Open access • Posted Content • DOI:10.1101/382465

Quantitative analysis reveals the basic behavioural repertoire of the urochordate Ciona intestinalis — Source link

Jerneja Rudolf, Daniel Dondorp, L Canon, S Tieo ...+1 more authors

Institutions: University of Bergen, École nationale supérieure de chimie de Montpellier, Paris Diderot University

Published on: 01 Aug 2018 - bioRxiv (bioRxiv)

Topics: Thigmotaxis

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

Jerneja Rudolf[1]#, Daniel Dondorp[1]#, Louise Canon[1,2]§, Sonia Tieo[1,3]§, Marios Chatzigeorgiou[1]*

# These authors contributed equally to this work. § These authors contributed equally to this work.

[1] Sars International Centre for Marine Molecular Biology, University of Bergen, Thormøhlensgate 55, 5006 Bergen, Norway. [2]École Nationale Supérieure de Chimie de Montpellier, 240 Avenue du Professeur Emile Jeanbrau, 34090 Montpellier, France, [3] University Paris Diderot-Paris7, 5 rue Thomas Mann, 75013 Paris, France

* Marios.Chatzigeorgiou@uib.no

Abstract

Quantitative analysis of animal behaviour in model organisms is becoming an increasingly essential approach for tackling the great challenge of understanding how activity in the brain gives rise to behaviour. In addition, behavioural analysis can provide insight on the molecular basis of nervous system development and function as demonstrated by genetic screens focused on behavioural phenotyping in some genetically tractable model organisms. The progress in building low-cost automated tracking setups, together with advances in computer vision machine learning have expanded the repertoire of organisms which are amenable to quantitative behavioural analysis. Here we used automated image-based tracking to extract behavioural features from an organism of great importance in understanding the evolution of chordates, the free swimming larval form of the tunicate *Ciona intestinalis* which has a compact and fully mapped nervous system composed of only 231 neurons. We analysed hundreds of videos of larvae and we extracted basic geometric and physical descriptors of larval behaviour. Most importantly, we used machine learning methods to create an objective ontology of behaviours for *C. intestinalis* larvae. We identified eleven behavioural modes using agglomerative clustering. This approach enabled us to produce a quantitative description of the basic larval behavioural repertoire. Furthermore, we tested the robustness of this repertoire by comparing different rearing conditions and ages. Using our pipeline for quantitative behavioural analysis, we successfully reproduced the known photoresponsive behaviour and the first demonstration to our knowledge that *C. intestinalis* larvae exhibit sensory arousal and thigmotaxis, both of which can be modulated by the anxiotropic drug modafinil. Remarkably, by comparing the behaviour between animals assayed individually or in small groups, we found that crowd size influences larval behaviour. This study shows that *C. intestinalis* larval behaviour can be broken down to a set of stereotyped behaviours that are used to different extents in a context-dependent manner. Furthermore, it raises exciting possibilities such as mapping behaviour to specific neurons of this compact chordate nervous system and it paves the way for comparative quantitative behavioural 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

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

in the water column. Following the hatching event larvae swim upwards towards the water surface by negative gravitotaxis using the otolith cell. Ablation experiments have shown that animals lacking the ocellus are also capable of this behaviour, indicating that the ocellus is not involved in gravitotaxis.(26, 27). Later on, larvae exhibit negative phototaxis, swimming away from the bright surface to deeper waters in a behaviour that possibly aims to identify suitable substrates for settlement (28, 29). The swimming larvae are thought to display three types of swimming activity: tail flicks, spontaneous swimming and shadow response(30). Larvae under constant illumination swim more frequently and for more extensive periods of time earlier in life, up to 2 hours post hatching. A behaviour that develops later in development is the shadow response, where dimming of light results in symmetrical swimming. After two hours post hatching the tail beating frequency increases(30). *C. intestinalis* larvae have also been shown to exhibit both sensitization and habituation to light (31, 32). A recent study reported that distinct groups of photoreceptors mediate negative phototaxis and dimming behaviour in *C. intestinalis* larvae(33). This functional insight was achieved through behavioural analysis on mutant lines, demonstrating the power of genetics in combination with behavioural analysis and knowledge of the larval connectome. However, this is not the first attempt to link behaviour to its genetic underpinnings in *C. intestinalis* as it has been shown that targeted knockdown of opsin1 results in a loss of the light OFF response in larvae(34). Beyond phototactic and gravitotactic behaviours there is evidence that hints to the possibility that the larvae can exhibit chemotactic(35) and mechanosensory behaviours(36-40). However, these have remained largely unexplored.

Over the past decades, the biological research community has used *C. intestinalis* as a model for uncovering the basic principles of chordate embryonic and post-embryonic nervous system development. We believe that by building on this work (e.g. taking advantage of the numerous characterised promoters, known cell lineage etc), we can use the compact nervous system of the larva in order to uncover the basic molecular and cellular mechanisms underlying chordate nervous system function. We have the tools required to deconstruct the molecular and cellular components

of the larval nervous system(41) and the opportunity to adopt existing toolkits to probe its functions(42). Here, we attempt to match the growing insights into the structure and function of the *C. intestinalis* nervous system with a detailed characterization of its behavioural output. To achieve this, we collected a large dataset of recordings of free-swimming *C. intestinalis* larvae on a custom IR-illuminated and temperature-controlled set-up. We analysed the recordings using ToxTrac(43) and custom written Python scripts to obtain positional data and descriptors like speed and its variability, turning angles, and path complexity. To describe behaviour both accurately and objectively, we used unsupervised clustering methods to identify distinct behavioural clusters in our dataset, ultimately arriving at a quantifiable distribution of detectable behavioural components. This approach enabled us to study how this distribution of behavioural components changed under the influence of different rearing conditions, at different ages and in response to different sensory cues. Strikingly, we show that *C. intestinalis* larvae exhibited a state of sensory arousal when first transferred into the behavioural arena. This sensory arousal state and thigmotaxis behaviour could be modulated by the anxiotropic drug modafinil.

