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9   "Quantitative analysis reveals the basic behavioural repertoire of the urochordate *Ciona intestinalis*."

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27 **Abstract**

28 Quantitative analysis of animal behaviour in model organisms is becoming an increasingly essential

29 approach for tackling the great challenge of understanding how activity in the brain gives rise to

30 behaviour. In addition, behavioural analysis can provide insight on the molecular basis of nervous

31 system development and function as demonstrated by genetic screens focused on behavioural

32 phenotyping in some genetically tractable model organisms. The progress in building low-cost

33 automated tracking setups, together with advances in computer vision machine learning have

34 expanded the repertoire of organisms which are amenable to quantitative behavioural analysis. Here

35 we used automated image-based tracking to extract behavioural features from an organism of great

36 importance in understanding the evolution of chordates, the free swimming larval form of the

37 tunicate *Ciona intestinalis* which has a compact and fully mapped nervous system composed of only

38 231 neurons. We analysed hundreds of videos of larvae and we extracted basic geometric and

39 physical descriptors of larval behaviour. Most importantly, we used machine learning methods to

40 create an objective ontology of behaviours for *C. intestinalis* larvae. We identified eleven behavioural

41 modes using agglomerative clustering. This approach enabled us to produce a quantitative

42 description of the basic larval behavioural repertoire. Furthermore, we tested the robustness of this

43 repertoire by comparing different rearing conditions and ages. Using our pipeline for quantitative

44 behavioural analysis, we successfully reproduced the known photoresponsive behaviour and the first

45 demonstration to our knowledge that *C. intestinalis* larvae exhibit sensory arousal and thigmotaxis,

46 both of which can be modulated by the anxiotropic drug modafinil. Remarkably, by comparing the

47 behaviour between animals assayed individually or in small groups, we found that crowd size

48 influences larval behaviour. This study shows that *C. intestinalis* larval behaviour can be broken down

49 to a set of stereotyped behaviours that are used to different extents in a context-dependent manner.

50 Furthermore, it raises exciting possibilities such as mapping behaviour to specific neurons of this

51 compact chordate nervous system and it paves the way for comparative quantitative behavioural

52 studies as a means to reconstruct the evolution of behaviour, especially in the chordate lineage.

Introduction

Close observation of living animals can reveal the large repertoire of behaviours they use to interact with the world. Animals can crawl, swim, run and fly to move from one place to another. Many animals perform extremely complex behaviours to attract mates, exhibit parental care and establish their position in social hierarchy. Numerous species are able to build elaborate structures, ranging from spider webs for catching preys to bird nests for shelter and raising of offspring. Some can even make and operate tools. These observations have led to two important challenges for the scientific community to pursue.

The first is to obtain a detailed understanding of how nervous systems generate behaviour. Modern approaches to tackle the first challenge include techniques for recording and targeted manipulation of neuronal activity using a wealth of molecular and cell type information (1). However, to fully understand the function of neural circuits, we need to obtain an equally precise and detailed understanding of behaviour (2, 3).

Behaviour is a process that is characterised by dynamic changes, and complex sequences of events that are often convoluted with noise. Therefore, measuring animal behaviour using manual approaches can be time consuming, and prone to errors, the latter especially in cases where a behavioural event is taking place over a very short or very long time scale, making it difficult to be detected by the experimenter. Modern computational analysis methods and accessible hardware for recording videos with high temporal resolution make it possible to observe and quantify behaviour in a more comprehensive, accurate and automated approach (4-7).

Automated behavioural analysis has been used to divide and classify behaviour into distinct modules, and has been extensively demonstrated in several organisms, including worms (8, 9), flies(10), zebrafish (11, 12) and mice (13). Despite the morphological and locomotor differences between these organisms, automated tracking systems coupled to machine learning can transform what appears as complex behaviours into a sequence of more basic motor patterns that are

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78   executed in a particular frequency and order. Overall, this approach can result in a simplified

79   description of how behaviour is organised and carried out.

80   The second challenge is to understand how behaviours and nervous systems co-evolved through

81   time. Most efforts to achieve this goal have focused on the dissection of the developmental and

82   genetic mechanisms driving the evolution of neuronal circuits linked to species-specific behaviours

83   (14, 15). The advent of automated tracking and analysis methods that allow for the recognition and

84   segmentation of morphologically diverse organisms, offer the opportunity to perform quantitative

85   behavioural analysis of key organisms across different taxa (16-18). This approach could provide

86   important insight into conserved, as well as novel behavioural programs and how they are linked to

87   major evolutionary transitions in nervous system structure and cell type composition.

88   In this study, we used the tunicate *Ciona intestinalis* to tackle these two challenges. Tunicates are the

89   closest relatives of vertebrates and have been successfully opted as models to study the evolution of

90   chordates(19). They have larvae with a chordate body plan and development(20). The two most

91   intensively studied tunicate species are *Ciona intestinalis* and *Ciona robusta*. *Ciona intestinalis*

92   features a defined cell lineage, an extensive genetic toolkit and a sequenced genome that shares a

93   high number of homologous genes to its vertebrate counterparts. It has been extensively used in

94   studying the chordate origins of many biological processes and has been particularly successful as a

95   model for studying the development and evolution of the chordate nervous system (21). It possesses

96   a dorsal central nervous system for which a documented synaptic connectome of the 177 CNS

97   neurons has recently been published. Together with a publication of the peripheral nervous system

98   connectome demonstrating the presence of an additional 54 neurons, these studies have laid out the

99   foundations for future functional studies (22-24). This makes *C. intestinalis* the second organism with

100   a complete connectome available after *Caenorhabditis elegans*(25).

101   An adult *C. intestinalis* animal can release hundreds of eggs together with sperm. Gametes undergo

102   fertilization and through a series of stereotyped developmental steps, with the embryos taking the

103   form of hatching lecithotrophic larvae. When the larvae hatch from the chorion, they find themselves

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104   in the water column. Following the hatching event larvae swim upwards towards the water surface

105   by negative gravitotaxis using the otolith cell. Ablation experiments have shown that animals lacking

106   the ocellus are also capable of this behaviour, indicating that the ocellus is not involved in

107   gravitotaxis.(26, 27). Later on, larvae exhibit negative phototaxis, swimming away from the bright

108   surface to deeper waters in a behaviour that possibly aims to identify suitable substrates for

109   settlement (28, 29). The swimming larvae are thought to display three types of swimming activity:

110   tail flicks, spontaneous swimming and shadow response(30). Larvae under constant illumination

111   swim more frequently and for more extensive periods of time earlier in life, up to 2 hours post

112   hatching. A behaviour that develops later in development is the shadow response, where dimming of

113   light results in symmetrical swimming. After two hours post hatching the tail beating frequency

114   increases(30). *C. intestinalis* larvae have also been shown to exhibit both sensitization and

115   habituation to light (31, 32). A recent study reported that distinct groups of photoreceptors mediate

116   negative phototaxis and dimming behaviour in *C. intestinalis* larvae(33). This functional insight was

117   achieved through behavioural analysis on mutant lines, demonstrating the power of genetics in

118   combination with behavioural analysis and knowledge of the larval connectome. However, this is not

119   the first attempt to link behaviour to its genetic underpinnings in *C. intestinalis* as it has been shown

120   that targeted knockdown of opsin1 results in a loss of the light OFF response in larvae(34). Beyond

121   phototactic and gravitotactic behaviours there is evidence that hints to the possibility that the larvae

122   can exhibit chemotactic(35) and mechanosensory behaviours(36-40). However, these have remained

123   largely unexplored.

124   Over the past decades, the biological research community has used *C. intestinalis* as a model for

125   uncovering the basic principles of chordate embryonic and post-embryonic nervous system

126   development. We believe that by building on this work (e.g. taking advantage of the numerous

127   characterised promoters, known cell lineage etc), we can use the compact nervous system of the

128   larva in order to uncover the basic molecular and cellular mechanisms underlying chordate nervous

129   system function. We have the tools required to deconstruct the molecular and cellular components

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130   of the larval nervous system(41) and the opportunity to adopt existing toolkits to probe its

131   functions(42). Here, we attempt to match the growing insights into the structure and function of the

132   *C. intestinalis* nervous system with a detailed characterization of its behavioural output. To achieve

133   this, we collected a large dataset of recordings of free-swimming *C. intestinalis* larvae on a custom IR-

134   illuminated and temperature-controlled set-up. We analysed the recordings using ToxTrac(43) and

135   custom written Python scripts to obtain positional data and descriptors like speed and its variability,

136   turning angles, and path complexity. To describe behaviour both accurately and objectively, we used

137   unsupervised clustering methods to identify distinct behavioural clusters in our dataset, ultimately

138   arriving at a quantifiable distribution of detectable behavioural components. This approach enabled

139   us to study how this distribution of behavioural components changed under the influence of

140   different rearing conditions, at different ages and in response to different sensory cues. Strikingly, we

141   show that *C. intestinalis* larvae exhibited a state of sensory arousal when first transferred into the

142   behavioural arena. This sensory arousal state and thigmotaxis behaviour could be modulated by the

143   anxiotropic drug modafinil.

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145   **Results**

146   **Defining behavioural parameters and modes**

147   In order to probe the behavioural dynamics of *C. intestinalis* larvae, we built an inexpensive tracker,

148   with temperature control and the ability to deliver light stimuli with accurate spatiotemporal control.

149   To maximize the open-source accessibility and modularity of the trackers we used Arduino based

150   circuits for controlling the various tracker components and 3D printed housing for the agarose plated

151   behavioural arenas. The agarose arena was housed inside a 3D printed ring fitted with infrared (IR)

152   LEDs (Fig 1A).IR LEDs were used in order to visualise the animals in a dark-field configuration, while at

153   the same time avoiding stimulating their photoreceptors with visible light, which they can detect and

154   modify their behaviour (31-33). We imaged approximately 550 *C. intestinalis* animals behaving freely

155   at two different frame rates: During the first 15 minutes after transferring animals to the setup we

156   imaged at 10 frames/s and subsequently we imaged the same animals for 5 minutes at 30 frames/s.

157   Following acquisition and processing, the videos were analysed on the tracking program ToxTrac(43)

158   (Fig 1C, D). Due to the lack of previous attempts to track *C. intestinalis* larvae with automated image

159   tracking software, we tried a series of available programs. We found that ToxTrac worked best in our

160   hands especially because it did not require specific knowledge of the animal's body geometry and

161   because it was particularly robust against false positives and identity switches. The position of the

162   animal was tracked by the centre of its detected shape, as identified by ToxTrac. From all the videos

163   tracked we obtained around 850 traces for further analysis and quantification.

164   Below we introduce some of the descriptive parameters used and the reasoning behind their

165   definition.

166   *C. intestinalis* larvae have been reported to modulate their locomotor activity levels through "bursts"

167   of spontaneous activity (26, 30, 44). We decided to introduce a parameter that would provide a

168   quantitative descriptor of locomotory activity, termed Activity coefficient (AC) and defined as the

169   fraction of time an animal spent locomoting. Filtered speed values of 200 μm/s and above were

170   considered as active, which in practical terms includes all actively swimming animals as well as

171   movement of the animals' centre-point due to tail flicks and twitching.

