% protein, 15.67% ash, 4.39% fat, 18.56% carbohydrates, and 4.73 Cal./g (French et al. 1957)). During experimental observations, feeding amounts were determined based on a tanks' respective biomass. Each tank was fed an approximate 1-2% respective tank biomass of chopped worms 4-5 days out of a given week. Feeding occurred during morning only.

#### 2.3 Treatment Setup

All tanks experienced the same temperature regime throughout the experiment regulated by three different digital PXR4 Micro-controller X (Fuji Electric Co. Tokyo, Japan). I identically programmed each of the blenders to mimic diel fluctuations in temperature between a minimum and maximum that did not exceed typical temperature patterns reported in Oak Creek (Kerst & Anderson, 1974) or the thermal tolerances of either species (Dwyer & Kramer, 1975; Bury, 2008). The minimum and maximum temperatures changed each week, first starting at the experimental minimum values of 10-14 °C, increasing to a peak 13-18 °C for two weeks, and then decreasing to 12-15 °C over the course of seven weeks (**Figure 1**).

Three treatments of changing pool depth over time simulated conditions of increasing synchronization between flow minima and temperature maxima (**Table 1**). All 15 tanks were equipped with a 40 cm long (initial depth), 2.54 cm diameter polyvinylchloride (PVC) standpipe that regulated the depth of the tank, and was marked according to one of the three changing

depth treatments. Depth treatments were created using an exponential decay function with three different coefficients associated to each treatment (**Table 1; Figure 1**) and standpipes were marked to indicate where the pipe needed to be cut to achieve the needed pool depth for a given week. Changes in depth for each treatment eventually reached a flow minimum after which pool depth no longer changed. Treatment names indicate the level of synchrony with temperature maxima: **Async\_T + Q**<sub>ref</sub> experienced less pool-depth change and no synchrony between low-flow and temperature maxima as a reference condition while **Async\_T + Q**<sub>drought</sub> experienced low-flow during temperature maxima (**Table 1; Figure 1**).

**Table 1:** Weekly changes in depth (cm) according to each treatment, and weekly temperature °C minimum and maximum for all treatments.

|        | Depths (cm)       |   |                      | Tempera | ture (°C) |
|--------|-------------------|---|----------------------|---------|-----------|
|        | Async_T +<br>Qref | $\begin{array}{c} \mathbf{Async}_T + \\ \mathbf{Q}_{drought} \end{array}$ | Sync_T +<br>Qdrought | minimum | maximum   |
| Week 1 | 40                | 40  | 40                   | 10      | 14        |
| Week 2 | 37                | 32  | 28                   | 12      | 15        |
| Week 3 | 35                | 26  | 20                   | 13      | 16        |
| Week 4 | 32                | 21  | 12                   | 14      | 18        |
| Week 5 | 30                | 17  | 12                   | 14      | 18        |
| Week 6 | 28                | 12  | 12                   | 13      | 16        |
| Week 7 | 26                | 12  | 12                   | 12      | 15        |



**Figure 1:** Experimental regimes. Inset depicts the diel fluctuation in temperature, bottom graph depicts the entire experiment duration where min and max temperatures for the diel fluctuation change weekly. The green (Async\_T +  $Q_{ref}$ ), orange (Async\_T +  $Q_{drought}$ ), and grey (Sync\_T +  $Q_{drought}$ ) lines indicate changing depth treatments in relation to temperature maxima.

2.4 Tank Setup

A total of 15 100L (80.0cm-l x 54.6cm-w x 40.7cm-h) tanks were equipped with aeration and water tubing (**Appendix A**). Each tank had artificial habitat consisting of a 25 cm section of 7.6 cm diameter PVC pipe with a rock in the center, and two 4x4 cm tiles placed next to the PVC cylinder, so that it was unable to roll. All tanks experienced the same artificial lighting conditions that lasted from approximately 07:00-19:00 each day. Each of the 15 tanks were stocked with five trout and two salamanders selected from the holding tanks on 17 July. Animals were randomly selected by similar size to reduce predation within a tank, measured for length and weight, and randomly assigned to one of the three treatments (**Table 2**).

**Table 2:** Initial weight (g) attributes by treatment and species. N refers to the number of individuals within each treatment, IQR represents the interquartile range, and SD for standard deviation.

| Treatment                | Ν  | Median | IQR   | Mean  | SD    |
|--------------------------|----|--------|-------|-------|-------|
| Trout                    |    |        |       |       |       |
| $Async\_T + Q_{ref}$     | 25 | 9.93   | 4.97  | 24.74 | 30.47 |
| $Async\_T + Q_{drought}$ | 25 | 34.60  | 28.61 | 36.96 | 21.82 |
| $Sync\_T + Q_{drought}$  | 25 | 31.35  | 17.75 | 30.83 | 11.08 |
| Salamander               |    |        |       |       |       |
| $Async\_T + Q_{ref}$     | 10 | 14.53  | 10.14 | 34.57 | 46.52 |
| $Async\_T + Q_{drought}$ | 10 | 32.52  | 31.68 | 29.46 | 17.88 |
| $Sync_T + Q_{drought}$   | 10 | 19.36  | 11.21 | 27.95 | 20.98 |

#### 2.5 Sampling

Video recordings of behavior began 10 days after animals were assigned to experimental tanks (27<sup>th</sup> July) and took place over the duration of the experiment. Recordings occurred in random order and at two sample times of the day (morning & afternoon) for each tank, and were intended to assess behavioral activity for 15 minutes before temperature maximum and during temperature maximum of a given day. Morning recordings occurred between 07:00 - 12:00, and afternoon recordings occurred between 12:00 - 16:00. Three tanks, one from each treatment, were video recorded simultaneously using three Aqua-Vu cameras (Aqua-Vu; Crosslake, MN) and homemade structures to house the camera above the tank. The housing structure is best described as a quadripod pyramid with translucent plastic covering, aside from the base that sits over the tank surface and a camera hole at the top (**Appendix A**). The plastic covering was implemented to diffuse light entering the tank.