Results

Defining behavioural parameters and modes

In order to probe the behavioural dynamics of *C. intestinalis* larvae, we built an inexpensive tracker, with temperature control and the ability to deliver light stimuli with accurate spatiotemporal control. To maximize the open-source accessibility and modularity of the trackers we used Arduino based circuits for controlling the various tracker components and 3D printed housing for the agarose plated behavioural arenas. The agarose arena was housed inside a 3D printed ring fitted with infrared (IR) LEDs (Fig 1A).IR LEDs were used in order to visualise the animals in a dark-field configuration, while at the same time avoiding stimulating their photoreceptors with visible light, which they can detect and modify their behaviour (31-33). We imaged approximately 550 *C. intestinalis* animals behaving freely at two different frame rates: During the first 15 minutes after transferring animals to the setup we

imaged at 10 frames/s and subsequently we imaged the same animals for 5 minutes at 30 frames/s. Following acquisition and processing, the videos were analysed on the tracking program ToxTrac(43) (Fig 1C, D). Due to the lack of previous attempts to track *C. intestinalis* larvae with automated image tracking software, we tried a series of available programs. We found that ToxTrac worked best in our hands especially because it did not require specific knowledge of the animal's body geometry and because it was particularly robust against false positives and identity switches. The position of the animal was tracked by the centre of its detected shape, as identified by ToxTrac. From all the videos tracked we obtained around 850 traces for further analysis and quantification.

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

C. intestinalis larvae have been reported to modulate their locomotor activity levels through "bursts" of spontaneous activity (26, 30, 44). We decided to introduce a parameter that would provide a quantitative descriptor of locomotory activity, termed Activity coefficient (AC) and defined as the fraction of time an animal spent locomoting. Filtered speed values of 200 µm/s and above were considered as active, which in practical terms includes all actively swimming animals as well as movement of the animals' centre-point due to tail flicks and twitching.

Planktonic larvae from different species exhibit a wide range of paths during locomotion(45), often dictated by their biomechanics (44, 46-48). Notably, reduced food availability can result in nonlinear paths, while directionality in trajectories can often be indicative of behavioural responses to environmental stimuli (49, 50). While recording animals in the behavioural arenas, we observed that they exhibited a large repertoire of trajectories while swimming in the arena. Some of the animals were swimming in straight lines with relatively few turns and changes in speed, representing what an observer would describe qualitatively as low complexity trajectories. In contrast, other animals moved in a more "complex" fashion, exhibiting sudden changes in direction and moving in spiral or circular paths (see Fig 1E for example trajectories). We decided to quantify the local path complexity using a method presented by Roberts et al. (51). In summary, this method uses embedding matrixes

for positions in a specific time window, over which the local path complexity is calculated in bits of entropy. Minimal complexity values calculated by this method correspond to the most predictable trajectory or, in other words, the most invariable movement in terms of represented speeds and directions in the time window (see Fig 1F for example traces with decreasing local complexity). While observing the animal trajectories, we noticed that a significant fraction of animals swam near the edges of the arena. This behaviour has been previously identified in other organisms (13, 52-54) including humans(55-57) and has been termed thigmotaxis. We decided to quantify the thigmotactic behaviour of the larvae. To define thigmotaxis, we divided the arena into two concentric zones of equal surface area. Animals in the outer zone were considered thigmotactic (Fig 1B). The amount of thigmotaxis is quantified in two measures: “Total Time spent in Outer zone” (TTO) and “Total Distance travelled in Outer zone” (TDO), as utilised for zebrafish previously (58).

A long-term goal of quantitative animal behaviour analysis is to identify the behavioural strategies that animals use to solve tasks and to dissect the underlying neuronal mechanisms. One approach to reach this goal is to generate a structured quantitative description of behaviour, by identifying stereotyped behavioural components. In order to identify these behavioural components in behaving *C. intestinalis* larvae, we used unsupervised clustering methods on a small feature- set (ρ- speed, Δρ-acceleration and Δϕ-turn angle values; see methods section for details), and we were able to identify and classify 11 distinct clusters in our data set. We assigned a relevant descriptor of the behavioural mode to each cluster according to its biological interpretation. This was done by inspecting the original videos and superimposing corresponding cluster data. When unable to resolve a biological difference between two clusters, we assigned them the same descriptive name. We list the behavioural modes in approximate order of speed of movement in Table1 below (see Fig 1G for some example traces of different modes and S1-S5 Video for example videos). This simple ontology of the objectively detectable behavioural modes in our data set served as an extra tool in assessing the effects of different stimuli and genetic or pharmacological perturbations on the behavioural repertoire of the larvae.

