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METHODS AND TECHNIQUES

A machine vision system for zooplankton behavioural studies: a case study on the phototactic behaviour of sea lice (*Lepeophtheirus salmonis*) during sound and ultrasound stimuli

Torfinn Solvang* and Andreas Hagemann

ABSTRACT

Machine vision represents an accurate and easily verifiable method for observing live organisms and this technology is constantly evolving in terms of accessibility and cost. Motivated by the complexity of observing small-sized aquatic organisms in experimental systems, and the difficulties related to real-time observation, sampling and counting without interfering with the organisms, we here present a new method for observing behaviour and dispersion of non-sessile zooplankton organisms using a custom-made tank with an associated machine vision system. The system was used in an experiment where the aim was to assess the effect of sound and ultrasound on the phototactic behaviour of copepodite stages of the salmon louse (*Lepeophtheirus salmonis*). The experimental set-up is described, including a triangular test tank designed to create a sound pressure gradient, a mechanized camera movement system and a vision system with dedicated image processing software.

KEY WORDS: Zooplankton behaviour, Observation system, Salmon lice, Phototaxis

INTRODUCTION

Exploring the behaviour of, for example, zooplankton with minimal interference is key to understanding natural behaviour (Price et al., 1988). Video analysis of zooplankton behaviour has been in use since cathode ray imaging was the state of the art technology (Buskey, 1984). Machine vision and imaging are becoming increasingly accessible because of improved software, development tools and hardware. Image analysis allows for the collection and processing of larger numbers of samples (here, images) than conventional methods, such as microscopy or counting chambers, normally associated with zooplankton studies. Using machine vision for behavioural studies is hence cost effective, reduces the risk of influencing the behaviour of the test organisms and allows for continuous sampling/observation during the experiment. Moreover, spatiotemporal development (e.g. speed of migration) can be extracted from one experiment iteration which can be used to refine the experimental set-up and save both time and the number of test organisms used. Such systems allow for non-biased data interpretation as the image analysis algorithm is mathematically objective. Machine vision as a methodological tool is easily verifiable as all images can be saved and reanalysed later using different approaches, if necessary.

3D systems are commonly used for zooplankton studies, at the expense of a small observational volume (field of view). These systems often use high frame rates (e.g. to capture water flow fluctuations), and may not store images over long periods of time. For some applications, 2D imaging is not only sufficient but also can increase the number of parameters to be studied. A good example is dispersion and migration, which requires a larger volume to be observed, usually over longer extents of time. 2D imaging systems have similar limitations to a 3D system (e.g. focal depth), but can be combined with dynamic camera systems to cover larger volumes, as will be demonstrated here.

The salmon louse, *Lepeophtheirus salmonis* (Krøyer 1837) has been a persistent challenge in salmon aquaculture for decades (Igboeli et al., 2014), presenting an increased production cost in the range of billions of Norwegian krone (Iversen et al., 2015). The copepodite stages of the salmon louse are positively phototactic (Johannessen, 1977; Bron et al., 1993). Bron et al. (1993) argue that in addition to light, chemical stimuli, water pressure and water flow affect copepodite behaviour. During infestation (attachment), the anterolateral flow field from a swimming host is expected to be the most important cue for successful contact with a host (Heuch and Karlsen, 1997). This field is a low-frequency, hydrodynamic dipole field derived from water being pushed ahead of and along the sides of the advancing fish, and this signal is mainly below 20 Hz (Kalmijn, 1988, 1989). Although this suggests that low frequencies can be used to mask the water pressure signature from a potential host, it also reveals that the copepodites are triggered by sound. Higher frequencies are easier to use in a study, because of sound source accessibility, but will suffer from stronger attenuation while propagating through the water, a possible disadvantage for field use.

