Technical Progress Report #2: August 28, 2006

Physiological and behavioral responses of *Eurycea sosorum* to variation in levels of conductivity

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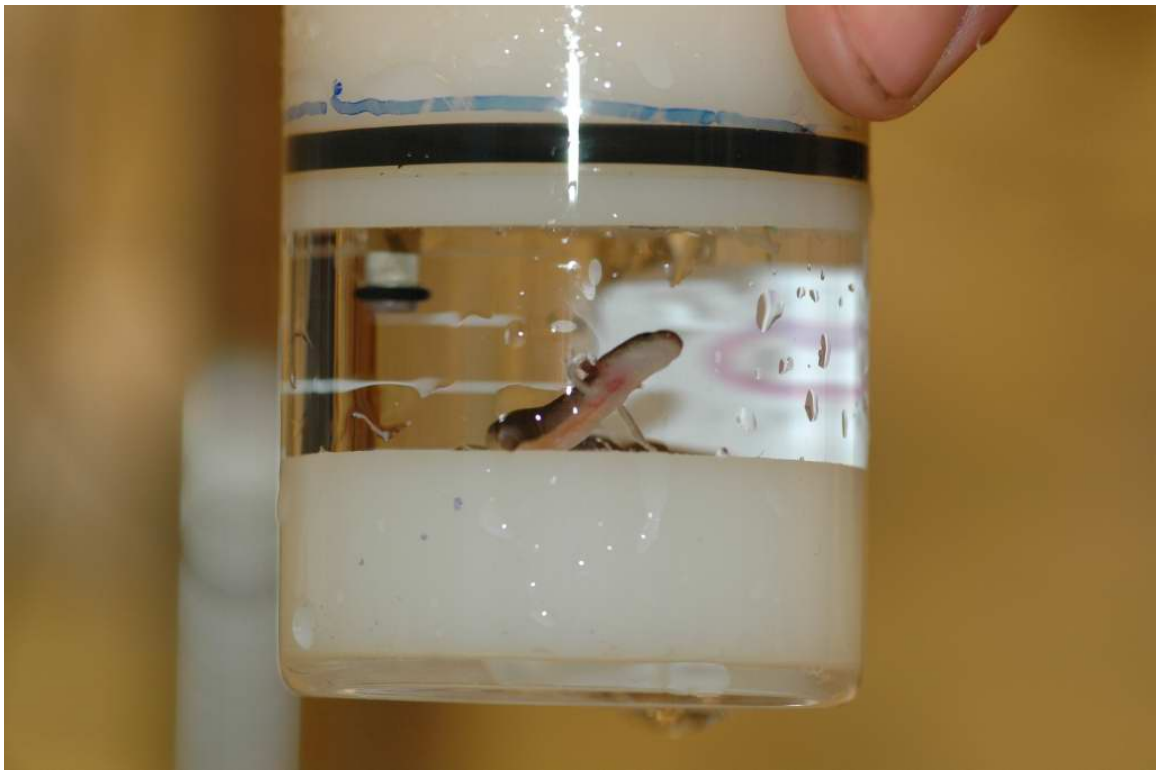


Summary
This report summarizes our findings on the response of adult salamanders to conductivity and covers a portion of our work between February 21 and August 23, 2006. We also include our results on salamander activity levels in response to dissolved oxygen (DO) in order to justify the oxygen levels we are currently testing in the 28 day oxygen toxicity experiment. Our next report will provide a detailed discussion of the effects of DO on salamander behavior, physiology and mortality. In this report, we cover all aspects of adult salamander response to conductivity, but we do not address the response of juvenile salamanders to conductivity. The 60 day juvenile growth response to conductivity will begin by the end of this month.

The experiments reported here include the 28-day conductivity toxicity tests, righting response experiments, activity levels in response to conductivity and DO, and metabolic rates across conductivity ramps.

General Collections and Activities:
We made our 2nd and 3rd sets of water collections at Eliza Spring and St. Alban's well on March 2nd and on August 2nd. We collected *Eurycea nana* from Spring Lake on March 9 (n = 66) and again on August 7 (n = 81). Most of the *E. nana* survived the initial experiments, and on May 15 we donated 56 *E. nana* to the captive breeding program at the San Marcos National Fish Hatchery and Technology Center.

In early August, Joe Fries, San Marcos National Fish Hatchery and Technology Center, notified us that he had juvenile *E. nana* available for our studies. We received 44 juvenile salamanders from Joe Fries on August 10, 2006. 15 of these salamanders were oviposited on June 26, 2006, and 29 were oviposited on June 3, 2006. All of these juveniles will undergo 60 day juvenile growth experiments in response to conductivity.

Overall Conclusions:
All of the conclusions reported here are for adult *E. nana*. There was no discernable effect of conductivity on adult salamanders, even when placed in 100% bad water (ca. 2800 µS/cm):

- mortality did not differ across levels of conductivity
- salamander metabolic rates did not differ across conductivity ramps
- activity patterns did not differ predictably with conductivity levels

In contrast, activity patterns did differ predictably with DO levels

Caveats:
All of these experiments were conducted with water collected from St. Alban's well and then stored for up to 2 months. During this time, the conductivity of the St. Alban's water decreased from ca. 2800uS/cm to ca. 2400uS/cm. In addition, the sulphur smell of this water dissipated within days. If sulphur in saline water is toxic to salamanders, we would not detect this with our long term experiments. We did run 2 righting response experiments in St. Alban's water that was collected no more than 2 days before the experiments. Salamanders did not appear to behave differently in this water and they did

not die. However, long-term effects of sulphur on these salamanders remains unknown. We could only test this if long-term experiments were set up near the source of the saline water.

Although adult salamanders do not appear to be affected by high levels of conductivity, the effects of salts on juvenile salamanders is just now being tested. Since juvenile amphibians are generally more sensitive to harsh environmental conditions, the data presented below should be used only for modeling the responses of adult salamanders to increasing levels of conductivity.

The rest of the report is broken into sections focusing on particular aspects of the conductivity results as summarized in the table on the next page.

Table Summary

	Status	**Starting Page**
Escape Response to Levels of Conductivity	Completed	5
Activity in Response to Conductivity and Oxygen	Completed	18
28 day toxicity test: conductivity	Completed	42
Metabolic Response to Conductivity Ramps	6 replicates completed	47
In Progress	Summarized	52
Appendix 1	S-Plus code to test activity levels in response to DO	54
Appendix 2	S-Plus code to test activity levels in response to conductivity	61
Appendix 3	S-Plus code to model changes in metabolic rate in response to conductivity ramps	65

Escape response to a range of conductivities

Summary findings

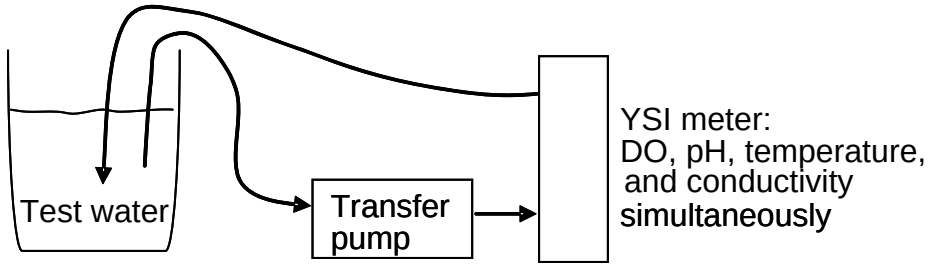
These experiments provide information about salamander escape response under ramped levels of conductivity. Results from these experiments were used to set conductivity levels in the 28 day conductivity toxicity study.

Short-term exposure to high conductivities had no effect on the type of escape response or duration of escape response of adult *Eurycea nana*. Salamander escape response was initially measured at conductivity levels that approximated 1-5% bad water. We found no effect of these levels of conductivity on *E. nana*. Thus, all salamanders were subjected to much higher conductivity levels to test for an upper threshold of conductivity. Salamanders were eventually subjected to 100% bad water (i.e., straight St. Alban's well water at 2738 uS/cm). Salamander activity levels were not affected even at these levels.

Caveat: All but two of these experiments were conducted with St. Alban's well water that had been collected 1 month prior. After sitting for 1 month, the saline water loses its sulphate component. Thus the majority of these experiments were conducted on "bad water" that had lost its sulphate component. However, Salamanders 18 and 19 were tested in St. Alban's water that had been collected the day before the experiments. These salamanders did not behave differently in the conductivity treatments as compared to the control treatments. There was also no detectable difference in their behavior as compared to the other salamanders.

Methods – These tests were originally designed as righting response tests in which each salamander would be flipped onto its back and the time to righting would be measured. In preliminary tests, we used small pocket-shaped squares of soft mesh to enclose and manipulate the salamander (see picture below). Salamanders were able to right themselves immediately at all levels of conductivity. In addition, catching and flipping the salamanders was exceptionally stressful for the animal. Instead of flipping, we redesigned the experiments so that each salamander was touched, but not flipped.

Ten salamanders were placed individually in ten 1.5 L aquaria that contained Eliza Spring water with 6 mg/l of oxygen. The test aquarium was placed in a cooled waterbath to maintain the temperature at 20°C. The gas settings for each experiment were 13% oxygen saturated water (~6 mg/l O_2) and 22% (2 mg/l) of CO_2 (to maintain a pH at 7.55). The aquarium was continuously and simultaneously tested for DO, pH, temperature and conductivity using a flow through system on the YSI meter. Salamanders were allowed to equilibrate for 20 minutes before manipulations begin. The behavior and response of each salamander was captured on digital tape throughout each test.



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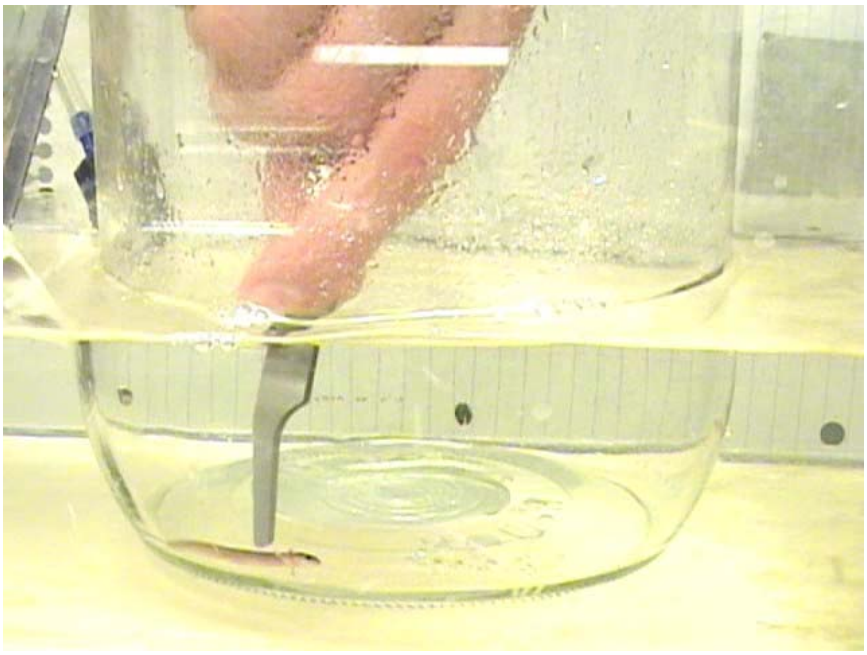
After 20 minutes, the salamander underwent 5 manipulations to test the escape response and activity level of the salamander. For each manipulation, we gently touched the salamander with entomology forceps (spring metal that does not hurt the animal). The touching was repeated five times. Touches 2-5 were done only after the animal remained stationary for 5 seconds.



The conductivity was immediately ramped up after each set of manipulations. This was done by removing a known volume of water from the test aquarium and adding back the same volume of St. Alban's water. The ramping schedule with approximate conductivity levels was as follows:

Trial	Eliza H2O removed (ml)	St. Alban's H2O added (ml)	initial conductivity uS/cm	final (trial level) conductivity (uS/cm)
1	0	0	445	445
2	100	100	445	738
3	40	40	737	833
4	40	40	833	928
5	40	40	928	1015
6	40	40	1015	1108
7	50	50	1108	1200
8	100	100	1200	1393
9	200	200	1393	1697
10	200	200	1697	1949
11	200	200	1949	2119
12	300	300	2119	2344

Ramping and stabilization of conductivity was immediate. After each increase in conductivity, the salamander was given a 20 minute rest period before manipulations were commenced.

Since each salamander underwent manipulations for several hours, we were concerned that any effects found could stem from salamander fatigue and not conductivity treatment. To account for this, we ran a set of control tests on each salamander two weeks after the conductivity tests. In these controls, we manipulated the salamander in the same way as in the experiments, except that conductivity was not increased. These controls were also filmed.

Each replicate was filmed and analyzed "blind", i.e., the person watching the video did not know the conductivity level that the salamander was undergoing. For each salamander, we collected the following data for each trial from the experiment and control videos:

- total time of activity following each manipulation
- # of body undulations during that time (as a measure of vigor)
- # of laps across the aquarium
- # of loops around the aquarium
- time to unconsciousness

Results

The escape behavior and time or escape in *Eurycea nana* was not affected by conductivity. Salamanders never lost consciousness or died during conductivity ramps, even when placed directly in St. Alban's well water (2738 uS/cm). There was no

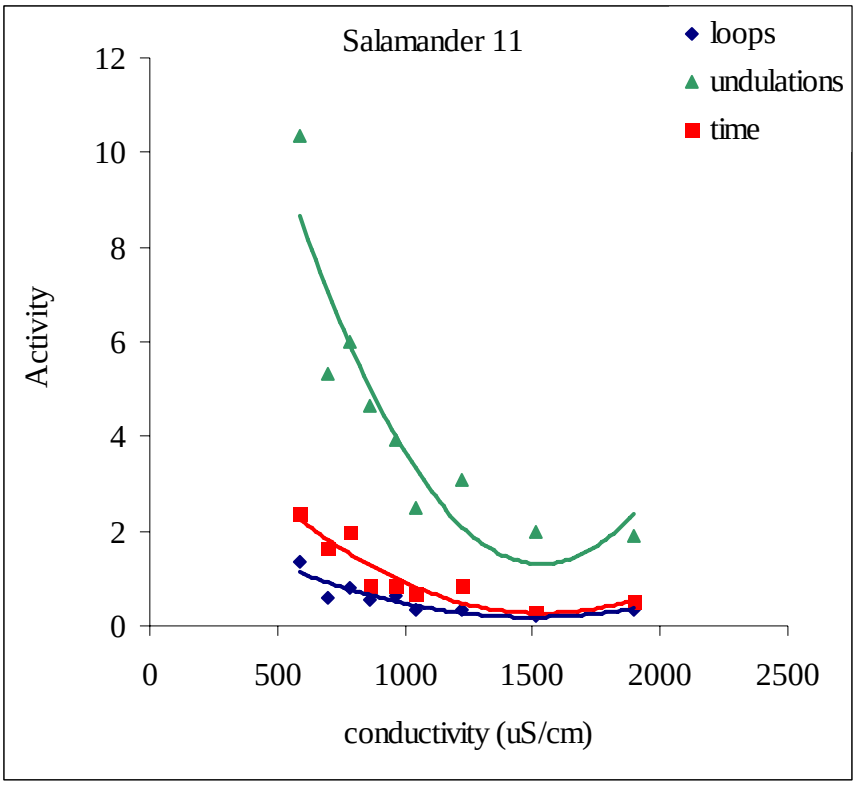
significant effect of conductivity level on salamander behavior, or on the total time that salamanders were active following perturbation. Salamanders behaved the same in control treatments (manipulation but no change in conductivity) as they did in ramped conductivity treatments.

Salamanders 11 through 17 were tested in St. Alban's water that had been held for 1 month. We were concerned that this water had lost its sulphate component, which may be toxic to salamanders. On March 6, we collected fresh St. Alban's well water and Eliza spring water. On March 7, we ran two more escape response tests with new salamanders (#s 18 and 19) to test whether the sulphate component of the St. Alban's water would affect salamander escape response in the short term. The conductivity of St. Alban's water for these experiments was 2883 uS/cm and the conductivity of the Eliza water was 667 uS/cm. Salamanders experienced conductivities ranging from 667 uS/cm to 2259 uS/cm (for salamander 18) and 2299 uS/cm (for salamander 19). Neither salamander 18 or 19 responded differently than the previously tested salamanders.

The following figures show paired response graphs for salamanders 11 through 19. Each set of graphs shows the response of the salamander when undergoing conductivity ramps followed by the response of the salamander when undergoing control manipulations (i.e., no conductivity). Although responses in control treatments did not always mirror those in conductivity treatments, there was no predictable pattern in salamander response to either treatment.

Activities in these graphs include:

- loops: the # of times the salamander swam around the circumference of its aquarium measured for every 1/8 loop.
- undulations: the # of full body undulations during the period of activity following manipulation
- time: the total time of activity following each manipulation.


Salamander 11
loops
undulations
time
Activity
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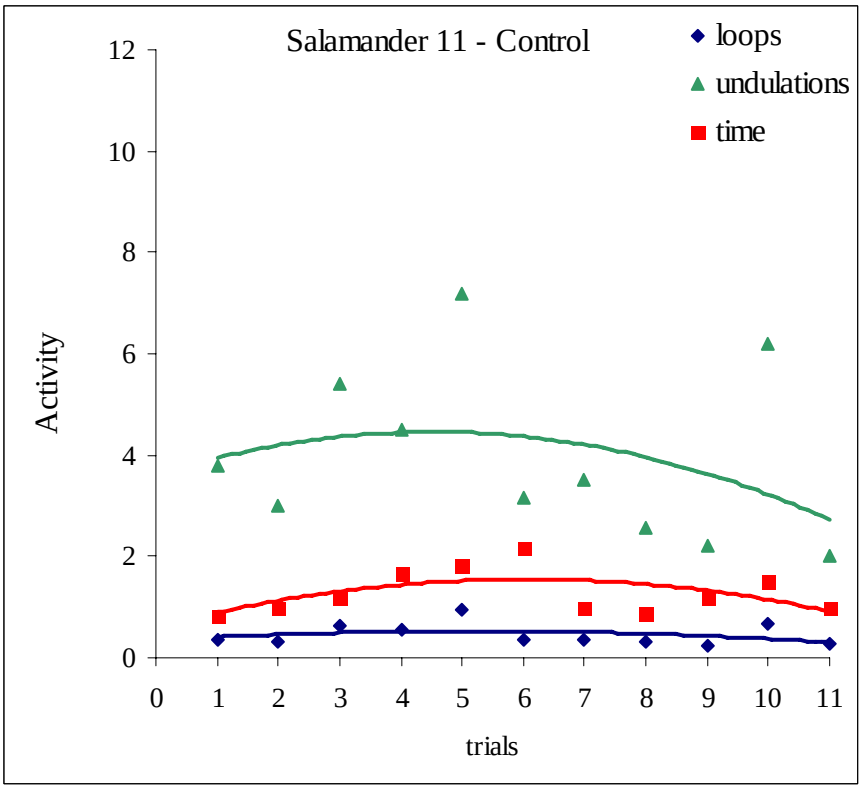
conductivity (uS/cm)


Salamander 11 - Control
loops
undulations
time
Activity
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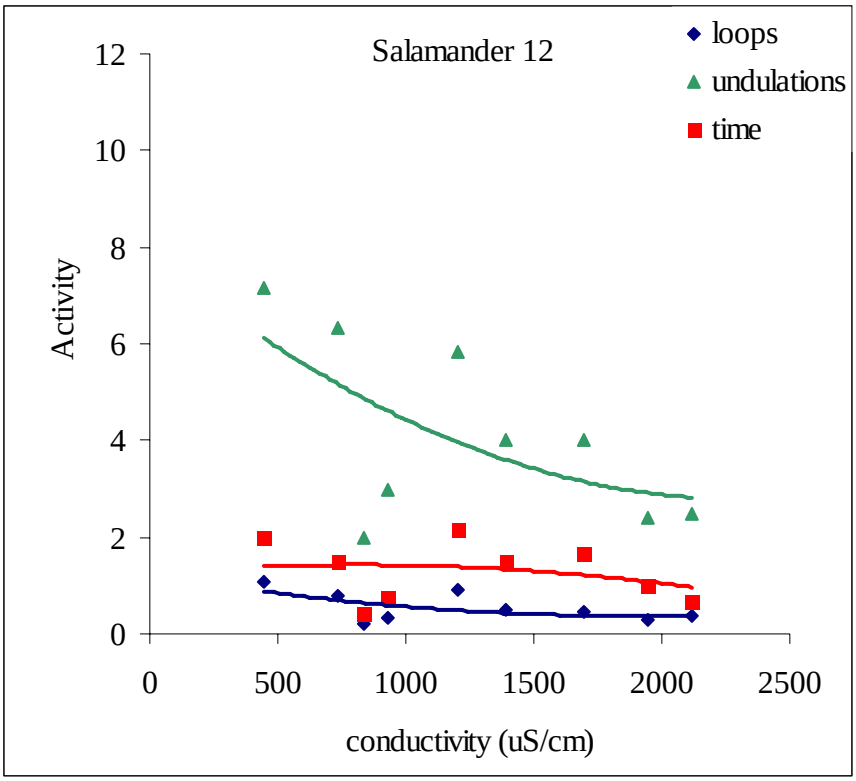
trials