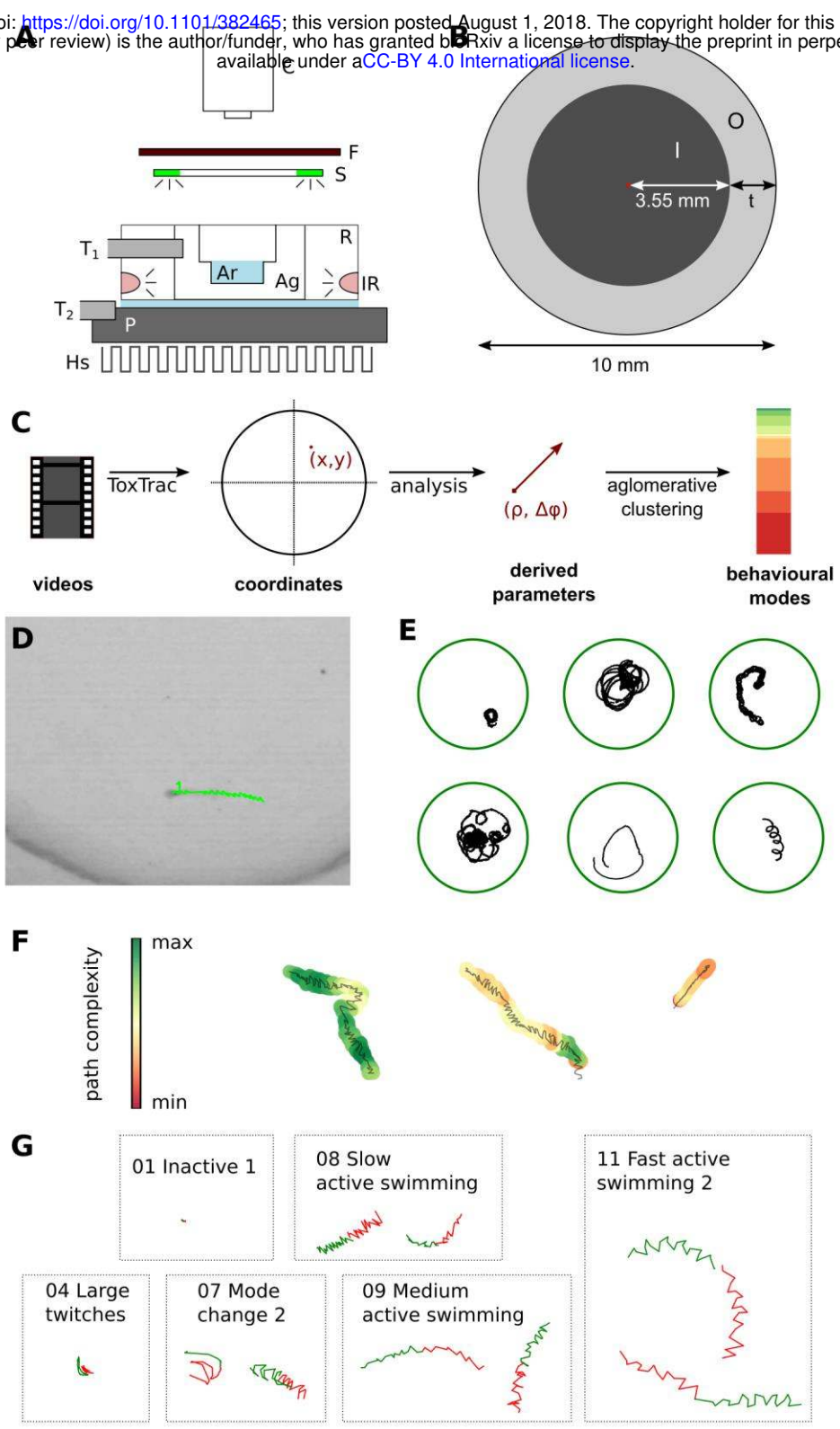

Fig 1. Setup and analysis methods.

(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

209

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-

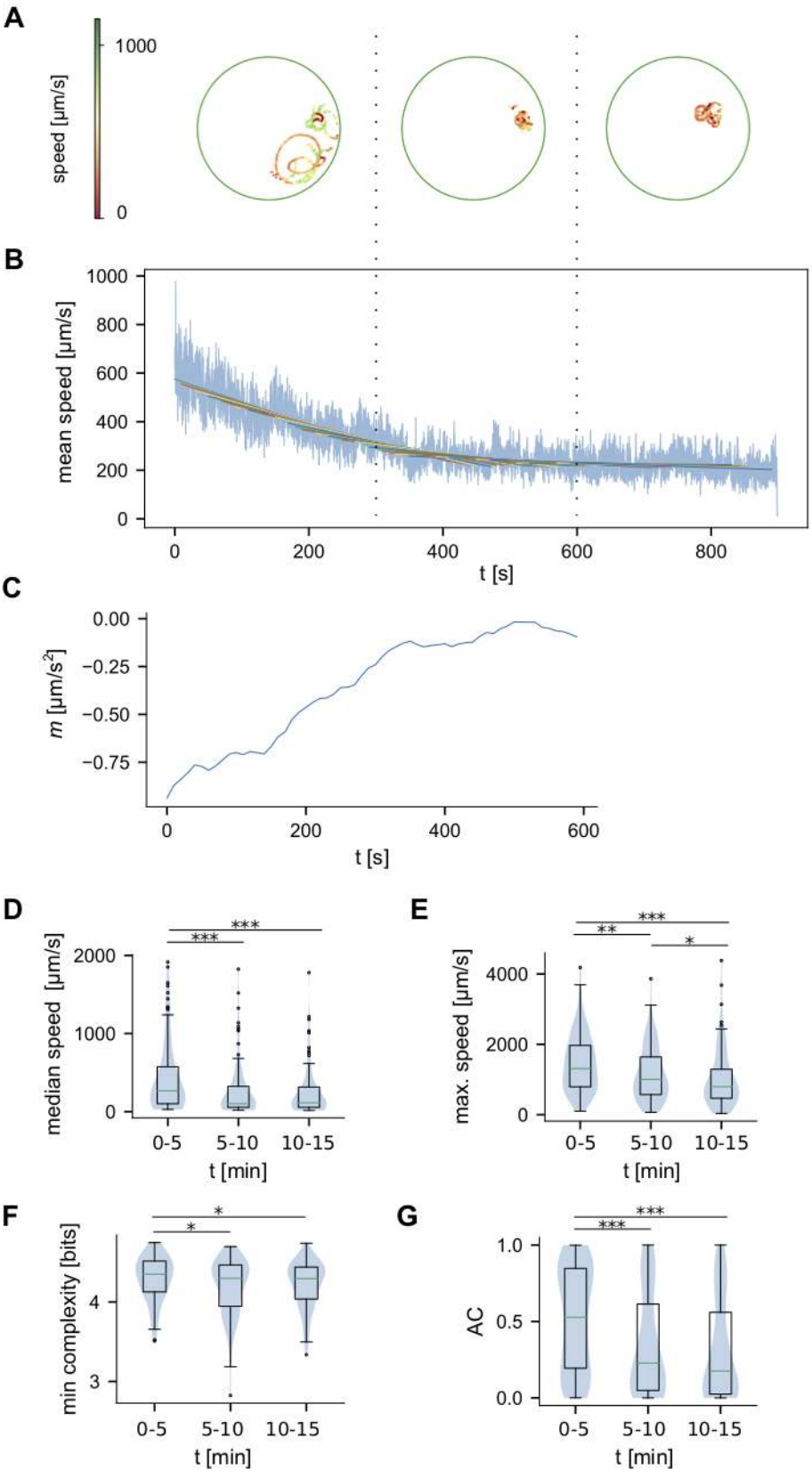

Fig 2. Adaptation of animals to the arena

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).

G). There are significant differences in both median speed (Fig 2D, median at 267 µm/s in the initial 5 min vs. 101 µm/s in the second and 113 µm/s in the last 5 minutes; $p < 0.001$ for both comparisons) and maximum speed of the animals (Fig 2E, median at 1310 µm/s in the initial 5 min vs. 1003 µm/s in 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)*= 0.048). Interestingly, as a result of sensory arousal, *C. intestinalis* larvae exhibited higher minimum path complexity in the first 5 minutes after they are placed in the arena (Fig 2F, 4.349 bit in the initial 5 min vs 4.297 and 4.293; *p(1. vs. 2.)* = 0.027*, p(.1 vs. 3.)* = 0.0038). This observation suggested that the animals followed a more unpredictable, or chaotic trajectory during the original arousal period. As they adapted they followed trajectories exhibiting lower entropy and thus higher degree of predictability. In other organisms such as birds the trajectory component showing higher entropy is thought to be associated with navigational uncertainty(65). Notably, the activity coefficient (Fig 2G, median at 0.53 vs. 0.23 and 0.18; $p < 0.001$ for both comparisons) of the aroused animals is also significantly higher in the first 5 minutes, suggesting that bouts of inactivity are suppressed during this period. Finally, unlike mice in open-field tests, which show high thigmotaxis during the initial period(63), we found no significant changes in thigmotaxis in the case of aroused larvae vs. animals that have adapted (data not shown).

