REPORT ARCTAQUA

INTRODUCTION:

In the following, outcome of activities of ***Work package 2 Nutrition***, ***Activity 2.1. Larvae and fry feeding behaviour and nutrition*** is reported. Originally three subtasks were included in the project plan, but for reasons lined out in the following, only two of these planned subtasks were ultimately carried out:

Initially, planned activities were scheduled to run from spring 2020 till summer 2021, but progress suffered from a series of unexpected events: Administrative and shipment delay of critical research equipment (behavioural tracking equipment) during late 2019 / early 2020 delayed installation, training and pre-testing of this equipment.
The Corona pandemic from March 2020, with its subsequent societal shut-down, further increased this delay and also seriously affected purchase of further technical components as well as the rigging and testing of experimental facilities and experimental designs.
Moreover, the first shipment of wolffish eggs in spring 2020 suffered fatal mortality due to corona-related shipment issues, and finally, in spring 2021, a fatal accident at our research facilities led to another event of total egg mortality.
Thus in sum, activities planned to be carried out on several egg batches over a time span of over two years, now had to be carried out using one single egg batch during late spring / early summer 2022. This led to limited time for preparation and handling of all planned activities. Together with practical issues with design of planned feed trials and a need to ensure manageable work load for staff during the three full weeks "experimental season", subtask 2.1.2 was removed from the research program, leaving the following sub tasks of Activity 2.1 to be carried out:

2.1.1. Optimize the visual environment at first feeding for wolffish
Here, we examined effects from light intensity and wavelength on chosen behavioural measures in two experiments:

- 2.1.1.a Micro scale light experiment
- 2.1.1. b Phototaxis light experiment

2.1.3 Chemo attractants in feed for wollfish
Here, we examined effects from chemo attractants - selected amino acids – to search for chemical cues that stimulate appetite to ultimately improve feed intake and production success.

- 2.1.3 a Micro scale scent experiment
- 2.1.3 b Small scale scent experiment*****

**Activity 2.1. 3 b suffered from practical design issues, with consequences for data analysis in terms of pseudo replication and dependency of observations. Though, also this design is shortly described and reported here, due to a few generally relevant findings.*

Generally, all of these listed activities aimed to support optimization of chosen environmental rearing factors from hatch till the juvenile stage by exploring effects from these factors on foraging related behaviour, to ultimately guide improvements in future production of viable juveniles. The approach was addressed by micro- and small scale early stage screening designs, using established software solutions to analyse behavioural responses from recorded videos.

BASIC DESIGN AND REARING OF EGGS AND WOLFFISH PRIOR TO ALL TRIALS

Eggs: Eggs were collected from 1 female and fertilized by sperm from 3 males by Nord University standard procedures for stripping and fertilization (Eggen, B, pers.com).

6000 fertilized eggs were placed in each of two 16 litre incubators and incubated at 7.0 °C in complete darkness and a water flow of 160l/h until hatching at approximately 890 °days the 10th of June 2022. Egg mortality during incubation was approximately 78 and 89 % respectively until hatch.

Larvae: The totally 750 hatched larvae were pooled together and transferred to a hatching tray (0.4 x 0.4 x 0.1 m LxBxH) placed in a light grey raceway (2.7 5x 0.42 x 0.16 m LxBxH) lit by rooflight, 24:0 light, prior to use in experiments (Figure 1; 1). The raceway was supplied with 33 ppt saltwater at temperature 9.3 +- 0.2°C until all experiments were complete at the 30th of June. During rearing, larvae were fed 6 times daily by hand distributing a thin layer of feed at the surface. Two experimental dry feeds, "A" (0.7-1.0 mm) and "B" (1.0-1.5 mm) were used, with A:B ratio of 30/70 the first week, 20/80 at week 2 and 10/90 during week 3, according to feeding protocols at Nord (Eggen, B, pers.com). The rearing compartment was cleaned daily in accordance with protocols.

Preparation for experiments: The day before experiments, the required no. of larvae was sampled by randomly picking healthy looking individuals from the hatching tray at about 4 PM and transporting them to temperature controlled experimental facilities (Figure 1; 2) in a light-tight container for acclimation overnight ,without feed and in darkness. Just prior to experiments on the next day, individual larvae were placed in petri dishes with sea water of the same quality and temperature as in the hatching tray, and stored in a light-tight container until taken one by one for experimental trials. Each experimental day, trials took place from 08:00 AM until completion.

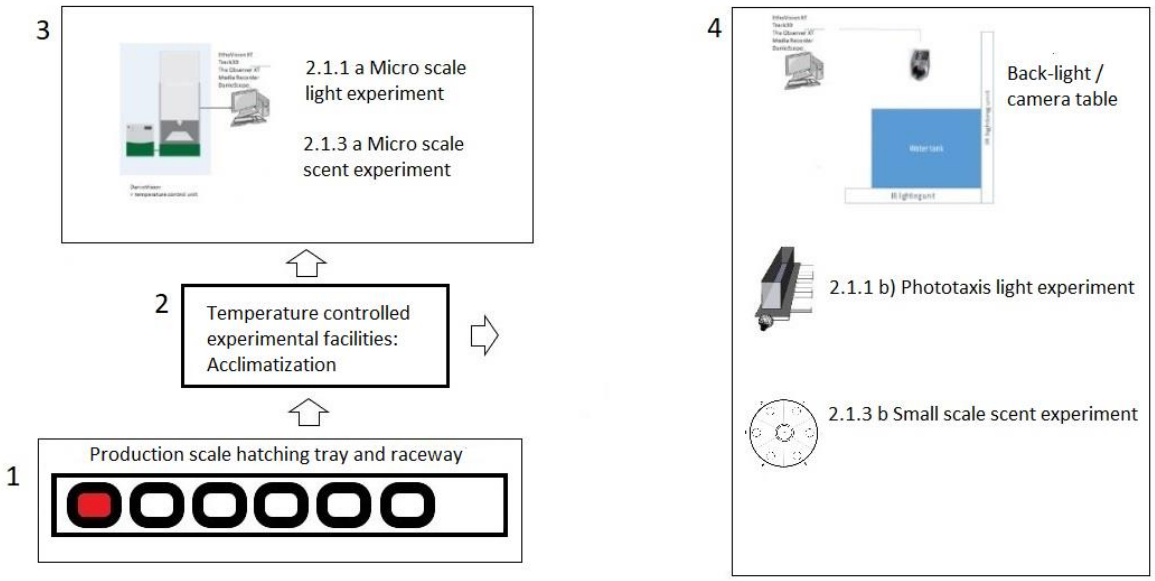

FIGURE 1: *Overview of research infrastructure and work flow: 1) Hatching tray (red), placed in a raceway (inner black rectangle). 2) Overnight storage in temperature controlled room 3) Microscale experiments (activities 2.1.1 a and 2.2.3 a) using DanioVision observation chamber, temperature control and EthoVision software. 4) Activities 2.1.1 b and 2.1.3), in dedicated experimental tanks, using UV back-light, external cameras end EthoVision software for recording trials for later analyses.*

OVERALL EXPERIMENTAL SCHEDULE:

To enable examining for potential age specific differences in larval responses to experimental treatments, each of the four experimental activities were repeated on a weekly during the three first weeks post hatch, starting with activity 2.1.1 a, at 3, 10 and 17 days post hatch (DPH) (Table 1).