Tracking data were collected using a manual tracking program within Fiji (Schindelin et al., 2012; v.2.0.0; Java 1.8.0) to record the 2-D position for each individual at each time-step throughout the video's length. Prior to analysis, all videos were condensed from 30 frames per second (fps) to one fps to make manual tracking more efficient. Tracking one individual in a 15 minute video at 30 fps would require approximately 27,000 (x,y) coordinate points, whereas the same video condensed to one fps would only require 900 (x,y) coordinate points. I used the first 300 seconds of an individual track (before feeding), rather than the full 900 seconds, to make comparisons of movement without the influence of morning feeding. This resulted in 509 videos collected on 18 separate days spanning from  $27^{\text{th}}$  July –  $11^{\text{th}}$  September, 2017 (**Appendix B**). Coordinates (x,y) were recorded at one-second intervals for each visible individual in a recording to derive movement metrics including cumulative distance and average speed. Distances were calculated on an individual track basis and because these were collected at one-second intervals,

speed and distance were equivalent. Cumulative distance was calculated as the sum of distance for an individual track within a unique tank observation.

Moribund animals were removed during the experiment to provide a humane endpoint, resulting in varying tank densities (**Table 4**). At the end of the seventh week, remaining animals were reflex assessed (RAMP), euthanized, and sampled for blood-glucose, weight, and length. Reflex assessments consisted of five measures that took less than 20 seconds to perform on each individual: tail grab, body flex, head complex, visual reflex, and orientation (see Raby et al., 2012 for details on each measure). Reflex responses were categorical (impaired = 0, unimpaired = 1) and a proportion between the five reflexes was calculated as the RAMP Index, with values closer to 0 indicating impairment and values closer to 1 indicating no impairment. Similar to Raby et al. (2012), if the reflex was questionably impaired, I categorized it as impaired.

Blood-glucose concentrations were determined using a conventional diabetic bloodmonitor (OneTouch, model Ultra2) (Wells & Pankhurst, 1999; Stoot et al., 2014; Ball & Weber, 2017), and animals were sampled within five minutes of euthanization. Trout were sampled by caudal-vein puncture with a sterile heparinized syringe, and the extracted blood sample was injected onto a sterile lancet strip. Salamanders were sampled by opening of the thoracic cavity and collecting blood with a sterile heparinized capillary tube near the shoulder girdle and applying it to the lancet strip in the same manner. Weight change and condition factor were derived from initial and final weight and length measurements. All animals were maintained and euthanized in accordance with OSU IACUC recommendations.

#### 2.6 Trout Bioenergetics

I utilized Fish Bioenergetics 4.0 (FB4, Deslauriers et al., 2017) through graphical user interface (Shiny) in RStudio programming and statistical software (R Core Team 2019, version 3.6.0).

FB4 is a complex bioenergetics package that is adapted from earlier "Wisconsin models" using energy balanced equations and thermodynamic principles to explain energy allocation in joules per day. The model functions in balancing consumption (C) into three components of energy fate: metabolism (R, A, SDA), wastes (F, U), and growth (G):

**Equation 1.**) 
$$C = R + A + SDA + F + U + G$$

Consumption (C) is balanced by metabolic demands such as standard metabolism (R), energy expenditure for activity (A), specific dynamic action, (SDA), energy losses in egestion (F) and excretion (U), and growth (G). I implemented Rainbow Trout (Adult) parameters in the model rather than Cutthroat Trout parameters because Cutthroat parameters were borrowed and slightly modified from Coho Salmon (*Oncorhynchus kisutch*), a common practice in bioenergetics (Ney, 1993). I deemed it appropriate to use adult Rainbow Trout (*Oncorhynchus mykiss*) parameters because it was not specified whether Coho Salmon were undergoing smoltification and Coho Salmon are less related to Cutthroat trout.

| Metric                  | Description  |
|-------------------------|--|
| Cumulative distance (m) | Derived from tracking coordinate (x,y) data                                    |
| Final weight (g)        | Final weight of an animal at day-48  |
| Percent weight change   | Percent relative change in weight from day-0 to day-48                         |
| Condition Factor        | Fulton's condition factor calculated as 100*(Weight/L <sup>3</sup> )           |
| RAMP Index              | Reflex action mortality predictors (RAMP). Reflex indicators of animal         |
|                         | impairment and delayed mortality.  |
| Blood-glucose (mg/dL)   | Concentration of blood-glucose (milligrams/deci-Liter)                         |
| Piconorgatias D valua   | Trout only: Proportion of daily consumption relative to $C_{\rm MAX}$ (Maximum |
| bioenergenes r-value    | possible consumption based on weight of animal and temperature regime)         |
| Disaparation a/a/d      | Trout only: Weight corrected daily consumption values in grams of food         |
| bioenergenes g/g/u      | per gram of fish per day.  |
| Final tank density      | Final number of individuals within a given tank.                               |

Table 3: Metrics used in analyses at the individual level.