Behavioural effects of larval crowd size

In a laboratory setting we often study behaviour using individual animals in isolation. However, in the wild, organisms are hardly ever acting in absolute isolation. Across the animal kingdom, locomotion and response to environmental stimuli are influenced by both direct and indirect interactions with conspecifics (66, 67). For example, interactions between individuals are important in mating(68), in the protection of colonies from pathogens(69), and in response to predators(70, 71). Previously it has been shown that mechanosensory interactions between desert locusts can lead to a dramatic phenotypic change where the animals switch from a cryptic solitary phase to a gregarious phase(72). More recently, in the genetically tractable organism *Drosophila melanogaster* it has been shown that mechanosensory interactions between adults drive collective response towards a sensory cue(73).

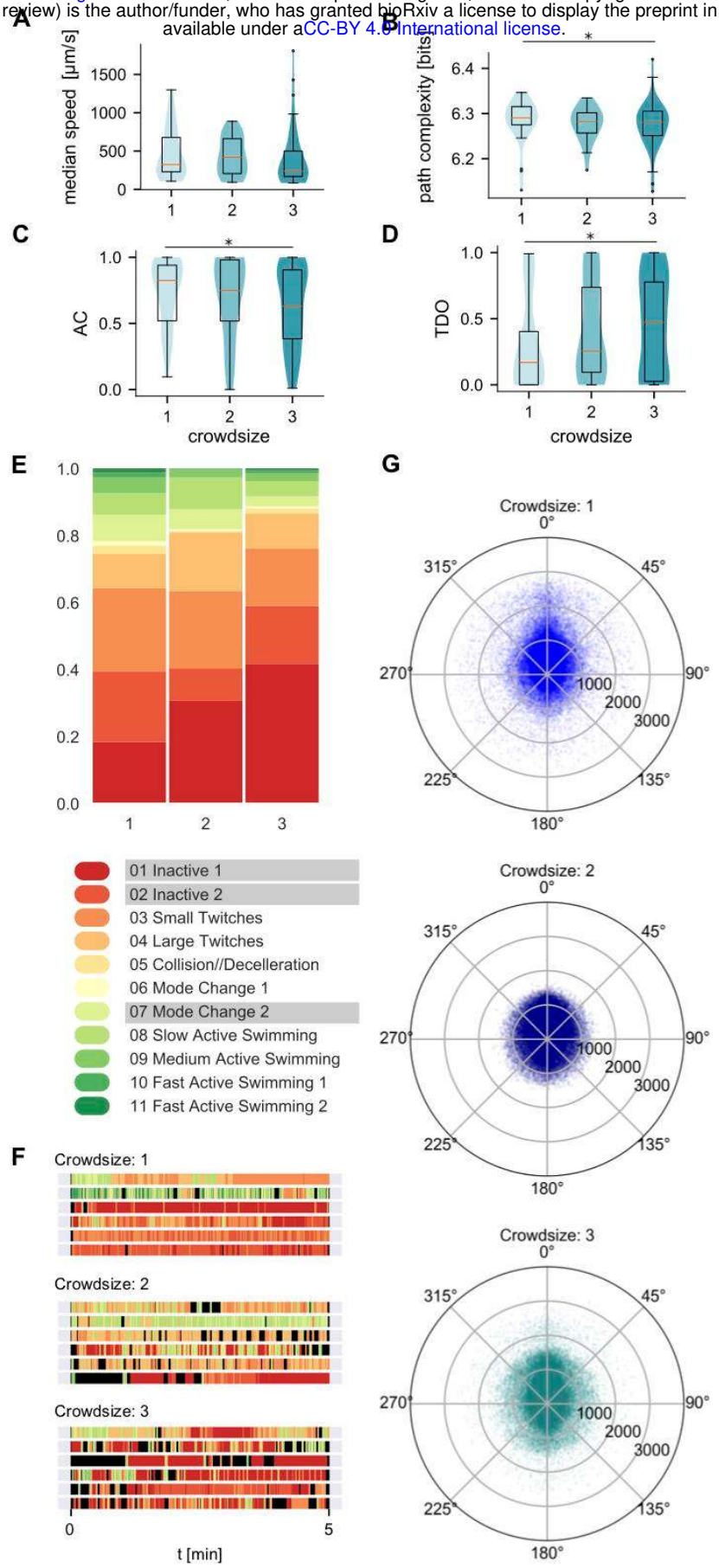

Fig 3. Crowdsize effects on behaviour

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).

The behaviour of *C. intestinalis* larvae in large groups has been studied to some extent, e.g. the change in distribution because of water agitation(29) or stimulation with light (30). In our study, we focused on the possibility of behavioural effects of small group interactions. To achieve this, we compared animals that were alone in the arena with animals recorded in pairs or groups of three. We found small differences in the basic behavioural parameters (Fig 3). Crowd size did not affect median (Fig 3A) or maximum speeds of movement significantly, but had a weak effect on the path complexity and activity coefficient (Fig 3B, C). The average path complexity was lowest for animals in the crowd size 3 group (6.281 bits, compared to 6.290 bits for animals in crowd size 1 group, p=0.05) and so was the median AC value (0.63 compared to 0.82 and 0.75 for animals in crowd size 1 and 2 groups respectively, $p(1.\ vs.\ 3.)$ =0.045). We also found slightly more animals with higher thigmotaxis values, with the difference only significant for TDO (Fig 3D) between crowd size 1 and 3 (median TDO is 0.17 for crowd size 1 vs. 0.47 for crowd size 3, p= 0.0227). The distribution of different behavioural modes shows higher representation of the less active modes corresponding to more time spent inactively in crowd size 3 animals (Fig 3E). In Fig 3F we present sample ethograms of 6 individual animals in different crowd size experiments. The smaller representation of higher speeds was also apparent when we plotted the distribution of speed values with the corresponding turn values on a polar scatterplot, presented for the different crowd sizes in Fig 3G.