Ultrasound technology has recently been suggested as a new preventive measure to reduce sea lice infestation in commercial salmon farming (e.g. Mortensen and Skjelvareid, 2015), and there are several companies providing industrial ultrasound systems for the salmon industry. The Norwegian Seafood Research Fund asked SINTEF to conduct initial screening experiments in 2016 on this matter (Solvang-Garten et al., 2016), leading to the development of the system described here. The conceivable effects from the ultrasound are to repel, attract, mask (drowning or jamming the sensory apparatus) or destruct the salmon louse. As high sound frequencies are proclaimed to influence salmon lice behaviour and infestation rates, we designed and tested a system for observing the behaviour and dispersion of the planktonic copepodid stage of the salmon louse. The aim of our study was to assess whether acoustic signals influence the copepodites' natural phototactic behaviour.

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Received 26 April 2018; Accepted 4 July 2018

Journal of Experimental Biology (2018) 221, jeb183277. doi:10.1242/jeb.183277

MATERIALS AND METHODS

Set-up

In order to create a sound gradient, the sound pressure level (SPL) had to decrease with distance from the sound source. Hence, the tank was designed as a triangle. As the sound waves propagated from the source, the shallow water level in the tank allowed principally for horizontal (2D) sound distribution. The sound energy dispersed over an increasing area as it left the speaker, resulting in a reduction in SPL proportional to the distance from the sound source. The end baffle was covered with a thick (20 mm) porous rubber material to dampen reflecting sound waves. To observe the copepodites, the volume in which they could move was restricted to a region corresponding to the camera observation field by constructing a corridor made from nylon mesh (64 µm opening; Sefar Nitex, Heiden, Switzerland). The mesh did not reduce sound waves or pressure significantly. In this 'observation corridor', the sea lice could move mainly in two directions: towards or away from the light source placed next to the speaker in the narrow end of the triangular tank. A water level of 25 mm was used for all experiments.

The stainless-steel bottom of the observation corridor was replaced by a transparent acrylic plate to allow for camera placement underneath the tank. Within the corridor, six equal observation sectors were defined (see the green quadrants in Fig. 1, top view). The camera system used for our experimental set-up gave a field of view of approximately 35 mm and a focal depth of approximately 3 mm. A camera glidetrack (150 cm, Digislider DS150, London, UK) was used to move the camera (Point Grey Grasshopper 3, 2.8MP, USB, 25 mm lens, FLIR, Richmond, BC, Canada) between the sectors. The camera sled was actuated using a stepper motor (Sanyo Denki SH1603-5240, Sapporo-shi, Japan) controlled using a single-board microcontroller (Arduino Uno, Italy) and a stepper motor drive (Astrosyn P403A, Chatham, Kent, UK). The images were collected using an application developed in LabVIEW (National Instruments Co., Austin, TX, USA). The synchronization between the camera and stepper motor was implemented using an I/O device (National Instruments USB6008) where the LabVIEW application sent a sync signal to the microcontroller. Please refer to Figs S1–S3 for details. Each of the six observation sectors were covered by two images, separated with a movement corresponding to the image width. After collecting the images in the first position, the camera moved to the next, and the procedure was repeated until all images were collected. At the end of the cycle, the camera returned to its initial position, pending the next sampling.

The sound signal was generated using a signal generator (GW Instek GFG-82149A, New Taipei City, Taiwan) producing a sinus with selectable frequency, connected to an audio amplifier (Harman Kardon Signature 1.5, Stamford, CT, USA), an impedance-matching