172   Planktonic larvae from different species exhibit a wide range of paths during locomotion(45), often

173   dictated by their biomechanics (44, 46-48). Notably, reduced food availability can result in nonlinear

174   paths, while directionality in trajectories can often be indicative of behavioural responses to

175   environmental stimuli (49, 50). While recording animals in the behavioural arenas, we observed that

176   they exhibited a large repertoire of trajectories while swimming in the arena. Some of the animals

177   were swimming in straight lines with relatively few turns and changes in speed, representing what an

178   observer would describe qualitatively as low complexity trajectories. In contrast, other animals

179   moved in a more "complex" fashion, exhibiting sudden changes in direction and moving in spiral or

180   circular paths (see Fig 1E for example trajectories). We decided to quantify the local path complexity

181   using a method presented by Roberts et al. (51). In summary, this method uses embedding matrixes

182   for positions in a specific time window, over which the local path complexity is calculated in bits of

183   entropy. Minimal complexity values calculated by this method correspond to the most predictable

184   trajectory or, in other words, the most invariable movement in terms of represented speeds and

185   directions in the time window (see Fig 1F for example traces with decreasing local complexity).

186   While observing the animal trajectories, we noticed that a significant fraction of animals swam near

187   the edges of the arena. This behaviour has been previously identified in other organisms (13, 52-54)

188   including humans(55-57) and has been termed thigmotaxis. We decided to quantify the thigmotactic

189   behaviour of the larvae. To define thigmotaxis, we divided the arena into two concentric zones of

190   equal surface area. Animals in the outer zone were considered thigmotactic (Fig 1B). The amount of

191   thigmotaxis is quantified in two measures: "Total Time spent in Outer zone" (TTO) and "Total

192   Distance travelled in Outer zone" (TDO), as utilised for zebrafish previously (58).

193   A long-term goal of quantitative animal behaviour analysis is to identify the behavioural strategies

194   that animals use to solve tasks and to dissect the underlying neuronal mechanisms. One approach to

195   reach this goal is to generate a structured quantitative description of behaviour, by identifying

196   stereotyped behavioural components. In order to identify these behavioural components in behaving

197   *C. intestinalis* larvae, we used unsupervised clustering methods on a small feature- set (ρ- speed, $\Delta\rho$-

198   acceleration and $\Delta\phi$-turn angle values; see methods section for details), and we were able to identify

199   and classify 11 distinct clusters in our data set. We assigned a relevant descriptor of the behavioural

200   mode to each cluster according to its biological interpretation. This was done by inspecting the

201   original videos and superimposing corresponding cluster data. When unable to resolve a biological

202   difference between two clusters, we assigned them the same descriptive name. We list the

203   behavioural modes in approximate order of speed of movement in Table1 below (see Fig 1G for

204   some example traces of different modes and S1-S5 Video for example videos). This simple ontology

205   of the objectively detectable behavioural modes in our data set served as an extra tool in assessing

206   the effects of different stimuli and genetic or pharmacological perturbations on the behavioural
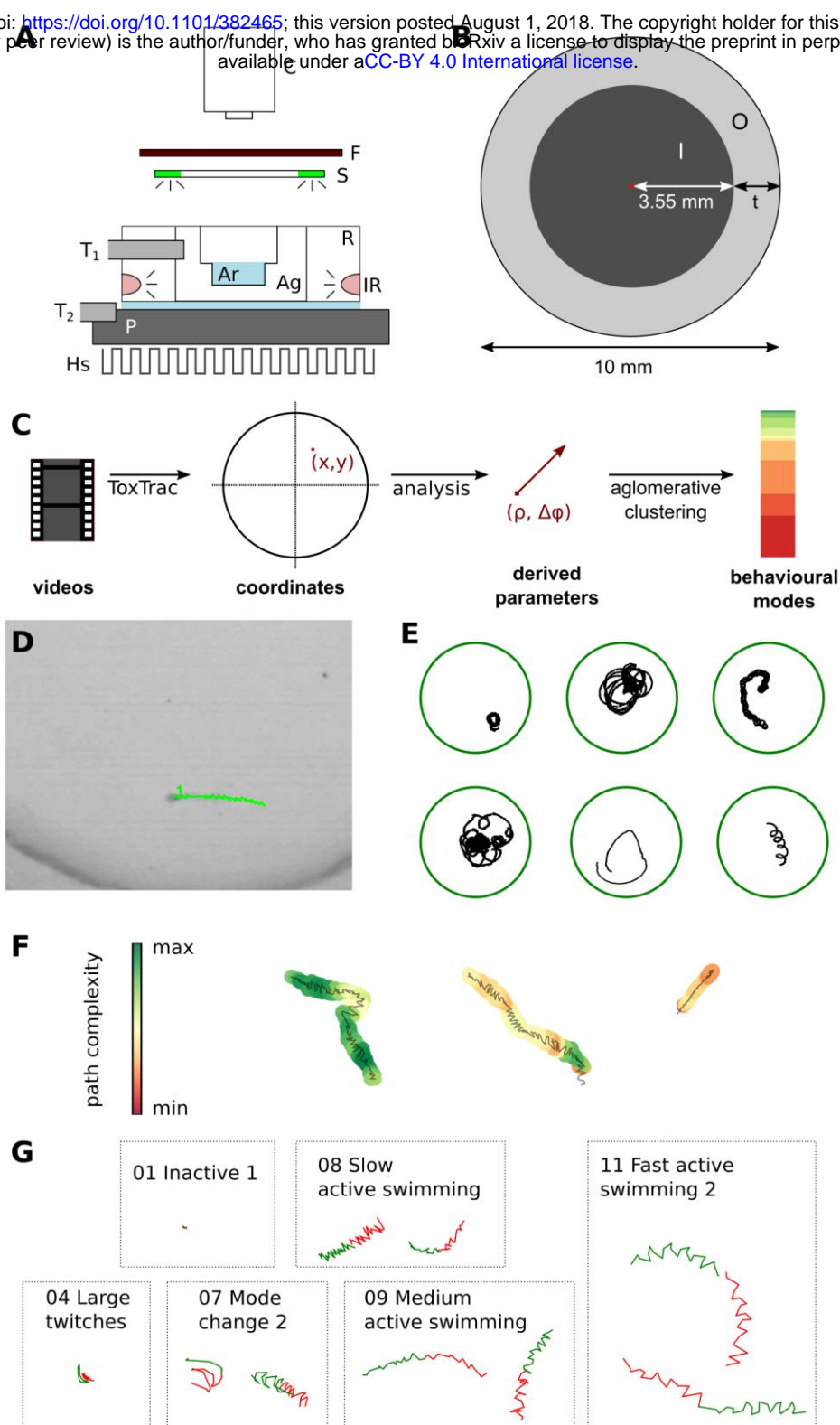
207   repertoire of the larvae.

Fig 1. Setup and analysis methods.

(A) Setup: C- camera, F- IR filter, S – ring with stimulation LED-s, T1 – Thermometer 1 measuring local temperature in the agarose, T2 – Thermometer 2 measuring cooling plate temperature, P – cooling plate, Hs – heat sink, R – PLA ring holding the arena, Ag– agarose, Ar– Arena with animals, IR – IR illumination. (B) Arena dimensions and areas used in thigmotaxis measures I- inner arena with radius 3.55 mm and O– outer thigmotaxis zone; with t= 1.45 mm the zones have equal surface area and the width of the thigmotaxis zone is above one animal body-length. (C) Workflow chart. (D) An example animal being tracked with the ToxTrac software (E) Some example trajectories of wild type animals swimming for 5 minutes (F) examples of different local complexity of a trace - each trajectory is coloured by local complexity which is calculated over a 3 s window and the total span of each trace is 6 s (G) Examples of traces spanning 50 frames based on which the current behavioural mode was calculated. In green are the 25 frames before the current time-point and in red the 25 frames later.

208   Table 1: Behavioural clusters with names.

Cluster	Descriptive name
01	Inactive 1
02	Inactive 2
03	Small twitches
04	Large twitches
05	Collision or deceleration event
06	Mode change 1
07	Mode change 2
08	Slow active swimming
09	Medium active swimming
10	Fast swimming 1
11	Fast swimming 2

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210   **Adaptation to the arena**

211   The introduction of an animal to a new environment, such as a tracking arena, is one of several

212   potential triggers of generalised nervous system arousal (59-61). Arousal can also be observed in

213   response to stimuli associated with harm, such as strong mechanical stimuli. In the case of the

214   nematode *C. elegans*, transferring an animal from one plate to another using a metal pick can result

215   in a temporally defined state of arousal demarked by higher motor activity that eventually returns to

216   baseline roaming locomotor activity(62). A similar observation has been made with mice when

217   placed in an open-field arena(63). This period is often termed the adaptation period. Given that

218   arousal mechanisms are evolutionarily conserved (64), we asked whether *C. intestinalis* larvae were

219   subject to generalised nervous system arousal as a result of the transferring process to the tracking

220   arena and the exposure to a new environment.

221   In order to answer this question, we decided that before recording the videos used for analysing

222   baseline behaviour, each animal would first be recorded for a 15 min period (Fig 2). In the first

223   minutes of the animal being exposed to the new environment its speed was generally higher (see Fig

224   2A for example traces), which we quantified as the slope of linear regression over the average speed

225   values of around 100 animals (Fig 2B, C). From these results, we inferred that the animals adapted to

226   the arena within approximately 6 minutes. We compared some basic behavioural parameters of