Age related changes in behaviour

Behavioural changes that result from aging have long been the focus of various senescence studies, but there are similarly important age related changes underlying the normal development of the animals' behavioural repertoire through time(74-79). For larval *C. intestinalis*, light and gravity dependent behaviours have already been shown to change with age (26, 31). Here we examined the development of baseline behaviour in the early hours post-hatching. In animals reared and recorded at 14 °C, we observed that in the first hour after hatching they were mostly inactive, followed by a period of time when the larvae twitched very actively and flicked their tails but would not cover a lot

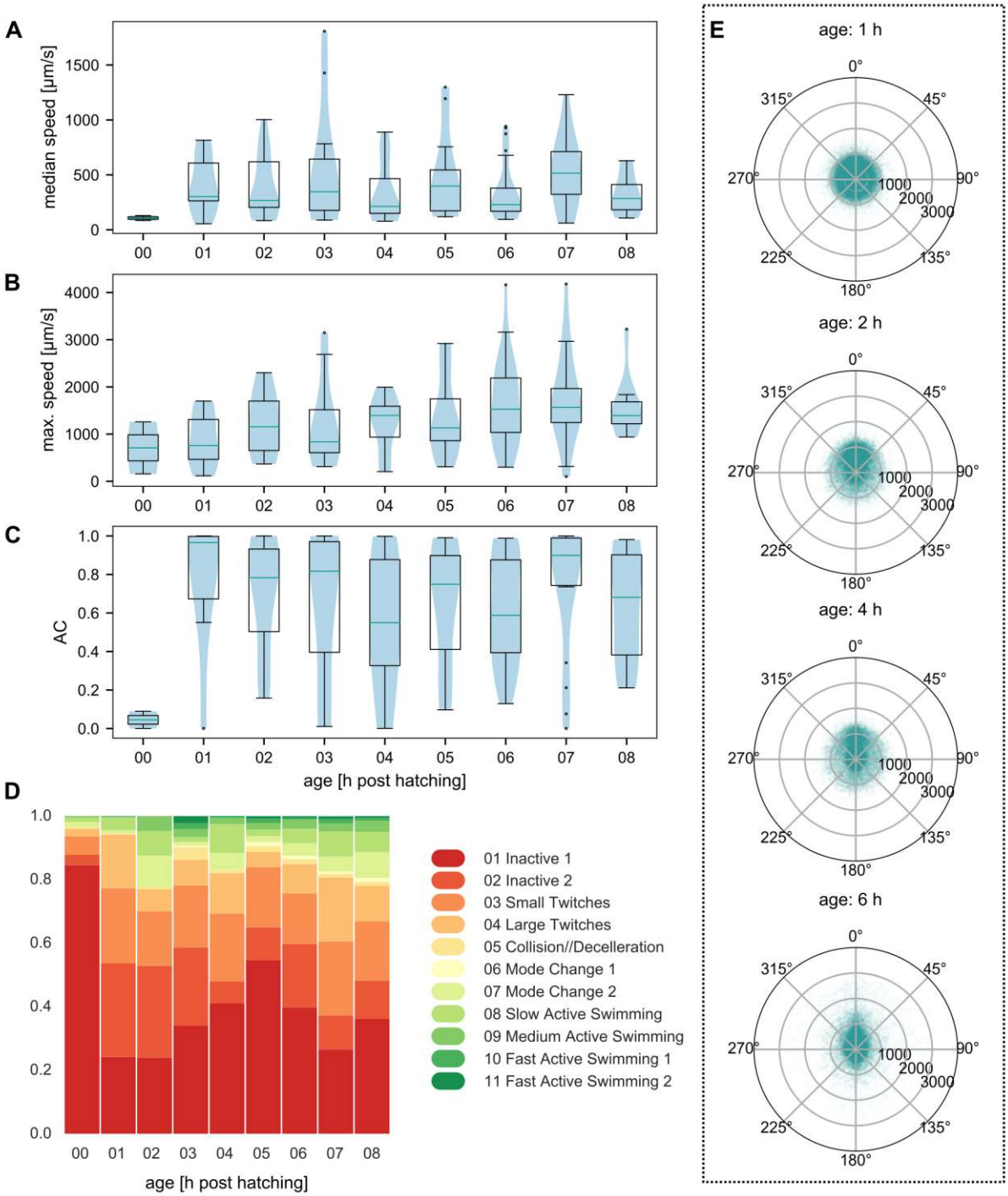

Fig 4. Age related changes in behavioural parameters

(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)

of distance swimming. In Fig 4 we show some of the basic behavioural descriptors compared between ages of WT animals reared at 14°C. Both maximum and median speeds were lowest for animals immediately after hatching (Fig 4A, B), accompanied by very low AC (Fig 4C). While animals 1h post fertilisation already achieved higher median speeds and generally had a very high AC, their movement was less directional as can be seen by the distribution of turn values versus speed values (Fig 4E) and the high representation of twitching modes in their behavioural repertoire (Fig 4D). To minimise any potential skewing of the data because of age-dependent changes, we used animals of 2-8 hours post hatching age for all later comparisons, unless specified otherwise.

Rearing temperature effects on a developmentally regulated behaviour

We (Fig 4) and others have shown that as the *C. intestinalis* larvae go through the post-hatching motile phase of their life cycle they change their behavioural responses at multiple levels. Such behavioural changes are thought to be tightly linked to developmental changes taking place in the larvae. An interesting question that arises is whether this developmental regulation of locomotor and sensory behaviours is robust to different environmental circumstances, possibly through a mechanism of canalization (80-83) or whether it shows plasticity (84-86). Temperature is one key physical parameter that has been shown to affect the speed of most biological processes, acting as a major environmental factor influencing the rate of animal development (87-89) and behaviour(90, 91). The two Ciona species (*Ciona intestinalis* and *Ciona robusta*) (92)occupy a very large part of the world's coastline from high to low latitudes(93) and they show great adaptability to a range of temperatures. Published studies have used 18°C as rearing and assay temperature for *C. intestinalis* and *Ciona robusta* larvae. However, our local animals belong to the *C. intestinalis* species and develop best at lower rearing temperatures, possibly due to an adaptation to the lower water temperatures in the Norwegian Fjords. We tested whether the lower rearing temperature of 14°C affected the onset of the light-off response that has previously been described by Nakagawa et al.(28). The increase in swimming speed immediately after a light stimulus is considered a hallmark of the older larva that will in its later age seek to settle utilising negative phototaxis. In animals reared