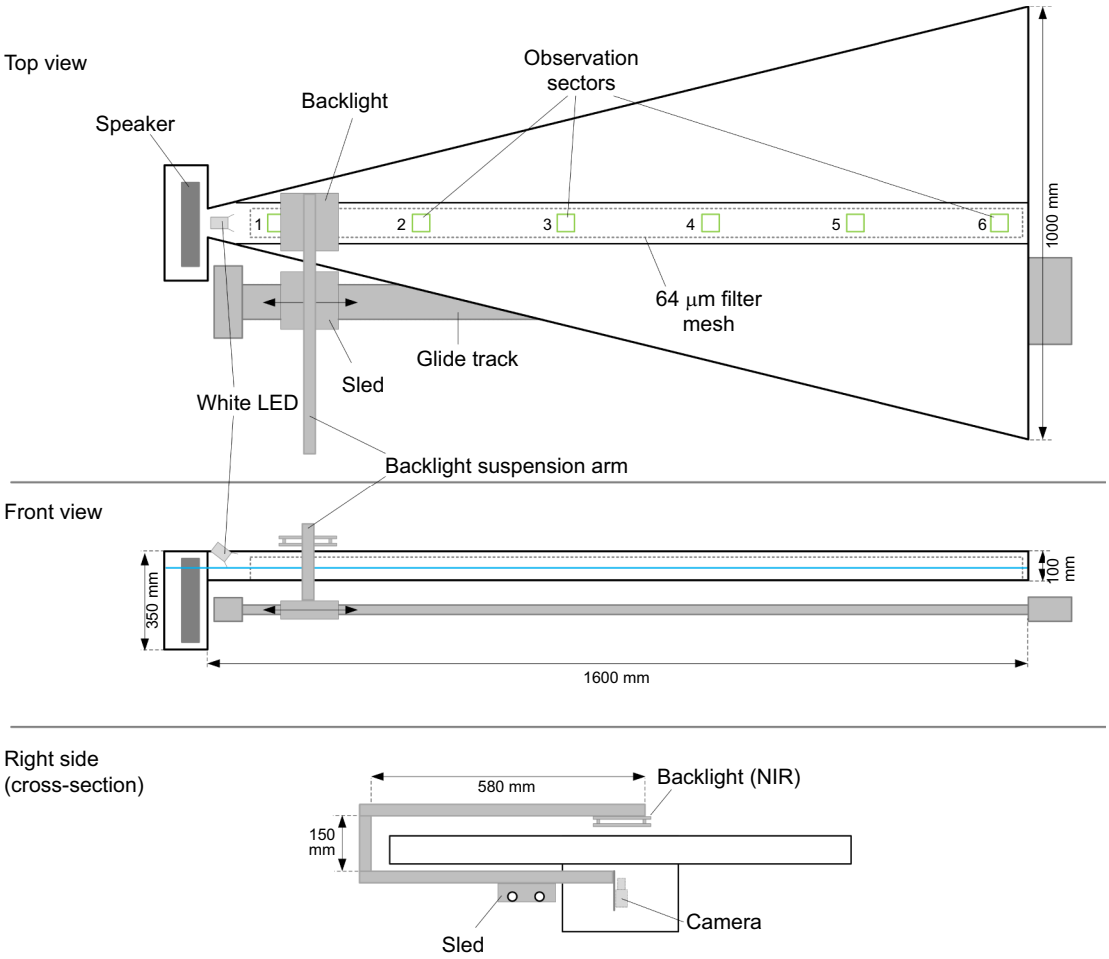

Fig. 1. Diagrammatic projections of the experimental apparatus. The apparatus consisted of a test tank with a camera glidetrack underneath. A speaker was placed in a water-filled chamber at the end of the narrow part of the triangular tank, and a white LED was placed in front of the speaker. The sea lice were confined to an 'observation corridor' by a plankton mesh (64 µm). The camera moved along the longitudinal axis of the observation corridor, which was divided into sectors (*n*=6), and each sector was covered by two images.

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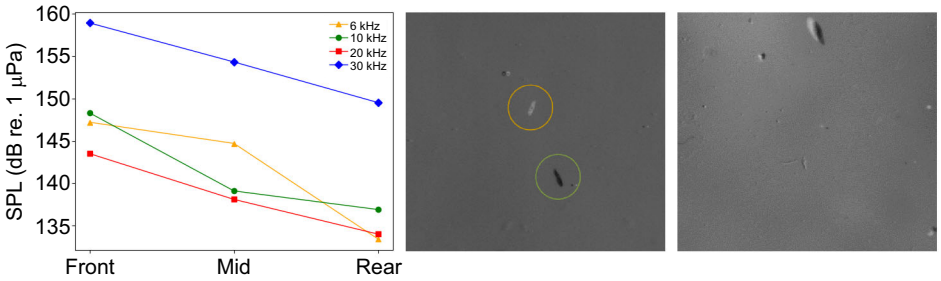