TABLE 1: Detailed overview of experimental activity schedule showing age in days post hatch (DPH):

DPH	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
2.1.1 a Micro scale light experiment																				
2.1.1 b Phototaxis light experiment																				
2.1.3 a Micro scale scent experiment																				
2.1.3 b Small scale scent experiment																				

BEHAVIOURAL REGISTRATION SOFTWARE AND HARDWARE:

All individual trials of all four experiments were video recorded using MediaRecorder software. Ethio Vision XT 15 video tracking software (Noldus IT, Wageningen, Netherlands) was used to track movements of individual larvae in videos and extract behavioural measures within the defined experimental units - “arenas” – of the different experiments.

Micro scale experiments (2.1.1 a and 2.1.3 a), were carried out in petri dishes in a Daniovision Observation Chamber equipped with a Danio Vision Toplight Unit to manipulate light settings in experiment 2.1.1 a. A Danio Vision TCU turbo cooling unit controlled water temperature during trials.

The experiments 2.1.1 b and 2.1.3 b were carried out in different dedicated experimental units, and were recorded from above using an external GigE industrial standard IR sensitive camera with Kowa 4.1-11 mm lens. Experimental units were placed on an 80x80 cm IR Backlight array to provide contrast and enable “dark” recording. Both experiments took place in a temperature controlled room to control water temperature. Walls were black to reduce light reflections.

POST EXPERIMENT PROCEDURES, ALL EXPERIMENTS:

Following all trials, individual fish were euthanized with an overdose of buffered MS 222, weighted to the nearest mg and photographed for later length measurement to the nearest 0.1 mm using ImageJ software.

REPORTING OF SPECIFIC RESEARCH ACTIVITIES

ACTIVITY 2.1.1 a MICRO SCALE LIGHT EXPERIMENT

GOAL: Explore if / how light intensity and wavelength affects measures of activity and spatial positioning of wolffish larvae.

DESIGN AND METHODS:

This was a micro scale pilot study / screening approach with factorial designs, repeated weekly at ages 3, 10 and 17 DPH. Treatment factors were light intensity at 3 levels; 10, 100 and 1000 lux, and wavelength at four levels; white (mixture), red (623nm), green (525nm) and blue (470nm). Each treatment factor combination was duplicated. Light was provided by the Daniovision Top Light unit.

The experimental units ("arenas") were 90 mm diameter, 27 mm height petri dishes filled with appr. 100 ml 9°C seawater, containing one single fish. In Ethio Vision *Arena Settings*, a mid-point and an inner zone constituting 50 % of the total petri dish area were defined, to enable assessing larval position relative to these locations.

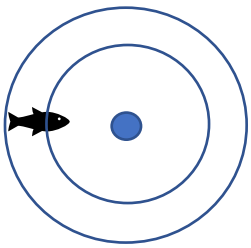

FIGURE 2: *Experimental arena schematic. Outer circle shows walls of the 90 mm diameter petri dish, and delimits the area from which behavioural registration took place. Central filled circle is the mid-point of petri dish, and Inner circle shows the 50% innermost area of the dish (radius 31.8 mm), as well as defines the outer zone between inner and outer circle.*

In Ethio Vision *Trial Control settings*, recording was set to 15 minutes per single trial; the first 5 minutes with light OFF, then 5 minutes with light ON and at last 5 more minutes OFF. This enabled examination of change in behaviour due to both introduction and removal of light for the different combinations of intensity and wavelength of the light source.

At each age, a complete design of 24 individual trials - one at the time - was run in random order. At onset, petri dishes with fish was transferred in dark to the Danio Vision Observation Chamber one at the time, and recordings were then performed according to the design scheme and stored for later data extraction.

Data acquisition and pre-treatment: Two different *Data Profiles* were set in Ethio Vision for extracting trial statistics: 1) Extraction per 1 min. period throughout a trial and 2) Extraction per each of the three 5 min. periods defined by changed light conditions. Initially, 6 response variables were included in the Analysis Profile and extracted. As several of these correlate due to arriving from the same fundamental registrations (spatial position and time) correlation analysis was done to reduce the no. of variables used further in analysis. Non-correlated variables representative of main aspects of behaviour that may affect feeding behaviour, were then chosen for further analyses: Distance moved (measure of general activity), Absolute angular velocity (measure of turning per unit time / searching activity) and Time Outer zone (measure of cumulative time in outer zone / thigmotaxis).

To illustrate larval positioning in experimental units, heat maps were generated in Ethio Vision. Data extracted per 5 min. period were used for statistical analyses. Heat maps and initial graphical exploratory analyses by age showed high between-trial variability of response variables already before exposure to treatments, so response variables were standardized by re-calculating from the observed raw values per 5 min. period to *change in values* between these 5 min. periods, as follows (T= time period, n=numbering of 5 min. time periods from onset of trial (T1=first period,T2=second):

- Change, Absolute Angular Velocity: Absolute angular velocity Tn+1 – Absolute angular velocity Tn (positive change is regarded indication of increased searching activity).
- Change, Distance moved (mm): (Distance moved T_{n+1} - Distance moved T_n) / Distance moved T1 (change standardized relative to movements first 5 min, positive change = increased movement)
- Change, Time outer Zone (s): Time in outer zone T_{n+1}-Time outer zone T (positive change = increased presence in outer zone)

Statistical analyses: To establish quantitative factorial designs for analyses, trials using white light (a mixture of wavelengths) as treatment were kept out, giving 3x3 duplicated designs of total size N=18. Analyses used graphical exploration options and statistical analyses of the statistical package Minitab 21. The *DOE – Factorial* procedures, were used separately for each of the three response variables, at the different larval ages. Model assumptions of normality and within group variability were assessed from normal plots and residual vs. fit plots, the direction of main effects was judged by factorial plots, and significance of treatment factors and 1. order interactions was evaluated by pareto plots and ANOVA tables t at α=0.05. Gross change in response variables due to turning on or off light – when ignoring treatment factors - was evaluated at all ages by paired samples t-tests, H0: Difference=0, α=0.05.

ACTIVITY 2.1.1 b PHOTOTAXIS LIGHT EXPERIMENT

GOAL: Explore effects from light intensity and wavelength on phototaxis and spatial positioning

DESIGN AND METHODS:

This was a small scale pilot study / screening approach using factorial designs, repeated weekly at ages 4, 11 and 18 DPH. Treatment factors were light intensity; approx. 10, 100 or 1000 lux set at centre of the experimental tank), and wavelength; white (mixture), red (625nm), green (530nm) and blue (470nm). Each factor combination was duplicated. At each age, a complete design of 24 individual trials - one at the time - was run in random order.

Light was provided by a 24 V DC narrow bandwidth led light source; MOBL -150x150-RGBW, Smart Vision Lights™), and wavelength was set by a 4WMD control unit (Smart Vision Lights™). Light intensity was set in advance of each individual trial by adjusting distance of the light source vs. experimental unit, to obtain the intensity of incoming light that gave intended intensity levels at the centre of the unit.