Salamander 12
loops
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Activity
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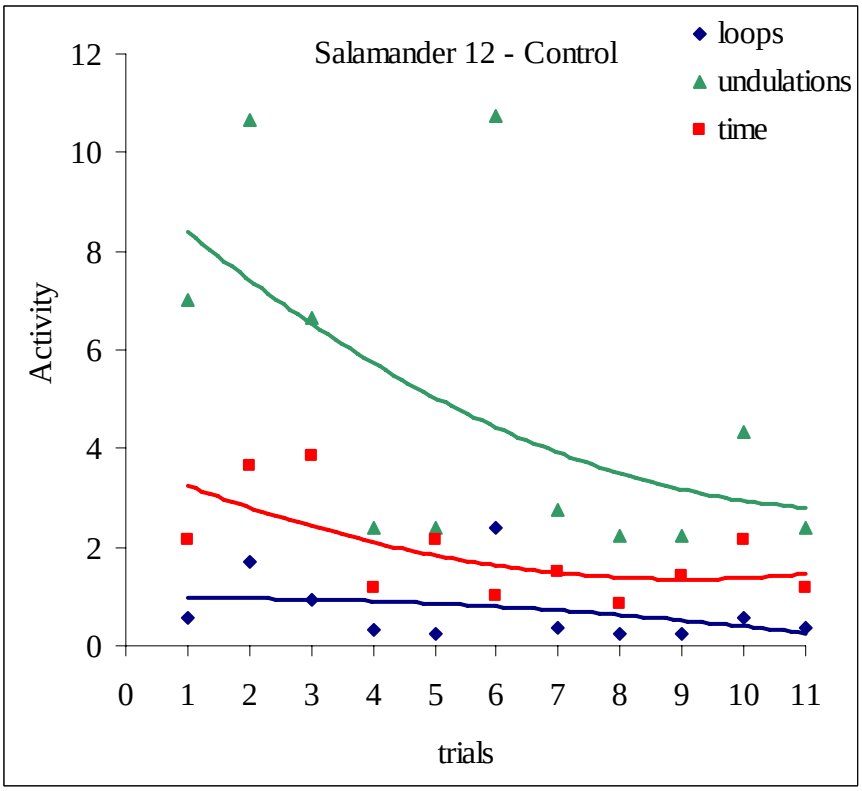
conductivity (uS/cm)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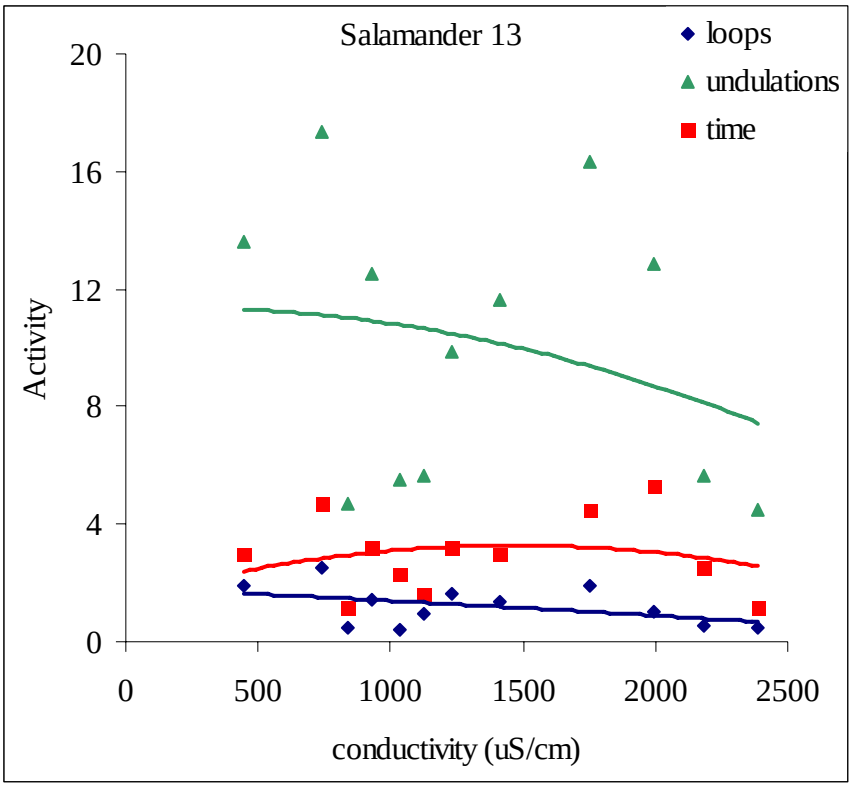
Salamander 13
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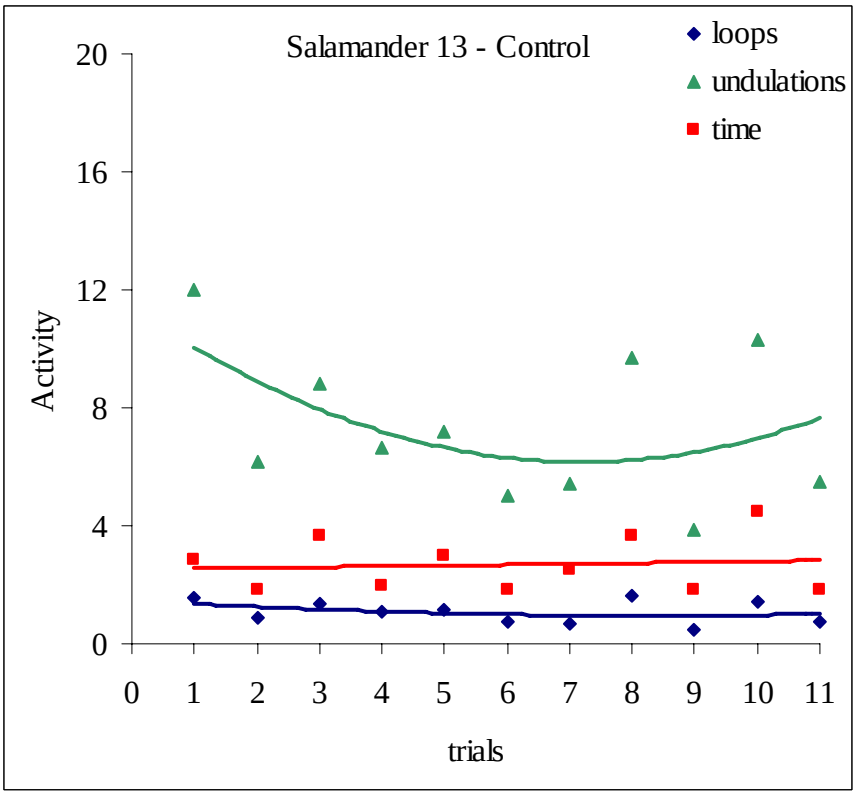
conductivity (uS/cm)


Salamander 13 - Control
loops
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Activity
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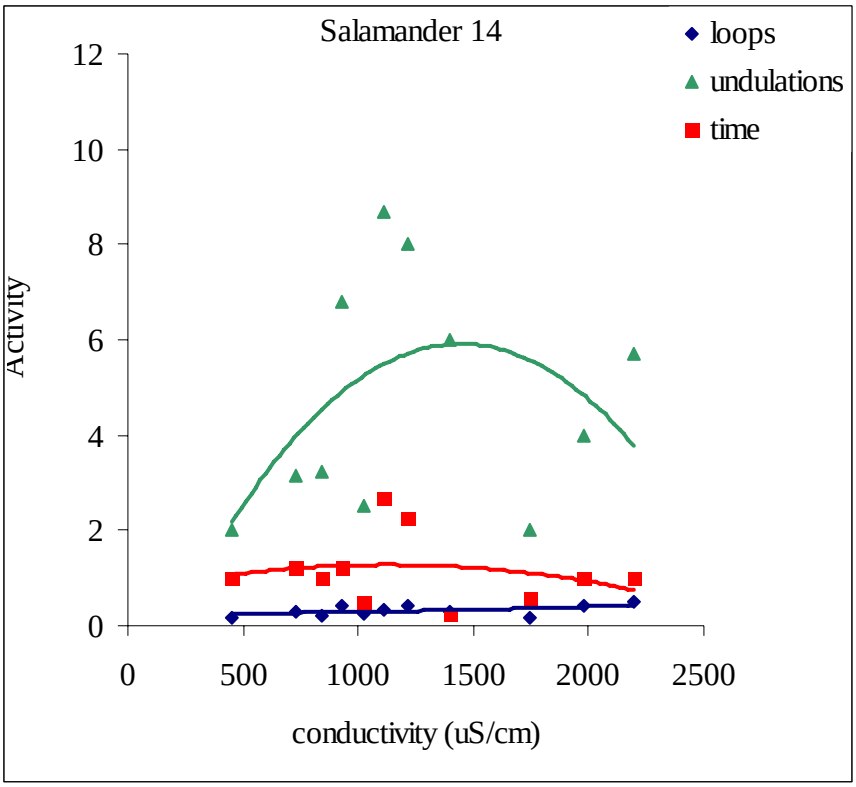
Salamander 14
loops
undulations
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Activity
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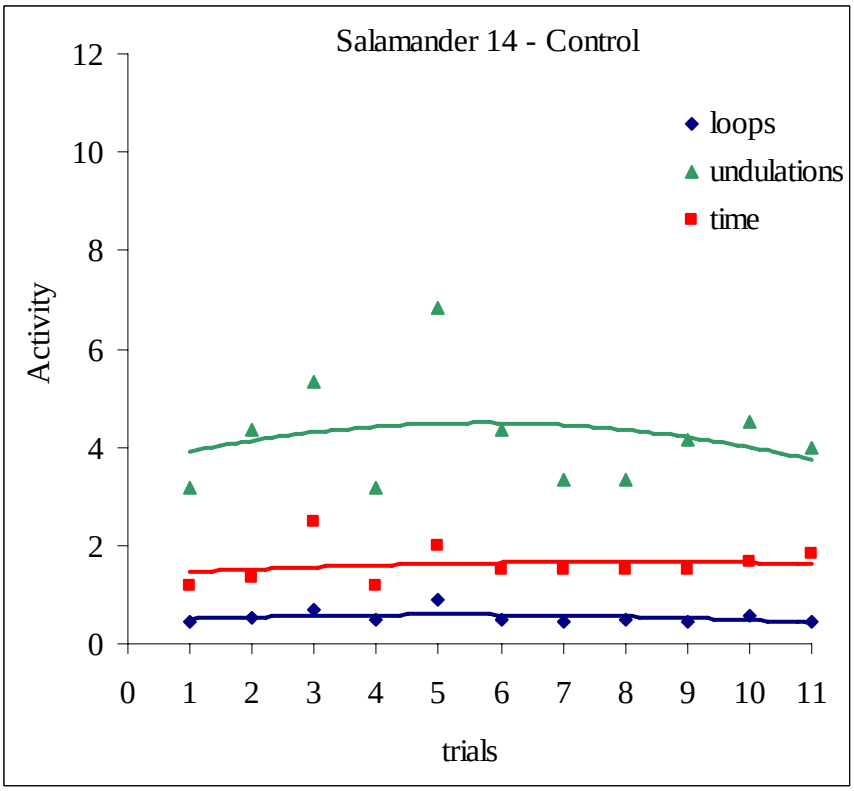
conductivity (uS/cm)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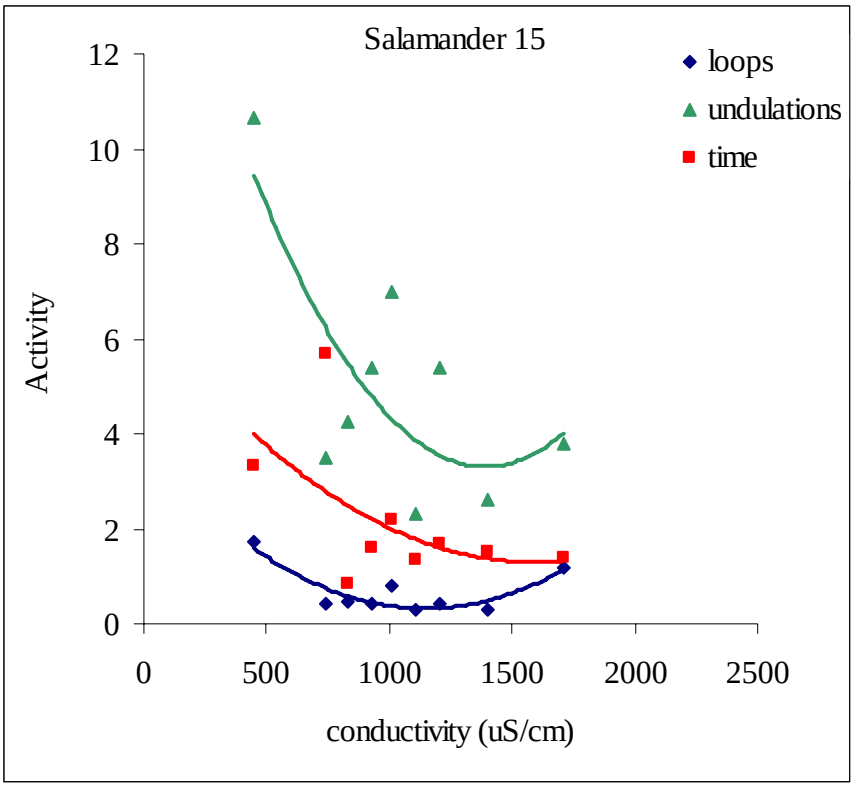
Salamander 15
loops
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Activity
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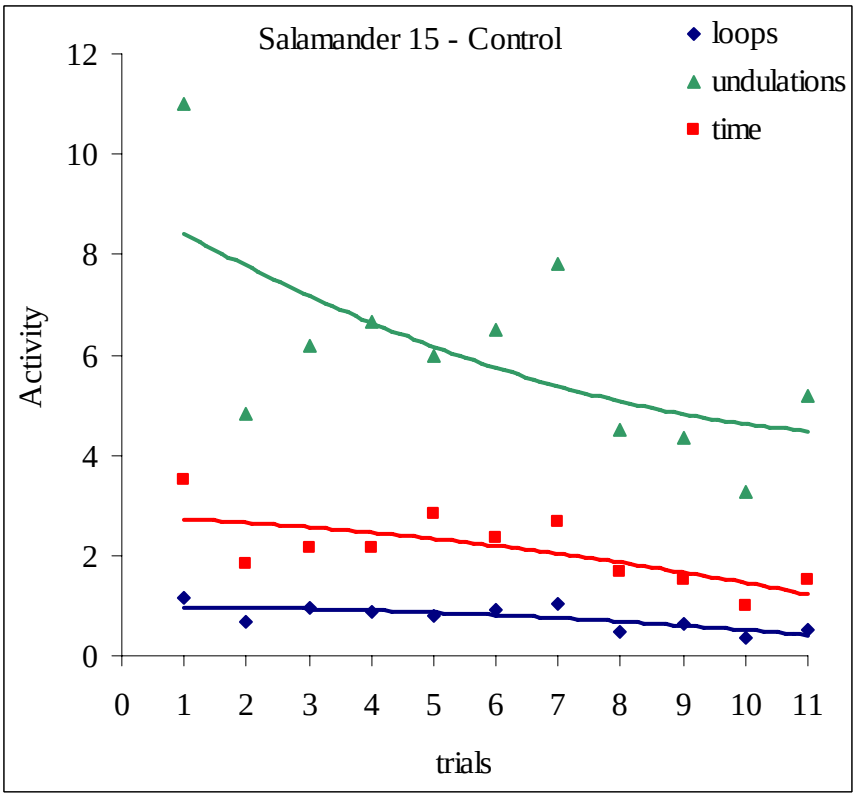
conductivity (uS/cm)


Salamander 15 - Control
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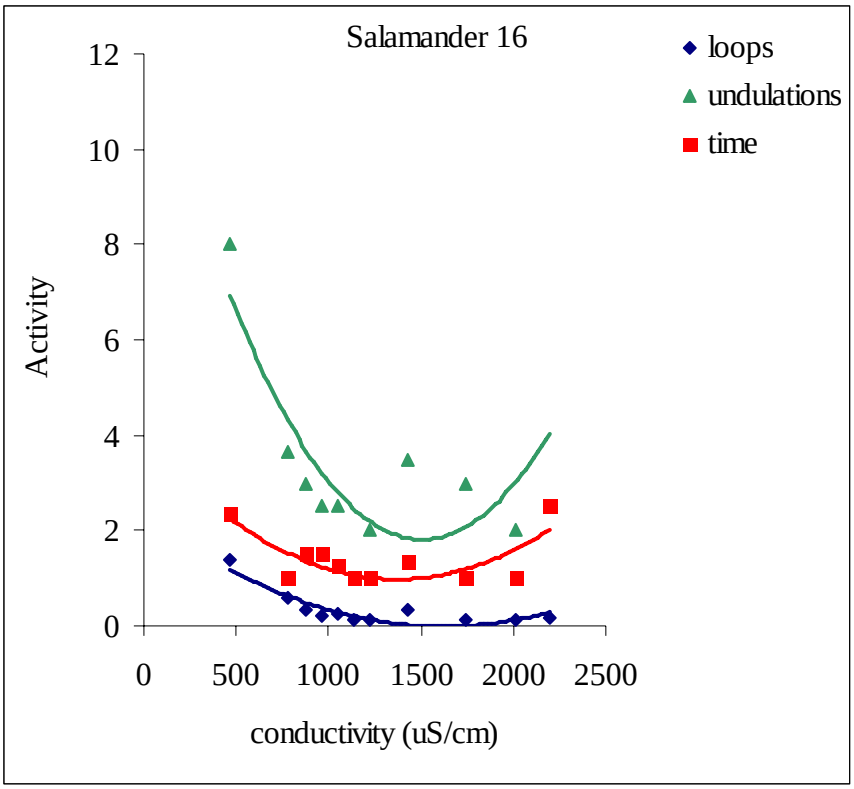
Salamander 16
loops
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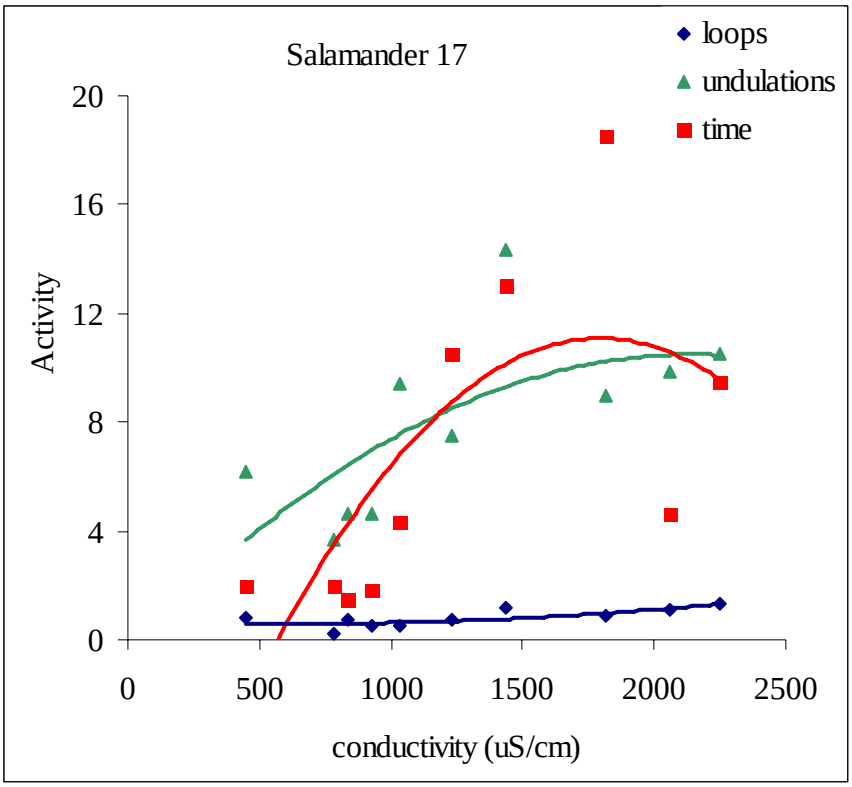
Salamander 17
loops
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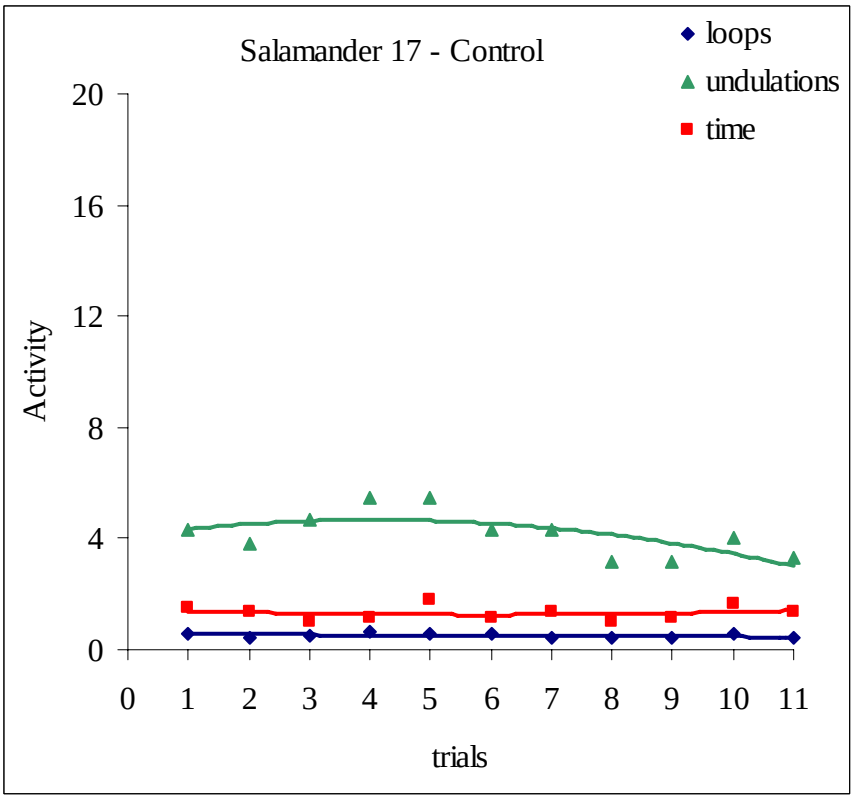
conductivity (uS/cm)


Salamander 17 - Control
loops
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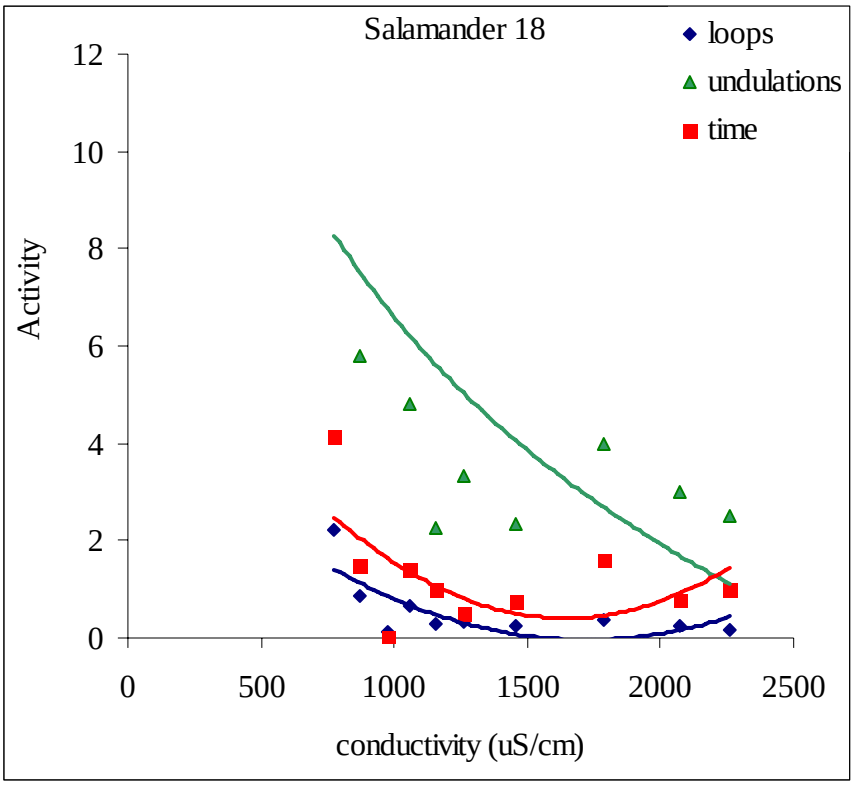
Salamander 18
loops
undulations
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Activity
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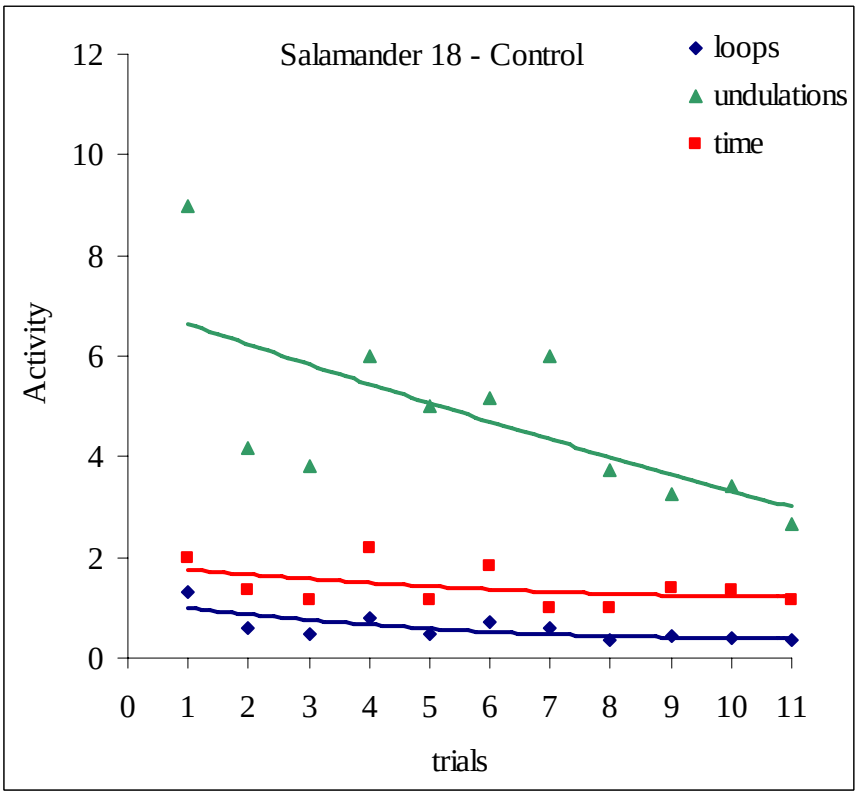
conductivity (uS/cm)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time
Activity
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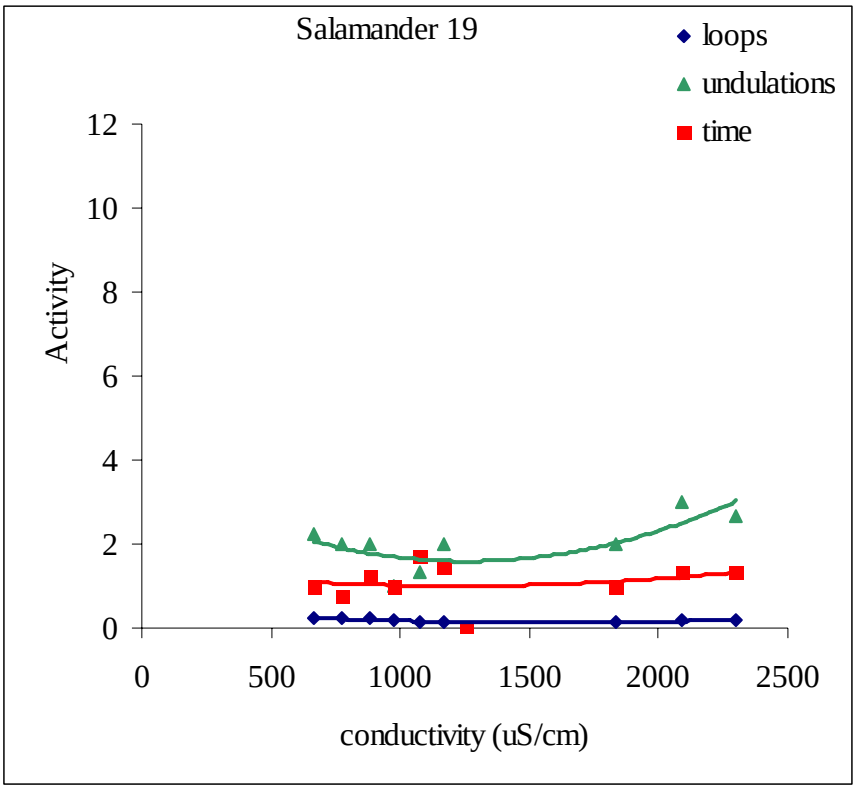
trials