Fig. 2. SPL and image analysis. Left: sound pressure level (SPL) at different positions in the tank. Middle: example from image analysis. The orange circle indicates the copepod from the latest run; the green circle indicates the copepod from the previous run. Right: these objects have not moved since the last run (60 s) and were regarded as immobile and therefore discarded.

filter and an underwater speaker (Ocean Engineering DRS-8, North Canton, OH, USA) submerged into a chamber in the tank (Fig. 1). The SPL was measured using a hydrophone (Bruel & Kjaer type 8104, Nærum, Denmark) and an amplifier (Nexus 2692, Bruel & Kjaer). The amplified signal was recorded using a PC and a USB PICO-scope. The SPL was calculated by measuring the AC true root mean square value and applying the hydrophone calibration data. For the SPL recordings, the hydrophone was placed at three different locations within the observation corridor; close to sector 1 (in front), between sectors 3 and 4 (middle) and close to sector 6 (rear), as depicted in Fig. 2. The white LED lamp was placed immediately in front of the speaker, above the water, angled downwards.

Experiments

Before each experimental run, copepodites (n=500) were counted and collected in plastic cups and stored in the dark. The tank was filled with sand-filtered, natural seawater (9°C, salinity 34 ppt and pH 8.1) collected at 70 m depth. Upon initiation of each run, the copepodites were introduced in sector 3 of the test tank, whereupon the sound signal, light source and image collection system were switched on. The image sampling procedure was repeated every 60 s and took approximately 30 s to fulfil. Each experimental run lasted 15 min and generated 180 images. The tank was thoroughly cleaned and refilled with fresh seawater between each run.