The experimental unit was a rectangular aquarium in transparent polycarbonate, 60Lx15Hx10W cm, of max volume 9 L. Walls were covered with dark plates except for at the tank floor and the short-end receiving the light. Before trials, the unit was cleaned, re-filled with 5 L salt water and placed at a fixed marked position on the IR Backlight array for filming from above. At each trial, individual fish were transported in dark (by covering the light source) to the acclimation chamber; a non-

transparent, thin walled PVC pipe, (D= 6.3 cm, height ≈15cm) at the tank center. Recording started automatically when fish were detected by the EthioVision software in the acclimation chamber.

FIGURE 3: Schematic drawing of experimental unit design. Treatment (light) enters through one end of the aquarium only. For each trial, an individual fish was placed in the centered, circular acclimation chamber for 5 minutes, then released and its movements recorded by video tracking.

In *Trial Control settings*, recording was set to 15 minutes totally per trial; First 5 minutes in dark in the acclimation chambers, then fish were released and filmed for 10 minutes during light treatment.

In EthioVision Arena Settings, three different *zone groups* were established to register and evaluate positioning of larvae in response to the light source position. The different zones were constructed by dividing the aquarium area into 2, 3 or 10 equally sized areas respectively, by dividing the aquarium length as follows:

Zone group 1: Two zones. Light zone vs. Dark Zone (Light zone towards light source)
Zone group 2: Three zones. Light Zone vs. Neutral vs. Dark Zone (Light zone towards light source)
Zone group 3: Ten zones, numbered 1-10 (Zone 10 closest to light source, zone 1 furthest apart)

Data acquisition and pre-treatment:

The EthioVision Data Profile was programmed to start recording by auto-detection of larval movements. For individual trials, recorded data was split between the acclimation (5 min) and treatment period (10 min) by nesting. In the Analysis Profile, response variables for raw data, were chosen as follows:

TABLE 2: Raw data variables obtained by EthioVision XT.

Dependent variable	Description
Distance moved	Total distance moved (mm) of center-point of individual larvae, obtained separately for each of the two time sequences
In zone, frequency	No. of times centre point of an individual larvae is observed in a given zone of a given zone group, obtained separately for each two time sequences
In zone, time	Cumulative time the centre point of individual larvae is observed in a given zone of a given zone group, obtained separately for each two time sequences

Response variables: Their examination, transformations and selection:

Introductory graphical analysis of the variable Distance Moved indicated high inter-individual variability already before treatments were applied. Thus, to reduce effects from initial variability, raw values were re-calculated to a measure of change: *Relative change distance moved = ((Distance moved Light/2) – Distance moved acclimation) / Distance moved acclimation.*

The two “In zone” variables included only observations *during the last 10 min. of each trial*. For analyses, derived variables of spatial distribution were calculated from these variables - for the different zone groups - as follows:

TABLE 3: Derived response variables calculated from EthioVision XT raw data variables.

Dependent variable	**Zone group**	**Derived variables:**
In zone, frequency	1	**Relative fraction observations in Light zone** = No. observations, Light zone / Sum observations, both zones. Outcome range 0-1, values >0.5 indicate positive, <0.5 indicate negative taxis.
	2	Relative fraction observations in Light zone = No. observations, Light zone / Sum observations, all three zones (Light/ Neutral/ Dark)
	3	**Index, Frequency**: For each 10 zones, a zone score value from -5 to 5 (position relative to light source) is multiplied by no. of observations. Outcome over all zones summed and divided with the summed no. of observations over all zones. Outcome range -5 to 5, >0 indicates positive taxis, <0 negative.
In zone, time	1	**Relative time in Light zone** = Cumulative time, Light zone / Sum cumulative time, both zones. Outcome range 0-1, values >0.5 indicate positive, <0.5 indicate negative taxis.
	2	Relative time in Light zone = Cumulative time, Light zone / Sum cumulative time, all three zones (Light/ Neutral/ Dark)
	3	**Index, Time:** For each 10 zones, a zone score value from -5 to 5 (position relative to light source) is multiplied by time in zone. Outcome over all zones summed and divided with the summed time registered over all zones. Outcome range -5 to 5, >0 indicates positive taxis, < 0 negative.

Derived variables of Zone group 2 correlated strongly with distance moved (indicator of individual activity level), and were discarded from further analyses. The remaining derived variables of zone groups 1 and 3 (in bold in table) showed no significant correlations with distance moved and were kept for further statistical analyses.

Statistical analyses: To establish quantitative factorial designs for analyses, trials using white light (a mixture of wavelengths) as treatment were kept out, giving 3x3 duplicated designs of total size N=18. Analyses used graphical exploration options and statistical analyses of the statistical package Minitab 21. The *DOE – Factorial* procedures, were used separately for all response variables at the different larval ages. Model assumptions of normality and within group variability were assessed from normal plots and residual vs. fit plots, and outliers were tested by Grubb´s test. The direction of main effects was judged by factorial plots, and significance of treatment factors and 1. Order interactions was evaluated by pareto plots at $\alpha=0.05$. Gross change in distance moved between the acclimation and treatment periods was assessed by Wilcoxon Signed rank test, H0: Difference=0, $\alpha=0.05$. To test for overall signs of taxis behaviour from derived variables, when disregarding treatment factor effects, one-sample t-tests or a non-parametric median test equivalent (Wilcoxon signed rank test) at $\alpha=0.05$, testing the following hypotheses of overall mean values:

- Relative fraction observations / relative time in Light Zone: H0: $\mu=0.5$, H1: : $\mu\neq0.5$
- Index frequency /index time: H0: $\mu=0.0$, H1: : $\mu\neq0.0$

ACTIVITY 2.1.3 a MICRO SCALE SCENT EXPERIMENT

GOAL : Explore if / how chosen amino acids added to water affect measures of activity and spatial positioning of wolffish larvae.

DESIGN AND METHODS:

This was a micro scale pilot study / screening approach with factorial designs, repeated weekly at ages 3, 10 and 17 DPH. The treatment factor was the addition of different amino acids; alanine, arginine, glutamic acid and glycine, pure saltwater (control) or no addition (treatment control). This was thus a one-factor design at 6 factor levels, each replicated with n= 6, giving overall N of 36 per age. Within ages, individual trials were run in randomized order.

In Ethio Vision *Arena Settings*, a mid-point and an inner zone constituting 50 % of the total petri dish area was defined, to enable assessing larval position relative to these locations (see Figure 2).

The experimental units ("arenas") were prepared in the morning the days of the experiments. These were 90 mm diameter, 27 mm height petri dishes filled with appr. 90 ml 9°C seawater and containing a single fish per trial.

All trials took place in darkness. In Ethio Vision *Trial Control settings*, recordings in IR lighting were set to 15 minutes per trial. At onset of each trial, an experimental unit was transferred in dark to the Danio Vision Observation Chamber and recording started. After 5 min. of recording, treatment was added by opening the front lid of the Danio Vision chamber, and injecting 10 ml pre-made solutes (10^{-3} M) amino acids, pure saltwater. Injection was done by carefully pipetting the solute centrally into the petri dish in one ml portions over a 1 min. time period. For treatment controls, recordings were done without opening the chamber lid. This approach enabled later examination of change of behaviour due to introduction of treatment, change during the 10 min. treatment period as well as between-treatment comparisons. Recordings were performed according to the design scheme and stored for later data extraction and analysis.