227   individual animals between the first, second and last third of the 15 min adaptation period (Fig 2 D-
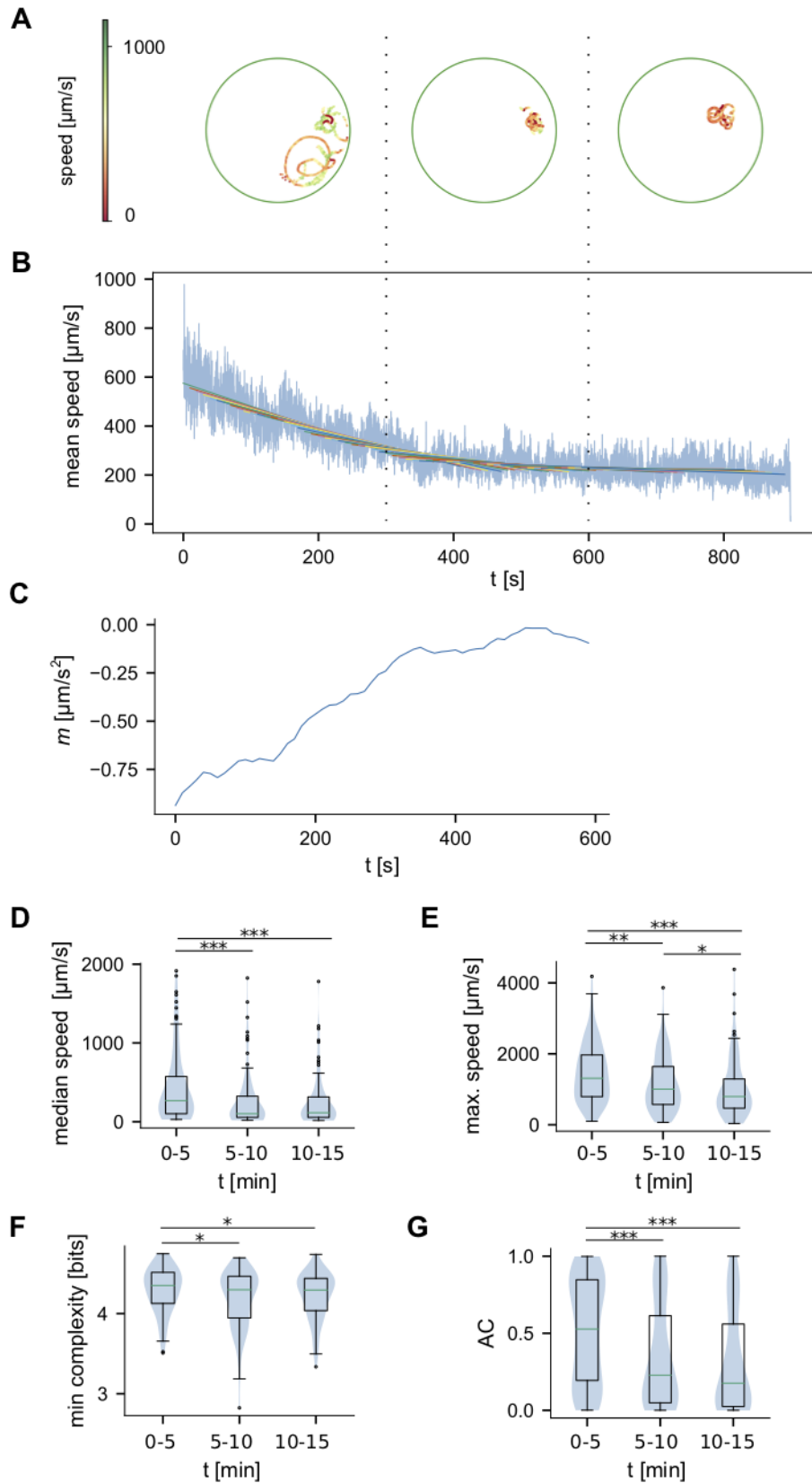
Fig 2. Adaptation of animals to the arena

Analysis of adaptation behaviour of larvae in the first 15 minutes after introduction to the arena. (A) An example trajectory of a path during the adaptation period. The path is plotted in thirds each corresponding to 5 minutes of the recording as illustrated by the dotted lines linking this and the next panel (B) Average speed of 105 animals during the 15 minutes after introduction to the arena shows higher speeds immediately after the animals were exposed to the new environment. (C) Slope of linear regression fitted to the average speed (window 5 min, step size 10 s) shows the stabilisation of speed after ca 6 min. We compared (D) median speed, (E) maximum speed, (F) minimal reached path complexity and (G) activity coefficient between the first, second and last third of the 15 min adaptation period (N_1= 105, N_2=102, N_3=92).

228   G). There are significant differences in both median speed (Fig 2D, median at 267 μm/s in the initial 5

229   min vs. 101 μm/s in the second and 113 μm/s in the last 5 minutes; $p < 0.001$ for both comparisons)

230   and maximum speed of the animals (Fig 2E, median at 1310 μm/s in the initial 5 min vs. 1003 μm/s in

231   the second and 795 μm/s in the last 5 minutes*; p(1 vs. 2) = 0.0013, p(1 vs. 3) < 0.001*, p*(2 vs. 3)*=

232   0.048). Interestingly, as a result of sensory arousal, *C. intestinalis* larvae exhibited higher minimum

233   path complexity in the first 5 minutes after they are placed in the arena (Fig 2F, 4.349 bit in the initial

234   5 min vs 4.297 and 4.293; *p(1. vs. 2.) = 0.027, p(.1 vs. 3.) = 0.0038*). This observation suggested that

235   the animals followed a more unpredictable, or chaotic trajectory during the original arousal period.

236   As they adapted they followed trajectories exhibiting lower entropy and thus higher degree of

237   predictability. In other organisms such as birds the trajectory component showing higher entropy is

238   thought to be associated with navigational uncertainty(65). Notably, the activity coefficient (Fig 2G,

239   median at 0.53 vs. 0.23 and 0.18; $p < 0.001$ for both comparisons) of the aroused animals is also

240   significantly higher in the first 5 minutes, suggesting that bouts of inactivity are suppressed during

241   this period. Finally, unlike mice in open-field tests, which show high thigmotaxis during the initial

242   period(63), we found no significant changes in thigmotaxis in the case of aroused larvae vs. animals

243   that have adapted (data not shown).

244   **Behavioural effects of larval crowd size**

245   In a laboratory setting we often study behaviour using individual animals in isolation. However, in the

246   wild, organisms are hardly ever acting in absolute isolation. Across the animal kingdom, locomotion

247   and response to environmental stimuli are influenced by both direct and indirect interactions with

248   conspecifics (66, 67). For example, interactions between individuals are important in mating(68), in

249   the protection of colonies from pathogens(69), and in response to predators(70, 71). Previously it has

250   been shown that mechanosensory interactions between desert locusts can lead to a dramatic

251   phenotypic change where the animals switch from a cryptic solitary phase to a gregarious phase(72).

252   More recently, in the genetically tractable organism *Drosophila melanogaster* it has been shown that

253   mechanosensory interactions between adults drive collective response towards a sensory cue(73).
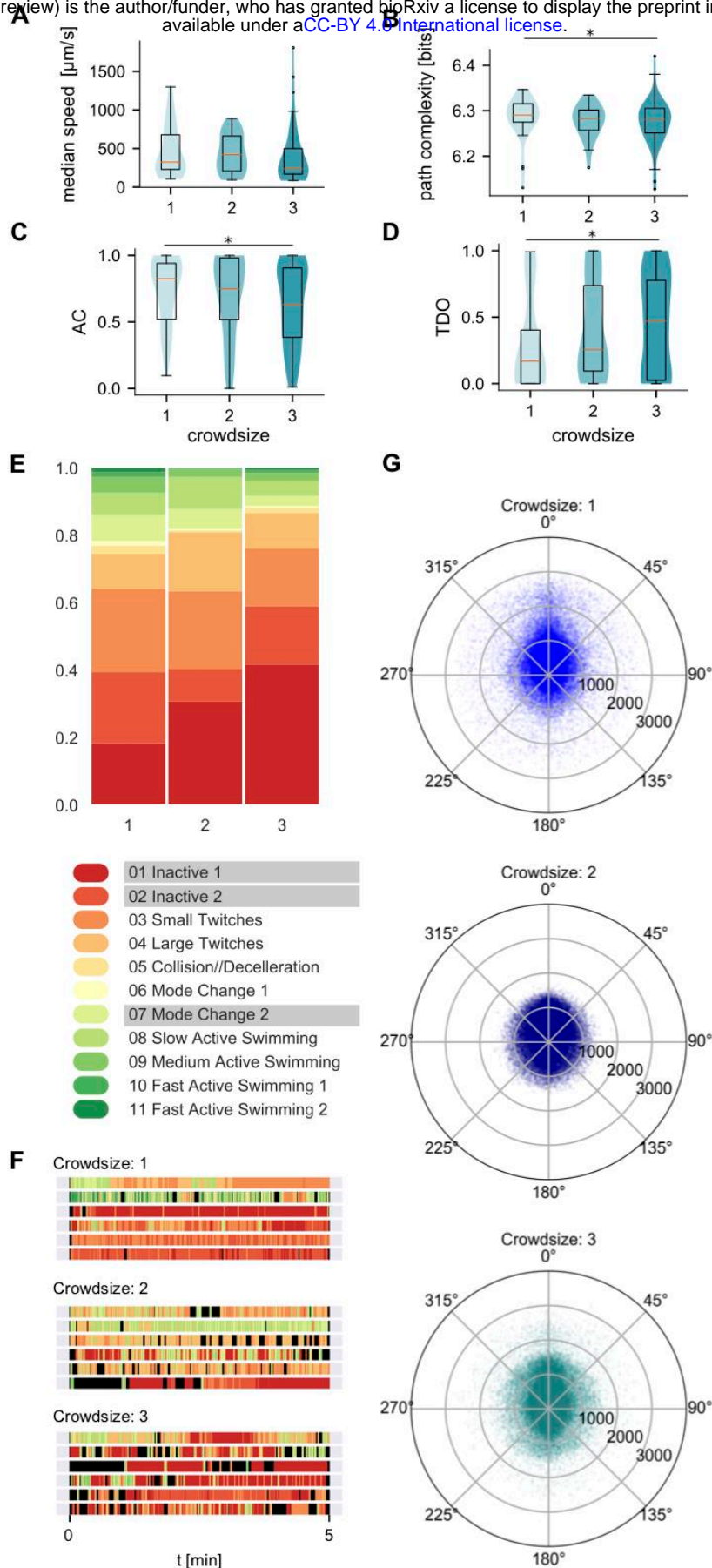
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Fig 3. Crowdsize effects on behaviour
Crowdsize effects on (A) median speed, (B) path complexity, (C) activity coefficient and (D) TDO. (E) Distribution of behavioural modes in animals in experiments with different crowdsizes. The modes underlined in the legend are the biggest contributors to the Chi2 statistics (data in S1 Table) (F) Example ethograms of individual animals in crowdsize experiments. Each line represents a 5 min recording and is coloured based on the assigned behavioural mode, black colour represents missing frames where modes could not be assigned. (G) Polar scatterplots of filtered speed values vs turn values for different crowdsizes (N1=33, N2=22, N3=46; number of points per polar plot is 100000).

254   The behaviour of *C. intestinalis* larvae in large groups has been studied to some extent, e.g. the

255   change in distribution because of water agitation(29) or stimulation with light (30). In our study, we

256   focused on the possibility of behavioural effects of small group interactions. To achieve this, we

257   compared animals that were alone in the arena with animals recorded in pairs or groups of three. We

258   found small differences in the basic behavioural parameters (Fig 3). Crowd size did not affect median

259   (Fig 3A) or maximum speeds of movement significantly, but had a weak effect on the path complexity

260   and activity coefficient (Fig 3B, C). The average path complexity was lowest for animals in the crowd

261   size 3 group (6.281 bits, compared to 6.290 bits for animals in crowd size 1 group, *p*=0.05) and so was

262   the median AC value (0.63 compared to 0.82 and 0.75 for animals in crowd size 1 and 2 groups

263   respectively, *p(1. vs. 3.)* =0.045). We also found slightly more animals with higher thigmotaxis values,

264   with the difference only significant for TDO (Fig 3D) between crowd size 1 and 3 (median TDO is 0.17

265   for crowd size 1 vs. 0.47 for crowd size 3, *p*= 0.0227). The distribution of different behavioural modes

266   shows higher representation of the less active modes corresponding to more time spent inactively in

267   crowd size 3 animals (Fig 3E). In Fig 3F we present sample ethograms of 6 individual animals in

268   different crowd size experiments. The smaller representation of higher speeds was also apparent

269   when we plotted the distribution of speed values with the corresponding turn values on a polar

270   scatterplot, presented for the different crowd sizes in Fig 3G.