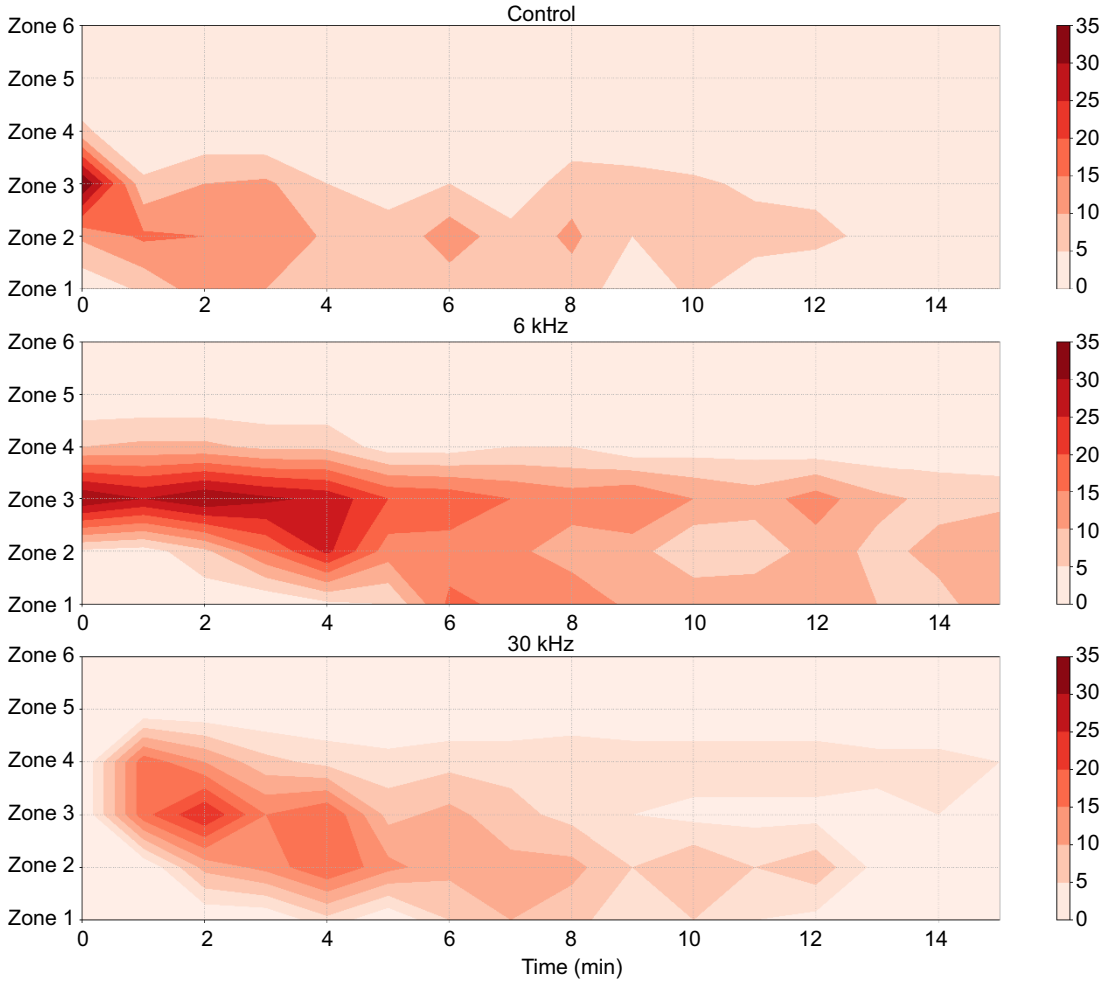

Fig. 3. Migration patterns due to sound exposure. The *y*-axis corresponds to observation sectors 1–6 in Fig. 1. The experiment started at time 0. Sampling was done every 1 min. The contour colour corresponds to the lice count indicated by the bars on the right.

Image analysis

A dedicated LabVIEW application was developed to analyse the collected images. The application sorted the images by sector (1–6) and combined the last collected image as an overlay to the previous image from the same sector. This technique left the objects from the last image bright, and particles from the previous image darker (Fig. 2, middle). Hence, stagnant particles (e.g. dead copepodites and debris) could easily be discarded from our recordings by both their position and silhouette. The number of copepodites in each image was manually counted and recorded in a table for each treatment and plotted in contour plots (Fig. 3).

RESULTS AND DISCUSSION

The frequency response of the speaker (including the effects from the tank environment) was not linear over the frequency range used for this experiment. The SPL ranged between 134 and 170 dB (re. 1 µPa) for the different frequencies (Fig. 2). However, all frequencies declined in amplitude with distance from the speaker, thus creating a gradient. Whether the SPL gradient throughout the rig was sufficient for the copepodites to differentiate between source and escape directions is a relevant question. Internal acoustic reflections were probably present in the tank and the shallow water represented an efficient route for sound propagation. To create a higher SPL loss and hence a steeper SPL gradient, the ideal design is an infinitely large tank without reflecting surfaces. However, a larger system would make the detection and counting of zooplankton difficult, even using a vision system.

The focal depth of the camera was approximately 3 mm, while the water column depth in the tank was 25 mm. Consequently, a large share of the water body remained hidden for the camera during the experimental runs. This did not cause any problems for our experiment because the copepodites mainly resided and swam along the bottom of the tank, where the focal point was set. At the end of each experiment, many lice were observed crowding at the nylon mesh just in front of the LED lamp, outside the field of view of the camera.

The control (no sound) treatment showed that most of the copepodites took 9–11 min to swim the distance from sector 3 to sector 1 (approximately 600 mm), towards the light source (Fig. 3). There were no significant differences in swimming distance or response time between the control and the sound and ultrasound treatments.

In summary, we successfully demonstrated a system for behavioural studies of salmon lice copepodites using machine vision and automated camera movement. Sound and ultrasound treatments in the range from 6 kHz to 30 kHz did not significantly counteract or decelerate the positive phototaxis of the copepodites.

Finally, this system is not limited to sound stimuli as shown for this study. Interchangeable light sources can serve to assess response variables in zooplankton to different light spectra and intensities, and chemical stimuli can be added to investigate behavioural responses to olfactory cues. Moreover, the system can be operated as a closed system as demonstrated, or as a flow-through system by adding a water inlet at one end and an outlet at the other.