Data acquisition and pre-treatment: Two different *Data Profiles* were set in Ethio Vision for extracting trial statistics: 1) Extraction per 1 min. period throughout the trial and 2) Extraction per each of three 5 min. periods: 0-5 min (acclimation), and 5-10 / 10-15 min. (both post-treatment).The same raw variables as in activity 2.1.1 were extracted from recordings for the time periods defined by data profiles: Distance moved (general activity), Relative angular velocity (circularity of movements) and Time Outer zone (cumulative time in outer zone / thigmotaxis).

Statistical analyses:
Data profile1 (per. minute) data for distance moved was examined graphically to check for possible method effects from injection of solutes and to examine distributional patterns of data. For statistical inference of between time-interval differences, Kruskal-Wallis analyses were run at each age.
Data profile 2 response variables (first 5 min. data only) showed no correlation when analysed by age (Pearson correlation), so all three variables were kept for further analyses..
To account for an injection (method) effect and variability between individuals before injections, response variables were re-calculated to measures of change between the first and last 5 min. period, excluding the mid period. Relative angular velocity was transformed to absolute velocity, a measure of search activity. Following assumption tests (Kolmogorov-Smirnoff and Levene tests), treatment effects were tested by one-way ANOVAs or Kruskal-Wallis tests, respectively. Overall change in responses, when not taking treatment effects into account, was examined by one-sample t-test or Wilcoxon signed rank tests. Significance level was set at 5 % for all tests.

ACTIVITY 2.1.3 b SMALL SCALE SCENT EXPERIMENT

GOAL: Explore if solutes / scents from different feed sources cause chemo-attraction in wolffish larvae, using an experimental scale choice chamber

DESIGN AND METHODS:

This was a small scale pilot study, repeated weekly at ages 6, 13 and 20 DPH. It was a choice / preference study, formally designed as a "quasi" non-replicated randomized block. In all individual trials (blocks), treatment levels were randomly distributed among defined areas (sectors) to allow fish to choose preferred areas. The treatment was addition of six solutes; 4 amino acids (alanine, arginine, glutamic acid and glycine), a solute of the fish feed used for rearing and a control (pure seawater). Seawater was used for all solutes. Concentration of amino acid solutes was 10^{-3} M. The feed solution was prepared from 1 g fish feed per L, dissolved in seawater overnight. All solutes were kept at 9 C prior to use.

The experimental arena was a 23 cm diameter glass crystallization disk with transparent walls and flat bottom. A 63 mm Φ, 1 cm high PVC tube was placed in the center. The bottom area was further divided into six 60 ° sectors (numbered 1-6 in figure 4) by attaching 1 cm height plexiglass barriers from disk walls towards the central PVC tube. At each trial, an acclimation chamber (50 mm Φ PVC tube, 15 cm height) was placed inside the center tube. Within the six 60° sectors, 32 mm PVC tubes with closed bottoms were placed, to distribute scent solutions to sectors. Each tube had a 1.5 mm hole at the base, covered with 200 µm plankton net, to allow solutions to gently distribute from the tube down into its respective bottom sector.

At each individual trial, the experimental arena was filled with 1 L of 9 ° C seawater and placed at a preset position on the IR light-board. Individual fish were transported in dark to the acclimation chamber, and a lid was put on to allow working conditions to distribute scents. Scents were randomly assigned to the different sectors by pipette. After 5 min. acclimatization, light was then adjusted according to experimental plan (see own section on light), and the acclimation tube was removed to allow fish to move freely between sectors. Recording started when fish were detected by the EthioVision software. The arena was cleaned and refilled with new seawater between each run.

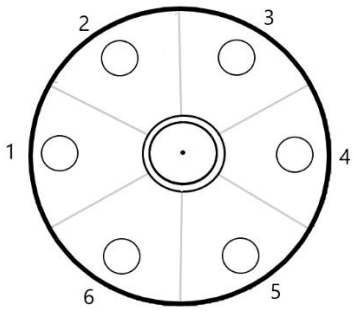

FIGURE 4 : Schematic drawing of experimental arena, seen from above: Outer circle: Crystallization disk walls. Inner, larger circle: 63 mm Φ PVC tube. Inner, small circle: 50 mm Φ PVC acclimation chamber. Sectors 1-6: 60 ° sections of bottom area. Six peripheral circles: 32 mm PVC tubes to distribute scent solutions to the sectors.

Light: Originally, it was planned to run experiments in dark (only IR light) to avoid confounding effects from visual environment on responses to scents. Though, at onset of the first experiment (6 DPH), minimal movement was observed, so for this first experiment it was decided to alternate the presence of light (ambient ceiling light) and darkness between trials, to enable to examine effects of the presence of light. Based on observations from this experiment, the remaining two experiment (13 and 20 DPH) were run primarily with light on (16 trials), but including 4 trials as dark control.

In *Trial Control settings*, EthioVision was programmed to start recording when detecting larval movements, and then record over a time period of 10 min. per trial.

In Arena Settings, a zone group of 6 zones, constructed in accordance with the sectors of the experimental arena, was constructed, to enable data extraction for all individual sectors.

Data acquisition and pre-treatment:

In *Data Profile*, the following statistics for whole tracks (10 minutes of recording) were extracted of from recordings of all individual trials at each age: *Cumulative time in zone (s), No. of times entering zone, Distance moved in zone (cm).*

To analyse for gross effects from light (on / off), disregarding treatment effects, *Distance moved* data for all zones was summed within individual trials prior to analyses.

To analyse for treatment effects, visits of very short duration in a zone (passing through, cumulative time in zone < 5 sec.) were not included in estimates of no. of times entering a zone. No adjustments were made to the variable Distance moved in zone.

Statistical analyses:
Gross effects due to light conditions (on / off) on distance moved was first examined graphically at all ages using dot plots, and tested statistically by Kruskal-Wallis tests. Due to reduced activity in dark, all further analyses included data from trials run in light only.
Number of zones visited by individual larvae at the different ages was examined by dot plots.
Possible dependency in responses between neighbour zones was examined by dot plots of distance moved per. zone position for all individuals trials, at each age.

Effects both from zone position and from treatment on the two response variables *Distance moved in zone* and *No. of times entering zone* was analysed graphically by dot plots and statistically by Kruskal-Wallis tests.