271   **Age related changes in behaviour**

272   Behavioural changes that result from aging have long been the focus of various senescence studies,

273   but there are similarly important age related changes underlying the normal development of the

274   animals' behavioural repertoire through time(74-79). For larval *C. intestinalis*, light and gravity

275   dependent behaviours have already been shown to change with age (26, 31). Here we examined the

276   development of baseline behaviour in the early hours post-hatching. In animals reared and recorded

277   at 14 °C, we observed that in the first hour after hatching they were mostly inactive, followed by a

278   period of time when the larvae twitched very actively and flicked their tails but would not cover a lot
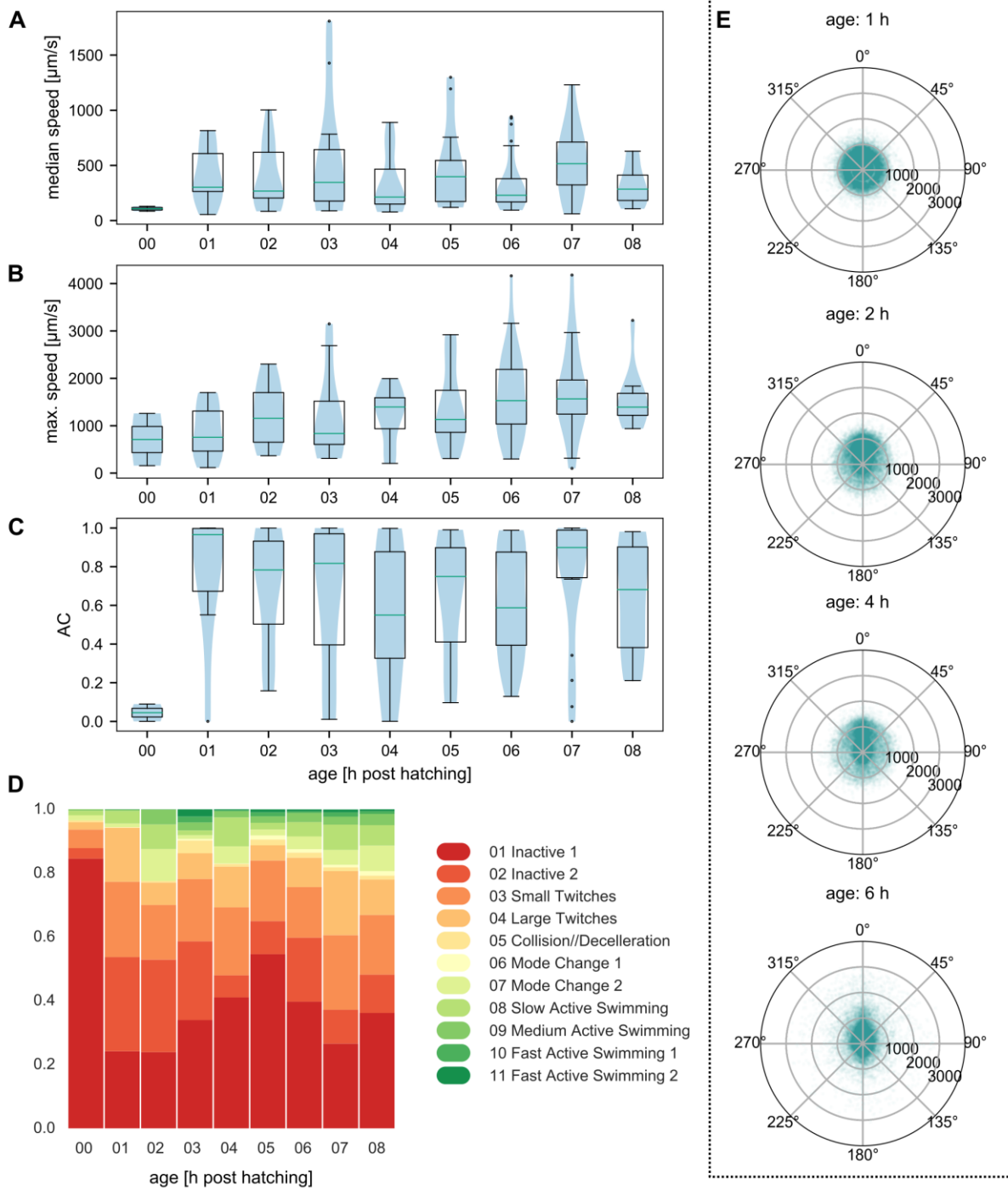
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Fig 4. Age related changes in behavioural parameters

(A) Median speeds, (B) maximum speeds and (C) Activity coefficient (AC) for WT animals reared at 14°C at different age post hatching. (D) Distribution of behavioural modes in animals of different age. (E) Speed-turn plots for ages 1, 2, 4 and 6 h post hatching. (N(00)=2, N(01)=8, N(02)=14, N(03)=12, N(04)=15, N(05)=12, N(06)=29,N(07)=18, N(08)=10, number of points per polar plot is 50000)

279   of distance swimming. In Fig 4 we show some of the basic behavioural descriptors compared

280   between ages of WT animals reared at 14°C. Both maximum and median speeds were lowest for

281   animals immediately after hatching (Fig 4A, B), accompanied by very low AC (Fig 4C). While animals

282   1h post fertilisation already achieved higher median speeds and generally had a very high AC, their

283   movement was less directional as can be seen by the distribution of turn values versus speed values

284   (Fig 4E) and the high representation of twitching modes in their behavioural repertoire (Fig 4D). To

285   minimise any potential skewing of the data because of age-dependent changes, we used animals of

286   2-8 hours post hatching age for all later comparisons, unless specified otherwise.

287   **Rearing temperature effects on a developmentally regulated behaviour**

288   We (Fig 4) and others have shown that as the *C. intestinalis* larvae go through the post-hatching

289   motile phase of their life cycle they change their behavioural responses at multiple levels. Such

290   behavioural changes are thought to be tightly linked to developmental changes taking place in the

291   larvae. An interesting question that arises is whether this developmental regulation of locomotor and

292   sensory behaviours is robust to different environmental circumstances, possibly through a

293   mechanism of canalization (80-83) or whether it shows plasticity (84-86). Temperature is one key

294   physical parameter that has been shown to affect the speed of most biological processes, acting as a

295   major environmental factor influencing the rate of animal development (87-89) and behaviour(90,

296   91). The two Ciona species (*Ciona intestinalis* and *Ciona robusta*) (92)occupy a very large part of the

297   world's coastline from high to low latitudes(93) and they show great adaptability to a range of

298   temperatures. Published studies have used 18°C as rearing and assay temperature for *C. intestinalis*

299   and *Ciona robusta* larvae. However, our local animals belong to the *C. intestinalis* species and

300   develop best at lower rearing temperatures, possibly due to an adaptation to the lower water

301   temperatures in the Norwegian Fjords. We tested whether the lower rearing temperature of 14°C

302   affected the onset of the light-off response that has previously been described by Nakagawa et

303   al.(28). The increase in swimming speed immediately after a light stimulus is considered a hallmark of

304   the older larva that will in its later age seek to settle utilising negative phototaxis. In animals reared

Fig 5. Light-Off response in animals reared and recorded at 14°C

(A) Experimental set-up. Light stimulus lasted 1 minute and the average speed of the animal in the last 10 s of the stimulation (L) was compared to the expected peak of speed upon onset of darkness (D) measured as average speed over 2.5 s starting from 0.5 s after light-off (B) Change in speed between L and D for different aged animals. (C) Distribution of behavioural modes in the last 10 s during a 1 min light stimulus – L and the onset of darkness – D for different aged animals (N_2=11, N_4=7, N_6=8)

305   at 18°C, the first notable response to a light-off signal was detected at 4 h post fertilisation,

306   coinciding with a reduced average speed of the animals in absence of the stimulus.

307   The sensitivity of this response has been shown to peak in the green part of the spectrum (28), so we

308   tested for light-off response in our animals using a green light (515-530 nm). We calculated the

309   change of speed (i.e. Δ speed (Off), Fig 5B) between the last 10 s during a 1 min light stimulus and the

310   first 2.5 s after the light was turned off (after a 0.5 s latency period). In Fig 5C we present the

311   distribution of the different behavioural modes for the same periods at the end of the light stimulus

312   (L) compared to the onset of the dark (D) for animals at three different post-hatching ages. At 2 and

313   even 4 h post hatching the animals reared at 14°C did not show a significant light-off reaction, with

314   the first notable response observed only at 6 h. The response was notable both as a slight general

315   increase in speed (Fig 5B) and more specifically as higher representation of twitching and medium

316   speed swimming at the expense of inactive modes (Fig 5C). It appears that *C. intestinalis* has the

317   potential to be a great genetically tractable model to answer the question of developmental stability

318   of behaviour.

319   **Rearing temperature effects on behaviour**

320   Animals reared at 18°C had a much narrower time window after hatching in which we could observe

321   active swimming behaviour, with the majority of animals being highly inactive by age 4 h post

322   hatching (data in S2C). We therefore only compared animals of age 0-3 h reared at 18°C to the 14°C

323   reared animals of ages 2-8 h, since we assumed they correspond to the same post hatching

324   development stages. Even when comparing what we assumed to be animals at a similar

325   developmental stage, animals reared and recorded at 18°C still exhibited some differences compared

326   to the ones at 14°C (Fig 6). Their traces were similar in median speed values (Fig 6A) but reach

327   significantly higher maximum speeds (Fig 6B, median at 1864 µm/s for animals reared at 18°C vs.

328   1440 µm/s for animals reared at 14°C, *p* >0.001). The difference in AC and path complexity is not