#### 2.7 Statistical Analysis

To answer my research questions, I first made comparisons between treatments for the behavioral movement data and the individual metrics. I treated each tank observation as independent for both behavior and individual metric data. I utilized non-parametric methods for analyses on all metrics because each data subset was non-normally distributed (behavioral movement long-tailed; **Figures 3-4**) or sample sizes were small, causing non-normal distributions and heterogeneous variances (individual metrics summarized at tank level; **Figures 6-8**). In addition, medians rather than means were calculated for each metric to reduce the influence of outliers. Analyses including all three treatments were made using Kruskal-Wallis Rank Sum tests, while Mann-Whitney U-tests were used for comparisons between two

treatments, specifically  $Async_T + Q_{drought}$  and  $Sync_T + Q_{drought}$ . All analyses were conducted through RStudio software (R Core Team 2019).

#### 3. Results

For trout, initial and final weights were not evenly distributed among treatment groups from the randomization procedure; this resulted in the reference  $Async_T + Q_{ref}$  treatment having smaller animals (**Table 4**). Kruskal-Wallis Rank Sum tests indicated significantly different final weights for trout among treatments (p = 0.006), however, salamanders exhibited no evidence of differences (p = 0.67). Additionally, trout mortality among treatments was far greater in the  $Async_T + Q_{ref}$  treatment, which lost 16 individuals, compared to seven and nine individuals lost from  $Async_T + Q_{drought}$  and  $Sync_T + Q_{drought}$  treatments, respectively. The weight and density disparity among the  $Async_T + Q_{ref}$  treatment and other two treatments (**Table 4**) may affect comparisons of all three groups; thus, statistical tests hereafter focused on comparisons between  $Async_T + Q_{drought}$  and  $Sync_T + Q_{drought}$  treatments for all response variables.

| <b>Table 4:</b> Final weights (g) summary table of remaining individuals within each treatment.   |
|---|
| Percent mortality calculated from starting N from Table 1, where N=25 for trout and N=10 for      |
| salamander. N refers to the number of individuals, IQR represents the interquartile range, and SD |
| for standard deviation.   |

| Treatment                | N  | Percent<br>mortality | Median | IQR   | Mean  | SD    |
|--------------------------|----|----------------------|--------|-------|-------|-------|
| Trout                    |    |                      |        |       |       |       |
| $Async_T + Q_{ref}$      | 9  | 64.0                 | 11.44  | 7.20  | 18.99 | 25.09 |
| $Async\_T + Q_{drought}$ | 18 | 28.0                 | 32.97  | 22.98 | 35.26 | 18.66 |
| $Sync\_T + Q_{drought}$  | 16 | 36.0                 | 33.42  | 20.73 | 29.80 | 12.21 |
| Salamander               |    |                      |        |       |       |       |
| $Async_T + Q_{ref}$      | 9  | 10.0                 | 14.68  | 12.17 | 33.14 | 39.70 |
| $Async\_T + Q_{drought}$ | 8  | 20.0                 | 29.29  | 26.62 | 27.81 | 15.56 |
| $Sync\_T + Q_{drought}$  | 9  | 10.0                 | 20.30  | 17.10 | 27.81 | 20.29 |



Treatment

Figure 2: Final weight (g) distributions for trout (2a) and salamander (2b). Outliers denoted with bold point '•'.

#### 3.1 Movement Data

Summarized tracking data resulted in approximately 550,000 coordinate points (x,y) pertaining to 2,144 individual tracks, of which 1,840 tracks belonged to trout and 304 belonged to salamanders. Median cumulative distances (m) were estimated on a species basis within each unique tank-observation, resulting in 504 trout and 210 salamander tank-observations (**Table 5**); an additional five observations could not be attributed to either species due to difficulty in identifying animals in the video tracking and were thus excluded from the analysis. For trout, morning recordings accounted for 248 observations while afternoon recordings accounted for 256 observations. Salamanders exhibited a similar pattern, with morning recordings accounting for 107 observations and afternoon recordings accounting for 103 observations. Cumulative movement data for trout and salamanders were non-normally distributed (long-tailed) within each treatment group split by morning or afternoon observations (**Table 5; Figure 3 & 4**).

| Treatment Time           |           | Ν  | Median (m) | IQR (m) |
|--------------------------|-----------|----|------------|---------|
| Trout                    |           |    |            |         |
| $Async_T + Q_{ref}$      | Morning   | 81 | 5.58       | 6.15    |
| $Async\_T + Q_{ref}$     | Afternoon | 84 | 4.48       | 5.60    |
| $Async\_T + Q_{drought}$ | Morning   | 85 | 1.75       | 1.56    |
| $Async\_T + Q_{drought}$ | Afternoon | 87 | 1.71       | 1.76    |
| $Sync_T + Q_{drought}$   | Morning   | 82 | 1.26       | 1.81    |
| $Sync\_T + Q_{drought}$  | Afternoon | 85 | 1.14       | 1.40    |
| Salamander               |           |    |            |         |
| $Async_T + Q_{ref}$      | Morning   | 34 | 0.09       | 1.14    |
| $Async\_T + Q_{ref}$     | Afternoon | 36 | 0.07       | 1.08    |
| $Async\_T + Q_{drought}$ | Morning   | 36 | 0.06       | 0.30    |
| $Async\_T + Q_{drought}$ | Afternoon | 30 | 0.02       | 0.19    |
| $Sync\_T + Q_{drought}$  | Morning   | 37 | 0.12       | 0.47    |
| $Sync\_T + Q_{drought}$  | Afternoon | 37 | 0.11       | 0.29    |

**Table 5:** Movement (m) data summary based on species, treatment, and morning or afternoon observations. N represents the number of unique tank-observations within each group. IQR pertains to the interquartile range of the median within the group.







35-

30-

25

20-

15

10-

5-

0.

Async\_ $T + Q_{ref}$ 

a

## Treatment

Figure 3: Cumulative distance (meters) moved on a weekly basis for trout (3a & 3b) and salamanders (3c & 3d) grouped by treatment and by timing of observations. Weeks arranged from left to right (1-7) for each plot. Outliers denoted with bold point '•'.