Acknowledgements

We thank the mechanical workshop M-Tech for production of the triangular test tank, and Åsmund Johansen and Arnstein Johannesen at SINTEF Ocean for technical assistance.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: T.S., A.H.; Methodology: T.S., A.H.; Software: T.S.; Writing - original draft: T.S., A.H.; Writing - review & editing: T.S., A.H.; Visualization: T.S., A.H.; Funding acquisition: T.S.

Funding

This work was funded by The Norwegian Seafood Research Fund (Fiskeri - og havbruksnæringens forskningsfond) (project number 901160).

Data availability

Data are available from the Norwegian Seafood Research Fund digital repository: http://www.fhf.no/prosjektdetaljer/?projectNumber=901160.

Supplementary information

Supplementary information available online at http://jeb.biologists.org/lookup/doi/10.1242/jeb.183277.supplemental

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Fig. S1. Motor control circuit schematic

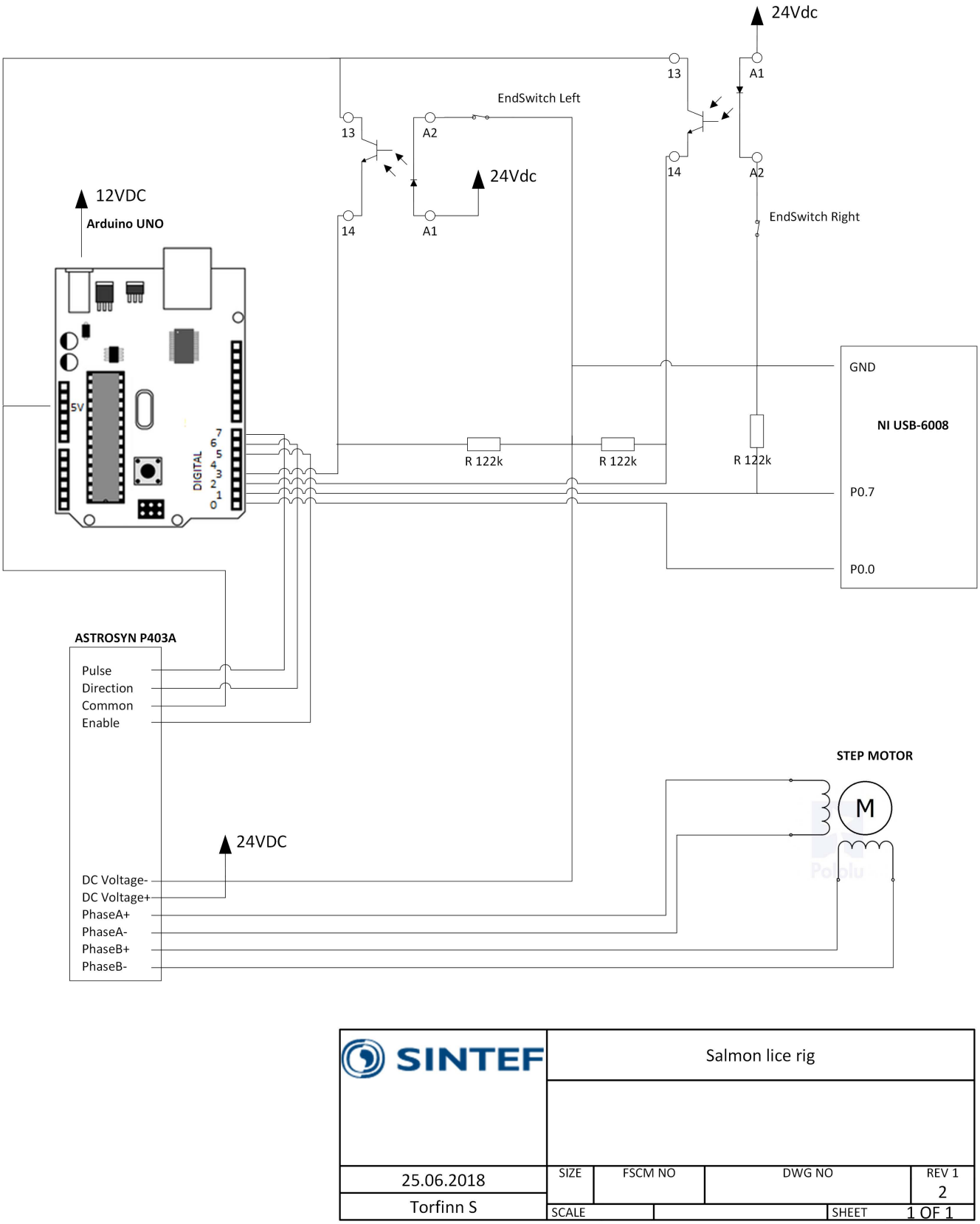

Fig. S2. Arduino code

Programmer's Notepad - Arduino - StepMotor.iso

```
#define RIGHT     LOW
#define LEFT      HIGH

int Start_Move = 0;
int Pos_Reached = 1;
int EndSWRight = 2;
int EndSWLeft = 3;
int MOTORRESET = 4;
int MOTOREN   = 5;
int MOTORDIR  = 6;
int MOTORPULSE = 7;

int motorState = 0;
int microstep = 1;                                  // 1 - 250 via DIP-switch
int moves = 0;
long StepCounter;

void setup() {
  InitMotor();
}

void loop() {
  SetMotorDirection(RIGHT);

  while(1){
    if (digitalRead(Start_Move)){
      StepMotor(3300, 100);
      while (!digitalRead(Start_Move))
        delay(10);
      StepMotor(19200, 100);
      moves += 1;
    }

    if (moves == 5){                                // Last step
      if (digitalRead(Start_Move))
        StepMotor(3300, 100);
      moves += 1;
    }

    if (moves > 5){
      SetMotorDirection(LEFT);
      StepMotor(115800, 120);
      moves = 0;