RESULTS:

ACTIVITY 2.1.1 a MICRO SCALE LIGHT EXPERIMENT

Initial graphical exploratory analyses by age:

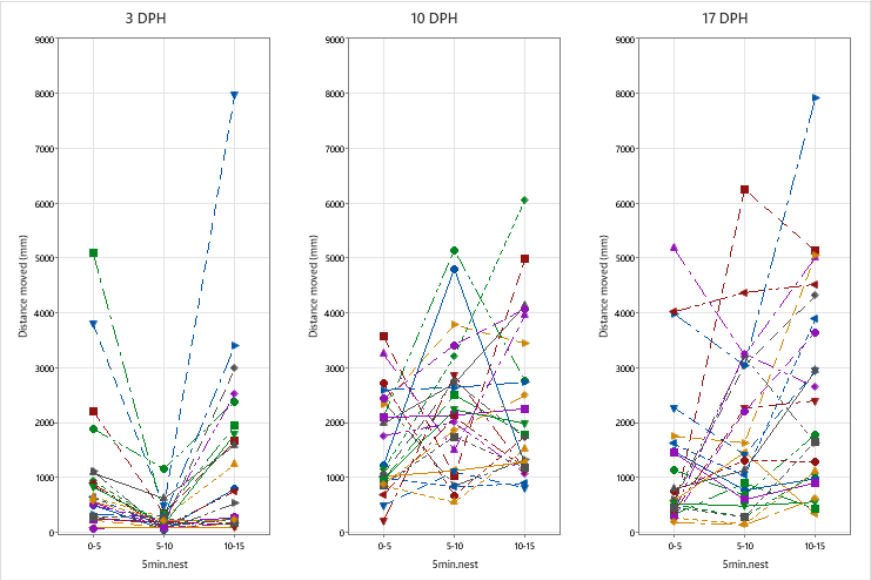

FIGURE 5: Distances moved for wolffish larvae during individual trials (N=18). Average values obtained at three 5 min. intervals; 0-5, 5-10 and 10-15 min., at age 3, 10 and 17 DPH respectively.

Graphical exploration of larval position in experimental units:

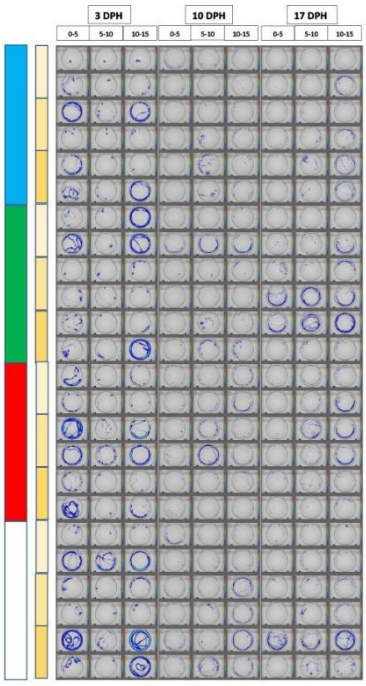

FIGURE 6: Heat-plot of larval position in circular experimental units at three time intervals (0-5=light off, 5-10=light on, 10-15=light off). Each row shows individual runs (N=24) at the different intervals, for three larval ages; 3, 10 and 17 DPH). Leftmost colour code indicates light wavelengths, while colors to the right, from top downwards, indicate light intensity (10, 100 and 1000 lux, respectively).

Factorial analyses of per age change in the response variables Absolute angular velocity, distance moved and time in outer zone in response to treatment factors wavelength and light intensity, are shown in table 4. The table also indicates the overall change when turning on / off light, when disregarding treatment factors (arrows):

TABLE 4: Change in response variables following onset and turning off the light, respectively, at different ages. The level of significance of treatment factors Wavelength and Intensity, and their 1. order interaction, as judged by factorial analysis, is indicated by red asterix ($p \leq 0.05$), filled circle (trend; $0.05 < p \leq 0.1$) or ns ($p > 0.1$). Arrows indicate increase upward) or decrease (downwards) of distance moved, time use in outer zone and absolute angular velocity, when effects from treatments are disregarded (red= significance, black= trend, no fill= p>0.1)

		LIGHT ON				LIGHT OFF		
	Wavelength	**Intensity**	**Interaction**	**Overall change**	**Wavelength**	**Intensity**	**Interaction**	**Overall change**
DPH	***CHANGE DISTANCE MOVED***							
3	ns	ns	ns	↓	ns	ns	ns	↑
10	ns	ns	ns	↑	ns	ns	ns	⇩
17	*	ns	*	↑	ns	ns	ns	⇧
	CHANGE TIME USE OUTER ZONE							
3	ns	ns	ns	⇩	ns	ns	ns	↑
10	ns	ns	ns	↑	ns	ns	ns	↑
17	•	ns	ns	↓	ns	ns	ns	↑
	CHANGE ABSOLUTE ANGULAR VELOCITY							
3	ns	•	ns	↑	ns	•	ns	⇩
10	ns	ns	ns	⇧	ns	ns	ns	↑
17	•	ns	*	⇩	ns	ns	ns	↑

ACTIVITY 2.1.1 b PHOTOTAXIS LIGHT EXPERIMENT

TABLE 5: Summary results for *Relative change distance moved* from acclimation to treatment period. For factorial analyses, significance level of treatment factors Wavelength and Intensity, and their 1. order interaction is shown by red asterix ($p \leq 0.05$), filled circle (trend; $0.05 < p \leq 0.1$) or ns ($p > 0.1$). For analyses of overall change due to treatment - disregarding factor levels - results are shown by arrows (upward arrow = positive change, black filled arrow= trend, red arrow= significant at p=0.05).

DPH	Wavelength	Intensity	Interaction	Overall change
4	ns	ns	●	⇧
11	ns	ns	ns	⇧
18	ns	ns	ns	⇧

TABLE 6: Summary results for *Fraction observations* and *Fraction time*. Values per trial were fractions of number of observation (a) and cumulative time (b), spent in the zone closest to the treatment light source, relative to the sum of both two zones. Significance levels of treatment factors and their 1. order interaction are shown by red asterix ($p \leq 0.05$), filled circle (trend; $0.05 < p \leq 0.1$) or ns ($p > 0.1$). Deviation from 1:1 ratio between the two zones – when disregarding treatment effects - are shown by arrows (upward = positive phototaxis - positioning towards light, black filled arrow=trend, red arrow=significant at p=0.05, open arrow=not significant).

a)	***Fraction observations***			
DPH	**Wavelength**	**Intensity**	**Interaction**	**Phototaxis**
4	**ns**	**ns**	**ns**	⇧
11	**ns**	**ns**	**ns**	⇩
18	**ns**	**ns**	**ns**	⇩
b)	**Fraction time**			
DPH	**Wavelength**	**Intensity**	**Interaction**	**Phototaxis**
4	**ns**	**ns**	**ns**	⇩
11	●	**ns**	**ns**	⇩
18	**ns**	**ns**	**ns**	⇧

TABLE 7: Summary results for *Index Frequency* and *Index time* at age Significance levels of treatment factors and their 1. order interaction are shown by red asterix ($p \leq 0.05$), filled circle (trend; $0.05 < p \leq 0.1$) or ns ($p > 0.1$). Deviation of indexes from 0 – when disregarding treatment effects - are shown by arrows (upward = positive phototaxis positioning toward light, black filled arrow=trend, red arrow=significant at p=0.05, open arrow=not significant).

a)	**Index Frequency**			
DPH	**Wavelength**	**Intensity**	**Interaction**	**Phototaxis**
4	*	ns	ns	⇧
11	ns	ns	ns	⇩
18	ns	ns	ns	⇧
b)	**Index Time**			
DPH	**Wavelength**	**Intensity**	**Interaction**	**Phototaxis**
4	ns	ns	ns	⇧
11	ns	ns	ns	⇩
18	ns	ns	ns	⇧

ACTIVITY 2.1.3 a MICRO SCALE SCENT EXPERIMENT

At all ages, injection of scents increased movement in larvae, seen as a generally strong increase of distance moved during interval 6, the one-minute time period when scents were physically injected into the experimental arena. Treating time intervals as factor levels, a strongly significant difference was seen at all ages (Kruskal-Wallis tests). Generally, at each age, activity levels in larvae divided into two main groups; a majority of modestly active larvae was seen, particularly at 5 and 12 DPH, while a smaller group of larvae were strongly active, already prior to treatment (FIGURE 7).