Treatment

Figure 4: Cumulative distance (meters) moved for trout (4a & 4b) and salamanders (4c & 4d) grouped by treatment, and morning or afternoon observations. Plots represent summary data in Table 4 (above), where movement is summarized over days and weeks. Outliers denoted with bold point '•'.

Kruskal-Wallis Rank Sum tests indicated that trout morning movement (p < 0.001) and trout afternoon movement (p < 0.001) significantly differed among treatments. Comparisons of trout movement between Async\_T + Q<sub>drought</sub> and Sync\_T + Q<sub>drought</sub> treatments showed significant differences in the morning (p = 0.047) as well as the afternoon (p = .005); Median cumulative distance was greater in the Async\_T + Q<sub>drought</sub> treatment than the Sync\_T + Q<sub>drought</sub> treatment for both morning and afternoon (**Table 6; Figures 4a, 4b**). However, when I compared trout morning and afternoon movement within each treatment, movement was not significantly different (**Table 6; Figures 4a, 4b**).

Salamander morning movement exhibited little evidence of differences among treatments (p = 0.8) while differences in afternoon movement was suggestive (p = 0.13). A Mann-Whitney U test between Async\_T + Q<sub>drought</sub> and Sync\_T + Q<sub>drought</sub> treatments showed that treatments differed significantly for afternoon movement (p = 0.047), where movement was greater in the Sync\_T + Q<sub>drought</sub> treatment (**Table 6; Figures 4c, 4d**). Furthermore, comparisons between morning and afternoon movement within each treatment had suggestive evidence for differences within the Async\_T + Q<sub>drought</sub> treatment (p = 0.09) while no evidence supported differences in the Sync\_T + Q<sub>drought</sub> treatment (p = 0.8) (**Table 6; Figures 4c, 4d**).

Although little evidence supported differences in morning or afternoon animal movement within each treatment, there was a similar pattern of variation between them (**Figure 3**). Trout morning movement was positively associated with afternoon movement within each treatment: (Async\_T +  $Q_{ref}$ : Spearman's rho=0.54, df = 75, *p* <0.001; Async\_T +  $Q_{drought}$ : Spearman's rho=0.33, df = 81, *p* =0.002; Sync\_T +  $Q_{drought}$ : Spearman's rho=0.43, df = 76, *p* <0.001). However, salamanders only exhibited significant positive associations between morning and

afternoon movement within the Async\_T +  $Q_{ref}$  treatment (Async\_T +  $Q_{ref}$  = Spearman's rho=0.62, df = 21, p =0.001).

**Table 6:** Results of Kruskal-Wallis and Mann-Whitney U tests on cumulative distances between indicated "Comparison" groups. Underlined text indicates the value or metric being tested in the comparison group. Values in bold and denoted with '\*' are statistically significant.

| Comparison   | Kruskal-Wallis            | Mann-Whitney U | p-value |
|--|---------------------------|----------------|---------|
| Trout  |                           |                |         |
| Morning:   |                           |                |         |
| All treatments                                     | $\chi^2 = 58.72, df = 2$  |                | <0.001* |
| $Async\_T + Q_{drought}vs.\ Sync\_T + Q_{drought}$ |                           | 4104           | 0.048*  |
| Afternoon:   |                           |                |         |
| All treatments                                     | $\chi^2 = 56.78$ , df = 2 |                | <0.001* |
| $Async\_T + Q_{drought}vs.\ Sync\_T + Q_{drought}$ |                           | 4614           | 0.005*  |
| Morning vs afternoon:                              |                           |                |         |
| $Async\_T + Q_{ref}$                               |                           | 3054           | 0.26    |
| $Async_T + Q_{drought}$                            |                           | 3770           | 0.83    |
| $Sync_T + Q_{drought}$                             |                           | 3308           | 0.57    |
| Salamander   |                           |                |         |
| Morning:   |                           |                |         |
| All treatments                                     | $\chi^2 = 0.29,  df = 2$  |                | 0.87    |
| Afternoon:   |                           |                |         |
| All treatments                                     | $\chi^2 = 4.08$ , df = 2  |                | 0.13    |
| $Async\_T + Q_{drought}vs.\ Sync\_T + Q_{drought}$ |                           | 397            | 0.047*  |
| Morning vs afternoon:                              |                           |                |         |
| $Async\_T + Q_{ref}$                               |                           | 596            | 0.085   |
| $Async_T + Q_{drought}$                            |                           | 408.5          | 0.092   |
| $Sync_T + Q_{drought}$                             |                           | 671            | 0.89    |

3.2 Individual Metrics

Analyses on metrics at the individual level (**Table 3**) comprised of 43 trout and 26 salamanders (**Table 4**) and were grouped by tank (experimental unit) for statistical comparisons. At the end of the experiment, median percent weight change for trout significantly differed among all treatments (p = 0.003) while no difference existed for salamanders (p = 0.8; **Figures 6a, 6b**). For trout, comparisons between Async\_T + Q<sub>drought</sub> and Sync\_T + Q<sub>drought</sub> treatments indicated significant differences in median percent weight change (p = 0.007), however, the distributions of these two groups appears to violate the non-parametric assumptions that the distributions are approximately similar (**Figure 6a**). Due to a low sample size and a constricted distribution in the Async\_T + Q<sub>drought</sub> treatment, I am unable to statistically justify the difference in median weight change.