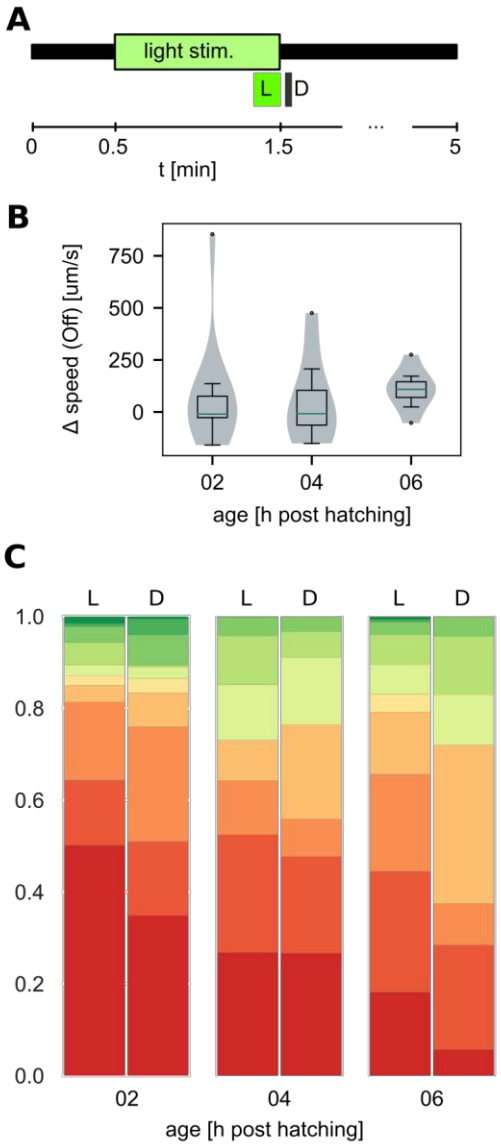

Fig 5. Light-Off response in animals reared and recorded at 14°C
(A) Experimental set-up. Light stimulus lasted 1 minute and the average speed of the animal in the last 10 s of the stimulation (L) was compared to the expected peak of speed upon onset of darkness (D) measured as average speed over 2.5 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)

at 18°C, the first notable response to a light-off signal was detected at 4 h post fertilisation, coinciding with a reduced average speed of the animals in absence of the stimulus. The sensitivity of this response has been shown to peak in the green part of the spectrum (28), so we tested for light-off response in our animals using a green light (515-530 nm). We calculated the change of speed (i.e. Δ speed (Off), Fig 5B) between the last 10 s during a 1 min light stimulus and the first 2.5 s after the light was turned off (after a 0.5 s latency period). In Fig 5C we present the distribution of the different behavioural modes for the same periods at the end of the light stimulus (L) compared to the onset of the dark (D) for animals at three different post-hatching ages. At 2 and even 4 h post hatching the animals reared at 14°C did not show a significant light-off reaction, with the first notable response observed only at 6 h. The response was notable both as a slight general increase in speed (Fig 5B) and more specifically as higher representation of twitching and medium speed swimming at the expense of inactive modes (Fig 5C). It appears that *C. intestinalis* has the potential to be a great genetically tractable model to answer the question of developmental stability of behaviour.

Rearing temperature effects on behaviour

Animals reared at 18°C had a much narrower time window after hatching in which we could observe active swimming behaviour, with the majority of animals being highly inactive by age 4 h post hatching (data in S2C). We therefore only compared animals of age 0-3 h reared at 18°C to the 14°C reared animals of ages 2-8 h, since we assumed they correspond to the same post hatching development stages. Even when comparing what we assumed to be animals at a similar developmental stage, animals reared and recorded at 18°C still exhibited some differences compared to the ones at 14°C (Fig 6). Their traces were similar in median speed values (Fig 6A) but reach significantly higher maximum speeds (Fig 6B, median at 1864 µm/s for animals reared at 18°C vs. 1440 µm/s for animals reared at 14°C, p >0.001). The difference in AC and path complexity is not statistically significant (Fig 6 C, D), but there was a slight but significant effect on thigmotaxis (Fig 6E,