Salamander 19
loops
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Activity
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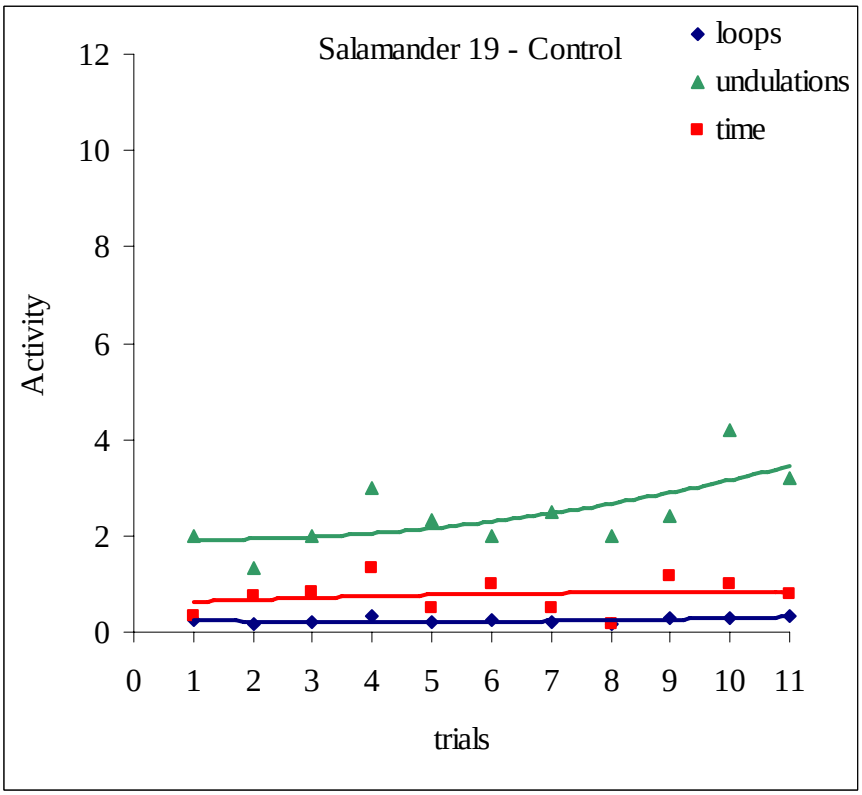
conductivity (uS/cm)


Salamander 19 - Control
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trials

Activity of *Eurycea nana* in response to ramped DO and conductivity.

Summary findings.

Eurycea nana were confined to small, flow-through glass chambers, and were allowed to acclimate for 4 hours before the start of the experiments. Once the acclimation period was complete, water characteristics were manipulated in one of two ways. Either DO was driven down, by bubbling source water with N_2 (and CO_2 to maintain pH). Or conductivity was raised, by gradually adding St. Albans well water to Eliza Springs water. During experiments we continuously monitored pH, DO, conductivity, and salamander activity.

Salamanders responded in different ways to the two manipulations. In response to low DO, salamanders showed distinctly higher activity levels. During the DO ramp down, activity switched on at relatively high levels of DO. We analyzed this effect with logistic regression, finding that DO at which half the salamanders were active was 49.3% of air saturation (with 95% CI of 43.6 – 55%). This level corresponds to ~ 4.3 mg oxygen/L. Salamanders continued to be highly active as DO dipped to its low point (about 15% of air saturation). As DO was gradually ramped back up, salamander activity began to fall off. The DO level at which half were again inactive was 34% of air saturation (with 95% CI of 26 – 42%). This level corresponds to ~ 3 mg oxygen/L.

DO conclusions: *E. nana* can sense DO. Individual salamanders respond to low DO by increasing activity. When DO is falling, activity becomes pronounced at relatively high levels of DO. When DO is rising, activity stopped at somewhat lower levels of DO. Salamanders were almost always active at DO < 34 % of air saturation (corresponding to ~ 3 mg oxygen/L).

Why become active at low DO? Salamanders likely were either trying to escape deteriorating conditions or were trying to minimize boundary layers around the gills in order to promote oxygen flux into the blood.

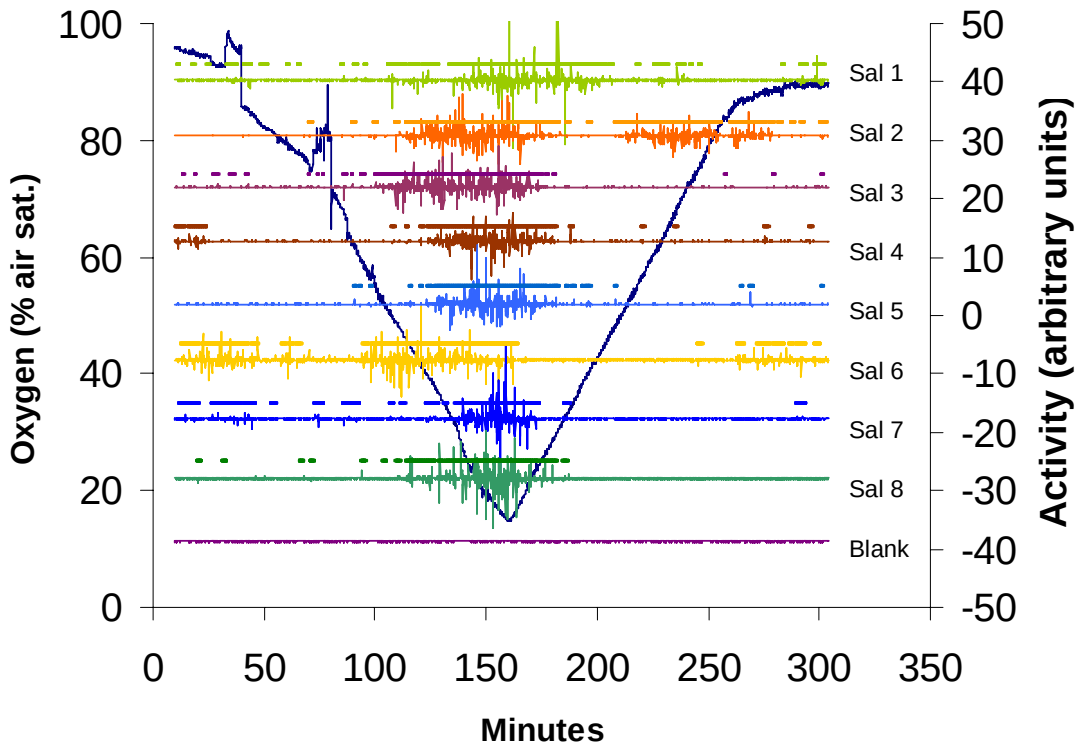
Conductivity conclusions: *E. nana* may be able to sense conductivity, although our experiment gave only equivocal support to this idea. At the end, once conductivity was < 1000 uS/cm, most salamanders became active. However, there was no distinct onset or cessation of activity during most of the conductivity ramp.

Analysis of dissolved oxygen (DO) ramp effect on salamander (*Eurycea nana*) activity. *E. nana* had been collected below the Spring Lake dam. Salamanders were set up in the morning in the metabolic circuits and allowed to acclimate for 4 hours prior to the start of the ramp. Activity was measured using a modified version of Sable Systems' AD-1 activity detector. The modification involved putting the LED emitters and detectors on 2-ft long wires, so that the LEDs could be placed underwater around the glass chambers holding salamanders. Movement by salamanders was detected by the

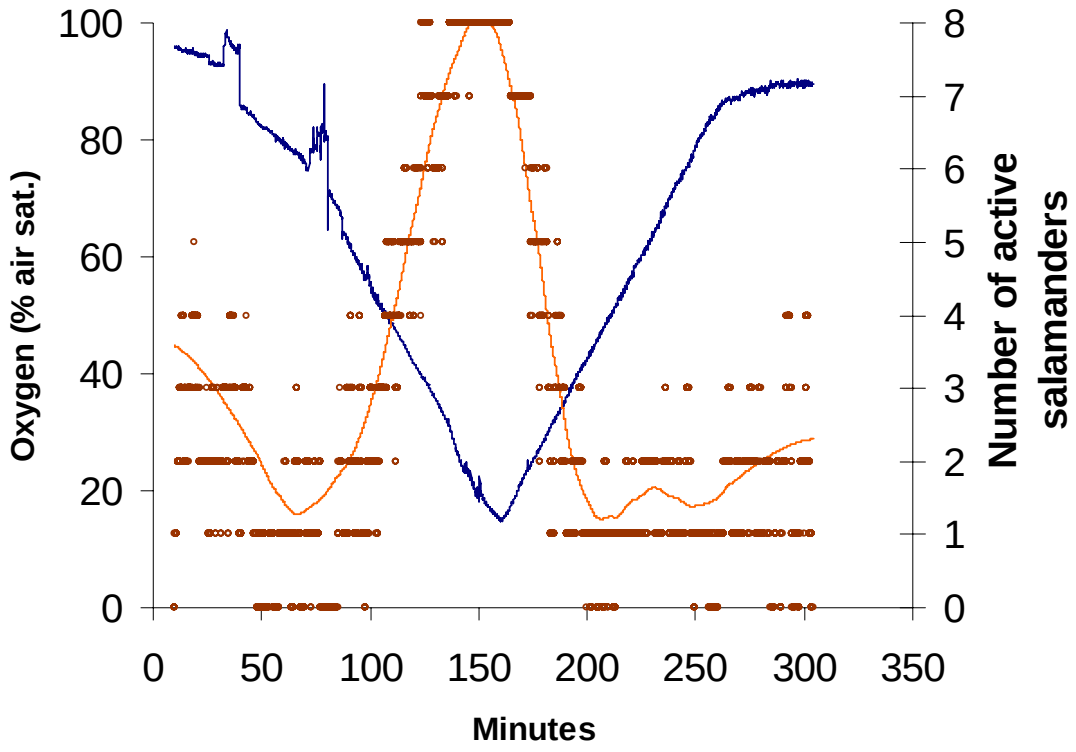
hardware, which puts out a voltage spike that we data logged. Data were sampled once per second, then the data were subsampled (choosing every fifth data point).

Simultaneously, we measured DO using the YSI meter, with water recirculated from the main chamber using the VWR chemical transfer pump.

Here's the main result, which shows dissolved oxygen levels (100% = air saturation = approx. 8.3 mg/L oxygen). Each salamander's activity is shown as a different colored trace (units are in voltage and are arbitrary). Associated with each voltage trace is a statistical estimation of when the salamander was in or out of activity bouts (log survivorship analysis; see below).



The salamanders had a clear onset of activity as the oxygen level dropped to between 30 – 60% of air saturation. Salamander activity ceased during the ramp back up at a lower level, approximately 20 – 45% of air saturation. We also can look at total activity across all 8 salamanders (summed activity):



In the plot above, each point is a second by second summary of # of salamanders active. The smooth line is a loess fit (local regression, with smoothing, see section starting p. 31 for an explanation) that describes the trace. Clearly activity is somewhat high at the beginning before trailing off. Then as DO falls below 60% of air saturation increases steeply until at around 20% of air saturation all salamanders are active. Then as DO is ramped back up, salamander activity trails off steeply and, in fact, begins to disappear immediately as DO goes up. It thus appears that salamanders are sensitive both to absolute level so oxygen and to the direction of change.

Statistical analysis

We used a technique called log survivorship analysis to divide each activity trace into within & between bout times (Slater & Lester 1982). The rest of this section shows how we performed this log survivorship analysis. All data manipulations and analyses were done using S-Plus v. 6.1 (Insightful Corp.) software, and the S-Plus code for the DO analysis is given in Appendix 1 (p. 54).

1. The first step was to filter the raw activity (voltage traces) so that events are classified either as 'no activity' (0) or 'activity' (1). We do this transformation, rather than simply using the raw voltage values, because there is no direct linear relationship between magnitude of voltage spike and amount of activity (advice from Sable Systems). However, this step was not straightforward either—the trick was to distinguish real activity spikes from background noise. We did this by establishing, for each salamander and its associated activity detector (AD-1), by picking out sections from each trace that clearly contained no activity spikes. For these sections we calculated a standard deviation. Subsequently the entire trace was filtered using the following criteria: if a spike was less than 5 standard deviations from the mean, it was considered background and converted to a zero; if it was greater than 5 standard deviations from the mean, it was considered activity and was converted to a 1. Sensitivity analyses showed that downstream results were not particularly sensitive to the choice of number of standard deviations.
2. The second step was to perform log survivorship analysis on the filtered traces. The basic procedure was to calculate the interval between every subsequent activity event. These intervals were then plotted on a histogram--of the log of the number of intervals longer than a specified time. In data traces in which there were specific bouts separated by longer inter-bout intervals, the log plots showed a characteristic concave shape (see plots starting p. 23), arising from two different event timings. Within bouts, there is a high probability of subsequent activity (short intervals), and thus at the left side of the graph the slope is steep (corresponding to a probability of subsequent activity). The shallower part of the trace, to the right, corresponds to the between-bout times—i.e., the slope is shallow because the probability of a subsequent event is low.
3. Historically the 'bout criterion'—the time that distinguishes within bout from between intervals—has been identified by eye, as the point at which the slope changes most rapidly. However, a number of authors have argued for more quantitative methods for estimating this point. We used Lester & Slater's (1982) method, which they show minimizes the total number of intervals that are misclassified. They define the optimal bout criterion as:

$$t' = \left(\frac{1}{\lambda_W - \lambda_B}\right)\log\left(\frac{\lambda_W N_W}{\lambda_B N_B}\right)$$

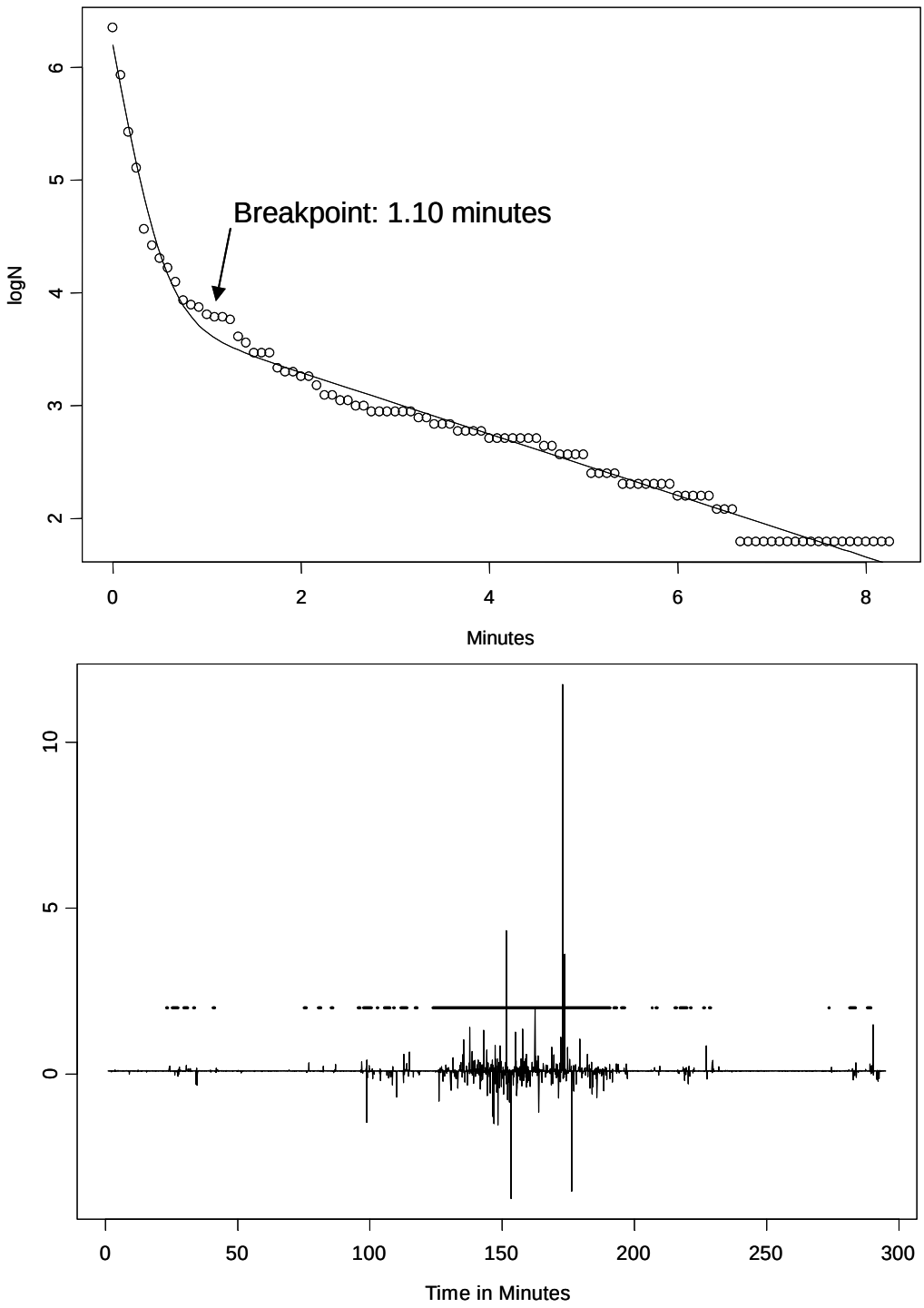
where λ_W and λ_B are the slopes of the within- and between-bout parts of the log survivorship graph. The four parameters were obtained for each individual

salamander by fitting a double exponential equation to the log survivorship plot, using a non-linear least squares fitting function in S-Plus. Mean bout criterion for the 8 salamanders was 1.60 minutes (range 0.82 – 2.56).

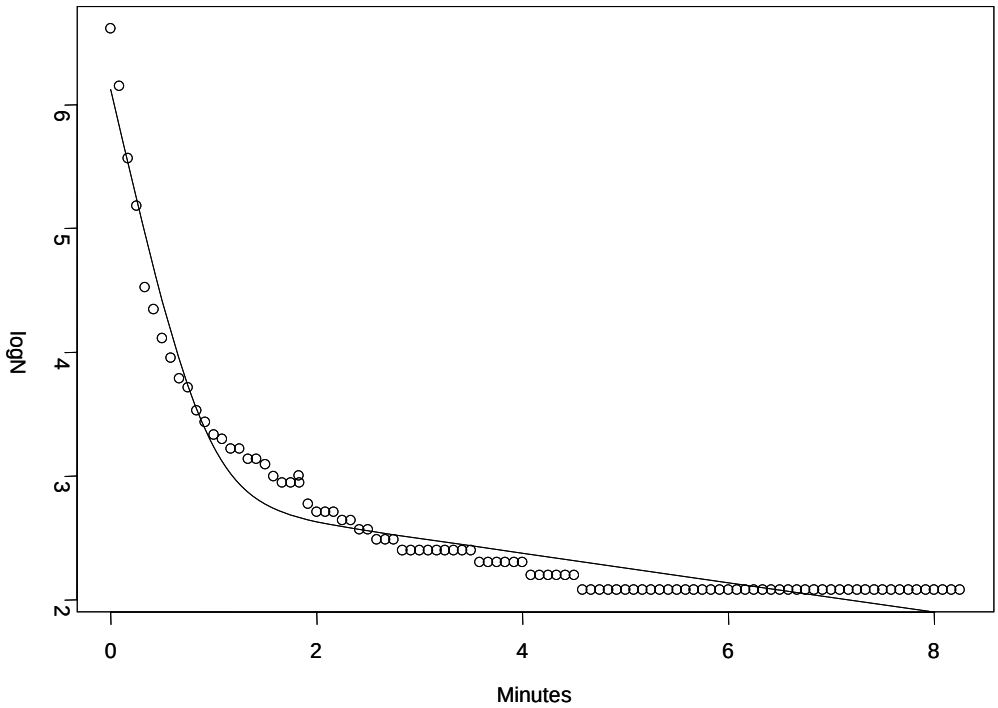
4. Once the bout criterion was identified for each salamander (see pages below), the activity vector was filtered once again to identify regions that were within and those that were between bouts. Within-bout regions are shown as solid lines above each voltage trace in the color figure on p. 19.

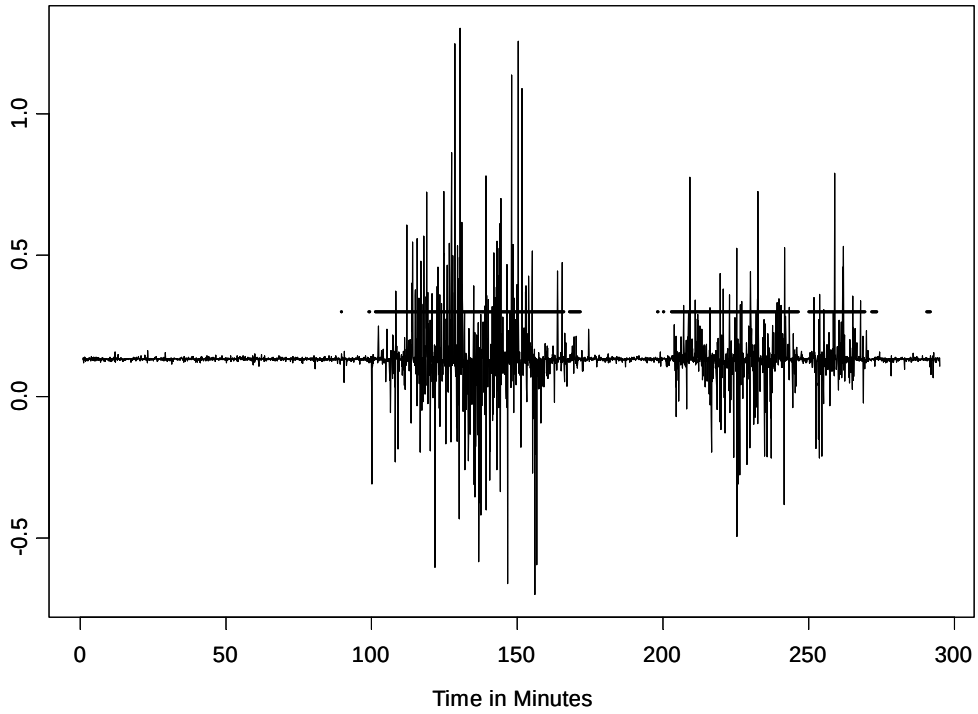
In the pages that follow we show two graphs for each salamander. The first one shows the low-survivorship plot (data as points, fitted double exponential as a line), along with the identified bout criterion determined from Lester & Slater's equation above. The second graph for each salamander superimposes the bout determinations onto the raw voltage graph.