Similarly, trout RAMP assessments, condition factor (K), and blood-glucose concentrations did not statistically differ across all three treatments (p > 0.1), although suggestive evidence occurred in RAMP scores between Async\_T + Q<sub>drought</sub> and Sync\_T + Q<sub>drought</sub> treatments (p = 0.08; Figures 6a, 6c, 7a, 7c). Salamander condition factor did not differ among treatments (p = 0.5) and tests on RAMP and blood-glucose were not performed on salamander due to the lack of empirical support relating the metrics to amphibian health; however, I included visual plots of these data (Figures 6d, 7b, 7d).

Estimates of P-value (proportion of realized consumption to  $C_{MAX}$ ) from the bioenergetics models showed little evidence of differences among treatments (p = 0.28) and only suggestive evidence for the comparison between  $Async_T + Q_{drought}$  and  $Sync_T + Q_{drought}$ treatments (p = 0.09) (**Figure 8a**). Weight corrected daily consumption (g/g/d) also exhibited little evidence of differences among treatments (p = 0.17) and virtually no evidence for differences between  $Async_T + Q_{drought}$  and  $Sync_T + Q_{drought}$  treatments (p = 0.8; **Figure 8b**).



# Treatment

Figure 5: Individual weight changes (5a & 5b) and individual condition factor changes for trout and salamanders over the course of 48 days. Red triangles ' $\blacktriangle$ ' in (5b & 5d) indicate salamanders that transformed into terrestrial morphs during the experiment. Outliers denoted with an asterisk ' $\ast$ '.



## Treatment

Figure 6: Summarized percent weight change (6a & 6b). Horizontal dotted line indicates no change from initial to final weight. Summarized condition factor for trout and salamanders (6c & 6d). Outliers denoted with bold point '•'.



Treatment

Figure 7: Summarized blood glucose concentrations (7a & 7b) and summarized RAMP assessment scores (7c & 7d). Outliers denoted with bold point '•'.



Figure 8: Estimated consumption relative to maximum possible consumption  $(C_{MAX})$  (8a) and trout weight corrected daily consumption in grams of food per gram of fish per day (8b). Outliers denoted with bold point '•'.

| Comparison   | Kruskal-Wallis            | Mann-Whitney U | p-value |
|--|---------------------------|----------------|---------|
| Trout  |                           |                |         |
| % Weight Change:                                     |                           |                |         |
| All Treatments                                       | $\chi^2 = 11.18$ , df = 2 |                | 0.0037* |
| $Async\_T + Q_{drought} vs. \ Sync\_T + Q_{drought}$ |                           | 0              | 0.0079* |
| Bioenergetics P-value:                               |                           |                |         |
| All Treatments                                       | $\chi^2 = 2.48$ , df = 2  |                | 0.29    |
| $Async\_T + Q_{drought}vs.\ Sync\_T + Q_{drought}$   |                           | 4              | 0.095   |
| Bioenergetics g/g/d:                                 |                           |                |         |
| All Treatments                                       | $\chi^2 = 3.50, df = 2$   |                | 0.17    |
| $Async\_T + Q_{drought}vs.\ Sync\_T + Q_{drought}$   |                           | 11             | 0.84    |
| RAMP Assessment:                                     |                           |                |         |
| All Treatments                                       | $\chi^2 = 4.59$ , df = 2  |                | 0.1     |
| $Async\_T + Q_{drought}vs.\ Sync\_T + Q_{drought}$   |                           | 21             | 0.087   |
| Condition Factor (K):                                |                           |                |         |
| All Treatments                                       | $\chi^2 = 4.34$ , df = 2  |                | 0.11    |
| $Async\_T + Q_{drought}vs.\ Sync\_T + Q_{drought}$   |                           | 12             | 1.0     |
| Glucose:   |                           |                |         |
| All Treatments                                       | $\chi^2 = 2.35$ , df = 2  |                | 0.31    |
| $Async\_T + Q_{drought}vs.\ Sync\_T + Q_{drought}$   |                           | 6              | 0.22    |
| Salamander   |                           |                |         |
| % Weight Change                                      |                           |                |         |
| All Treatments                                       | $\chi^2 = 0.38$ , df = 2  |                | 0.83    |
| Condition Factor (K):                                |                           |                |         |
| All Treatments                                       | $\chi^2 = 1.26, df = 2$   |                | 0.53    |

**Table 7:** Results of comparisons between treatments. All comparisons were based on medians of the listed value. Underlined text indicates the value or metric being tested in the comparison group. Values in bold and denoted with '\*' are statistically significant.

### 4. Discussion

Due to the imbalances in weight and density from the randomization procedure that may draw misleading inferences in the reference treatment (Async\_ $T + Q_{ref}$ ), hereafter, I focus the discussion on comparing two treatment scenarios of synchronous (Sync\_T + Q<sub>drought</sub>) and asynchronous  $(Async_T + Q_{drought})$  conditions. Following my initial prediction, I show that from these two treatments of flow minima with temperature maxima, trout consistently move less under low-flow and temperature maximum synchrony during both morning and afternoon. Salamanders tend not to move as much as trout and show some evidence of afternoon differences between treatments. Surprisingly, trout lose more weight under the asynchronous scenario compared to the synchronous one, and salamanders show no large differences in weight change between treatments, which is opposite of my original expectations. I found little evidence to support differences in blood-glucose concentrations for trout as the hematologic indicator of stress between treatments. Reflex assessment indices on trout exhibit more evidence of impairment in the synchronous condition. Bioenergetics calculations show little differences in theoretical consumption over the experiment duration between the synchronous and asynchronous treatments; however, some evidence supports higher P-values (consumption relative to  $C_{MAX}$ ) in the synchronous condition.