329   statistically significant (Fig 6 C, D), but there was a slight but significant effect on thigmotaxis (Fig 6E,
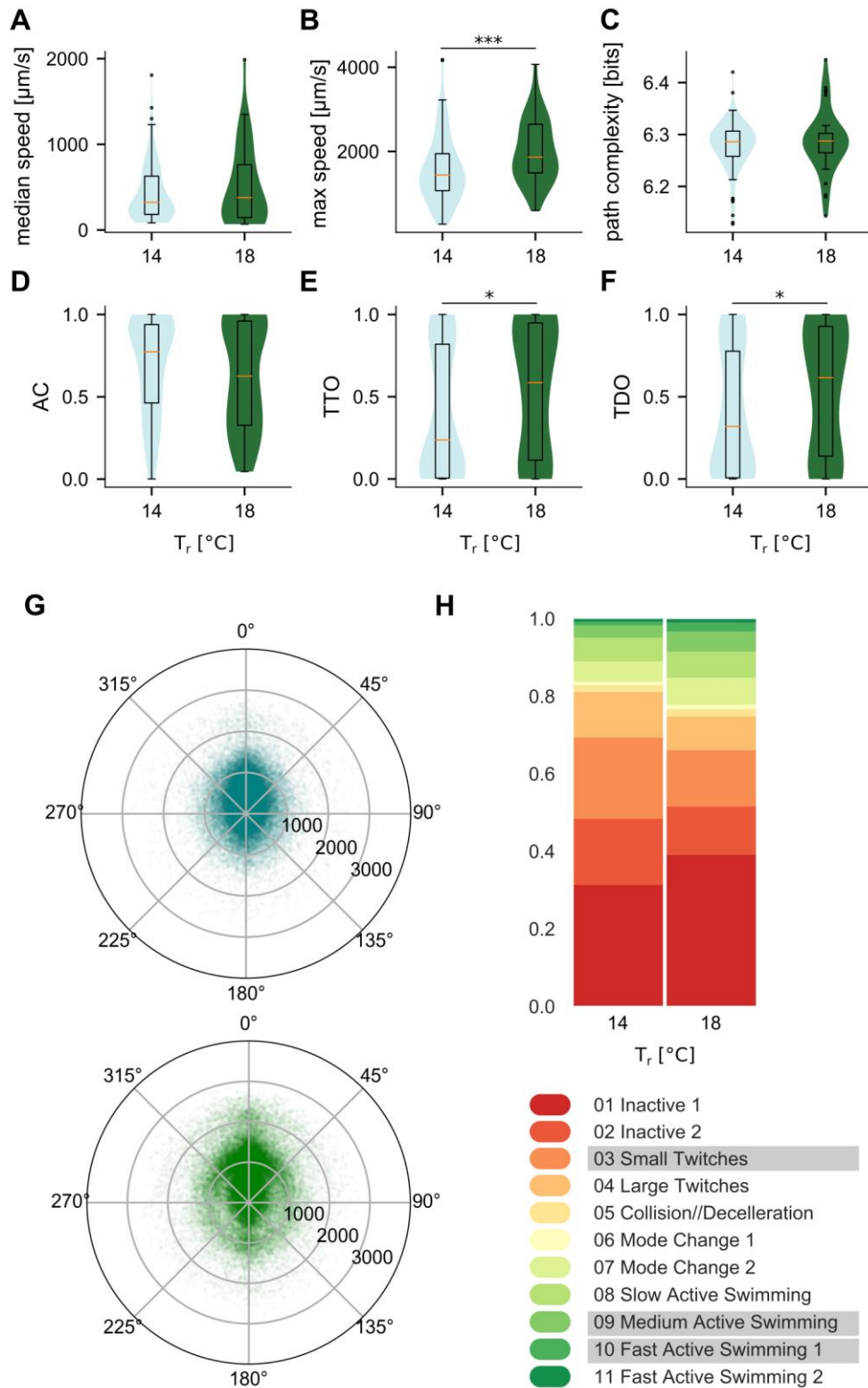
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Fig 6. Effect of rearing temperature on behaviour

Rearing temperature effects on (A) median speed, (B) maximum speed, path complexity (C), activity coefficient (D) and thigmotaxis measures (E) TTO and (F) TDO. (G) Polar scatterplots of filtered speed values vs turn values for animals reared and recorded at 14°C (in teal) and at 18°C (in green). (H) Distribution of behavioural modes for the two groups. The modes underlined in the legend are the biggest contributors to the Chi^2 statistics (data in S1 Table) (N(14°C) = 101, N(18°C) = 36; number of points per polar plot is 100000)

330   F). The median TTO for animals reared at 18°C was 0.585 vs 0.237 at 14°C (Fig 6E, p = 0.035) and the

331   median TDO at 18°C was 0.616 vs 0.319 at 14°C (Fig 6F, p = 0.038). At 18°C we also observed a higher

332   representation of medium-high speeds (around 1000-1500 μm/s) in combination with a wider range

333   of turn values, while at lower speeds the variability of turns was smaller (Fig 6G). This was matched

334   with a lower representation of twitching modes and more occurrences of the modes representing

335   swimming at medium speeds (Fig 6H).

336   **Dechorionation effects**

337   The eggs of *C. intestinalis* are nested in a chorion surrounded by follicle cells (Fig 7A) and the normal

338   development of left-right asymmetry in the embryo has been shown to be disrupted by

339   dechorionation (94). However, transient transgenesis of *C. intestinalis* is predominantly achieved by

340   electroporations, which do require the dechorionation of eggs. We therefore set out to test if

341   dechorionation has specific effects on behaviour. Being aware of any potential effects of

342   dechorionation will be vital for the future interpretation of behavioural phenotypes in transgenic

343   animals. The dechorionated larvae achieved a higher median (Fig 7B, 477 μm/s median vs 324 μm/s

344   in chorionated animals, p=0.0125) and maximum speeds (Fig 7C, 1784 μm/s vs 1440 μm/s for

345   chorionated animals, p=0.0024). The differences in AC and path complexities were not statistically

346   significant in our set (Fig 7 D, E). There was however a slight but significant effect on thigmotaxis (Fig

347   7 F, G), resulting in a higher median TTO (0.56 vs 0.23 for chorionated animals, p= 0.035) and TDO

348   (0.58 vs 0.32 for chorionated animals, p= 0.033) values for dechorionated animals. The differences in

349   distribution of turns and speeds were less apparent compared to the effect of temperature, but the

350   increased representation of higher swimming speeds in dechorionated animals was evident in the

351   polar scatterplots (Fig 7H) as well as form the distribution of the behavioural modes (Fig 7I).
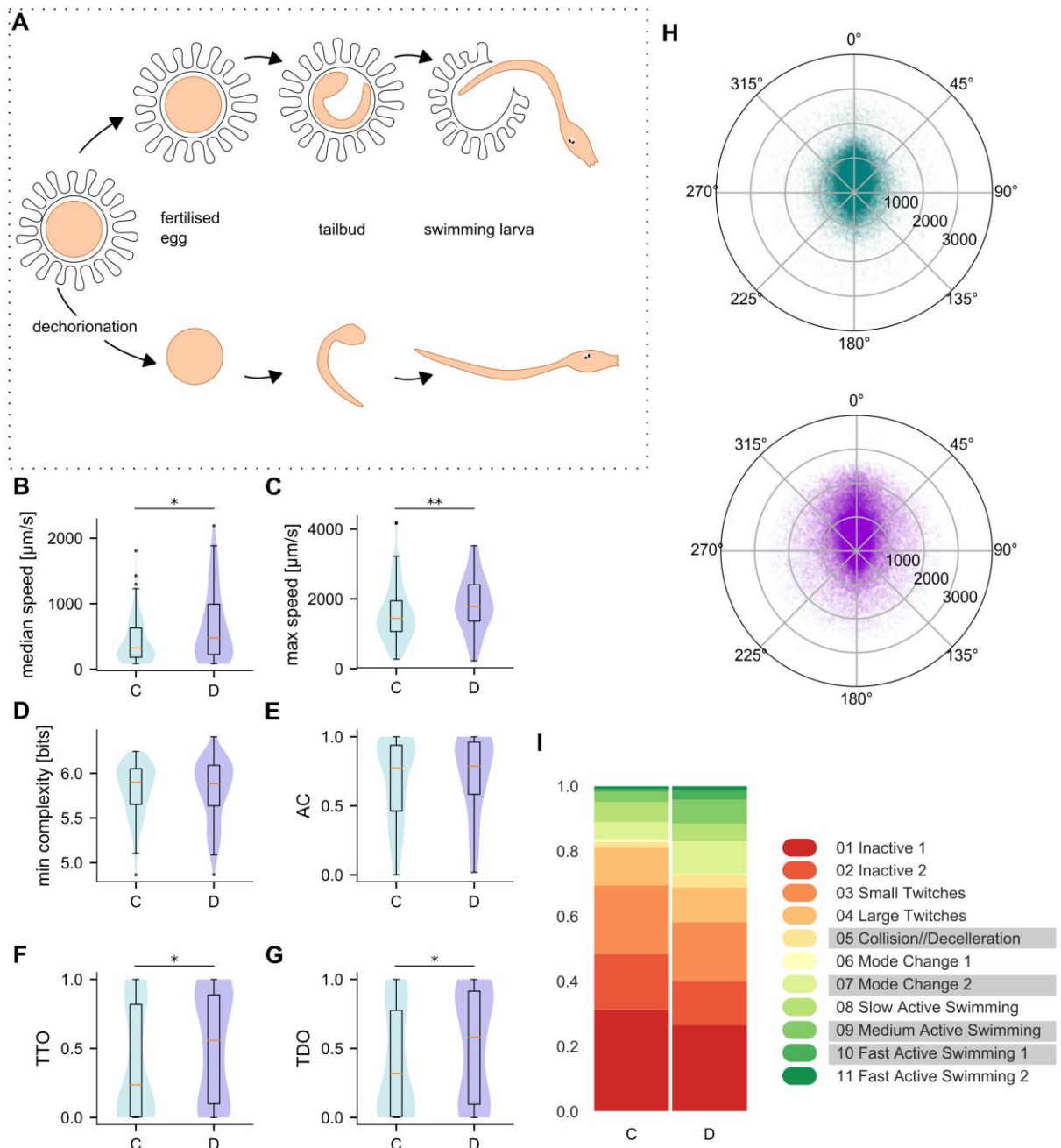
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Fig 7. Effect of dechorionation on behaviour

(A) Schematic representation of the *C. intestinalis* embryo development inside the chorion (top) in untreated animals compared to dechorionation and subsequent development of a dechorionated embryo (bottom). We present dechorionation effects on (B) median speed, (C) maximum speed, path complexity (D), activity coefficient (E) and thigmotaxis measures f) TTO and (G) TDO. Chorionated animals are denoted as C, dechorionated as D. (H) Polar scatterplots of filtered speed values vs turn values for dechorionated (purple) animals compared to chorionated animals (teal). (I) Distribution of behavioural modes for the two groups. The modes underlined in the legend are the biggest contributors to the Chi^2 statistics (data in S1 Table) (N(C) = 101, N(D) = 74; number of points per polar plot is 100000)

355 **Thigmotaxis and effect of modafinil**

356 Finally, we attempted to influence thigmotaxis in our animals by exposing them to an anxiotropic

357 drug modafinil. In this set of experiments, we compared two groups of animals swimming in 20 mg/l

358 and 2 mg/l solution of modafinil respectively to a control group in DMSO and to the untreated WT

359 set (Fig8, S4). We determined the dosage based on preliminary tests and previously published

360 literature on modafinil effects on larval zebrafish (95). The effect of 20 mg/l modafinil on thigmotaxis

361 was very pronounced and statistically significant (Fig 8E, F). While the median TTO value for the

362 DMSO control was 0,427, it reached 0,669 in the 20 mg/l modafinil group signifying longer periods

363 spent in the outer zone of the arena (p =0.0015). Similarly, the TDO measure shows the modafinil-

364 affected animals travelled much bigger proportions of their total distance in the outer zone of the

365 arena (median TDO at 0.628 in the 20mg/l modafinil group compared to the DMSO control at 0.395,

366 $p<0.001$). For the 2 mg/l group the distribution for both thigmotactic measures was similar to the WT

367 state and was not statistically different from either of the controls.