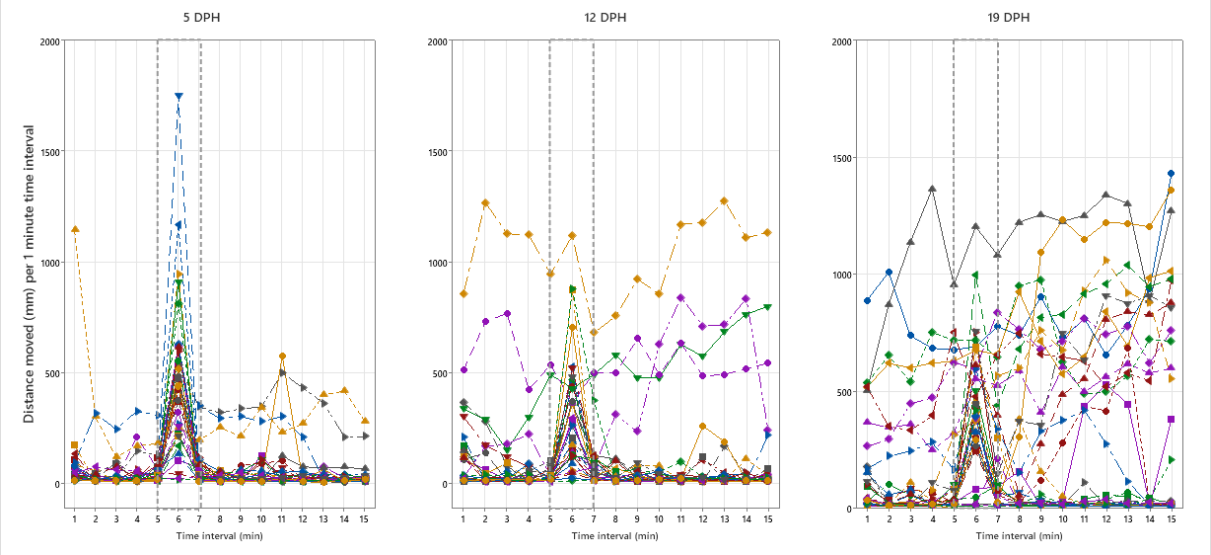

FIGURE 7: Distance moved (mm) in individual trials at ages 5, 12 and 19 DPH, plotted for one-minute time intervals 1-15. Within each age, individual trials have unique symbol x colour combinations. The area between vertical dashed lines complies with the time intervals of administration of the treatment by injection of scent solutes by pipette.

TABLE 8: Analysis of treatment effects on response variables at all three ages. Kruskall-Wallis test

RESPONSE VARIABLE	DPH	DF	H-Value	P-Value
Change of cumulative distance moved (mm)				
	5	5	9,23	0,100
	12	5	2,05	0,842
	19	5	6,02	0,305
Change of absolute angular velocity (deg)				
	5	5	2,18	0,824
	12	5	1,06	0,958
	19	5	4,06	0,541
Change of time used in outer zone(s)				
	5	5	7,61	0,179
	12	5	5,59	0,349
	19	5	5,64	0,343

Overall change, disregarding treatment effects, was not significant at any age for distance moved, while absolute angular velocity showed highly increased values at all ages ($p<0.001$). Time in outer zone was significantly reduced only at 12 DPH($p<0.001$), with no significant changes at 5 and 19 DPH.

ACTIVITY 2.1.3 b SMALL SCALE SCENT EXPERIMENT

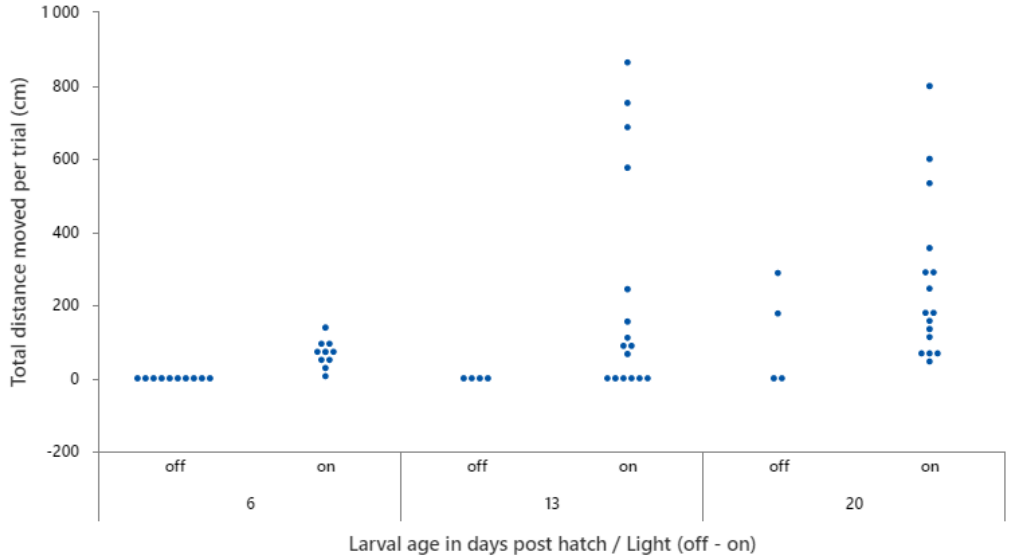

FIGURE 8: Distance moved at different ages in response to light conditions (on / off).

The plot shows increased activity with light on at all ages. At days 6 and 13 DPH, no movement was seen in trials run in dark. Kruskal-Wallis analyses showed highly significant effect from light on distance moved at 6 DPH (Df=1, H= 14.3, p<0.001), significant also at 13 DPH (Df=1, H=4.1, p=0.043), while at 20 DPH the effect was not statistically significant (Df=1, H=1.75, p=0.186).