Trout within the predicted favorable asynchronous treatment lost more weight than in the synchronous treatment. Although this outcome is opposite of my initial hypotheses, it provides support that less movement in the synchronous scenario can lead to lower energy expenditure and thus smaller changes in weight. In the reference condition, small and large trout gain weight compared to the other two treatments, suggesting that lower densities may reduce both competition for available food and aggressive encounters (Chapman, 1966). Trout bioenergetics

support observed changes in weight due to changes in activity; P-values in the synchronous treatment are higher than the asynchronous treatment. Three alternative bioenergetics scenarios can explain these findings: animals experiencing different thermal regimes leading to differences in  $C_{MAX}$ , increasing consumption relative to  $C_{MAX}$  to mitigate weight change, or modifying their behavioral activity (A in equation 1). I applied the same temperature regime to all treatments, and weight distributions are similar between the asynchronous and synchronous treatments. In addition, increases in consumption are unlikely because I maintained constant feeding proportions during the experiment, and evidence supports less movement in the synchronous treatment. Assuming that the other components of metabolism fate are approximately similar among trout in each treatment (i.e. R, SDA, F, U in equation 1), and growth is different between treatments, then we can deduce that activity levels likely influenced weight changes. This finding suggests that trout may modify their behavioral activity as a short-term adaptive mechanism to cope with synchronous conditions of flow minima and temperature maxima.

Although short-term trout weight loss is less evident in the predicted unfavorable scenario (synchronous treatment), RAMP and glucose responses suggest synchronous conditions could be detrimental to trout under prolonged exposure. Reflex scores were consistently lower in the synchronous condition indicating that impairment is present and that delayed mortality is more likely to occur in this treatment (Raby et al., 2012). Similarly, blood-glucose levels at the end of the experiment were slightly higher in the synchronous treatment, suggesting the mobilization of energy reserves in response to stress. Endpoint measurement of blood-glucose certainly does not capture the changes that occur on a weekly basis, but can provide insights into the relative cumulative stress of treatments (Martínez-Porchas et al., 2009). This could have survival implications in that a chronically stressed individual may display indicators of delayed

mortality (RAMP indices) and be less able to respond to an immediate stressor such as predation due to physiological exhaustion (allostatic overload) (Mommsen et al., 1999; Schreck, 2000). In prolonged drought conditions, changes in weight may not reflect long-term implications for animals. Rather, physiological measures such as RAMP indices and glucose levels may better indicate an individual's chronic exposure to environmental extremes (Raby et al., 2012; Madliger & Love, 2014).

Little information exists for Coastal Giant Salamanders regarding stress responses to environmental conditions. From the existing research on other amphibians, we do know that glucose relates to energy mobilization and behavioral activity may reflect underlying physiological states. Decreased locomotory activity is often observed in response to stress in other salamander species and is sometimes accompanied with increased levels of corticosterone (Wack et al., 2013; Woodley, 2017). From my results, salamanders within the asynchronous treatment exhibited elevated levels of glucose, less afternoon movement, and RAMP scores indicating impairment. Differences between the two treatments are apparent and may suggest that the asynchronous treatment was more stressful for salamanders, but these metrics have not been validated for this species and limits inferences. Lastly, metamorphoses (aquatic to terrestrial morphotypes) occurred during the experiment, but did not appear related to any treatment condition. The synchronous and asynchronous treatments each had two transformations while the reference treatment had one. These metamorphoses do not appear related to the treatment conditions in this experiment, similar to work that has tried to link environmental conditions to transformation rates (Wagner, 2014).

The results from this experiment are more intricate than expected and individual variation in responses are apparent (**See Figure 5**). My study used wild animals that inherently vary physiologically and behaviorally more so than hatchery or domesticated animals (Johnsson & Näslund, 2018). This allows us to understand some range of natural variability in individual responses, although I acknowledge that the small animal sample size in this study may not fully account for it and it would be beneficial to repeat this in the future with a larger sample size. Trout in my study exhibited high prevalence of a natural fish pathogen, bacterial cold-water disease (BCWD, Flavobacterium psychrophilum), and parasitic copepods (Salmincola *californiensis*). Trout removed before the experimental endpoint were infected with both pathogen and parasite that affected mostly smaller individuals. Removals did not occur until the third week of the experiment, with the highest frequency of removals happening during the warmest weeks of the experiment (weeks 4 and 5). Stress from parasite and pathogens infections combined with increases in metabolic costs due to temperature likely led to allostatic overload for these animals. However, several animals survived extensive infestations of the parasitic copepod in the absence of BCWD, indicating their individual ability to maintain homeostasis under these conditions. These infections provide important insights regarding parasite-host interactions during environmental extremes. In isolated pool habitats, parasites are likely to spread more effectively due to increases in host density (May & Lee, 2011) and decreases in flow (Barndt & Stone, 2003). Extended periods in these conditions may lead to stress and lowered ability to avoid and/or resist infections. This is particularly problematic for smaller individuals and BCWD, while larger individuals may be more susceptible to copepod infection (Barndt & Stone, 2003). From my observations, smaller fish appear to be most susceptible to BCWD infection while copepod infestations were most prominent in larger fish. In spite of the inherent variability using wild animals, we still observed indicators of stress that corresponded with synchrony of flow minima and temperature maxima.

Capturing wild animals and housing them in laboratory settings likely induced stress across individuals with different tolerances to these stressors. Additionally, I utilized artificial mesocosms that allowed for the precise control of diel temperature and pool depth to simulate isolated pool habitats under summer conditions. These mesocosms are all the same in structure, but they still oversimplify natural pool habitat heterogeneity. I mimicked low flow regimes where animal densities can be increased due to seasonal availability of physical space and movements can be restricted to a pool (Harvey et al., 2011; May & Lee, 2011). This experimental reduction in space likely restricts movement behavior, more so for larger individuals (Polverino et al., 2016). Similarly, lack of habitat heterogeneity can increase aggression between trout because subordinates cannot stay out of sight from more aggressive conspecifics (Kalleberg, 1958).