Sal 1, subsampled, bout criterion = 1.10 minutes

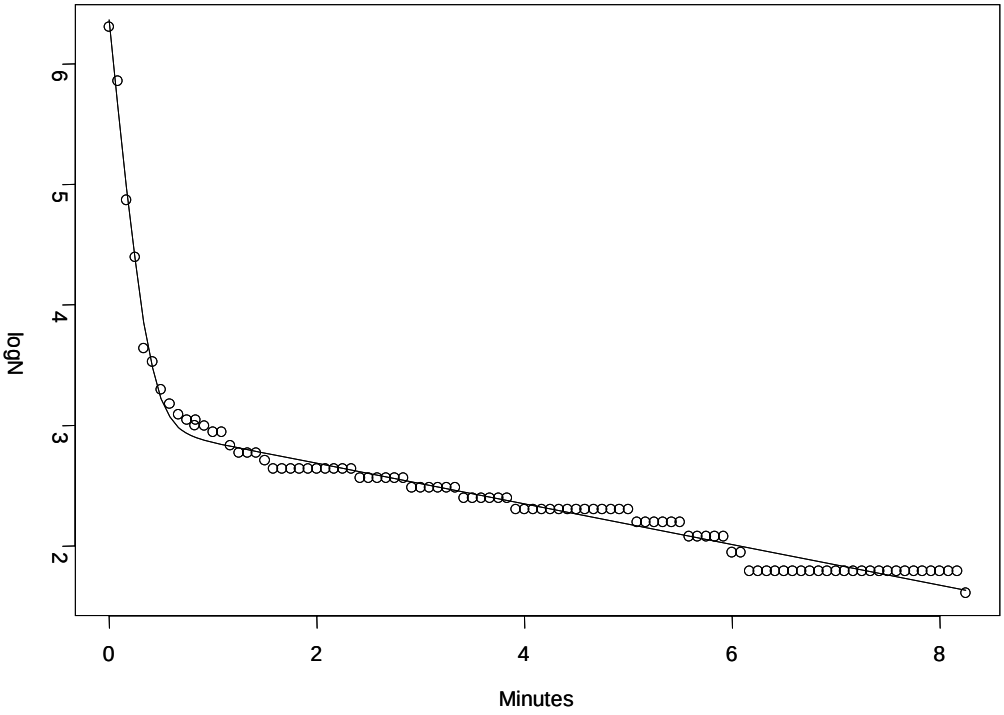


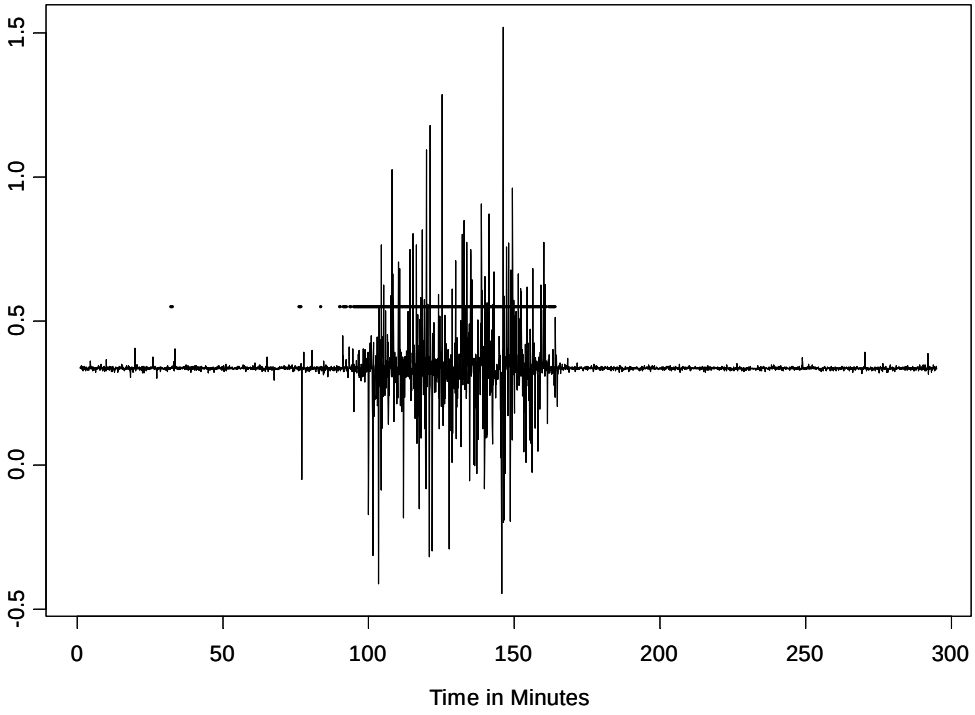
Salamander 2, breakpoint = 1.83 minutes

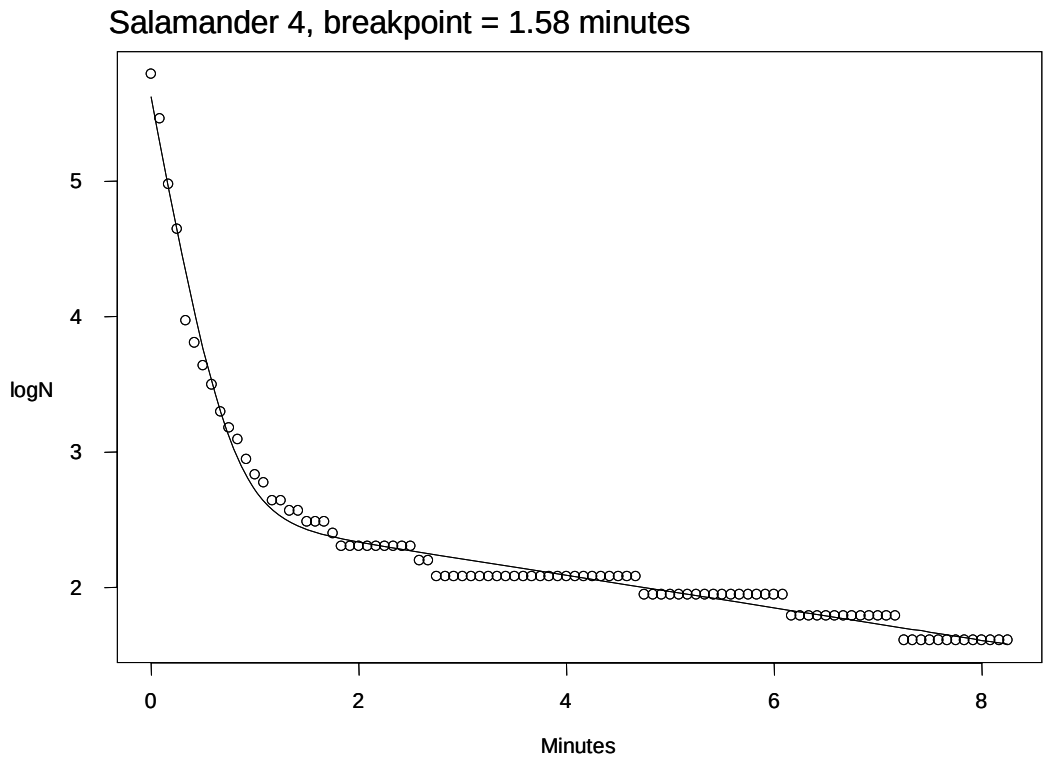


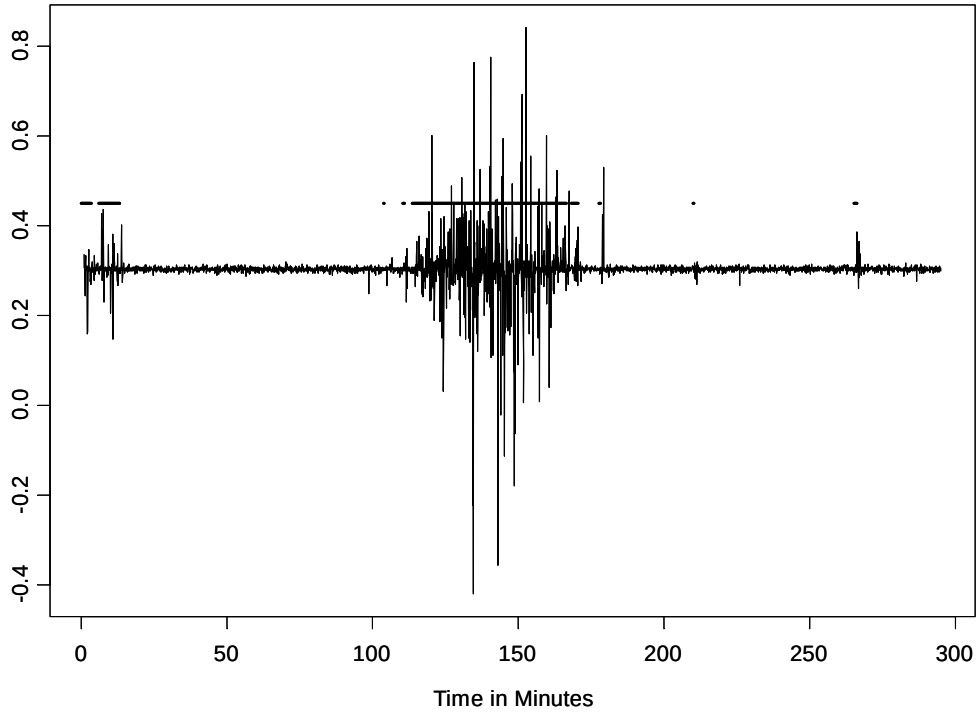


Salamander 3, breakpoint = 0.82 minutes

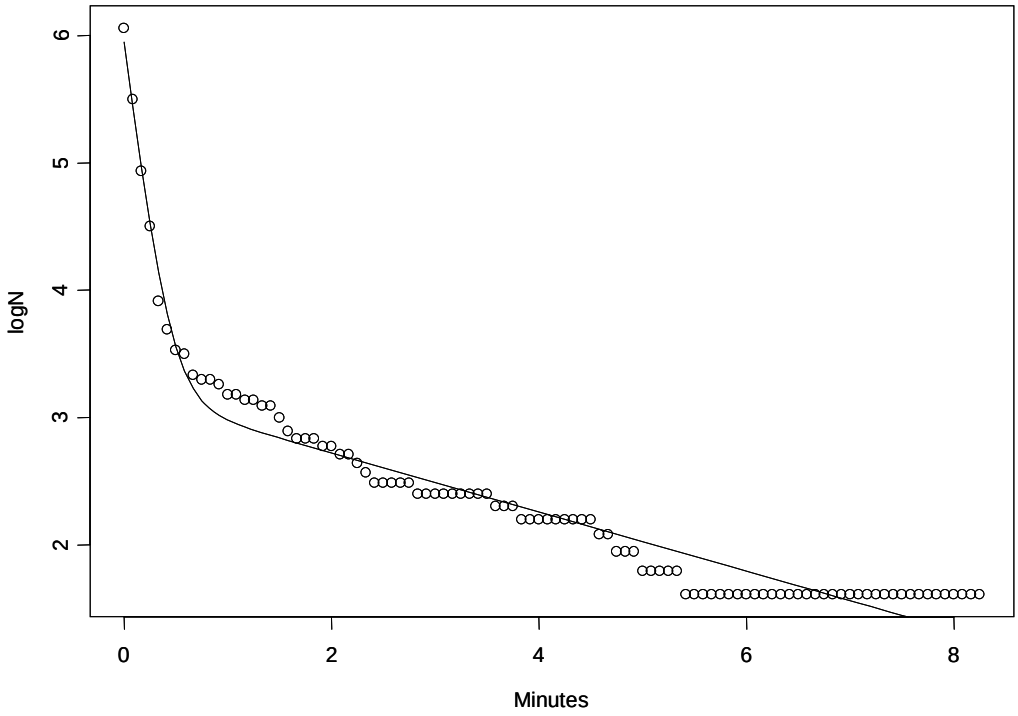


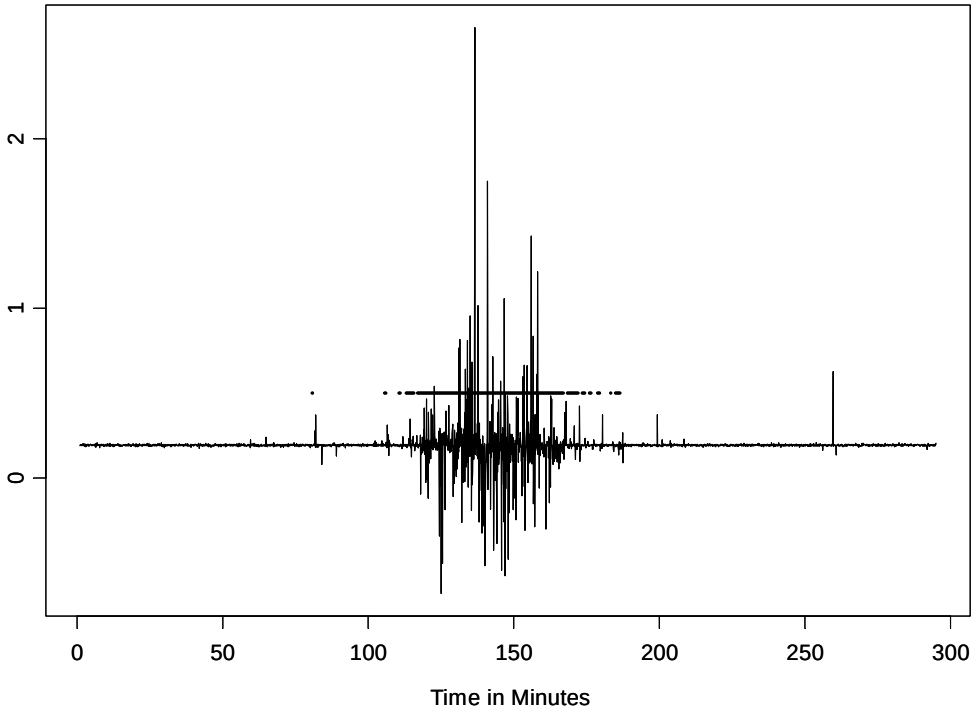



Salamander 4, breakpoint = 1.58 minutes
logN
5
4
3
2
0
2
4
6
8
Minutes

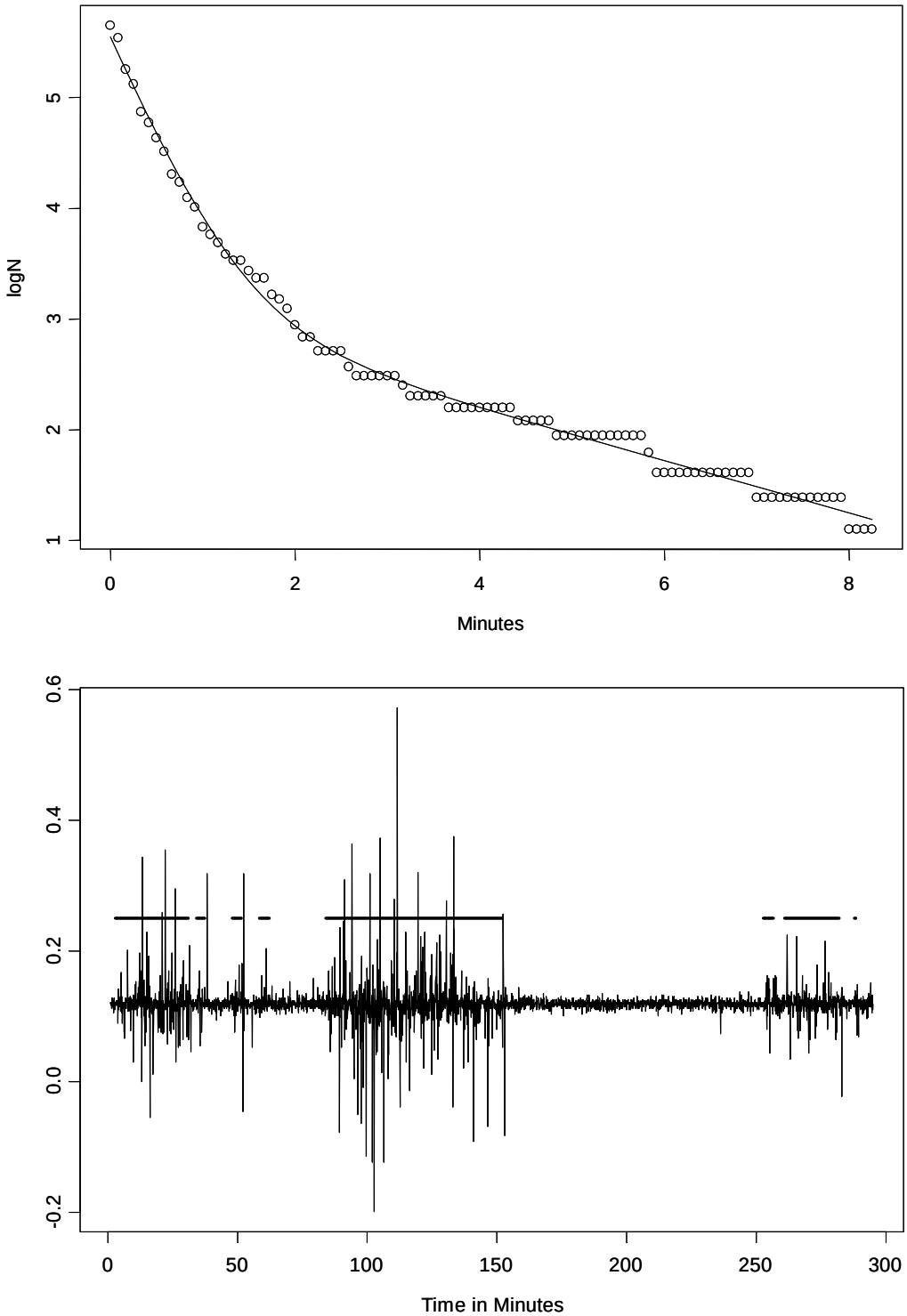

0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
0
50
100
150
200
250
300
Time in Minutes