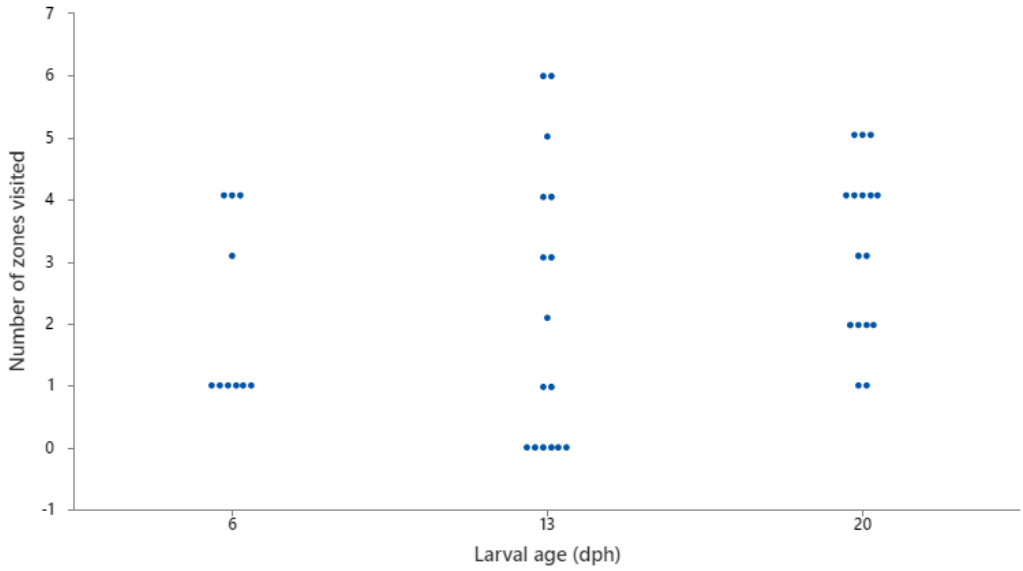

FIGURE 9: Number of zones visited per individual larvae / trial at the different ages. Only data from trials run in light are included. Number of trials included at ages: 10 / 16 / 16.
Plot shows a highly variable no. of zones visited at all ages, in most cases less than four - indicating it is questionable to use the design to study preference among zone specific scents: To make informed choices, it should be assured that individuals experience all options available.

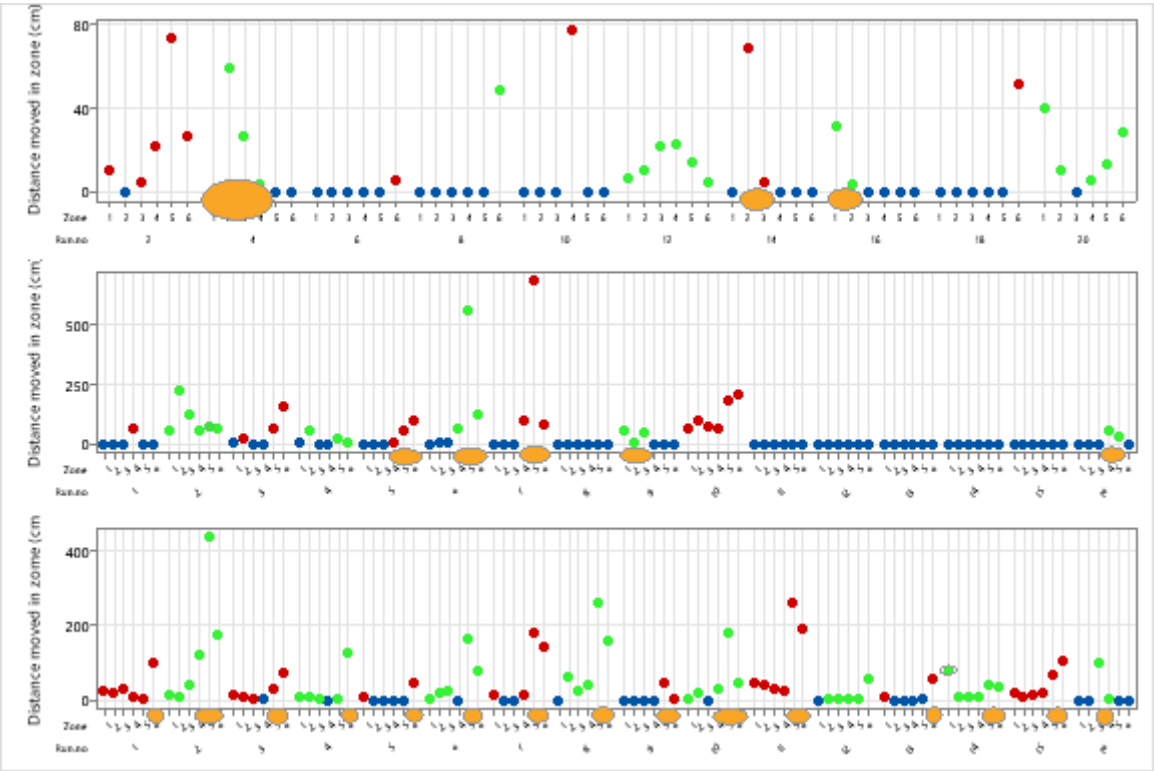

FIGURE 10: Distance moved in different zones (1-6) plotted per individual trial, at 6, 13 and 20 DPH. Orange coloured ellipses indicate possible spatial dependency of values between neighbour zones.

Due to design issues, results from statistical analyses of treatment effects should be treated with caution, but are briefly presented:

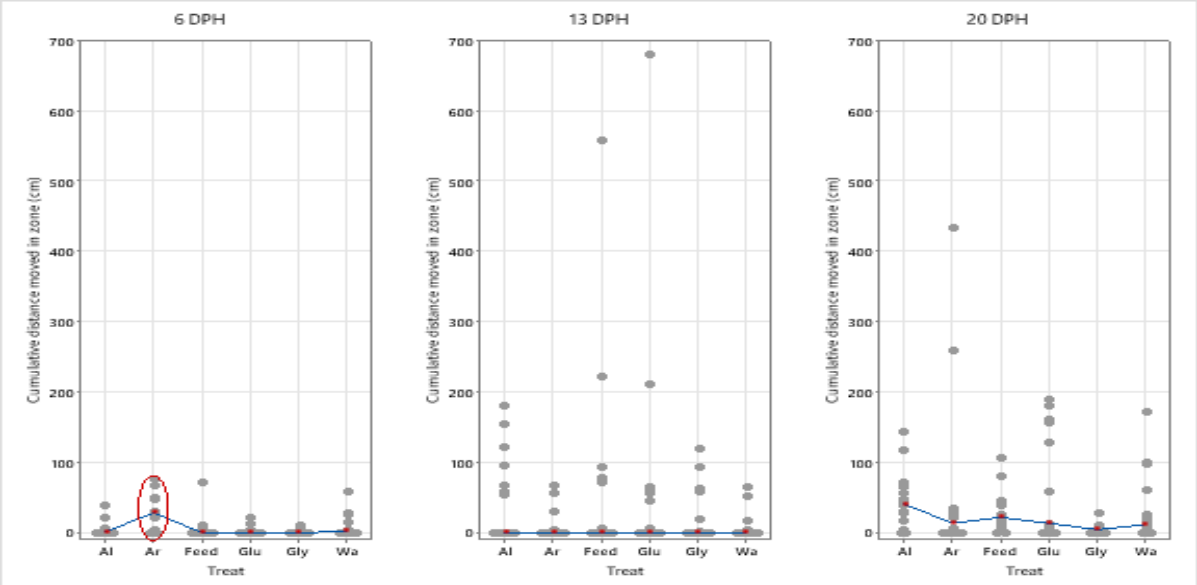

FIGURE 11: Cumulative distance moved in different treatment zones. For a given treatment zone, grey circles show distance of individual trials, red circles show median distance over all trials. Significant effect at 6 DPH is indicated with red ellipse.