Comparisons of metrics for trout and salamander are relative to each treatment rather than to empirically derived baselines. Establishing field baselines of blood-glucose, RAMP indices, and bioenergetics for Coastal Cutthroat Trout and Coastal Giant Salamander would help link the animals' health to the environmental condition (Madliger & Love, 2014). Bioenergetics calculations in this study are dependent on parameters for Rainbow Trout because parameters do not exist for Coastal Cutthroat Trout specifically, and are absent for salamanders. Surprisingly, FB4 borrows and adapts parameters from Coho Salmon for Coastal Cutthroat Trout. This 'parameter borrowing' is a common practice in bioenergetics modelling due to the difficulty in measuring these values for a new species (Ney, 1993), which limits the accuracy of results. However, because these are mathematical models, differences among treatments will remain, but at different scales. Short-term experiments such as this one may provide insights about adaptions of a species to environmental extremes at ecological time scales. However, the mesocosms approach used here simplifies environmental complexity, where natural settings may lead to different individual interactions and outcomes during this scenario. This experiment provided important insights regarding these species responses to environmental stress such as movement activity and pathogen infections. Future research on this topic would benefit from understanding behavior responses and pathogen infections under natural settings or in open field experiments (Johnsson & Näslund, 2018). Natural environmental conditions will likely mediate individual responses in a different manner than the mesocosms used here. More research is needed to understand individual responses under natural settings and to expand the temporal scope of these adaptions to population and evolutionary scales (Calow & Forbes, 1998).

### 5. Conclusion

Due to climate change, flow minima and temperature maxima will become more synchronous in headwater streams over time (Arismendi et al., 2013). This will be energetically costly for animals, but individual fates will depend on environmental and individual characteristics, as well as the duration of these extreme events. This experiment showed that individual variability is important in this sense, as it can provide a population-level buffer to environmental selection. We know that this natural variation is disrupted by anthropogenic activities such as dams and other stream barriers that limit natural movement throughout a stream network. Artificial barriers can reduce the genetic diversity of a population (Novinger & Rahel, 2003; Wofford et al., 2005; Underwood et al., 2016), which limits a population's ability to persist under environmental selection and should be considered. Weight loss may not indicate long-term consequences of environmental extreme exposure. Other metrics that relate to the animals' physiological condition, such as RAMP and glucose, may aid in understanding the implications of prolonged drought, but further research is necessary to establish baselines to understand these relationships. Investigation of other individual metrics related to an animal's physiology and behavior could also supplement this knowledge. Trout and salamander have persisted in these headwaters across millennia and likely possess individual variation in tolerance to environmental extremes, especially drought. Managing for the maintenance of this individual variation through population connectivity will ensure the continued persistence under anticipated drought conditions. Ultimately, investigating how individuals persist under environmental extremes in headwaters will allow better understanding of population-level responses to climate change.

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# 7. Appendices

**Appendix A:** Photos of the experiment in the Aquatic Animal Health Laboratory. **a**) Tank setup showing the standpipe regulating pool depth and PVC pipe as habitat. **b**) Camera holding contraption during a recording event. **c**) Tanks were supplied water through pipes (center) and were adjacent to each other. **d**) Aquatic (top) and terrestrial metamorphosed (bottom) salamanders.



| Appendix B:   | Video trackin | g data for the | experiment   | duration. ( | Observations | s are grouped | by treatment,  | tank, day, | and morning | $(\mathbf{M})$ or |
|---------------|---------------|----------------|--------------|-------------|--------------|---------------|----------------|------------|-------------|-------------------|
| afternoon (A) | recordings. V | videos used in | analyses ind | icated with | h '1' while  | '0' indicates | videos not use | d.         |             |                   |