Salamander 5, breakpoint = 0.95 minutes

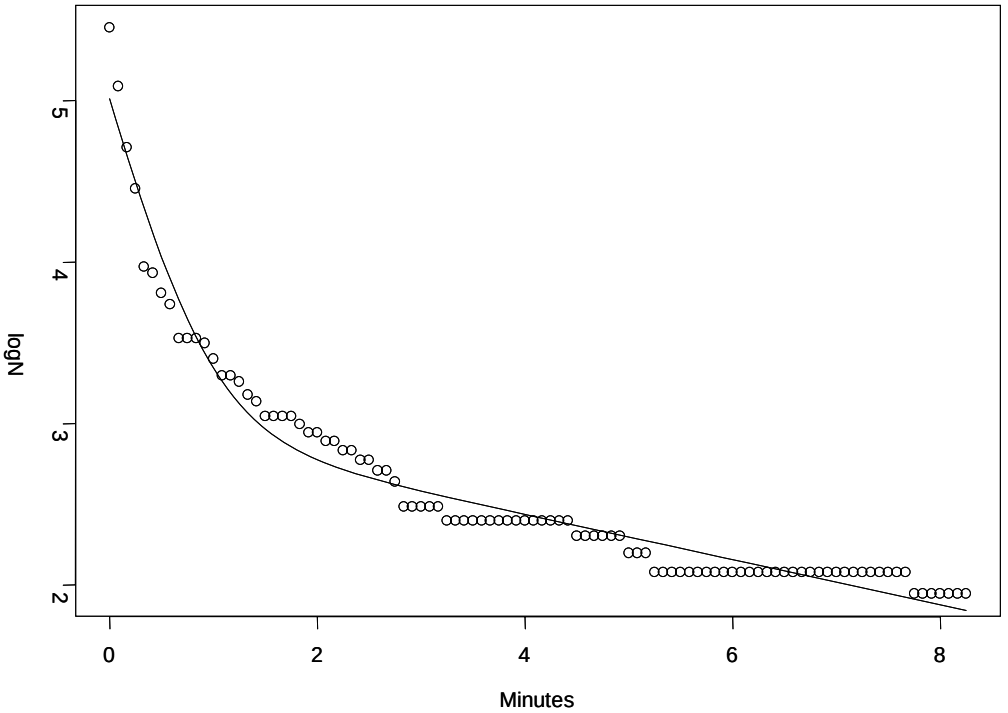


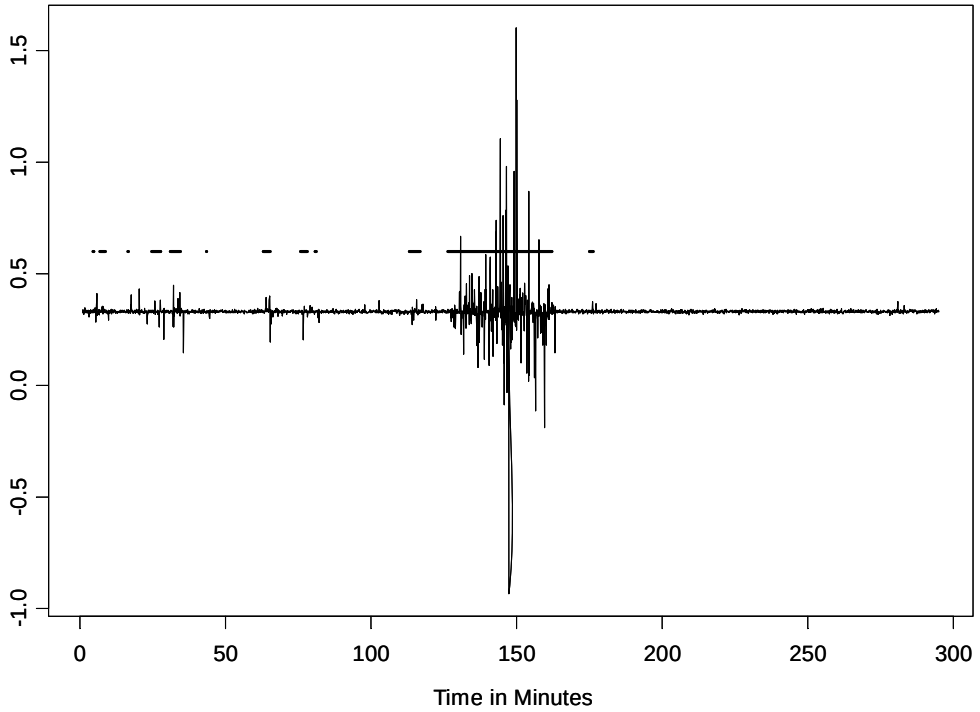


Salamander 6, breakpoint = 2.56 minutes

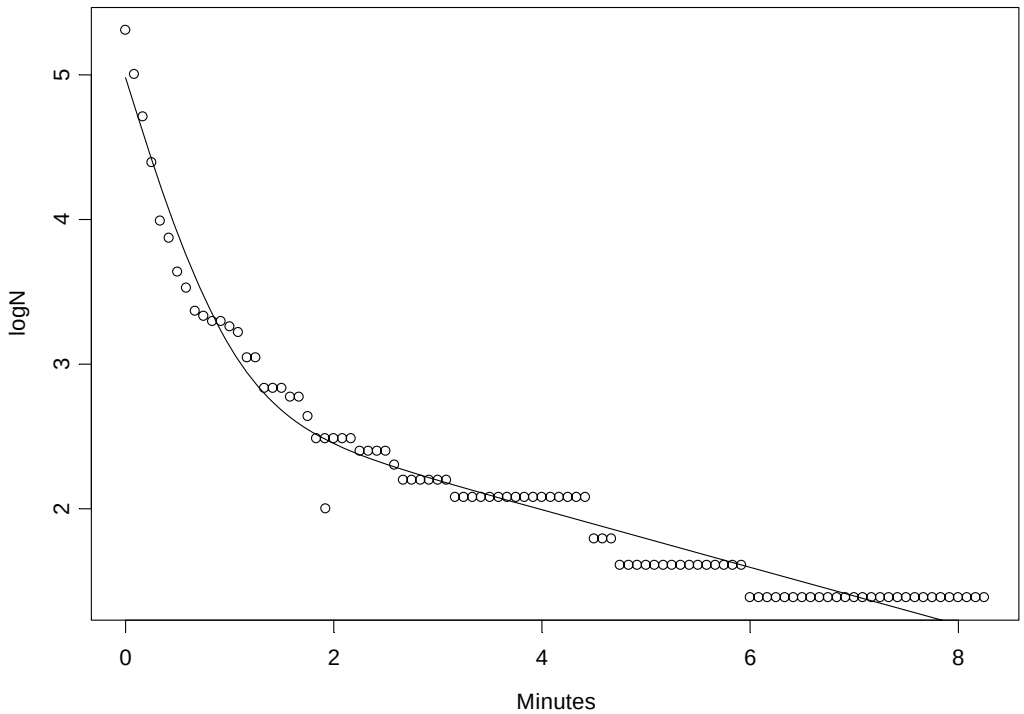


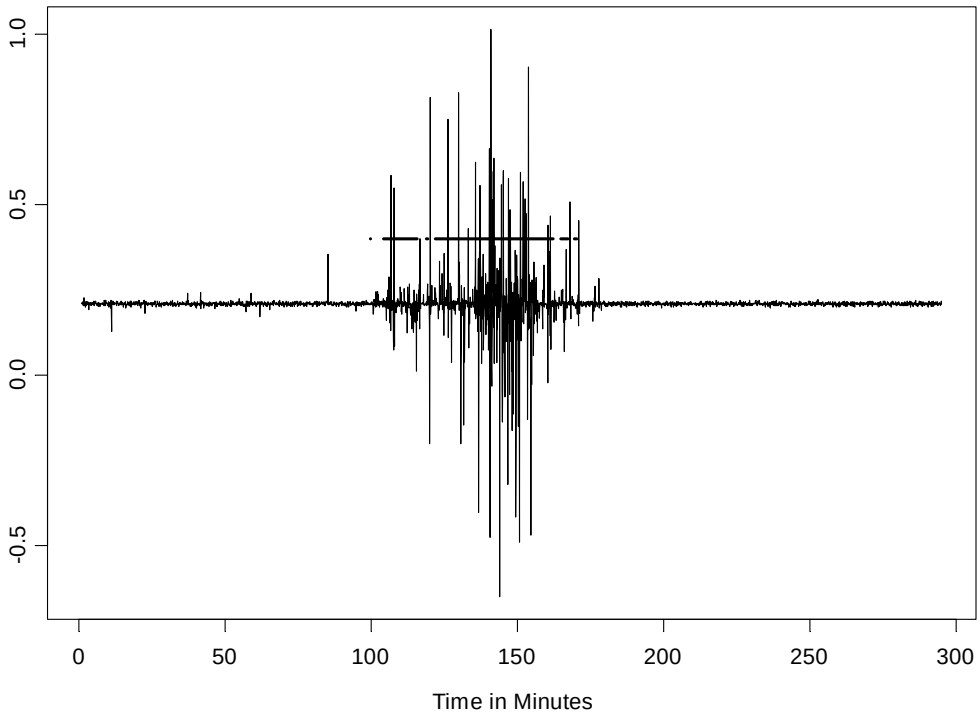
Salamander 7, breakpoint = 2.03 minutes



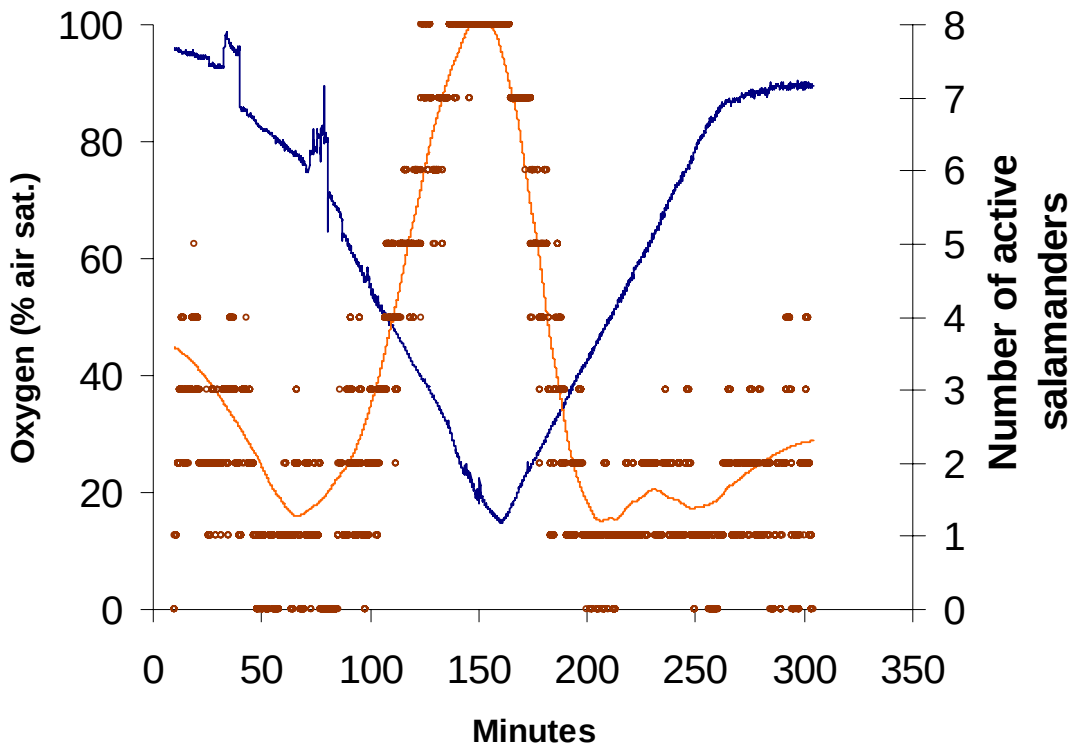


Salamander 10, breakpoint = 1.92 minutes





Here again is a graph showing summed salamander activity (dots) and oxygen level (blue line). The orange line is the loess (local regression, with smoothing) fit to the summed activity data.



The fitting was done to all activity data, on all 8 salamanders, simultaneously, with smoothing parameter set to 0.3. From the data, we were able to calculate the oxygen level giving 50% of salamanders active (which hereafter we will call AD50, or 'activity dose 50'). This measure is similar to an LD50 in toxicology and medicine—i.e., it gives the oxygen 'dose' at which 50% of the population responds. We performed this analysis for the rising and falling parts of the curve separately.

Since the response variable (activity) is binary, either 0 or 1, it is appropriate to use logistic regression. We used both probit and logit links in the analysis. To make best use of the data, we fitted the logistic regression to each salamander individually. Thus, we ended up with 8 estimates of the AD50, which allowed us to establish a mean and 95% CI.

Rising part of the curve, between 80 and 150 minutes:

	probit	**logit**
sal1	49.73158	49.70985
sal2	51.80382	51.36695
sal3	57.76966	57.4549
sal4	43.9509	43.86837
sal5	45.11959	44.57967
sal6	64.47505	63.85089
sal7	41.76121	41.38186
sal10	41.34704	40.9685
mean	49.49486	49.14762
stdev	8.231824	8.16246
N	8	8
95% CI	5.704258	5.656192

As you can see, both probit and logit links give virtually identical numbers. So for the rising part of the activity curve (declining oxygen), salamanders became active at 49.3% of air saturation (95% CI 43.6 – 55%).

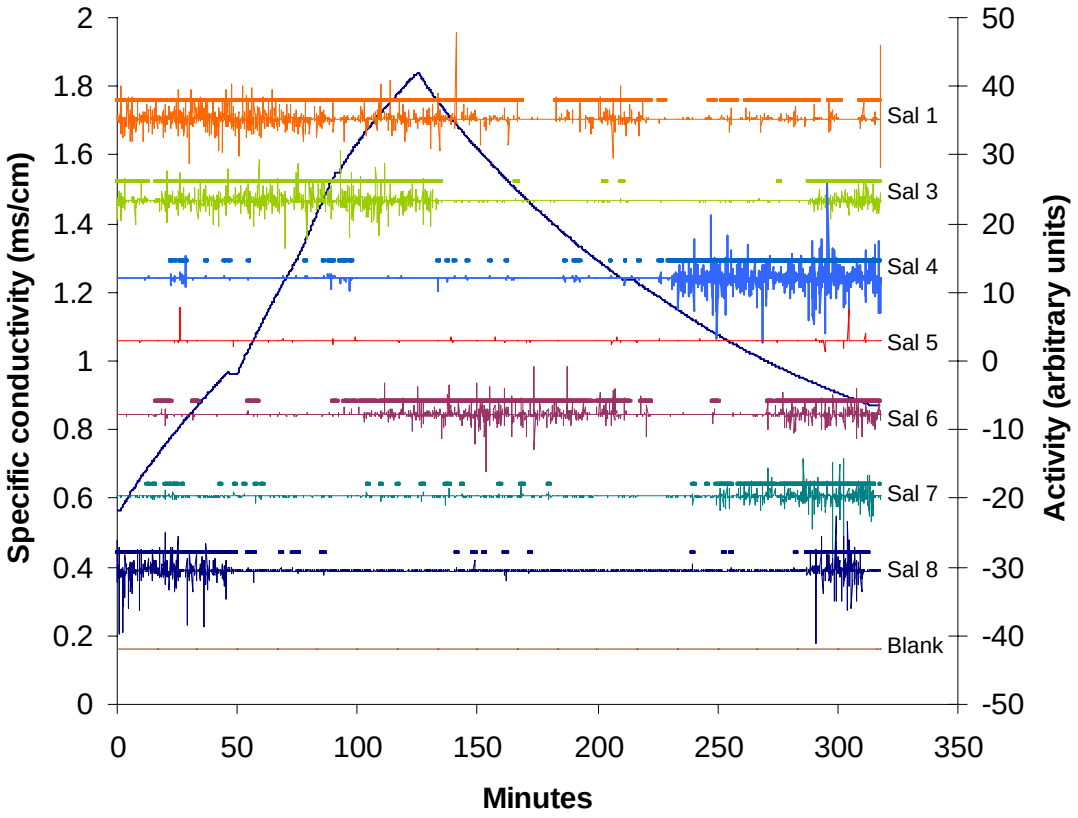
Falling part of the curve, between 150 and 220 minutes:

	probit	**logit**
sal1	50.82718	50.623
sal2	46.68295	46.66799
sal3	25.50505	25.44814
sal4	43.9509	43.86837
sal5	31.5003	31.3735
sal6	18.96997	18.91136
sal7	27.47956	27.10945
sal10	27.42039	27.04798
mean	34.04204	33.88122
stdev	11.54581	11.57327
N	8	8
95% CI	8.000688	8.01972

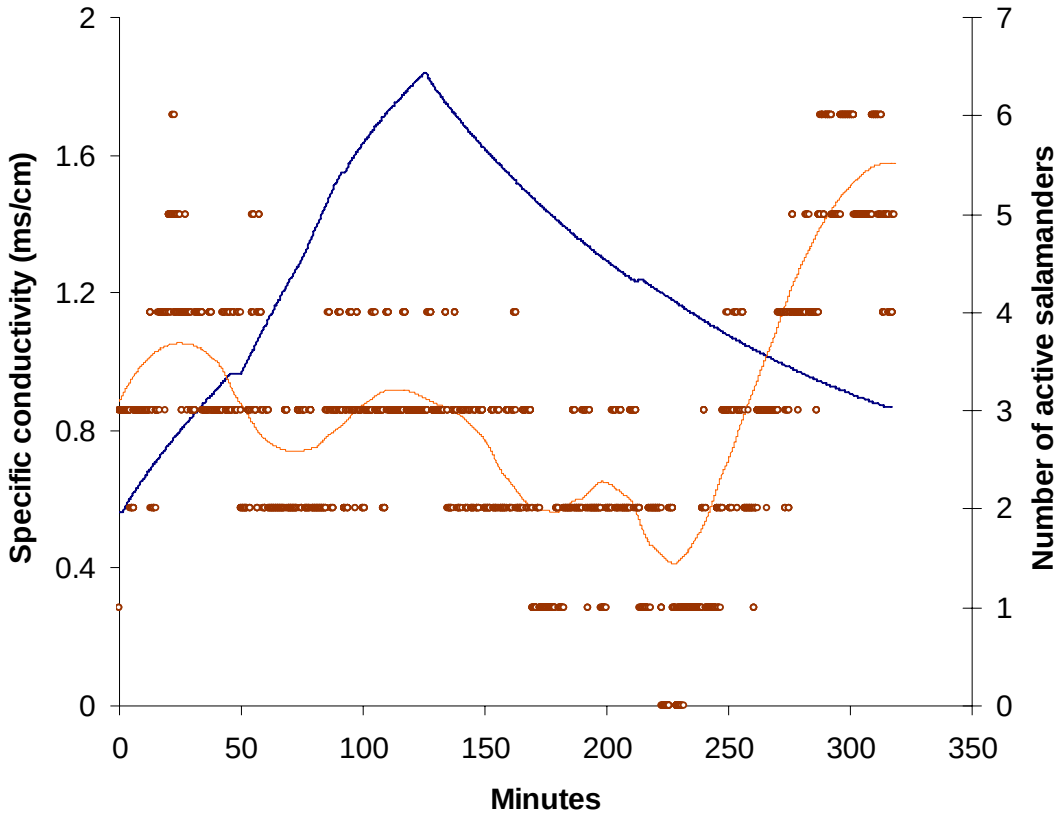
Here the probit and logit links again give very similar results. However, salamanders became inactive (LD50 for activity) at a substantially lower oxygen level: 34% of air saturation (95% CI 26 – 42%).

Breakpoint analyses for conductivity experiment in April 2006

Our analysis of activity during the conductivity ramp was very similar to the oxygen activity analysis (see above for description of statistical analysis). S-plus code for this analysis is presented in Appendix 2 (p. 61).



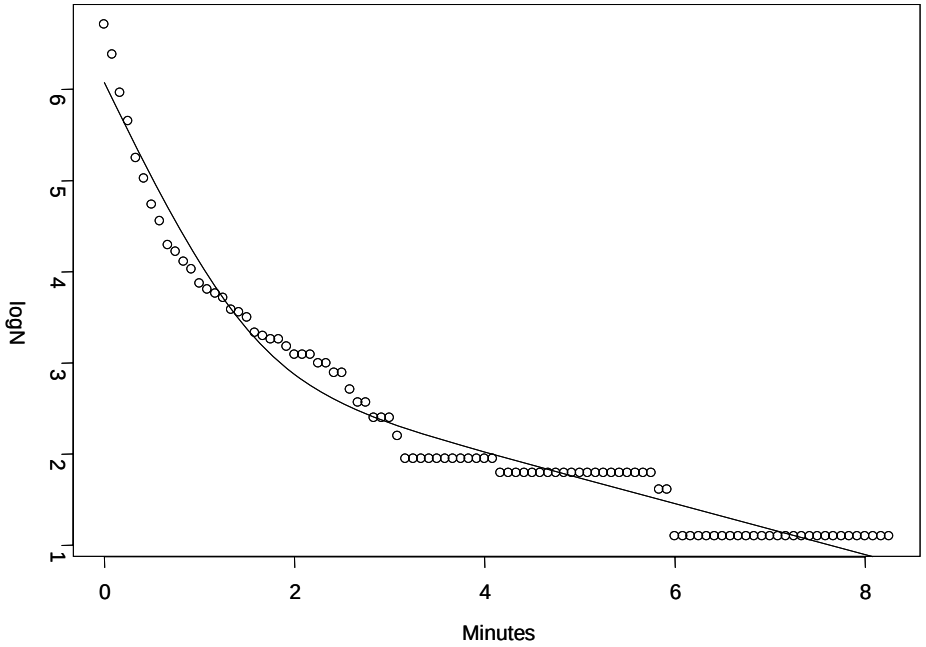
There is clearly less of a pattern here in response to conductivity than there was in response to DO. Another way to look at this is to plot # of salamanders active (in bouts, as decided by the bout criterion analysis) as a function of time:

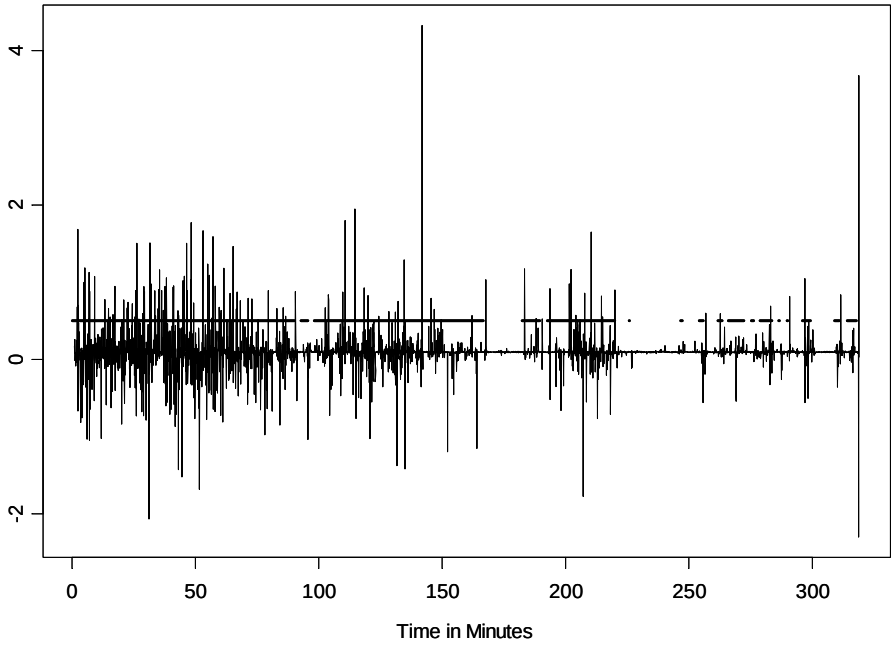


Again, the blue line is specific conductivity, brown dots are total # of salamanders active (out of 7) and the orange line is a fitted loess curve (local regression, with smoothing, smoothing parameter = 0.3). It appears that there may be some slight depression of activity up until about ¾ of the way through the experiment, though without any relation to conductivity. Then there is a spike in activity toward the end as conductivity nears its starting point. Why activity should increase then we don't know.

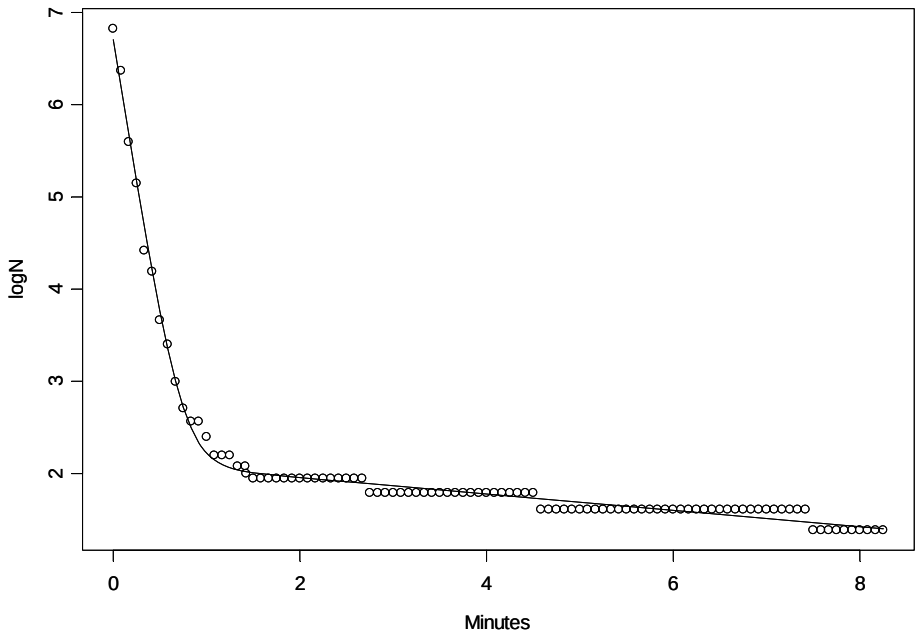
Below are plots of the bout criterion analysis for each salamander (same format as the DO analysis above).