368 The animals affected by modafinil also exhibited an overall more active set of behaviours with much

369 higher representation of the active-swimming modes and less time spent inactively (Fig 8G, H). This

370 resulted in increased median and maximum speeds (Fig 8A, B) and higher AC values (Fig 8D) for

371 animals in 20 mg/l modafinil. Path complexity (Fig 8C) showed fewer differences with the only

372 significant change being the higher minimal complexity values for 20 mg/l group (5.99 compared to

373 5.85 in DMSO and 5.78 in 2mg/l).
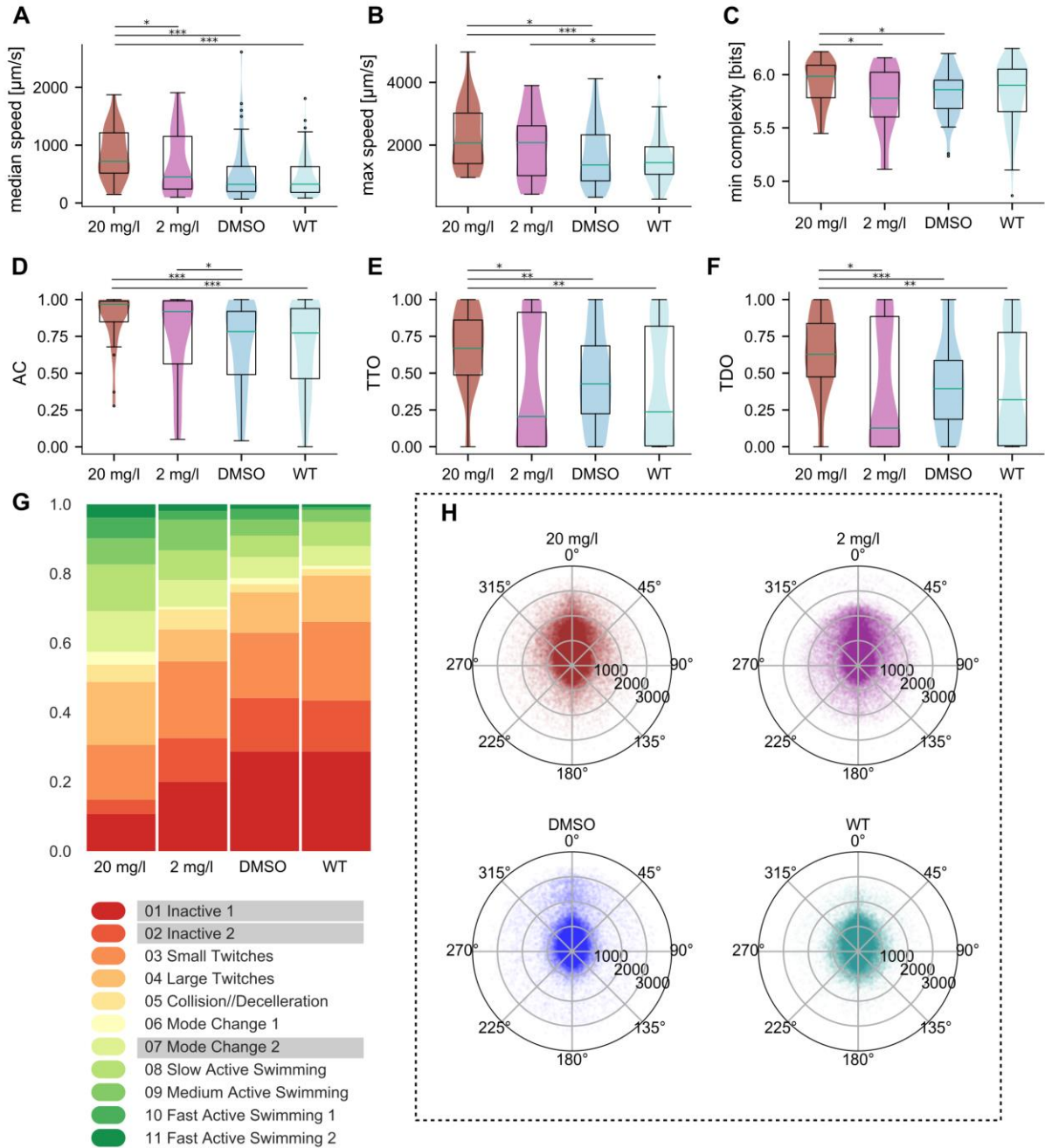
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Fig 8. Effect of modafinil on behaviour

Modafinil effects on (A) median speed, (B) maximum speed, (C) minimal path complexity, (D) activity coefficient and thigmotaxis measures (E) TTO and (F) TDO. The two groups affected by 20 mg/l and 2 mg/l modafinil are plotted in dark red and purple respectively, the control group in 0.1% DMSO in blue and wild type animals in light teal. (G) Distribution of behavioural modes for the groups. (H) Polar scatterplots of filtered speed values vs turn values the different groups. (N(20mg/l) = 28, N(20mg/l)= 27, N(DMSO) = 48, N(WT)= 101; number of points per polar plot is 100000)

381 **Discussion**

382 The chordate *Ciona intestinalis* in its larval form is emerging as a promising organism for

383 neuroethological studies. The present study provides a quantitative description of larval behaviour in

384 different contexts, using biologically relevant features. We performed unsupervised clustering of our

385 data and identify clusters, with which we generate a behavioural ontology. We uncovered some of

386 the behavioural effects of post-embryonic development and dechorionation on larval behaviour and

387 pinpoint the behavioural consequences of crowd size and sensory arousal. Furthermore, we provide

388 evidence that *C. intestinalis* larvae exhibit thigmotactic behaviour that can be modulated by the drug

389 modafinil.

390 **Quantitative description of *Ciona intestinalis* larval behaviour**

391 The potential of the *C. intestinalis* larva as an organism to perform neuroethological studies has been

392 noticed for several decades. There have been several efforts to perform behavioural studies with

393 increasing sophistication over the years (26-33). However, automated quantitative analysis of *C.*

394 *intestinalis* behaviour had been hindered by the lack of suitable open-source software with the ability

395 to follow the larvae providing precise positional information over long time series, with few or no

396 identity switches in case multiple animals are tracked simultaneously. In this study, we identified

397 Toxtrac (43) as a suitable open-sourced tracking software, we built customizable hardware and

398 developed an automated behaviour analysis pipeline for *C. intestinalis* larvae.

399 Our analysis suggests that *C. intestinalis* larvae show a surprising amount of complexity in their

400 spontaneous swimming behaviour. Notably, the larvae exhibited a large range of swimming patterns

401 showing significant variation in path complexity (Fig 1F), and individuals used a broad range of

402 behavioural mode sequences (e.g. Fig 3F). The advent of tracking methods has revealed the presence

403 of multiple characteristic scales of organisation in single and multiple animal traces that can be

404 explained only if we consider theoretical frameworks for mobility that extend beyond simple

405 diffusion mechanisms (96, 97). In the future it would be interesting to study the temporal structure

406 of spontaneous swimming in *C. intestinalis* larva in greater detail. Intriguing topics to investigate are

16

407   for example whether some of the swimming patterns of *C. intestinalis* exhibit a Lévy-like

408   behaviour(49, 98) and whether they may play a role in a dispersal strategy(99, 100) . In accordance

409   with our expectations, external sensory cues seemed to influence the swimming strategy of the

410   larvae. Interestingly, path complexity appeared to be modulated in opposite ways by sensory arousal

411   and crowd size (Fig 9).

412   The sensitivity of our measurements allowed us to quantify the activity levels of the animals in

413   different experimental contexts. We found that larvae showed both bursts of activity and bouts in

414   irregular intervals. Intermittent locomotion(101) is one of the various morphological (102)and

415   behavioural strategies(103, 104), often under evolutionary pressure(105), that have been

416   implemented by moving organisms, including marine larvae in order to conserve energy(106). An

417   alternative hypothesis is that fluctuations in activity levels may reflect an adjustment of motility to

418   small changes in flow taking place in the arena. Similar bouts and bursts of activity characterise

419   zooplankton motility in turbulent flow(107).

420   Quantitative behavioural analysis has been moving from subjective observation, and imprecise

421   annotation of behavioural data, towards the automated recognition and classification of behaviours

422   amongst very large data sets (2, 4, 7, 108). Some of the breakthroughs that have permitted this

423   progress have focused on generating low-level representations of behaviour for automated analysis

424   and automated classification algorithms of behaviours. The ultimate goal is to break down complex

425   behaviours into their constituent building blocks. In this study, we employed a clustering

426   methodology, using first unsupervised clustering of a minimal feature-set to identify behavioural

427   modes, followed by training a K-Nearest-Neighbours classifier in order to classify all of our data

428   corresponding to approximately 1.8 million observations from 850 data-frames. We found 11 distinct

429   clusters that were classified in an equal number of basic behavioural modes. These 11 behavioural

430   modes provide an unbiased way to dissect the structure of behaviour and will allow us to

431   systematically classify complex behavioural phenotypes that result from pharmacological, genetic or

432   optogenetic perturbations (109-112). However, our current automated image based tracking

433   approach is relying on marking each animal with a centroid rather than segmenting out the entire

434   shape of the animal in order to generate an outline or a skeleton. We therefore are lacking postural

435   information that would enrich our dataset significantly. This presents an important next step towards

436   obtaining a complete ethogram of *C. intestinalis* larval behaviour. We note however, that clustering

437   data points from centroid analysis already allowed us to describe a number of behavioural modes

438   accurately. Furthermore, we are testing our animals in an open field arena that is suitable for

439   recording a relatively small number of animals, possibly in a setting that is relatively distant to the

440   natural ecological niche of the larvae. This problem is faced by numerous experimenters who are

441   trying to obtain high quality tracking data in a controlled environment(5). We envision that in the

442   future the use of larger arenas and the ability to deliver multiple sensory stimuli reaping the benefits

443   of the open architecture of the behavioural setup, will allow us to study other ecologically relevant

444   behaviours such as settlement behaviour and metamorphosis more closely.

445

446   **Arousal from transfer to new environment**

447   Animals have the ability to modulate their readiness to react to sensory cues, in a phenomenon

448   known as arousal. This modulation is very obvious when comparing the states of sleep and

449   wakefulness. In addition, during the awake period, animals are able to enter short-term behavioural

450   states, during which they exhibit heightened activity and general or specific sensory stimulus

451   responsiveness and thus are able to anticipate and address sudden challenges (113). Here we report

452   that *C. intestinalis* larvae are in a state of arousal during the first minutes of being placed in the arena

453   (Fig 2, 9). Generalised arousal is thought to be widespread across vertebrates (59, 60) but the

454   detailed neuronal and molecular mechanisms are still poorly understood. In fact, amongst

455   invertebrates, there is evidence that sensory arousal is present in Aplysia(114) and in ecdysozoans,

456   like the nematode worm *C. elegans* (62, 115) and *Drosophila melanogaster*(116, 117). In the case of

457   *C. elegans*, sensory circuits involved in sensing high threshold mechanical and noxious stimuli are

458   implicated in the heightened state of arousal. Given that the arousal exhibited by the *C. intestinalis*

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459   larvae is likely due to mechanical stimulation from transfer to the arena it would be interesting to

460   study the contribution of mechanosensory circuits to this behavioural state. In the case of

461   Drosophila, acute sensory arousal is more apparent when comparing between states of wakefulness

462   and sleep(118). A first clue as to which circuits might be involved comes from the observation that

463   arousal in *C. intestinalis* is modulated by the drug modafinil (Fig.9). Modafinil has been classified as a

464   psychostimulant and has been extensively used in narcoleptic patients in order to address sleep

465   related disorders. It is thought to act as a selective dopamine (119) and norepinephrine transporter

466   inhibitor (120), thus raising the possibility that monoamine signalling plays an important role in

467   modulate the arousal state in *C. intestinalis* larvae. In this study we show that beyond modulating the

468   arousal state of the animals, modafinil appears to alter the activity coefficient. The larvae also

469   showed less quiescent periods with a higher activity coefficient. This has also been observed in mice

470   and zebrafish. In mice, modafinil results in wake-promoting action, possibly via dopamine

471   transporters(121), while in zebrafish modafinil shortens the periods of sleep(122).