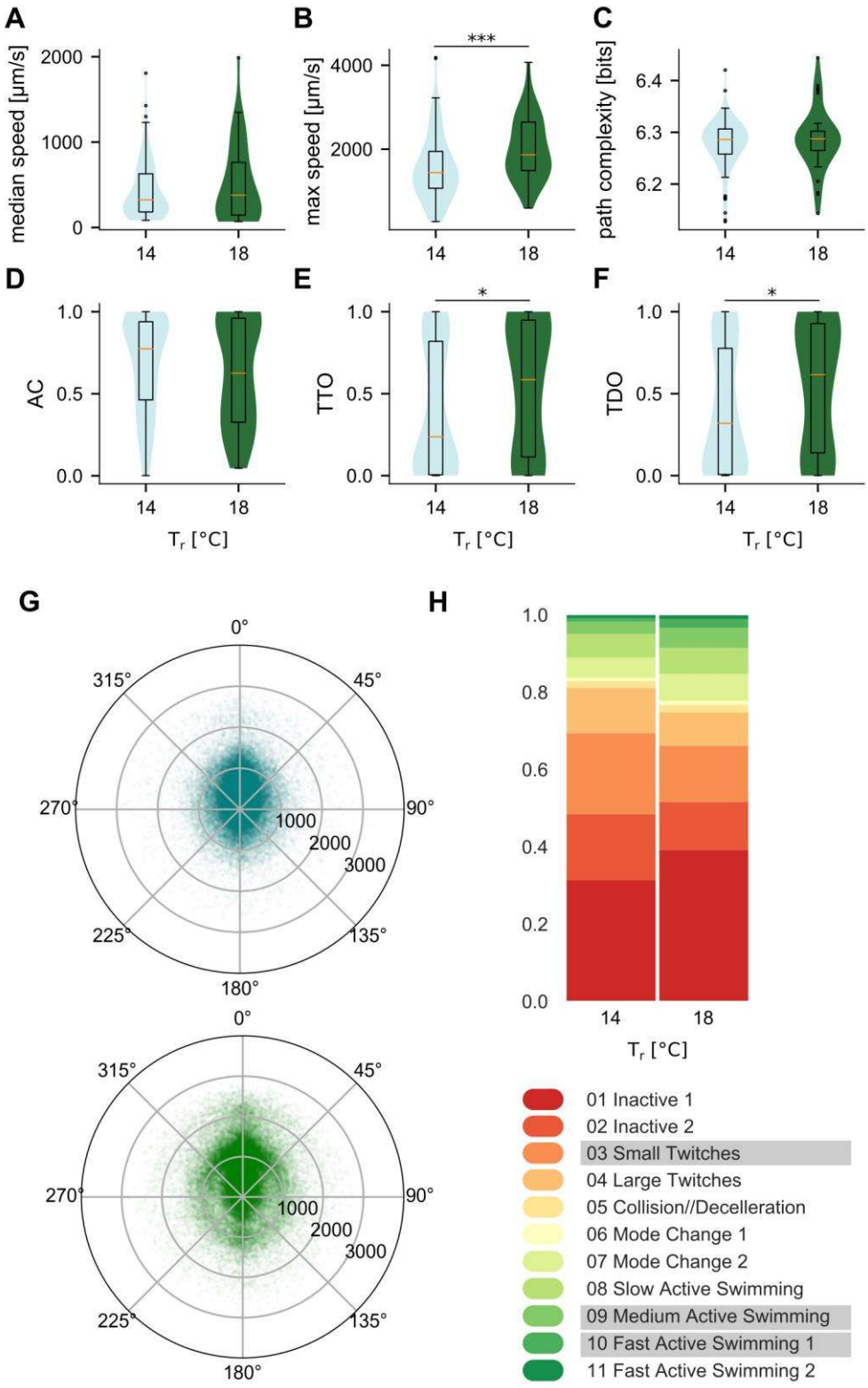

Fig 6. Effect of rearing temperature on behaviour

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)

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 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 representation of medium-high speeds (around 1000-1500 µm/s) in combination with a wider range of turn values, while at lower speeds the variability of turns was smaller (Fig 6G). This was matched with a lower representation of twitching modes and more occurrences of the modes representing swimming at medium speeds (Fig 6H).

Dechorionation effects

The eggs of *C. intestinalis* are nested in a chorion surrounded by follicle cells (Fig 7A) and the normal development of left-right asymmetry in the embryo has been shown to be disrupted by dechorionation (94). However, transient transgenesis of *C. intestinalis* is predominantly achieved by electroporations, which do require the dechorionation of eggs. We therefore set out to test if dechorionation has specific effects on behaviour. Being aware of any potential effects of dechorionation will be vital for the future interpretation of behavioural phenotypes in transgenic animals. The dechorionated larvae achieved a higher median (Fig 7B, 477 µm/s median vs 324 µm/s in chorionated animals, p=0.0125) and maximum speeds (Fig 7C, 1784 µm/s vs 1440 µm/s for chorionated animals, p=0.0024). The differences in AC and path complexities were not statistically significant in our set (Fig 7 D, E). There was however a slight but significant effect on thigmotaxis (Fig 7 F, G), resulting in a higher median TTO (0.56 vs 0.23 for chorionated animals, p= 0.035) and TDO (0.58 vs 0.32 for chorionated animals, p= 0.033) values for dechorionated animals. The differences in distribution of turns and speeds were less apparent compared to the effect of temperature, but the increased representation of higher swimming speeds in dechorionated animals was evident in the polar scatterplots (Fig 7H) as well as form the distribution of the behavioural modes (Fig 7I).

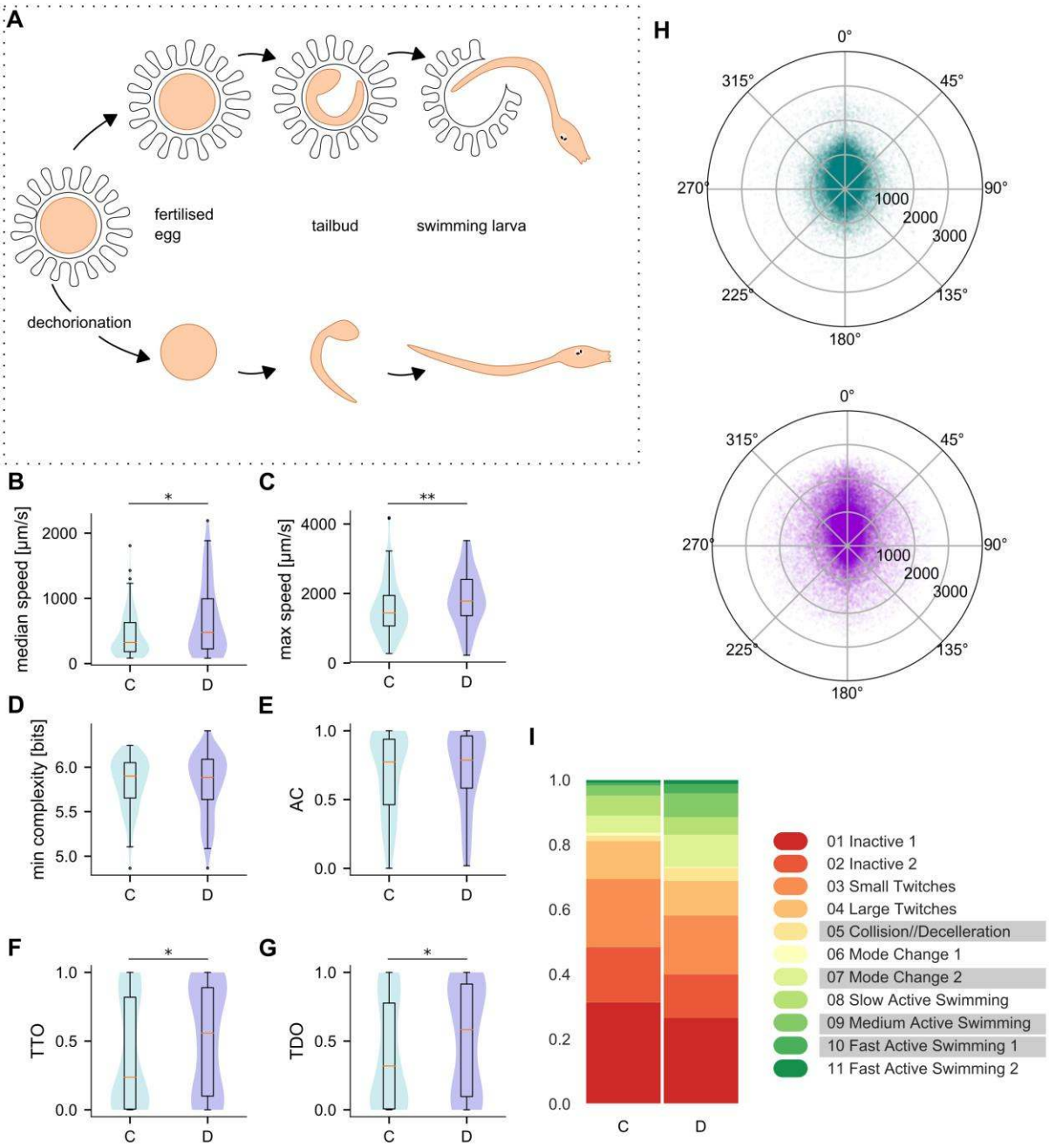

Fig 7. Effect of dechorionation on behaviour

(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)