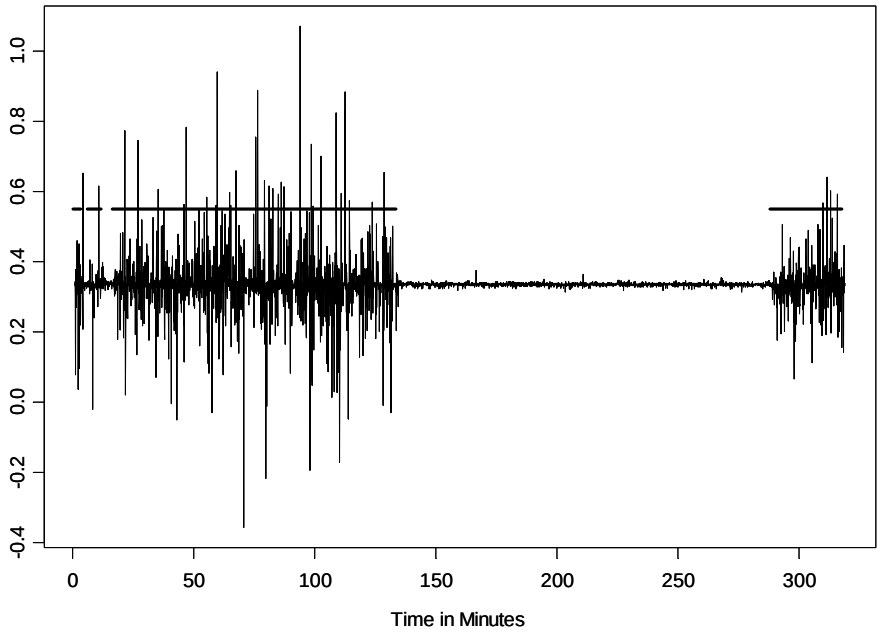
Salamander 1 conductivity, breakpoint = 2.52 minutes



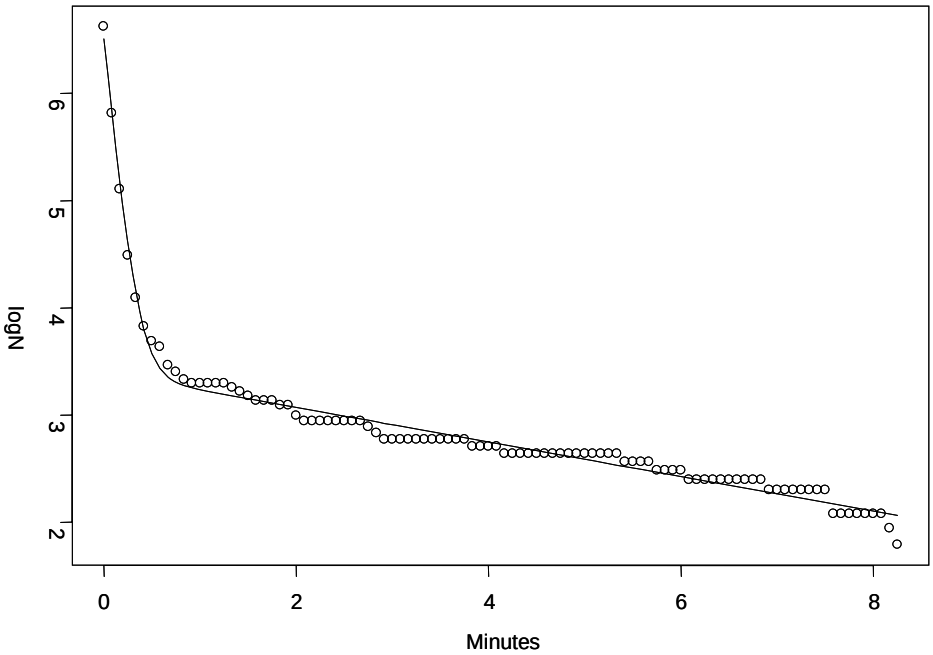


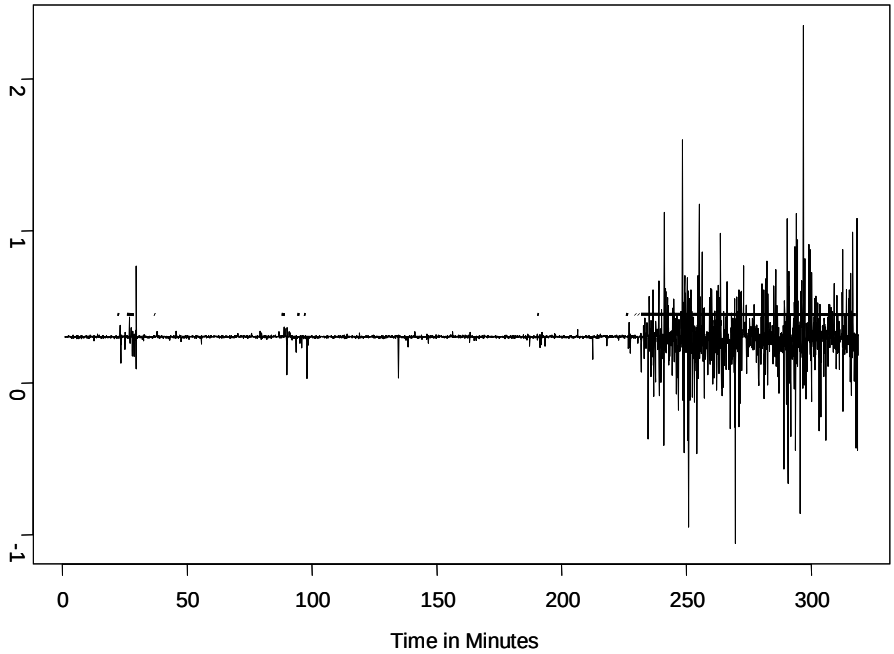
Salamander 3 conductivity, breakpoint = 1.42 min



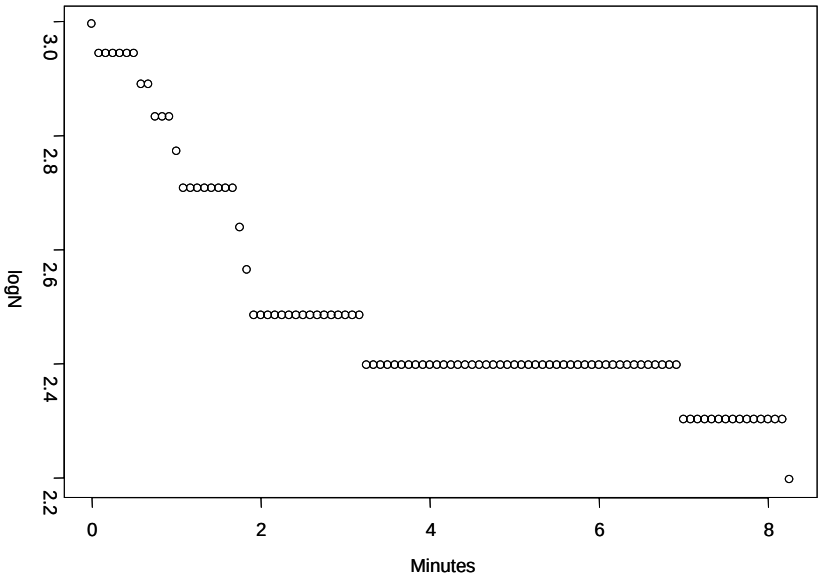


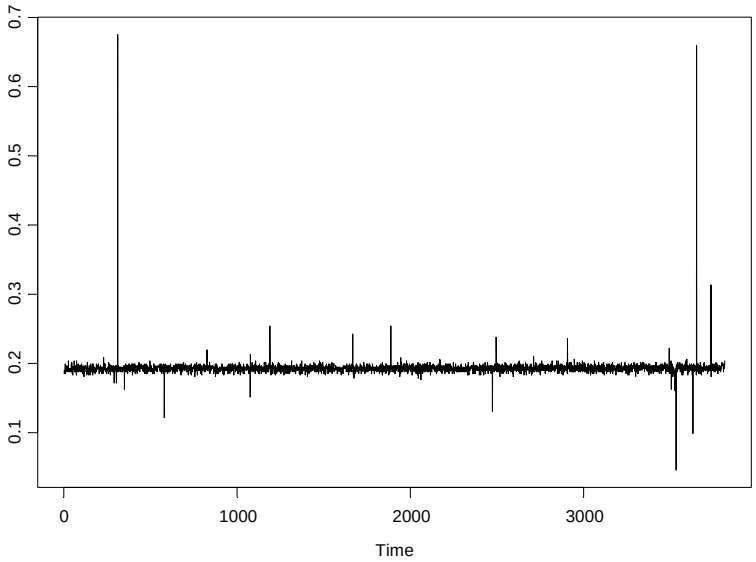
Salamander 4 conductivity, breakpoint = 0.82 min



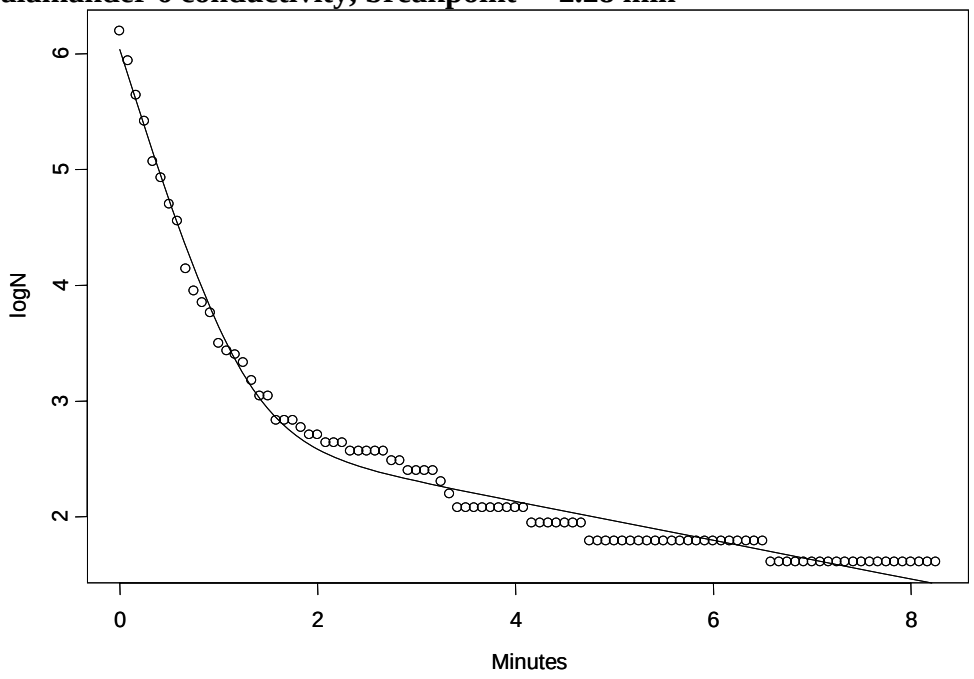


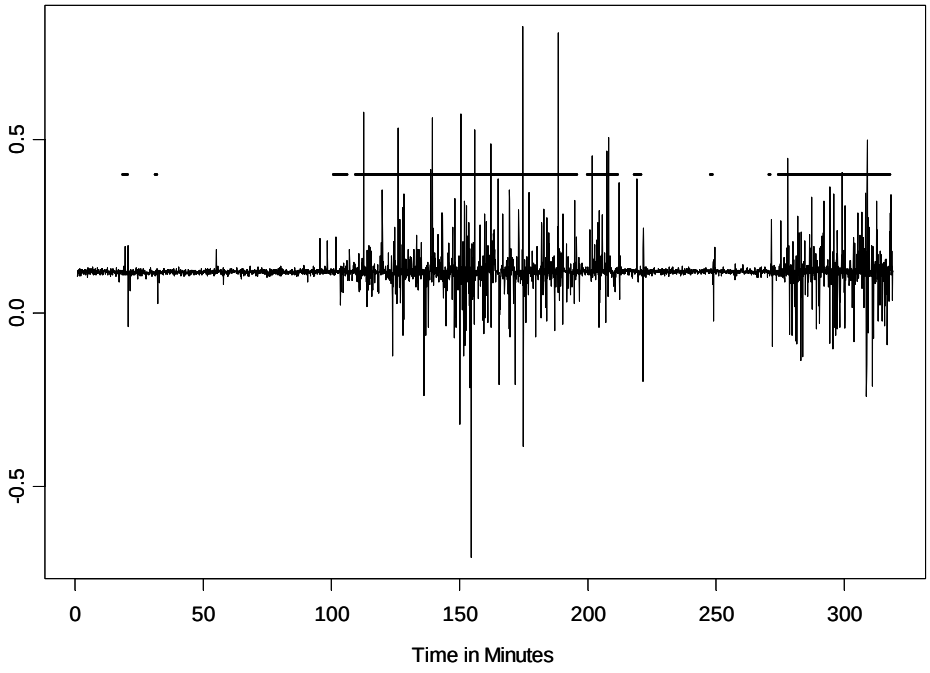
Salamander 5 conductivity, no breakpoint discernable in data, meaning that this salamander wasn't active in 'bouts'



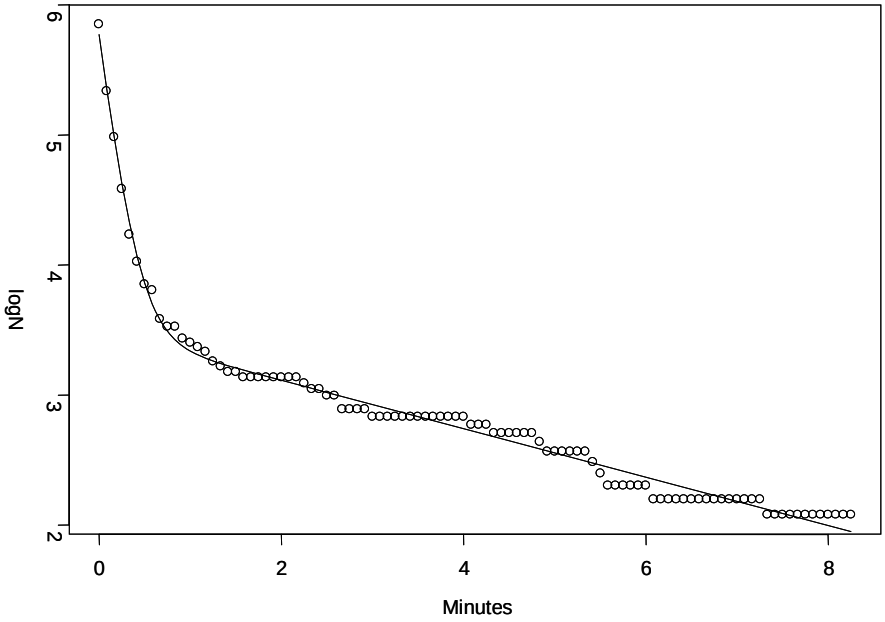


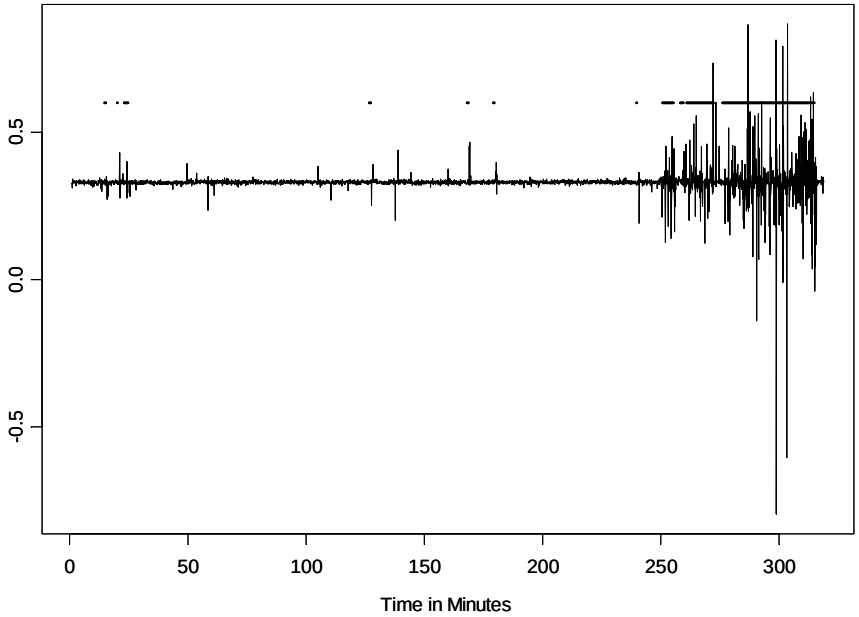
Salamander 6 conductivity, breakpoint = 2.28 min



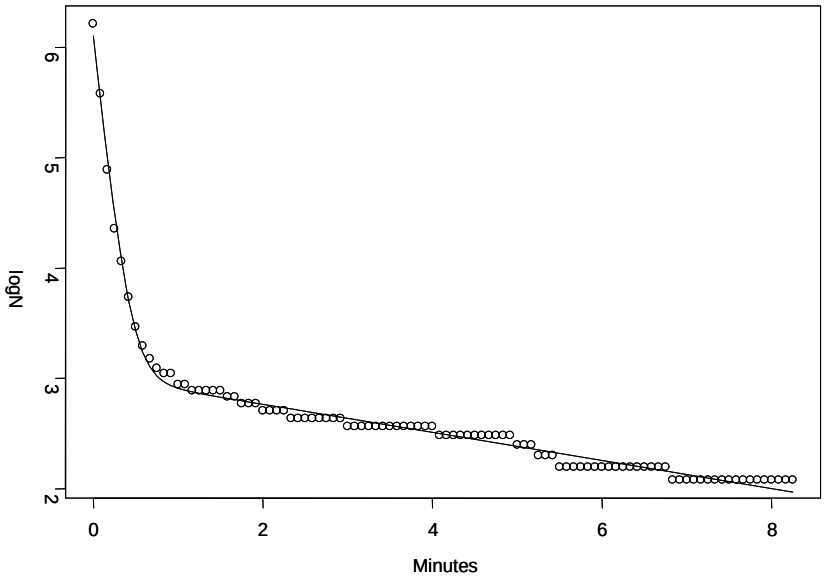


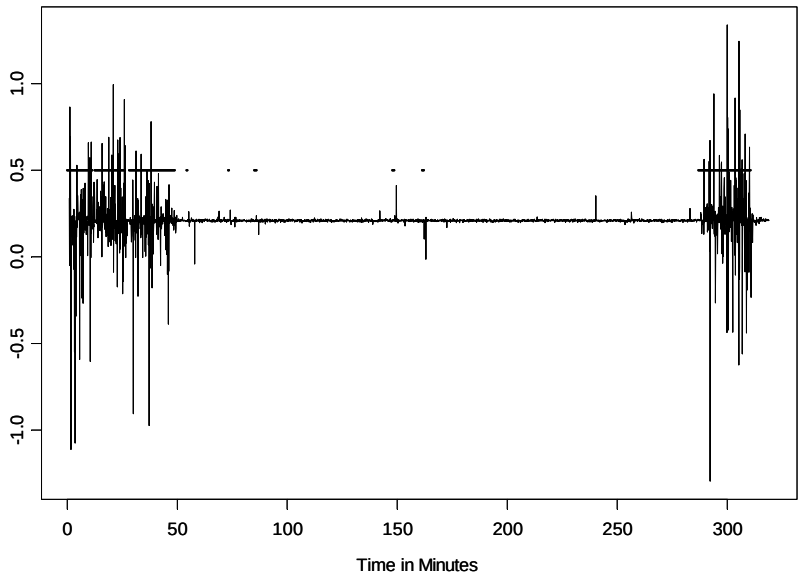
Salamander 7 conductivity, breakpoint = 1.04 minutes





Salamander 8 conductivity, breakpoint = 1.00 min





28 day toxicity experiments

Summary Findings

No salamander mortality was attributable to conductivity. Salamander condition, as measured by change in body mass during the experiment, was not significantly affected by conductivity. However, at 100% bad water, body condition did decline slightly.

Methods – We conducted 28 day conductivity toxicity tests to test for levels of conductivity that:

1. lead to specific % mortality of the salamanders. e.g. 100% mortality, 50% mortality, 25% mortality and 0% mortality
2. caused a decline in salamander condition as measured by body mass

Sixty salamanders were divided into 5 treatment levels of conductivity for a total of 12 salamanders per treatment. Conductivity levels were based on the expected intrusion of up to 5% bad water in Barton Springs. In addition, conductivity levels were based on the activity and escape response data that showed no significant effect of extremely high conductivity on salamander behavior. Conductivity levels were:

TRT 1	100% Eliza + 0% St. Alban's	597 uS/cm
TRT 2	92% Eliza + 8% St Alban's	778 uS/cm
TRT 3	87% Eliza + 13% St Alban's	955 uS/cm
TRT 4	80% Eliza + 20% St. Alban's	1044 uS/cm
TRT 5	0% Eliza + 100% St. Alban's	2887 uS/cm

(TRT = treatment)

Salamanders were held individually in 2L aquaria. Three aquaria were held in 1 box, for a total 4 boxes per treatment and 20 boxes total. Within each box, we circulated chilled water at the base of the aquaria to maintain aquaria temperatures at 20°C. We circulated CO_2 through each treatment to maintain pH at 7.6.

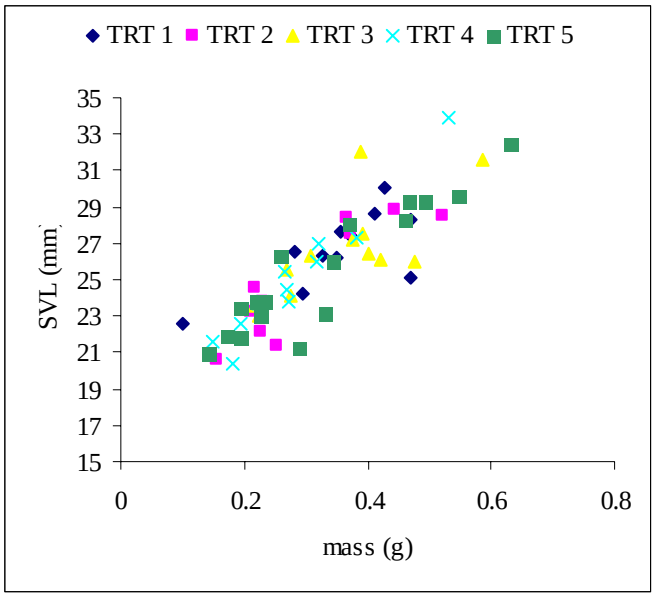
The following picture shows the general set-up of the boxes. The boxes were configured on 3 shelves with a common flow-through water cooling system. The green meters controlled pH in each treatment.

Boxes were assigned treatments based on a modified latin squares design. For treatments 1 through 5, the set-up was as follows with each row representing 1 rack of the experiment and each square representing 1 box:

1	2	3	4	5	1		
5	1	2	3	4	5		
2	3	4	5	1	2	3	4

Each salamander was provided with a section of pvc pipe for shelter and an air bubbler covered in a glass tube to create current within the aquarium and to prevent the salamander from coming into direct contact with gas bubbles.

Salamanders were randomly distributed among the treatments. We measured mass and SVL (snout vent length) of each salamander before placing them in the experiment. This graph shows that salamanders were randomly distributed among treatments with respect to size.



Salamanders were fed 2 Hikari brand "blood worms" (chironomid larvae) every other day. Uneaten blood worms were removed every other day. 75% of the water was changed every 4 days and water quality was also measured at this time.

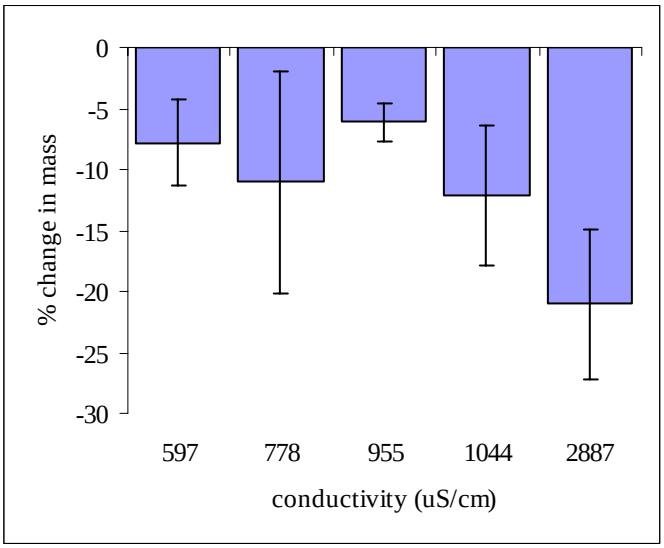
Results – No salamanders died as a result of conductivity. In addition, the percent change in salamander mass did not differ significantly across conductivity treatments.

Analysis of variance testing for the effects of treatment and block design on the % change in salamander mass. There was no significant effect of treatment or blocking on salamander mass at the end of the experiment.

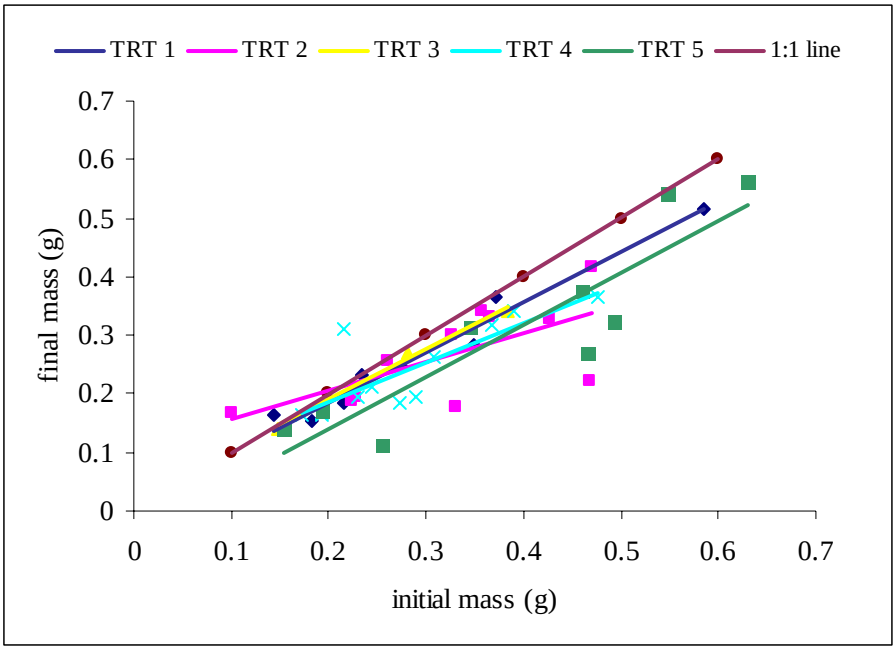
	Df	SS	MS	F	p
Conductivity Trt	4	1038.90	259.72	0.496	0.738
Box in Trt	13	2897.39	222.87	0.426	0.945
Residuals	27	14122.40	523.05		

There was a 20% decline in body mass of salamanders from the 100% St. Alban's treatment at the end of the 28 days. Although this was not statistically significant, the change appears to be biologically significant. Relative to the other treatments, the salamanders in 100% St. Alban's water had, on average, 50% more change in body mass than salamanders in the other treatments. However, the likelihood of Barton Springs salamanders naturally experiencing 100% bad water is very low.