TABLE 9: Test of preference for particular treatment zones. Kruskal-Wallis analysis (assuming independence of observations made in the different zones)

Larval age (DPH)	**6**			**13**			**20**		
Response variable	***Df***	***H-value***	***P-value***	***Df***	***H-value***	***P-value***	***Df***	***H-value***	***P-value***
Cumulative distance moved (cm)	5	11.72	**0.039**	5	2.42	0.788	5	10.39	0.065
Visits in zone > 5 seconds stay	5	7.51	0.186	5	1.63	0.897	5	8.5	0.131

CONCLUSIONS:

ACTIVITY 2.1.1 a MICRO SCALE LIGHT EXPERIMENT

Initial graphical exploratory analyses by age:

A generally high between-trial variability in distance moved was observed at all three ages (Figure 5). This finding formed the basis for standardizing raw data for response variables to measures of change relative to initial levels of these variables before conducting statistical analyses. A general increase in activity level was seen with increasing larval age. The plot indicated some general change in activity following switching on/off light.

Generally high inter-individual variability, as measured by *Distance moved*, was observed in this experiment (FIGURE 5), which may raise questions about larval quality. General activity increased with age, and at 3 DPH Distance moved was clearly reduced when light was turned on, but fell back to initial levels when light was once more turned off (FIGURE 5). At 3 DPH, the heat-plot indicated elevated larval activity concentrated near the experimental unit walls (thigmotaxis) during the two dark periods but not during light, possibly indicating anxiety reactions to totally dark conditions. No treatment factor effects were apparent (FIGURE 6).

Factorial analyses as a whole showed few incidences of significant treatment factor effects; only at 17 DPH, *change in distance moved* and *absolute angular velocity* showed significant interaction effects when light was turned on. Turning off light gave no indication of treatment effects.

When analysing overall change without taking treatment factors into account (gross overall change from one light condition to another), effects were more numerous: When light was turned ON, at 3 DPH, a significant negative change in *distance moved* (lower activity) was seen, while *absolute angular activity,* often assumed to be a measure of searching activity, increased. For 10 DPH, there was a trend for increase in values of all three response variables, but no significance, also for 17 DPH, no significance was seen. When light was turned OFF, there were signs of overall increase in both *distance moved*, *time use in outer zone* and *absolute angular velocity* in 7 of 9 cases, 5 of which showed significance.

ACTIVITY 2.1.1 b PHOTOTAXIS LIGHT EXPERIMENT

For the response variable *Relative change of distance moved*, no significance was seen at any age, for any of the treatment factors. Also, analyses of overall change when disregarding treatment effects showed no significance, but at all ages *distance moved* showed a slight overall increase during treatment.

Response variables *Fraction observations* and *Fraction time* showed no significant treatment effects or overall effects on phototaxis, and estimates of direction of taxis was non-significant and not consistent between these two variables.

For Index Frequency and *Index Time*, one significant treatment effect was seen (from Wavelength at 4 DPH, for the response Index Frequency). No other significant treatment effects were seen for these response variables, and also, no analyses of indexes - when disregarding treatment effects - showed significant difference from expectation at no taxis (index of 0). Contrary to for the responses *Fraction observations* and *Fraction time*, though, the direction of estimated phototaxis was identical at each age between these two indexes.

Overall, a slight but non-significant change in activity was seen between acclimation and treatment periods at all ages. Estimated direction of phototaxis was not consistent between the different response variables. To conclude, convincing treatment effects could not be claimed neither for

activity (*change in distance moved*) nor for any of the response variables addressing larval positioning relative to the light source.

ACTIVITY 2.1.3 a MICRO SCALE SCENT EXPERIMENT

The observed general increase of activity during injection of scent solutions appeared to be a mere method issue, which calls for refinement of future similar experiments.
The observation that a fraction of the larvae were particularly active already before treatment was added, and continuously kept this high activity level throughout trials, indicates high inter-individual variability in experimental animals. Objective means of assessing larval quality in this species are not yet established, but egg mortality was high, so larval quality may have been an issue here.

No significant treatment effects were seen at age for any of the three core response variables *Change distance moved*, *Change absolute angular velocity* or *Change time used in outer zone*.

Analysis of pooled values of response variables showed strongly significant overall increase in *Absolute angular velocity* between the first and last 5-minute interval, at all the three ages. This effect appeared also in the Water only and No action (method control) group. Thus, as no significant treatment effects were seen, this overall increase might have been a general temporal effect on aspects of behaviour during trials, e.g. an acclimation process following handling prior to trials.

ACTIVITY 2.1.3 b SMALL SCALE SCENT EXPERIMENT

The only significant difference between treatment groups was seen for *cumulative distance moved* at 6 DPH- apparently due to high levels of movement in the zone added arginine treatment. At no other age did any of the three response variables show significant differences due to treatments, and plots of *Distance moved* did not show consistent effects from treatments at any age.
An obvious problem with this design was a possible dependency of spatial observations due to design based limitations on movement patterns between zones, which actually questions the validity of assumptions for statistical testing. As experiments were re-defined to run at ambient light, possible confounding patterns between zone position and randomly assigned treatments might also arise due to the position of the light source, which was difficult to account for due to the circular design. Additionally, many of the zones were not visited, which by itself reduces the value of analyses in terms of analysing preferences, and raises questions about the reasons for the generally high individual variability. In conclusion, the most obvious results was the strong reduction in larval movement from running experiments in darkness, seen particularly for 6 and 13 DPH.

METHOD DISCUSSION:

There was considerable variability among individual larvae, which might be an indication of variable larval quality, and might thus affect the relevance of behavioural observations made. Additionally, this variability directly affects the accuracy of statistical analyses, by increasing error variance in parametric testing. To reduce this issue, means should be taken to reduce such variability, by finding ways to assess larval quality and remove larvae of poor quality from experiments.

Post hoc power estimates were carried out both for results from factorial analyses of the response variable *Change in distance moved* of Activity 2.1.1 a and for one-way ANOVA of all three response variables *Distance moved, Relative angular velocity* and *Time Outer zone* of Activity 2.1.3 a. These analyses showed low post hoc power at all ages, in the range 0.08-0.33, which weakens statistical inference made from non-significant test results. General means to improve power is either to reduce error variance (between experimental unit variability) or increase replication. Further a priori power calculations based on observed error variance from experiments indicated considerable increased replication should take place, both for the one factor and factorial designs, to achieve the commonly recommended statistical power of 0.8.

Of specific practical methodological issues that should be addressed in follow-up studies is first the injection method for scents applied in Activity 2.1.3 a, as injection itself induces current in the experimental unit which directly affects larval movements. Also, the design of Activity 2.1.3 b was not suited for its purpose, foremost due to physical restrictions to movement of larvae between the different treatment zones in the tank, which gave dependency between repeated observations.