|          |              | Week 1 Week 2 |   |     |             |   |     |    | Week 3 |   |        |   |     | Week 4 |     |     |     | Week 5 |     |     |     | Week 6 |     |         |   |     |       |   | Week 7 |   |     |     |        |   |   |   |   |
|----------|--------------|---------------|---|-----|-------------|---|-----|----|--------|---|--------|---|-----|--------|-----|-----|-----|--------|-----|-----|-----|--------|-----|---------|---|-----|-------|---|--------|---|-----|-----|--------|---|---|---|---|
|          | 27-Jul 1-Aug |               |   | 3-4 | 3-Aug 4-Aug |   | 8-A | ug | 10-Aug |   | 14-Aug |   | 15- | Aug    | 17- | Aug | 22- | Aug    | 24- | Aug | 29- | Aug    | 30- | Aug 1-S |   | Sep | 5-Sep |   | 7-Sep  |   | 8-5 | зер | эр 11- |   |   |   |   |
|          | Tank         | м             | Α | м   | Α           | м | Α   | м  | Α      | м | Α      | м | Α   | м      | Α   | м   | Α   | м      | Α   | м   | Α   | м      | Α   | м       | Α | м   | Α     | м | Α      | м | Α   | м   | Α      | м | Α | М | Α |
| <b>.</b> | A1           | 1             | 1 | 1   | 0           | 1 | 1   | 1  | 1      | 1 | 1      | 1 | 1   | 1      | 1   | 1   | 1   | 0      | 1   | 1   | 1   | 1      | 1   | 1       | 1 | 1   | 1     | 1 | 1      | 1 | 1   | 1   | 1      | 1 | 1 | 1 | 1 |
| P Ore    | A4           | 1             | 0 | 1   | 1           | 1 | 1   | 1  | 1      | 1 | 1      | 1 | 1   | 1      | 1   | 0   | 1   | 1      | 1   | 1   | 1   | 1      | 1   | 1       | 1 | 1   | 1     | 1 | 1      | 1 | 1   | 0   | 1      | 1 | 1 | 1 | 1 |
| ÷.       | A5           | 1             | 1 | 1   | 1           | 1 | 1   | 1  | 1      | 1 | 1      | 1 | 1   | 1      | 1   | 0   | 1   | 1      | 1   | 1   | 1   | 1      | 1   | 1       | 1 | 1   | 1     | 1 | 1      | 1 | 1   | 1   | 1      | 1 | 1 | 1 | 1 |
| Asyn     | A9           | 1             | 1 | 1   | 1           | 1 | 1   | 1  | 1      | 1 | 1      | 1 | 1   | 1      | 1   | 1   | 1   | 1      | 1   | 1   | 1   | 1      | 1   | 1       | 1 | 1   | 1     | 1 | 1      | 1 | 1   | 1   | 1      | 1 | 1 | 1 | 1 |
|          | A15          | 1             | 1 | 1   | 1           | 1 | 1   | 1  | 1      | 1 | 1      | 1 | 1   | 1      | 1   | 1   | 1   | 1      | 1   | 0   | 1   | 0      | 1   | 0       | 0 | 1   | 1     | 1 | 0      | 1 | 1   | 1   | 1      | 1 | 1 | 0 | 1 |
|          |              |               |   |     |             |   |     |    |        |   |        |   |     |        |     |     |     |        |     |     |     |        |     |         |   |     |       |   |        |   |     |     |        |   |   |   |   |
| ght      | A2           | 1             | 1 | 1   | 1           | 1 | 1   | 1  | 0      | 1 | 1      | 1 | 1   | 1      | 1   | 1   | 1   | 1      | 1   | 1   | 1   | 1      | 1   | 1       | 1 | 1   | 1     | 1 | 1      | 1 | 1   | 1   | 1      | 1 | 1 | 1 | 1 |
| drou     | A6           | 1             | 1 | 1   | 1           | 1 | 1   | 1  | 1      | 1 | 1      | 1 | 1   | 1      | 1   | 1   | 1   | 1      | 1   | 1   | 1   | 1      | 1   | 1       | 1 | 1   | 1     | 1 | 1      | 1 | 1   | 1   | 1      | 1 | 1 | 1 | 1 |
| 0<br>+   | A7           | 1             | 1 | 1   | 1           | 1 | 1   | 1  | 1      | 1 | 1      | 1 | 1   | 1      | 1   | 1   | 1   | 1      | 1   | 1   | 1   | 1      | 1   | 1       | 1 | 1   | 1     | 1 | 1      | 1 | 1   | 1   | 1      | 1 | 0 | 1 | 1 |
| ις'      | <b>A</b> 8   | 1             | 1 | 1   | 1           | 1 | 1   | 1  | 1      | 1 | 1      | 1 | 1   | 1      | 1   | 1   | 1   | 1      | 1   | 1   | 1   | 1      | 1   | 1       | 1 | 1   | 1     | 1 | 1      | 1 | 1   | 1   | 1      | 1 | 1 | 1 | 1 |
| As)      | A10          | 1             | 1 | 1   | 1           | 1 | 1   | 1  | 1      | 1 | 1      | 1 | 1   | 1      | 1   | 1   | 1   | 1      | 1   | 1   | 1   | 0      | 0   | 1       | 1 | 0   | 1     | 1 | 1      | 1 | 1   | 1   | 1      | 1 | 1 | 0 | 1 |
|          |              |               |   |     |             |   |     |    |        |   |        |   |     |        |     |     |     |        |     |     |     |        |     |         |   |     |       |   |        |   |     |     |        |   |   |   |   |
| ght      | <b>A</b> 3   | 1             | 1 | 1   | 1           | 1 | 1   | 1  | 0      | 0 | 0      | 1 | 1   | 1      | 1   | 1   | 1   | 1      | 1   | 1   | 1   | 1      | 1   | 0       | 1 | 1   | 1     | 1 | 1      | 1 | 1   | 1   | 1      | 1 | 1 | 1 | 1 |
| lroug    | A11          | 1             | 1 | 1   | 1           | 1 | 1   | 1  | 0      | 1 | 1      | 1 | 1   | 1      | 1   | 1   | 1   | 1      | 1   | 1   | 1   | 1      | 1   | 0       | 1 | 1   | 1     | 1 | 1      | 1 | 1   | 1   | 1      | 1 | 1 | 1 | 1 |
| Ŭ        | A13          | 0             | 1 | 1   | 1           | 1 | 1   | 1  | 1      | 1 | 0      | 1 | 1   | 1      | 1   | 1   | 1   | 1      | 1   | 1   | 1   | 1      | 1   | 0       | 1 | 1   | 1     | 1 | 1      | 1 | 1   | 1   | 1      | 1 | 1 | 1 | 1 |
| L<br>L   | A14          | 1             | 1 | 1   | 1           | 1 | 1   | 1  | 1      | 1 | 0      | 1 | 1   | 1      | 1   | 1   | 1   | 1      | 1   | 1   | 1   | 1      | 1   | 0       | 1 | 0   | 1     | 1 | 1      | 1 | 1   | 1   | 1      | 1 | 1 | 1 | 1 |
| ş        | D5           | 1             | 1 | 1   | 1           | 1 | 1   | 1  | 1      | 1 | 1      | 1 | 1   | 1      | 1   | 1   | 1   | 1      | 1   | 1   | 1   | 1      | 1   | 0       | 1 | 1   | 1     | 1 | 1      | 1 | 1   | 1   | 1      | 1 | 1 | 1 | 1 |