In addition, all salamanders lost some weight, which suggests that the amount they were fed could have been increased.



This figure shows the relationship of the initial salamander mass to the final mass. The 1:1 line represents the expected relationship between initial and final mass if there was no treatment effect. TRT 1 is 100% Eliza water and TRT 5 is 100% St. Alban's water.



Conclusion:

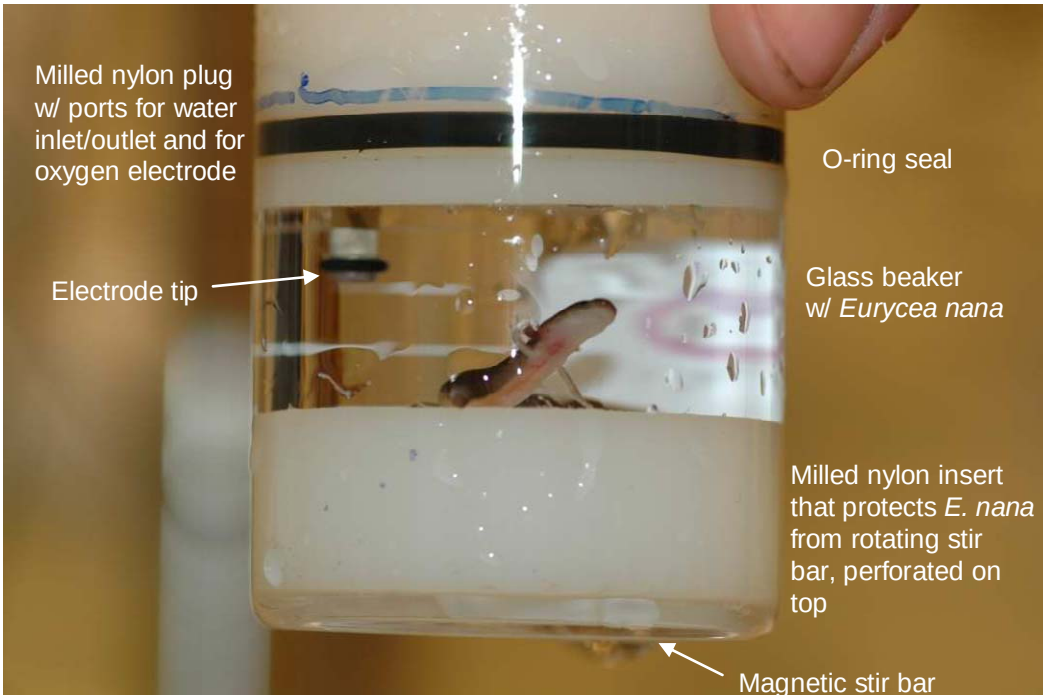
Longer term effects of conductivity levels within the range expected in Barton Springs (1-5% bad water) had no effect on adult salamander survival or mass. However, it appears that extremely high levels of conductivity, e.g. 100% bad water, do have an effect on salamander mass, but not survival over a 1 month period.

Metabolic rates of *Eurycea nana* in response to changing conductivity.

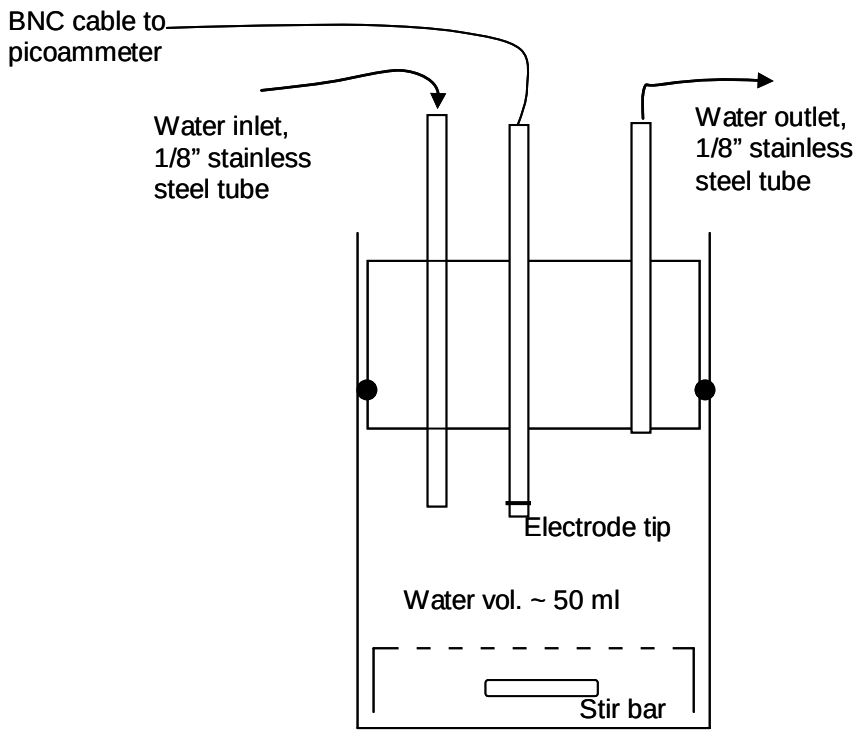
Summary finding

E. nana appears not to show a short-term metabolic response to conductivity.

Changes to procedure for measuring metabolic rate. As you know, we have had persistent problems getting clean measurements from the flow-through metabolic system. The main issue was the appearance of oxygen in nitrogen-purged water in the enclosed loop. We believe that the oxygen was not leaking into the system from outside—rather, it likely diffused into the water stream from small bubbles of air trapped in the circuit, possibly in association with exposed stainless steel threads on some of the parts. After an intensive round of trying to solve this problem in Montana (in July), we abandoned the setup and redesigned the metabolic system to be semi-closed. This redesign represented a substantial simplification of the equipment, and it allowed us to confirm visually whether or not bubbles were present. Here is a picture of a salamander in one of the new metabolic chambers:



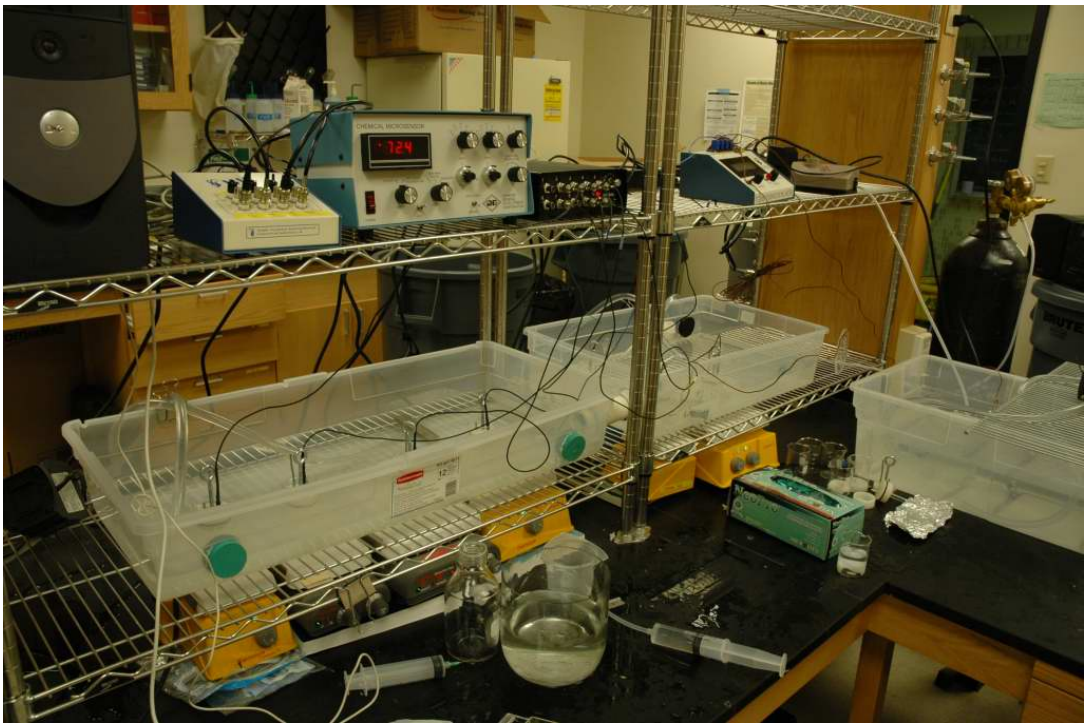
And on the top of the next page is a schematic showing how chamber works.



Water volumes on all chambers were measured gravimetrically (~50 ml total water volume). Stir bar rotation was set fast enough to mix all water in the chamber in 5 – 10 seconds, as estimated by dye injection. The inlet and outlet ports allowed us to flush chambers with new water, providing a ready means of ramping conditions for the salamanders. Flushing also introduced air-saturated water. When flushing was stopped, however, changes in oxygen in the enclosed volume were due only to biological activity. Extensive testing showed that the chambers were essentially leak-free: when nitrogen-purged water was introduced into the chamber, the oxygen levels would remain zero for several hours. Moreover, because the walls of the chamber were glass, we were able to readily see trapped air bubbles and to remove them with noninvasive techniques (gentle tapping and flushing).

The downside of the semi-closed technique is that it provides less control over rates of water movement, and water movement is turbulent rather than laminar. However, we felt, under the circumstances, that giving up this control was highly preferable to dealing with leak-ridden data. We are in the process of estimating, from dye injection experiments, the approximate water speeds experienced by salamanders.

Using an oxygen electrode multiplexer, we were able to keep track of multiple chambers at once (see following picture).



We used this system to measure metabolic rates of 36 *E. nana* during the week of Aug 13 – 21, 2006. The measurements comprised parts of two different experiments:

--we measured metabolic rates (in air-saturated water) of half (30) of the 60 salamanders going into the oxygen toxicity experiment. Our intention is to measure metabolic rates again at the end of the 28-day toxicity experiment, as a means of assessing whether metabolic rate undergoes any kind of acclimation.

--we measured metabolic rates (in air-saturated water) of 6 salamanders exposed to a conductivity ramp (from low to high, then back to low).

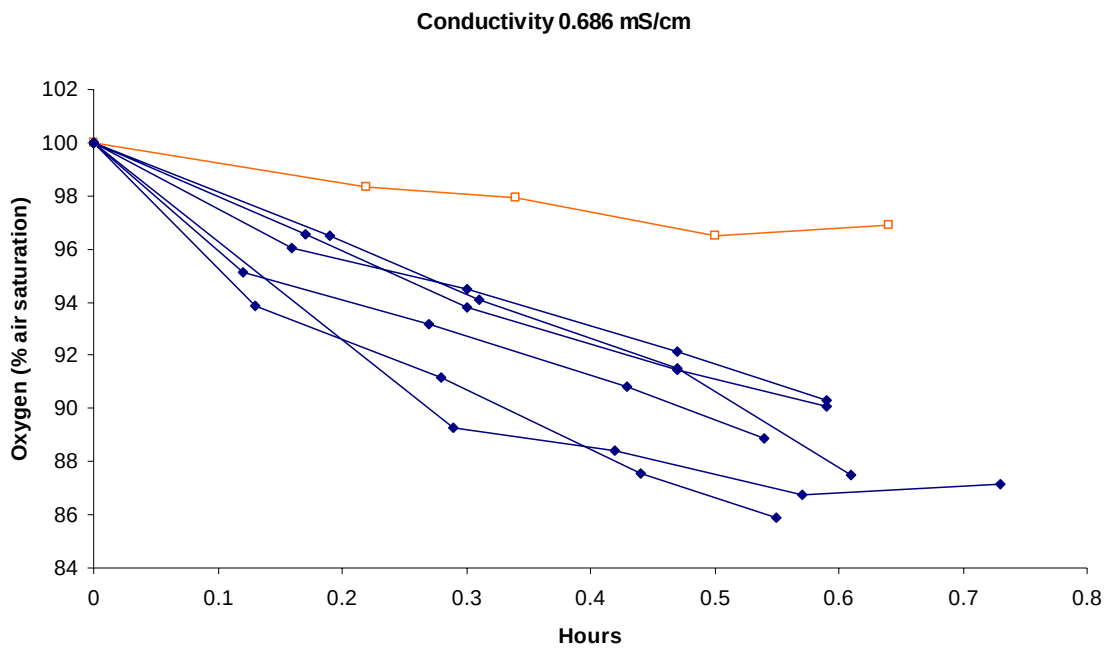
The details of the latter experiment are described below.

General procedures:

1. Wash all metabolic chamber parts in soap & hot water (to reduce bacterial biofilm formation), rinse thoroughly in tap water and distilled water.
2. Calibrate electrodes using nitrogen-purged and air-saturated water.
3. Weigh and photograph salamanders.
4. Place them one to a chamber, being careful to exclude all air bubbles.
5. Submerge most of chamber in a temperature-controlled water bath (see picture above) set to 20°C.
6. Allow salamanders ~ 45 minutes to become accustomed to the metabolic chambers.

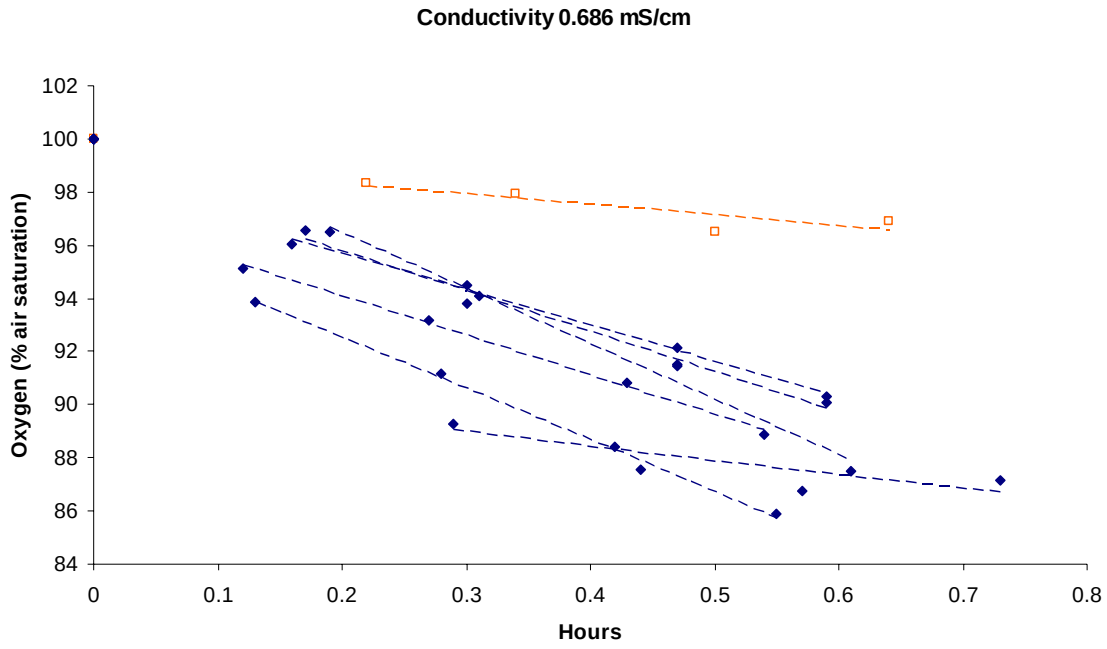
7. Flush each chamber with 5 volumes (~ 250 ml) of air-bubbled Eliza springs water (conductivity 0.686 mS/cm [value corrected to 25°C]).
8. Stop flushing, which 'closes' the chamber. Use the electrode multiplexer to step through the electrodes, measuring the oxygen concentration in each chamber for 1 – 2 minutes before moving on to the next chamber. Repeat the cycle 4 or 5 times in 30 – 45 minutes.
9. Flush each chamber with a new conductivity, mixed from different volumes of Eliza Springs water (0.686 mS/cm) and St. Alban's water (3.012 mS/cm). In this experiment we exposed salamanders to conductivities in the following order: 0.686, 0.903, 1.235, 2.080, and 0.686. This series represents increasing exposure to high conductivity followed by a return to pure Eliza water at the end. Remeasurement of metabolic rates in Eliza water is important to control for the confounding effects of time of day and length of time in the metabolic chambers.

Here is a graph showing typical measurements of change in oxygen content at ambient conductivity (0.686 uS/cm). The orange trace is a blank chamber (no salamander) and the six blue traces represent six different chambers each with a salamander.



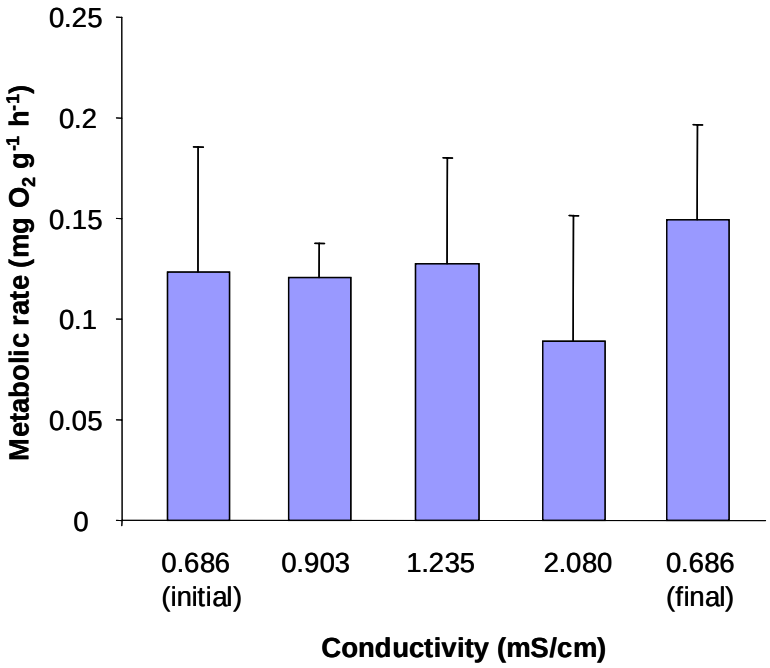
The blank chamber (orange) showed a slight apparent decline in oxygen content, which could arise from bacterial respiration, absorption of oxygen by the nylon parts, electrode drift, etc. The six salamanders (blue) all caused much more substantial declines in chamber oxygen levels.

We estimated metabolic rate by first fitting lines to each data trace (excluding the first point at 100%). The fitted lines are shown below:



The slopes provide estimates of metabolic rate, which we can calculate precisely from the known saturating DO levels in water of particular conductivity and temperature, and from the known water volume inside each metabolic chamber. We also corrected each set of salamander measurements by subtracting the apparent slope of the blank from the slopes of data from individual salamanders.

Here is a summary graph showing metabolic rate as a function of conductivity (error bars are standard deviations):



Although the data are from only six salamanders (measured repeatedly), there was no indication of any trend in metabolism with increasing conductivity, nor after the return to low-conductivity Eliza Springs water. A brief statistical analysis confirms this impression. A paired t.test indicates no difference between the first and last exposures to Eliza Springs water ($t = -1.07$, $df = 5$, p-value = 0.34). For the first four treatments it is possible to use linear regression, as the conductivities are ordered. A flexible kind of analysis that allows one to keep track of non-independent data from the same individuals is linear mixed-effects models. We used these models in S-Plus, modeling metabolic rate as a function of conductivity and specifying individual salamanders as random effects (see Appendix 3 for S-Plus code)
The summary above indicates that 'conductivity' was not a significant term, meaning that it provided no explanatory power with respect to metabolic rate.

Conclusion: The metabolic rates of *E. nana* appear not to be influenced by short-term changes in conductivity within a reasonable natural range.

In Progress

We are currently conducting 28 day oxygen toxicity tests. These experiments began on 24 August and include 5 levels of dissolved oxygen: 1.5%, 3%, 6%, 10%, and 21%. In addition, we have prepared a separate arena for testing the effects of conductivity on juvenile salamander growth over a period of 60 days. We plan to initiate these experiments on 29 August with the juveniles that we received from Joe Fries.

Once these experiments are running, we plan to measure metabolic rates of *E. nana* under ramped levels of oxygen. We hope to complete these experiments by the end of September. If all goes well, we will collect *E. sosorum* for oxygen and conductivity ramps to be conducted in October.

The 28 day oxygen toxicity tests will conclude at the end of September. Once these are finished, and if juvenile *E. nana* are available, we will begin the 60 day juvenile growth experiments under different levels of oxygen.

We plan to conclude the experimental phase of this project in December.