472

473   **Age related changes**

474   A large number of animals exhibit behavioural changes linked to post-embryonic development of

475   their nervous system. For example, in *Xenopus laevis*, locomotor activity patterns are modified as the

476   animals transition from sessile hatchlings to free-swimming larvae(76), through changes in the cell

477   properties of neurons(123) and a nitrogen oxide signalling mechanism (75). Other examples of post-

478   embryonic changes in behaviour include the photoresponses of the stick insect *Carausius morosus*

479   (77) and the chemotactic responses in *C. elegans*(124). *C. intestinalis* larvae are also subject to post-

480   embryonic developmental changes and associated behavioural modifications. It has previously been

481   reported that light and gravity dependent behaviours change during the larval life (26, 31). Here we

482   quantified how progression through larval life changes behaviour of the animals, and found age

483   dependent differences in the distribution of behavioural modes (Fig 4). This puts a quantitative angle

484   to previously reported findings in the literature(30). What may be the mechanisms that bring about

19

485 these changes in behavioural modes used in larvae of different ages? One possibility is that some

486 neurons of the larval system fully differentiate and connect to the nervous system only after

487 hatching. Indeed, there has been evidence for post embryonic terminal differentiation of

488 dopaminergic cells in *C. intestinalis* larvae (125). The authors of the study postulated that dopamine

489 might modulate the neural circuits involved in the age-dependent changes in swimming behaviour of

490 the larva. The genetically tractable *C. intestinalis* is proving to be a powerful model for providing in-

491 depth insight into developmental processes in the post-embryonic nervous system and the functional

492 basis of locomotor dynamics changes throughout larval life. Notably, the use of a fast-growing larval

493 animal with associated changes in body proportions and shape, provides us with the opportunity to

494 understand how alterations in biometrics and sensory capacity may relate to simultaneous changes

495 in locomotory behaviour.

496

497 **Crowd size effects on larval behaviour**

498 Many behaviours, such as mating, shoaling, schooling, aggressive encounters rely on the interaction

499 of two or more individuals. These interactions largely depend on achieving a coordinated movement

500 between individuals and the entire group(126).

501 Previous work in *C. intestinalis* has shown that larvae can aggregate into a column when placed in a

502 three dimensional chamber and that they can form swarms, especially upon agitation of the

503 water(29). Also it has been shown that larval distribution can change in the presence or absence of

504 light(30). Notably, it has been reported that ascidian behaviours prior to settlement are largely

505 influenced by conspecifics, while the larvae exhibit a form gregariousness(35, 127). However, these

506 are largely qualitative observations that were made in the course of experiments that were not

507 designed to specifically address the interactions between conspecifics. Further difficulties for

508 providing a quantitative description of larval interactions stem from the lack (until recently) of

509 automated tracking software that faithfully maintained the identity of each tracked animal(5). Taking

510 advantage of the ability of ToxTrac to maintain the identity of multiple animals in the same arena we

511   attempted to determine whether there are differences when single vs multiple individuals are placed

512   in the arena. We find that the presence of two or three animals in the arena can already result in a

513   few changes in the measured behavioural parameters (Fig 9). Notably, a significant change in one of

514   the thigmotactic indices is also observed (Fig 3D). Interestingly, an enhancement of thigmotaxis in

515   individual versus group context has been observed in the case of ants(128). Given the past literature

516   it will be interesting to determine if and how *C. intestinalis* larvae achieve coordinated movement. It

517   is believed that the type of distributed sensing required to generate robust collective behaviour is

518   rather simple, requires rudimentary circuits and thus it may be widespread across different animal

519   taxa(129). *C. intestinalis* has a small nervous system and thus is ideal to study the neural circuits

520   controlling pairwise and group-level behaviours. We note that a limitation of our method is that our

521   crowd size experiments were conducted in an extremely small volume of sea water compared to the

522   ethologically relevant volumes that these animals would encounter in the sea. Future experiments

523   should be conducted in larger arenas that may be ethologically more relevant for crowd size

524   experiments.

525

526   **Behavioural robustness to altered rearing conditions**

527   Temperature is a known modulator of key physiological processes and behaviours in numerous

528   animals(130). For example, thermal rearing conditions can affect the dispersal of adult spiders(131),

529   the host-seeking behaviour of parasitic nematodes(132), mating behaviour in Drosophila(133), and

530   the feeding behaviour in the mud snail *Heleobla australis*(134). To contextualise our data we

531   compared our main wild type group reared and recorded at 14°C to animals at 18°C, since behaviour

532   of *C. intestinalis* larvae has previously often been studied at 18°C e.g. (27, 31, 32) or even at room

533   temperature (29) . We describe the distinct difference in the speed of post hatching development at

534   different rearing temperatures (Fig 4, S2) At 18°C the period of higher spontaneous locomotor

535   activity coinciding with the lack of significant responses to light-off stimuli lasts for ca. 4 h post

536   hatching (28). In animals at 14°C we can detect the first significant, yet still weak, responses to the

537   light-off signal at 6 h post hatching (Fig 5), coinciding approximately with the period of higher

538   locomotor activity in animals up to 8 h post hatching. Apart from this clear influence in the rate of

539   post hatching development, the higher temperature seemed to have little effect on the animals'

540   behaviour, but we do note the higher representation of medium-high speeds in combination with a

541   wider range of turn values (Fig 6). It may be the case that rearing temperature has no strong

542   behavioural defects in *Ciona intestinalis* larvae, possibly through a buffering mechanism.

543   Alternatively, we may have not identified the behaviours and sensory modalities affected by rearing

544   temperature. This is a plausible explanation in light of significant evidence suggesting that not all

545   sensory modalities are affected equally by the rearing temperature or from deviations from that,

546   since olfaction appears to be particularly strongly affected (135-139) compared to other sensory

547   modalities in other organisms.

548   Yet another treatment that can challenge the behavioural robustness of *Ciona intestinalis* larvae is

549   the enzymatic removal of the chorion that envelopes the eggs, a process termed dechorionation.

550   This enzymatic treatment is an essential step in the generation of transgenics via electroporation.

551   However, it can potentially interfere with the establishment of brain asymmetry in the ascidian brain,

552   which is dependent on an intact chorion(140). Given that left-right asymmetries in behaviour and in

553   nervous system structure are abundant phenomena across different animal taxa(141), it was of

554   paramount importance to understand, in the first instance, the effects of dechorionation to

555   spontaneous larval swimming. The behavioural comparison of chorionated versus dechorionated

556   animals revealed differences in speed and thigmotaxis. Unexpectedly, dechorionated animals swam

557   faster and showed higher thigmotaxis levels (Fig 10D). These observations suggest that future

558   quantitative behavioural studies making use of electroporated transgenic animals need to use

559   dechorionated animals for 'wild-type' controls rather than larvae hatched from chorionated eggs.

560   Nonetheless, it is encouraging to see that dechorionated larvae are in many behavioural parameters

561   indistinguishable from chorionated egg derived larvae.

562

563 **Thigmotaxis and modafinil effects**

564 Using an open-arena to monitor our animals, we noticed that a large fraction of larvae exhibited

565 strong thigmotactic behaviour. This appears to be an adaptive behaviour that has been observed in

566 numerous organisms, where the circular wall of the arena allows the animals to exhibit a defensive

567 response (i.e. to hide from potential predators) and facilitates their orientation in space(142).

568 Therefore, it is not unlikely that thigmotaxis presents an evolutionarily conserved behaviour. One

569 may wonder what role thigmotaxis plays in the larvae of *C. intestinalis*. In fact, almost thirty years

570 ago it was hypothesised that thigmotaxis, amongst other behaviours, may be involved in the

571 selection of habitats for larval settlement(35). Interestingly, we have been able to demonstrate that

572 physiological (rearing temperature) and morphological changes (dechorionation) can affect the

573 animal's ability to perform thigmotaxis. Moreover, we found that modafinil increased thigmotaxis

574 levels in *C. intestinalis* larvae. This is interesting in light of the fact that the effects of modafinil

575 treatment in both humans and other animals has shown variable effects. In some cases, it acts as an

576 anxiogenic drug like in one study in humans (143)and in others as an anxiolytic drug such as in

577 marmosets(144). Notably, one study showed that modafinil increased the exploratory behaviour of

578 mice in a dose dependent manner (145). It has also been shown that Modafinil can reduce

579 thigmotaxis levels in zebrafish, (95). The strong effect that the anxiotropic drug modafinil has on *C.*

580 *intestinalis* larval thigmotaxis is evidence that a common mechanism might mediate thigmotaxis

581 across taxa(63, 95, 146). Future work should explore the molecular and cellular underpinnings of

582 thigmotactic behaviour in *C. intestinalis* larvae and aim to understand the ecological context in which

583 it may be used.

584

585 *Ciona intestinalis* **as a neuroethological model**

586 For years, cutting edge functional imaging and automated behavioural analysis was the privilege of a

587 few model organisms. However, there has been a turn of tide in recent years. For one, the

588 understanding that comparative studies are very important in order to draw conclusions on the

589   generality of biological phenomena and mechanisms, drew the attention to non-model organisms. At

590   the same time, functional imaging techniques and molecular toolkits started to became highly

591   adaptable, in order to meet the requirements of previously intractable nervous systems(147). The

592   same trend was observed with open-source machine vision and machine learning tools that could be

593   used to automatically track a large variety of organisms with different shapes and behavioural

594   repertoire (16, 18, 148). The larval form of *C. intestinalis* fulfils several criteria that would allow it to

595   ride this wave of change in neuroethology.