      SetMotorDirection(RIGHT);
    }

    delay(50);
  }
}

void InitMotor(){
 // Astrosyn P403A driver, 24 - 40V dc
 // 0,9deg/step and microstep = 1 -> 400 steps / rotation
  pinMode(Start_Move, INPUT);
  pinMode(Pos_Reached, OUTPUT);
  pinMode(EndSWRight, INPUT);
  pinMode(EndSWLeft, INPUT);
  pinMode(MOTORRESET, INPUT);
  pinMode(MOTOREN, OUTPUT);
  pinMode(MOTORDIR, OUTPUT);
  pinMode(MOTORPULSE, OUTPUT);
  digitalWrite(MOTORDIR, HIGH);
  digitalWrite(MOTOREN, HIGH);
}

void MotorPulse(){
  if(digitalRead(EndSWLeft) || digitalRead(EndSWRight)) // Normally low - closes to 5V (via optocouplers) and end switch
    StepCounter = 0;
  else
    motorState = !motorState;

  digitalWrite(MOTORPULSE,motorState);
}

void SetMotorDirection(int Status){
  digitalWrite(MOTORDIR, Status);
}

void StepMotor(long steps, int delayMicro){
  digitalWrite(Pos_Reached, LOW);                   // Signal to LabVIEW - moving
  StepCounter = steps;
  long rampStep = 4;
  int RampDelay = 2500;  // Startdelay
  int RampA = RampDelay/rampStep;
  long StepLen = RampDelay;

  while((microstep*StepCounter) >= 0){
    MotorPulse();
    delayMicroseconds(StepLen);

    if (StepCounter <= RampA){                      // ramp down
        StepLen += (rampStep*1.1);
        if(StepCounter <= 0){
          digitalWrite(Pos_Reached, HIGH);          // Signal to LabVIEW - start taking images
          delay(50);
        }
    }
    else{                                           // Normal drive mode
      if (StepLen < delayMicro)
        StepLen = delayMicro;

      else if (StepCounter >= (steps - RampA))      // ramp up
        StepLen -= rampStep;
    }
    StepCounter--;
  }
}
```

Fig. S3. LabView code

FHF UL mod 2 eng.vi

The Low-Level Sequence VI acquires a sequence of images and displays the images in an image control using low-level acquisition VIs. A sequence is appropriate for applications in which you want to process multiple images.