Appendix 1. S-Plus script used to analyze activity in response to DO

```
##salamander 1
tmp1<-na.omit(DO.activity$Sal1activity)
tmp5<-rts(tmp1)               #make a regular time series from the data
#tsplot(tmp5)
tmp2<-tmp1[c(700:900, 2900:3200)]

##salamander 2
tmp1<-na.omit(DO.activity$Sal2activity)
tmp5<-rts(tmp1)               #make a regular time series from the data
#tsplot(tmp5)
tmp2<-tmp1[c(400:950, 2100:2300)]
height<-.3                       #altitude of activity bout bar


##salamander 3
tmp1<-na.omit(DO.activity$Sal3activity)
tmp5<-rts(tmp1)               #make a regular time series from the data
#tsplot(tmp5)
tmp2<-tmp1[2100:2900]
height<-0.55

##salamander 4
tmp1<-na.omit(DO.activity$Sal4activity)
tmp5<-rts(tmp1)               #make a regular time series from the data
#tsplot(tmp5)
tmp2<-tmp1[200:1000]
height<-0.45

##salamander 5
tmp1<-na.omit(DO.activity$Sal5activity)
tmp5<-rts(tmp1)               #make a regular time series from the data
#tsplot(tmp5)
tmp2<-tmp1[c(0:700, 2600:2800)]
height<-0.5

##salamander 6
tmp1<-na.omit(DO.activity$Sal6activity)
tmp5<-rts(tmp1)               #make a regular time series from the data
#tsplot(tmp5)
tmp2<-tmp1[2000:2800]
height<-0.25

##salamander 7
tmp1<-na.omit(DO.activity$Sal7activity)
tmp5<-rts(tmp1)               #make a regular time series from the data
#tsplot(tmp5)
tmp2<-tmp1[2100:3000]
height<-0.6

##salamander 10
tmp1<-na.omit(DO.activity$Sal10activity)
tmp5<-rts(tmp1)               #make a regular time series from the data
#tsplot(tmp5)
tmp2<-tmp1[2200:3100]
height<-0.4


#######after calculating tmp2 for each salamander, then do the following
tmp2.mean<-mean(tmp2)
tmp2.stdev<-stdev(tmp2)
```

```
tmptmp2highCI<-tmp2.mean + 3*tmp2.stdev
tmp2lowCI<-tmp2.mean - 3*tmp2.stdev

sal1.filt<-rts(ifelse(((tmp1>tmp2highCI) | (tmp1<tmp2lowCI)), 1, 0), deltat =
   0.0833333, units = "Minutes")
tmp10<-seq(from = 0, length = length(sal1.filt), by = 0.0833333)      #create a
   time sequence that goes with activity
tmp11<-data.frame(tmp10, sal1.filt)
names(tmp11)<-c("minutes", "activity")
tmp12<-tmp11[tmp11$activity==1,]            ##collapse data frame to contain
   only rows in which activity ==1
tmp13<-tmp12$minutes[2:length(tmp12$minutes)] -
   tmp12$minutes[1:length(tmp12$minutes) - 1] ##calculate intervals
tmp20<-seq(from = 0, by = 0.0833333, length = 100)
tmp15<-c()
for(i in 1:100){
   tmp16<-length(tmp13[tmp13>tmp20[i]])
   tmp15<-c(tmp15, tmp16)
   i<-i+1
}

tmp21<-data.frame(tmp20, tmp15)
names(tmp21)<-c("Minutes", "N")
param(tmp21,"lambdaW") <- 25; param(tmp21,"lambdaB") <- .5; param(tmp21,"W") <-
   400; param(tmp21,"B") <- 50
fit <- nls(log(N)~log(W*exp(-lambdaW*Minutes) + B*exp(-lambdaB*Minutes)),
   data=tmp21)
plot(tmp20, log(tmp15), xlab="Minutes", ylab ="logN")
lines(tmp20, fitted(fit))
breakpoint<-(1/(fit$parameters[1] -
   fit$parameters[2]))*log((fit$parameters[3]*fit$parameters[1])/(fit$parameter
   s[4]*fit$parameters[2]))
points(breakpoint, 2)

breakpoint


########now draw graph that calculates actual activity bouts
tmp5<-rts(tmp1, deltat=0.0833333, units="Minutes")                #make a
   regular time series from the data
tsplot(tmp5)
tmp30<-sum(sal1.filt)         #total number of active points
par(lwd=3)                       #line width
i<-2
started<-F
for(i in 2:tmp30){

   if(((tmp12$minutes[i] - tmp12$minutes[i-1])>breakpoint) && (started==T)) {
      started<-F
      end<-tmp12$minutes[i-1]
      segments(beginning, height, end, height)
   }

   if(((tmp12$minutes[i] - tmp12$minutes[i-1])<breakpoint) && (started==F)) {
      started<-T
      beginning<-tmp12$minutes[i-1]
   }

   i<-i+1
}
par(lwd=1)
```

```
#######################here create vectors for each salamander that contain
   either zero or 1 for each timepoint,
#depending on whether the salamander is in the activity bout or not.

get.bouts<-function(filtered.vector, bout.criterion){
   tmp31<-array(dim=c(length(filtered.vector)), 0)
   tmp10<-seq(from = 0, length = length(filtered.vector), by = 0.0833333)      #create a time se
   tmp11<-data.frame(tmp10, filtered.vector)
   names(tmp11)<-c("minutes", "activity")

   act1.time<-0                     ##this loop finds the time of the first
   activity spike.
   for(i in 1:length(tmp11$activity)){
      if(tmp11$activity[i]==1){
         act1.time<-tmp11$minutes[i]
         start.i<-i
         break
      }
   }

   for(j in start.i:length(filtered.vector)){
      if(tmp11$minutes[j] - act1.time < bout.criterion){
         tmp31[j]<-1
      }
      if(tmp11$activity[j]==1) act1.time<-tmp11$minutes[j]
      j<-j+1
   }
   return(tmp31)
}



tmp2.mean<-mean(tmp2)
tmp2.stdev<-stdev(tmp2)
tmp2highCI<-tmp2.mean + 3*tmp2.stdev
tmp2lowCI<-tmp2.mean - 3*tmp2.stdev

sal1.filt<-rts(ifelse(((tmp1>tmp2highCI) | (tmp1<tmp2lowCI)), 1, 0), deltat =
   0.0833333, units = "Minutes")
sal10.filtered<-sal1.filt


tmp35<-get.bouts(sal1.filtered, 1.10)
tmp36<-get.bouts(sal2.filtered, 1.83)
tmp37<-get.bouts(sal3.filtered, 0.82)
tmp38<-get.bouts(sal4.filtered, 1.58)
tmp39<-get.bouts(sal5.filtered, 0.95)
tmp40<-get.bouts(sal6.filtered, 2.56)
tmp41<-get.bouts(sal7.filtered, 2.03)
tmp42<-get.bouts(sal10.filtered, 1.92)

#vector for minutes
tmp43<-seq(from = 10, by = 0.0833333, length = 3529)


#now add up all the traces to get total activity

tmp45<-tmp35 + tmp36 + tmp37 + tmp38 + tmp39 + tmp40 + tmp41 + tmp42

#make a data.frame to export to excel
```

```
DO.activity.all<-data.frame(cbind(tmp35, tmp36, tmp37, tmp38, tmp39, tmp40,
   tmp41, tmp42, tmp43, tmp45))
names(DO.activity.all)<-c("sal1", "sal2", "sal3", "sal4", "sal5", "sal6",
   "sal7", "sal10","Minutes","Summed")
#exportData(DO.activity.all, file="DO.activity.all.xls")



##try loess fit w/ SE

DO.loess <- loess(Summed~Minutes,data=DO.activity.all, span=.3)
plot.loess(DO.loess, confidence = 100, coverage=0.99, ylim=c(0,8))
points(DO.activity.all$Minutes, DO.activity.all$Summed)

exportData(fitted(DO.loess), "oxygen.loess.xls")


##Try spline; doesn't work
DO.spline<- spline(DO.activity.all$Minutes, DO.activity.all$Summed, n =
   length(DO.activity.all$Minutes))
plot(DO.activity.all$Minutes, DO.activity.all$Summed)
points(DO.spline)


###############fitting part with glm#####################

from http://biotech.embl-ebi.ac.uk:8400/sw/common/info/R-intro.info-4

To fit a binomial model using `glm()' there are two possibilities
for the response:

   * If the response is a _vector_ it is assumed to hold _binary_ data,
     and so must be a 0/1 vector.

   * If the response is a _two column matrix_ it is assumed that the
     first column holds the number of successes for the trial and the
     second holds the number of failures.

   Here we need the second of these conventions, so we add a matrix to
our data frame:

     > kalythos$Ymat <- cbind(kalythos$y, kalythos$n - kalythos$y)

   To fit the models we use

     > fmp <- glm(Ymat ~ x, family = binomial(link=probit), data = kalythos)
     > fml <- glm(Ymat ~ x, family = binomial, data = kalythos)

   Since the logit link is the default the parameter may be omitted on
the second call.  To see the results of each fit we could use

     > summary(fmp)
     > summary(fml)

   Both models fit (all too) well.  To find the LD50 estimate we can
use a simple function:

     > ld50 <- function(b) -b[1]/b[2]
     > ldp <- ld50(coef(fmp)); ldl <- ld50(coef(fml)); c(ldp, ldl)

##aw, same for me

tmp0<-cbind(DO.activity.all, oxygen)
```

```
tmp1<-tmp0[tmp0$Minutes>80 ,]
DO.rising<-tmp1[tmp1$Minutes < 148,]
DO.rising$ActMat<-cbind(DO.rising$Summed, 8 - DO.rising$Summed)

Activity.probit <- glm(ActMat ~ Oxygen, family = binomial(link=probit), data =
   DO.rising)
Activity.logit <- glm(ActMat ~ Oxygen, family = binomial, data = DO.rising)

ldp <- ld50(coef(Activity.probit)); ldl <- ld50(coef(Activity.logit)); c(ldp,
   ldl)

(Intercept) (Intercept)
    50.08442    49.95866      #####these are values of oxygen (LD50 for
   activity)


#now do the above analysis for each salamander individually

#salamander 1
Activity.probit <- glm(sal1 ~ Oxygen, family = binomial(link=probit), data =
   DO.rising)
Activity.logit <- glm(sal1 ~ Oxygen, family = binomial, data = DO.rising)
ldp <- ld50(coef(Activity.probit)); ldl <- ld50(coef(Activity.logit)); c(ldp,
   ldl)
(Intercept) (Intercept)
    49.73158    49.70985

#salamander 2
Activity.probit <- glm(sal2 ~ Oxygen, family = binomial(link=probit), data =
   DO.rising)
Activity.logit <- glm(sal2 ~ Oxygen, family = binomial, data = DO.rising)
ldp <- ld50(coef(Activity.probit)); ldl <- ld50(coef(Activity.logit)); c(ldp,
   ldl)
(Intercept) (Intercept)
    51.80382    51.36695

#salamander 3
Activity.probit <- glm(sal3 ~ Oxygen, family = binomial(link=probit), data =
   DO.rising)
Activity.logit <- glm(sal3 ~ Oxygen, family = binomial, data = DO.rising)
ldp <- ld50(coef(Activity.probit)); ldl <- ld50(coef(Activity.logit)); c(ldp,
   ldl)
(Intercept) (Intercept)
    57.76966     57.4549

#salamander 4
Activity.probit <- glm(sal4 ~ Oxygen, family = binomial(link=probit), data =
   DO.rising)
Activity.logit <- glm(sal4 ~ Oxygen, family = binomial, data = DO.rising)
ldp <- ld50(coef(Activity.probit)); ldl <- ld50(coef(Activity.logit)); c(ldp,
   ldl)
(Intercept) (Intercept)
     43.9509    43.86837

#salamander 5
Activity.probit <- glm(sal5 ~ Oxygen, family = binomial(link=probit), data =
   DO.rising)
Activity.logit <- glm(sal5 ~ Oxygen, family = binomial, data = DO.rising)
ldp <- ld50(coef(Activity.probit)); ldl <- ld50(coef(Activity.logit)); c(ldp,
   ldl)
(Intercept) (Intercept)
    45.11959    44.57967
```

```
#salamander 6
Activity.probit <- glm(sal6 ~ Oxygen, family = binomial(link=probit), data =
   DO.rising)
Activity.logit <- glm(sal6 ~ Oxygen, family = binomial, data = DO.rising)
ldp <- ld50(coef(Activity.probit)); ldl <- ld50(coef(Activity.logit)); c(ldp,
   ldl)
(Intercept) (Intercept)
    64.47505    63.85089
>

#salamander 7
Activity.probit <- glm(sal7 ~ Oxygen, family = binomial(link=probit), data =
   DO.rising)
Activity.logit <- glm(sal7 ~ Oxygen, family = binomial, data = DO.rising)
ldp <- ld50(coef(Activity.probit)); ldl <- ld50(coef(Activity.logit)); c(ldp,
   ldl)
(Intercept) (Intercept)
    41.76121    41.38186

#salamander 10
Activity.probit <- glm(sal10 ~ Oxygen, family = binomial(link=probit), data =
   DO.rising)
Activity.logit <- glm(sal10 ~ Oxygen, family = binomial, data = DO.rising)
ldp <- ld50(coef(Activity.probit)); ldl <- ld50(coef(Activity.logit)); c(ldp,
   ldl)
 (Intercept) (Intercept)
    41.34704     40.9685


##########now do cessation of activity for downward part of curve

tmp0<-cbind(DO.activity.all, oxygen)
tmp1<-tmp0[tmp0$Minutes>150 ,]
DO.falling<-tmp1[tmp1$Minutes < 220,]
DO.falling$ActMat<-cbind(DO.falling$Summed, 8 - DO.falling$Summed)

Activity.probit <- glm(ActMat ~ Oxygen, family = binomial(link=probit), data =
   DO.falling)
Activity.logit <- glm(ActMat ~ Oxygen, family = binomial, data = DO.falling)

ldp <- ld50(coef(Activity.probit)); ldl <- ld50(coef(Activity.logit)); c(ldp,
   ldl)

    (Intercept) (Intercept)
    33.37326    32.84751
   #####these are values of oxygen (LD50 for activity, on the falling part of
   the curve)


#now do the above analysis for each salamander individually

#salamander 1
Activity.probit <- glm(sal1 ~ Oxygen, family = binomial(link=probit), data =
   DO.falling)
Activity.logit <- glm(sal1 ~ Oxygen, family = binomial, data = DO.falling)
ldp <- ld50(coef(Activity.probit)); ldl <- ld50(coef(Activity.logit)); c(ldp,
   ldl)
Intercept) (Intercept)
     50.82718      50.623

#salamander 2
Activity.probit <- glm(sal2 ~ Oxygen, family = binomial(link=probit), data =
   DO.falling)
```

```
Activity.logit <- glm(sal2 ~ Oxygen, family = binomial, data = DO.falling)
ldp <- ld50(coef(Activity.probit)); ldl <- ld50(coef(Activity.logit)); c(ldp,
    ldl)
 46.68295    46.66799

#salamander 3
Activity.probit <- glm(sal3 ~ Oxygen, family = binomial(link=probit), data =
    DO.falling)
Activity.logit <- glm(sal3 ~ Oxygen, family = binomial, data = DO.falling)
ldp <- ld50(coef(Activity.probit)); ldl <- ld50(coef(Activity.logit)); c(ldp,
    ldl)
(Intercept) (Intercept)
    25.50505    25.44814


#salamander 4
Activity.probit <- glm(sal4 ~ Oxygen, family = binomial(link=probit), data =
    DO.falling)
Activity.logit <- glm(sal4 ~ Oxygen, family = binomial, data = DO.falling)
ldp <- ld50(coef(Activity.probit)); ldl <- ld50(coef(Activity.logit)); c(ldp,
    ldl)
(Intercept) (Intercept)
     43.9509    43.86837

#salamander 5
Activity.probit <- glm(sal5 ~ Oxygen, family = binomial(link=probit), data =
    DO.falling)
Activity.logit <- glm(sal5 ~ Oxygen, family = binomial, data = DO.falling)
ldp <- ld50(coef(Activity.probit)); ldl <- ld50(coef(Activity.logit)); c(ldp,
    ldl)
(Intercept) (Intercept)
    31.5003     31.3735


#salamander 6
Activity.probit <- glm(sal6 ~ Oxygen, family = binomial(link=probit), data =
    DO.falling)
Activity.logit <- glm(sal6 ~ Oxygen, family = binomial, data = DO.falling)
ldp <- ld50(coef(Activity.probit)); ldl <- ld50(coef(Activity.logit)); c(ldp,
    ldl)
(Intercept) (Intercept)
 18.96997    18.91136

#salamander 7
Activity.probit <- glm(sal7 ~ Oxygen, family = binomial(link=probit), data =
    DO.falling)
Activity.logit <- glm(sal7 ~ Oxygen, family = binomial, data = DO.falling)
ldp <- ld50(coef(Activity.probit)); ldl <- ld50(coef(Activity.logit)); c(ldp,
    ldl)
(Intercept) (Intercept)
     27.47956    27.10945

#salamander 10
Activity.probit <- glm(sal10 ~ Oxygen, family = binomial(link=probit), data =
    DO.falling)
Activity.logit <- glm(sal10 ~ Oxygen, family = binomial, data = DO.falling)
ldp <- ld50(coef(Activity.probit)); ldl <- ld50(coef(Activity.logit)); c(ldp,
    ldl)
 (Intercept) (Intercept)
    27.42039    27.04798
```

Appendix 2: S-Plus script for analyzing activity in response to conductivity

```
##import 'cond.activity.txt' and name object cond.activity


##salamander 1
tmp1<-na.omit(cond.activity$Sal1activity)
tmp5<-rts(tmp1)               #make a regular time series from the data
tsplot(tmp5)
tmp2<-tmp1[c(2050:2150, 2700:2900)]

##salamander 3
tmp1<-na.omit(cond.activity$Sal3activity)
tmp5<-rts(tmp1)               #make a regular time series from the data
tsplot(tmp5)
tmp2<-tmp1[2000:3000]
height<-0.55

##salamander 4
tmp1<-na.omit(cond.activity$Sal4activity)
tmp5<-rts(tmp1)               #make a regular time series from the data
tsplot(tmp5)
tmp2<-tmp1[500:1000]
height<-0.45

##salamander 5
tmp1<-na.omit(cond.activity$Sal5activity)
tmp5<-rts(tmp1)               #make a regular time series from the data
tsplot(tmp5)
tmp2<-tmp1[c(1200:1500, 2000:2300)]
height<-0.7

##salamander 6
tmp1<-na.omit(cond.activity$Sal6activity)
tmp5<-rts(tmp1)               #make a regular time series from the data
tsplot(tmp5)
tmp2<-tmp1[c(0:200, 800:1100)]
height<-0.4

##salamander 7
tmp1<-na.omit(cond.activity$Sal7activity)
tmp5<-rts(tmp1)               #make a regular time series from the data
tsplot(tmp5)
tmp2<-tmp1[c(800:1200, 2200:2600)]
height<-0.6

##salamander 8
tmp1<-na.omit(cond.activity$Sal8activity)
tmp5<-rts(tmp1)               #make a regular time series from the data
tsplot(tmp5)
tmp2<-tmp1[1100:1700]
height<-0.5


#######after calculating tmp2 for each salamander, then do the following
   [[which sets up for bout analysis]]
tmp2.mean<-mean(tmp2)
tmp2.stdev<-stdev(tmp2)
tmp2highCI<-tmp2.mean + 5*tmp2.stdev
tmp2lowCI<-tmp2.mean - 5*tmp2.stdev
```

```
sal1.filt<-rts(ifelse(((tmp1>tmp2highCI) | (tmp1<tmp2lowCI)), 1, 0), deltat =
   0.0833333, units = "Minutes")
tmp10<-seq(from = 0, length = length(sal1.filt), by = 0.0833333)      #create a
   time sequence that goes with activity
tmp11<-data.frame(tmp10, sal1.filt)
names(tmp11)<-c("minutes", "activity")
tmp12<-tmp11[tmp11$activity==1,]           ##collapse data frame to contain
   only rows in which activity ==1
tmp13<-tmp12$minutes[2:length(tmp12$minutes)] -
   tmp12$minutes[1:length(tmp12$minutes) - 1] ##calculate intervals
tmp20<-seq(from = 0, by = 0.0833333, length = 100)
tmp15<-c()
for(i in 1:100){
   tmp16<-length(tmp13[tmp13>tmp20[i]])
   tmp15<-c(tmp15, tmp16)
   i<-i+1
}

tmp21<-data.frame(tmp20, tmp15)
names(tmp21)<-c("Minutes", "N")
param(tmp21,"lambdaW") <- 25; param(tmp21,"lambdaB") <- .5; param(tmp21,"W") <-
   400; param(tmp21,"B") <- 50
fit <- nls(log(N)~log(W*exp(-lambdaW*Minutes) + B*exp(-lambdaB*Minutes)),
   data=tmp21)
plot(tmp20, log(tmp15), xlab="Minutes", ylab ="logN")
lines(tmp20, fitted(fit))
breakpoint<-(1/(fit$parameters[1] -
   fit$parameters[2]))*log((fit$parameters[3]*fit$parameters[1])/(fit$parameter
   s[4]*fit$parameters[2]))
points(breakpoint, 2)

breakpoint


########now draw graph that calculates actual activity bouts
tmp5<-rts(tmp1, deltat=0.0833333, units="Minutes")                 #make a
   regular time series from the data
tsplot(tmp5)
tmp30<-sum(sal1.filt)         #total number of active points
par(lwd=3)                       #line width
i<-2
started<-F
for(i in 2:tmp30){

   if(((tmp12$minutes[i] - tmp12$minutes[i-1])>breakpoint) && (started==T)) {
      started<-F
      end<-tmp12$minutes[i-1]
      segments(beginning, height, end, height)
   }

   if(((tmp12$minutes[i] - tmp12$minutes[i-1])<breakpoint) && (started==F)) {
      started<-T
      beginning<-tmp12$minutes[i-1]
   }

   if((i==tmp30) && (started==T)){
      segments(beginning, height, max(tmp12$minutes), height)
   }

   i<-i+1
}
par(lwd=1)
```

```
#######################here create vectors for each salamander that contain
   either zero or 1 for each timepoint,
#depending on whether the salamander is in the activity bout or not.

get.bouts<-function(filtered.vector, bout.criterion){
   tmp31<-array(dim=c(length(filtered.vector)), 0)
   tmp10<-seq(from = 0, length = length(filtered.vector), by = 0.0833333)      #create a time se
   tmp11<-data.frame(tmp10, filtered.vector)
   names(tmp11)<-c("minutes", "activity")

   act1.time<-0                     ##this loop finds the time of the first
   activity spike.
   for(i in 1:length(tmp11$activity)){
      if(tmp11$activity[i]==1){
         act1.time<-tmp11$minutes[i]
         start.i<-i
         break
      }
   }

   for(j in start.i:length(filtered.vector)){
      if(tmp11$minutes[j] - act1.time < bout.criterion){
         tmp31[j]<-1
      }
      if(tmp11$activity[j]==1) act1.time<-tmp11$minutes[j]
      j<-j+1
   }
   return(tmp31)
}


tmp2.mean<-mean(tmp2)
tmp2.stdev<-stdev(tmp2)
tmp2highCI<-tmp2.mean + 5*tmp2.stdev
tmp2lowCI<-tmp2.mean - 5*tmp2.stdev

sal1.filt<-rts(ifelse(((tmp1>tmp2highCI) | (tmp1<tmp2lowCI)), 1, 0), deltat =
   0.0833333, units = "Minutes")
sal8c.filtered<-sal1.filt


tmp35<-get.bouts(sal1c.filtered, 2.523642)
#tmp36<   ###this salamander (#2) died during the experiment, so data not
   analyzed.
tmp37<-get.bouts(sal3c.filtered, 1.424218)
tmp38<-get.bouts(sal4c.filtered, 0.8236778)
###tmp39<-get.bouts(sal5c.filtered, 0.95)  ##this salamander had no breakpoint,
   so no activity bouts
tmp40<-get.bouts(sal6c.filtered, 2.275069)
tmp41<-get.bouts(sal7c.filtered, 1.03553)
tmp42<-get.bouts(sal8c.filtered, 1.000058)

#now add up all the traces to get total activity

tmp45<-tmp35 +  tmp37  + tmp38 + tmp40 + tmp41 + tmp42

#vector for minutes
tmp43<-seq(from = 10, by = 0.0833333, length = 3816)


#make a datga.frame to export to excel
```

```
cond.activity.all<-data.frame(cbind(tmp35, tmp37, tmp38, tmp40, tmp41, tmp42,
   tmp43, tmp45))
names(cond.activity.all)<-c("sal1", "sal3", "sal4",  "sal6", "sal7",
   "sal8","Minutes","Summed")

exportData(cond.activity.all, file="cond.activity.all.xls")


##try loess fit w/ SE

cond.loess <- loess(Summed~Minutes,data=cond.activity.all, span=.3)
plot.loess(cond.loess, confidence = 100, coverage=0.99, ylim=c(0,7))
points(cond.activity.all$Minutes, cond.activity.all$Summed)

exportData(fitted(cond.loess), "cond.loess.xls")
```

Appendix 3: S-plus code to test for differences in metabolic rate as a function of conductivity . (Individual salamanders are specified as random effects)

```
tmp1<-MR.conductivity
tmp2<-tmp1[tmp1$subset!="c",]

tmp3<-lme(MR~cond, random=~1|salamander,data=tmp2)

summary(tmp3)
Linear mixed-effects model fit by REML
 Data: tmp2
        AIC      BIC   logLik
  -56.28007 -51.9159 32.14003

Random effects:
 Formula:  ~ 1 | salamander
          (Intercept)   Residual
StdDev: 0.00002663555 0.05015547

Fixed effects: MR ~ cond
                 Value  Std.Error DF   t-value p-value
(Intercept)  0.1472223 0.02669036 17  5.515938  <.0001
       cond -0.0267978 0.02055345 17 -1.303809  0.2097
 Correlation:
     (Intr)
cond -0.924

Standardized Within-Group Residuals:
       Min         Q1      Med        Q3      Max
 -2.164694 -0.4292405 0.056266 0.5233264 2.008579

Number of Observations: 24
Number of Groups: 6
```