596   Phylogenetically*, Ciona intestinalis* is positioned at a key evolutionary node, as a member of the sister

597   group of vertebrates(149). Its compact nervous system showcases numerous developmental

598   mechanisms and gene regulatory networks common to all chordates, including vertebrates(150). The

599   complete mapping of the larval wiring diagram using Electron Microscopy (22-24) allows for

600   structural framework upon which to generate testable hypotheses. Most importantly the genetic and

601   genomic toolkit (41, 151) together with the ease of transgenesis and the transparency of the larva

602   make this organism particularly amenable to optogenetics and functional imaging. Notably, the

603   Genetically Encoded Calcium Indicator (GECI) GCaMP6s, has already been used in *C. intestinalis* to

604   study developmental calcium dynamics (152-154). The adoption of GECIs in *C. intestinalis* combined

605   with our approach of automated behaviour recognition and analysis have allowed us to

606   systematically dissect the larval behavioural repertoire and image the entire nervous system in vivo,

607   with the ultimate aim to elucidate the neural networks underlying behaviour. With our approach, we

608   were able to show that we can quantify larval behaviours automatically and identify novel

609   behaviours (thigmotaxis) and behavioural states (arousal). This approach also allowed us to

610   investigate the robustness of the behavioural repertoire under diverse environmental,

611   developmental and pharmacological conditions. Future work, includes obtaining a more detailed

612   mechanistic understanding of the stimulus driven behaviours, social interactions and learning
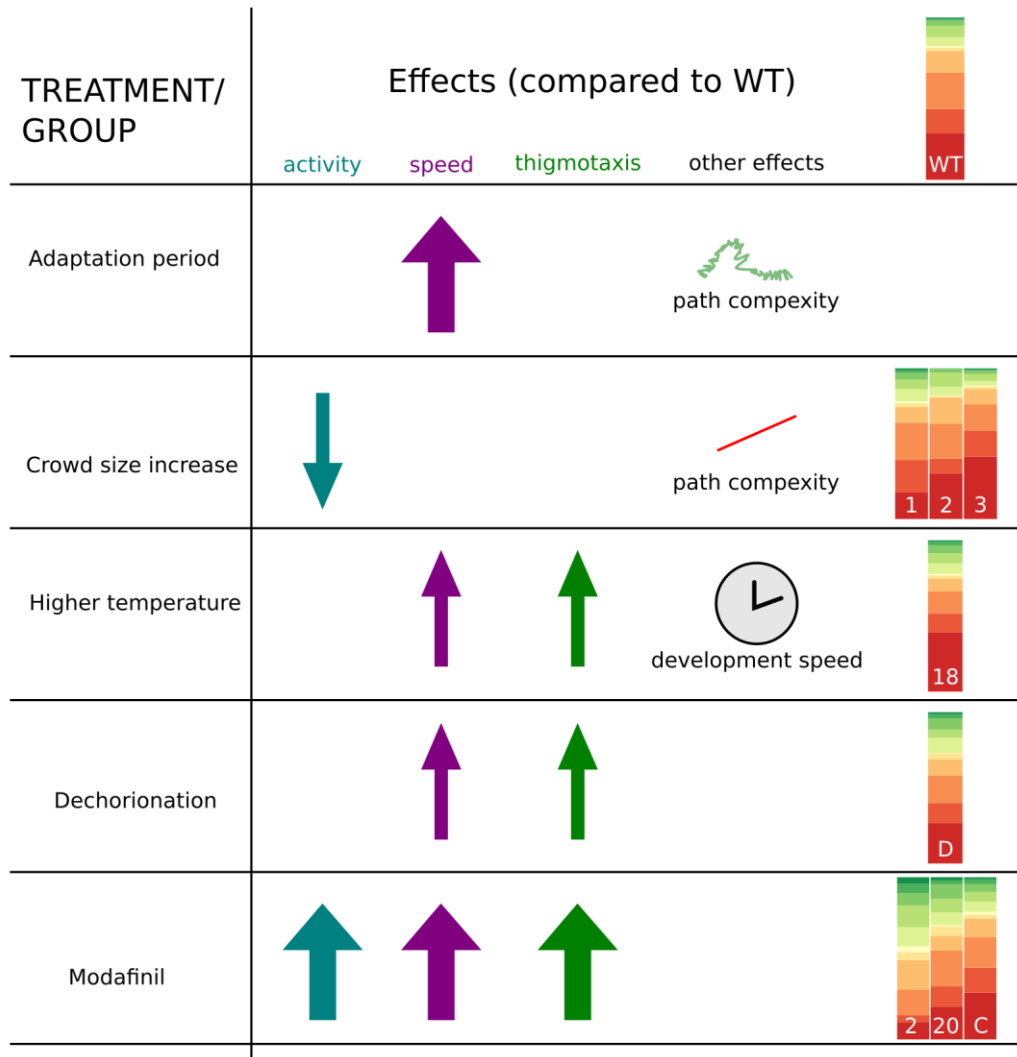
613   paradigms.

614

Fig 9. Summary

During the adaptation period (A) *C. intestinalis* larvae exhibited sensory arousal, which translated to higher speeds and increased path complexity. The presence of conspecifics in the arena (B), resulted in reduced locomotor activity, reduced path complexity and a change in the distribution of behavioural modes. We tested the robustness of behaviour in the context of rearing temperature (C) and dechorionation (D) treatments. Finally, the anxiotropic drug Modafinil (E) was able to modulate thigmotaxis, arousal and the overall state of animal activity, by changing the distribution of the behavioural modes.

615 **Methods**

616 **Animals**

617 Adult Ciona intestinalis were collected locally from the Bergen area and Sotra Island, Norway. We

618 incubated them in filtered seawater at 10°C under constant illumination to stimulate egg production.

619 Eggs and sperm were obtained from individual animals to perform in vitro fertilisation. Part of the

620 eggs were dechorionated using Na-Thioglycolate and mechanical dechorionation(155). Both eggs

621 with and without chorion were fertilized at the same time and incubated in artificial sea water (ASW,

622 Red Sea Salt) at either 14 or 18 °C. The post hatching age of animals is referred to relative to the

623 onset of hatching of larvae from the chorion.

624

625 **Set-up for behavioural experiments**

626 Animal behaviour was recorded in a custom-made setup developed in our lab (Fig 1A). Using a 3D-

627 printed PLA mould, we made single-use agarose arenas (0.8% in ASW, by Invitrogen, USA,). The arena

628 was nested inside a PLA ring with infrared (IR, peak emission 850 nm) LEDs, which provided dark-field

629 illumination of the animals without stimulating their photoreceptors. The ring also held a small

630 thermometer (DS18B20, Maxim Integrated) positioned close to the arena and was placed on top a

631 Peltier element with a thin layer of ASW underneath the agarose to improve heat conduction and

632 image quality. Light stimulation was performed using LED illumination (green LED in NeoPixel LED

633 array; emission 515-530nm) and an IR filter (cut-of at 780 nm) positioned in front of the camera.

634 Videos were recorded using an IR sensitive monochrome camera (DMK 33UP1300, The Imaging

635 Source, Germany) and IC Capture software. An Arduino based circuit, interfacing with a GUI written

636 in Python, provided stimuli and PID-controlled temperature control.

637

638 **Recordings**

639 1-3 animals were placed in an agarose arena (10mm in diameter and 3mm high, approximate volume

640 236 mm^3). Each animal in behavioural experiments was first filmed for a period of 15 minutes during

25

641   acclimatization to the arena (at 10 frames/s). Subsequently 1 to 3 videos of 5 min duration were

642   filmed at 30 frames/s to analyse either base line behaviour or effect of stimulation, rearing

643   temperature or added drugs on behaviour. For modafinil experiments the animals were first

644   transferred to a dish containing DMSO or modafinil and then immediately transferred to the arena

645   which also contained DMSO or modafinil (2 or 20 mg/l) as schematically presented in Fig 8.

646

647   **Video conversion and analysis**

648   Videos were analysed using ToxTrac software and custom-made software using OpenCV and python

649   environment (Fig 1C). For each video all frames were enhanced with Contrast Limited Adaptive

650   Histogram Equalization (CLAHE) with a clip limit of 1 and a tile grid size of 50x50 pixels. After

651   histogram equalization noise was reduced with a median blur with a tile grid size of 5x5 pixels. To

652   input bright-background videos into the ToxTrac software, all frames were inverted by subtracting

653   from a true white frame of equal size. Within the ToxTrac software, the ID algorithm used in our

654   study was 2TCM sel. by Hist (MEE).

655

656   **Data analysis and statistics**

657   All data analysis was performed with python using the numpy, pandas, scipy, scikit-learn and

658   matplotlib libraries. For every analysed video the position of the centre of the arena is determined

659   with a Hough Circle Transform algorithm in OpenCV in python. For every trace the [x,y]-positions are

660   corrected so that [0,0] was at the centre of the arena. All positions were then multiplied by the factor

661   of 11.56µm/pixel for the setup the recording originates from. From these positions distances, speeds

662   and subsequently all other parameters are derived. We excluded animals that were completely

663   immobile and hence indistinguishable from dead form further analysis by excluding all traces where

664   the maximal displacement from the starting position was less than one body-length (comprising

665   approximately 10% of all examined traces). Similarly, traces where the animal was tracked for less

666   than 2000 frames were considered unrepresentative and excluded from further analysis.

667   The tracked centre-point corresponded predominantly to the animal's head. To filter out noise

668   caused by the undulatory movement of the head during swimming the speed sequence values were

669   filtered with a 1 Hz low-pass filter. All speed values presented are therefore filtered speeds and turn

670   values refer to values calculated between coordinates 5 time-points apart for the same reason.

671   Whenever statistical significance was tested we used nonparametric test for all non-normally

672   distributed data, namely Levene's test for equality of variances, Kruskal-Wallis analysis of variance

673   and Mann–Whitney U test. To compare the similarity of distributions in the scatterplots of speed vs

674   turn values presented in this paper we compare the sorted Mahalanobis distances of these

675   distributions (data in S3). Where relevant, χ^2 statistic was calculated for behavioural modes

676   distributions and the greatest contributor underlined in the figure legend (see Supplemental material

677   for values).

678

679   **Clustering of behavioural modes**

680   To identify objective behavioural modes we attempted unsupervised clustering of a minimal feature-

681   set that describes the behaviour of the larvae. The featureset was created as follows:

682   For each good recording the velocity vectors (ρ, φ) were calculated from coordinates 5 frames apart.

683   Values ρ, Δρ and Δφ were used as measures for speed, acceleration and turns respectively. For each

684   point the mean of a sliding window of [-25:+25] frames was used to include information of past and

685   future movement. This results in a dataset of three features and ca. 1.8 million observations.

686   Clusters in the dataset were identified using an agglomerative clustering algorithm with ward-

687   linkage. To determine the optimal number of clusters we identified the point where adding more

688   clusters would not reduce the total distance of all points to their respective cluster centre drastically.

689

690   **Classifying**

691   Using the clustered dataset, we trained a K-Nearest-Neighbours classifier that takes a recording

692   expressed in the features mentioned above, and assigns each point in this recording to a cluster. We

693   classified all collected traces with this classifier, and inspected the original videos with the assigned

694   clusters superimposed in order to assess the biological relevance of each cluster. We found that

695   apart from cluster 0, which turned out to be the result of an artefact from data were there was

696   insufficient datapoints in the window for averaging, we could identify distinct behaviours for the

697   remaining eleven clusters. Several of these clusters described the same biological behaviour but

698   resulted in different clusters as a result of the tracking marker being placed in either the head or the

699   neck of the animal by the tracking software (For an illustration of the speed and turn values present

700   in the different clusters see data in S1).

701

702   **Data availability**

703   The designs for the behavioural setup components that were 3D printed can be found here:

704   https://github.com/ChatzigeorgiouGroup/Rudolf-Dondorp-2018/tree/master/3d%20Cad%20files

705   The dataset used for this study can be found here: http://doi.org/10.5281/zenodo.1298978

706   The analysis code is located in our GitHub repository:

707   https://github.com/ChatzigeorgiouGroup/Rudolf-Dondorp-2018/tree/master/Code

708

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713
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