Thigmotaxis and effect of modafinil

Finally, we attempted to influence thigmotaxis in our animals by exposing them to an anxiotropic drug modafinil. In this set of experiments, we compared two groups of animals swimming in 20 mg/l and 2 mg/l solution of modafinil respectively to a control group in DMSO and to the untreated WT set (Fig8, S4). We determined the dosage based on preliminary tests and previously published literature on modafinil effects on larval zebrafish (95). The effect of 20 mg/l modafinil on thigmotaxis was very pronounced and statistically significant (Fig 8E, F). While the median TTO value for the DMSO control was 0,427, it reached 0,669 in the 20 mg/l modafinil group signifying longer periods spent in the outer zone of the arena (p =0.0015). Similarly, the TDO measure shows the modafinil-affected animals travelled much bigger proportions of their total distance in the outer zone of the arena (median TDO at 0.628 in the 20mg/l modafinil group compared to the DMSO control at 0.395, p<0.001). For the 2 mg/l group the distribution for both thigmotactic measures was similar to the WT state and was not statistically different from either of the controls.

The animals affected by modafinil also exhibited an overall more active set of behaviours with much higher representation of the active-swimming modes and less time spent inactively (Fig 8G, H). This resulted in increased median and maximum speeds (Fig 8A, B) and higher AC values (Fig 8D) for animals in 20 mg/l modafinil. Path complexity (Fig 8C) showed fewer differences with the only significant change being the higher minimal complexity values for 20 mg/l group (5.99 compared to 5.85 in DMSO and 5.78 in 2mg/l).

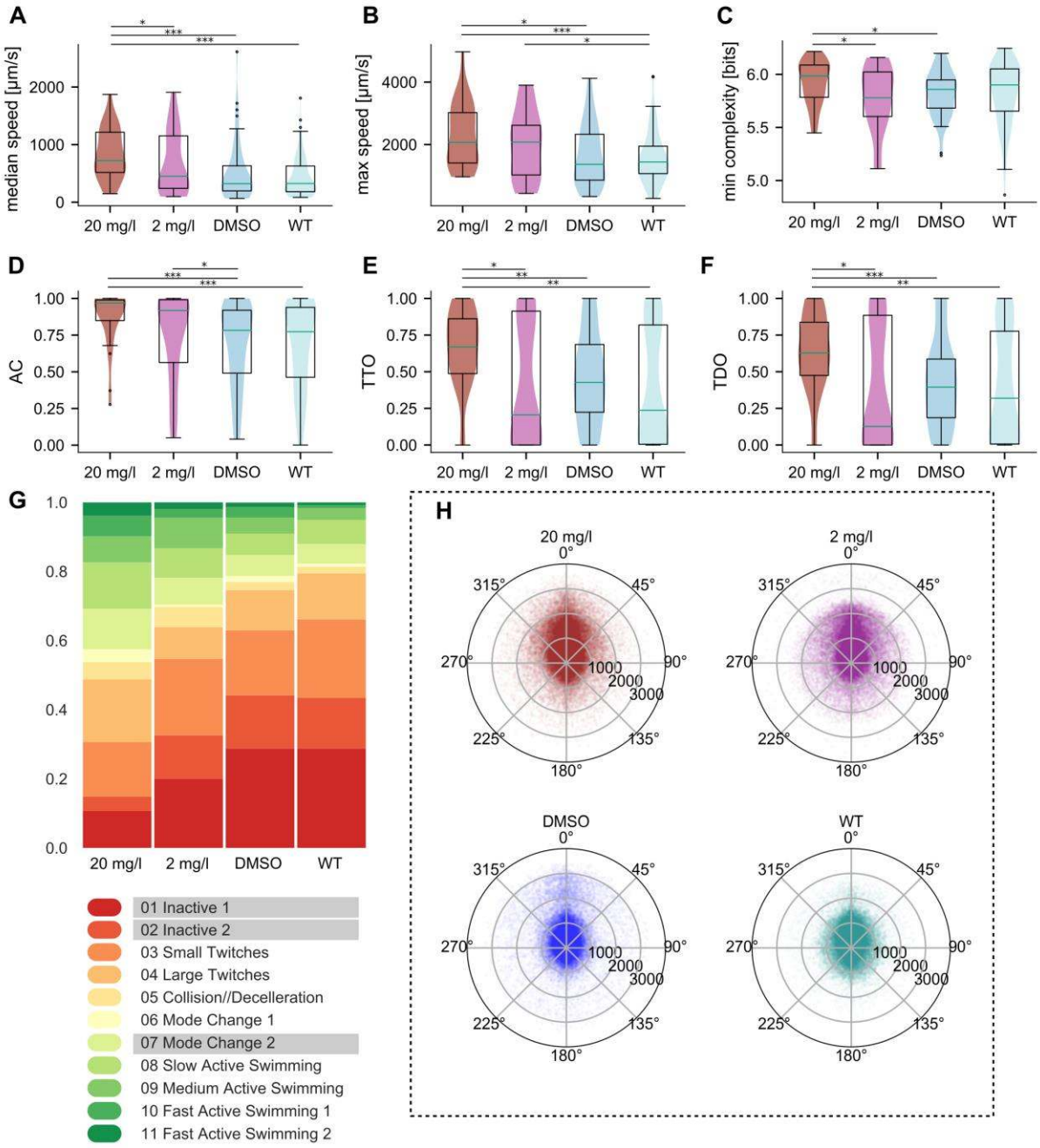

Fig 8. Effect of modafinil on behaviour

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)

Discussion

The chordate *Ciona intestinalis* in its larval form is emerging as a promising organism for neuroethological studies. The present study provides a quantitative description of larval behaviour in different contexts, using biologically relevant features. We performed unsupervised clustering of our data and identify clusters, with which we generate a behavioural ontology. We uncovered some of the behavioural effects of post-embryonic development and dechorionation on larval behaviour and pinpoint the behavioural consequences of crowd size and sensory arousal. Furthermore, we provide evidence that *C. intestinalis* larvae exhibit thigmotactic behaviour that can be modulated by the drug modafinil.

Quantitative description of *Ciona intestinalis* larval behaviour

The potential of the *C. intestinalis* larva as an organism to perform neuroethological studies has been noticed for several decades. There have been several efforts to perform behavioural studies with increasing sophistication over the years (26-33). However, automated quantitative analysis of *C. intestinalis* behaviour had been hindered by the lack of suitable open-source software with the ability to follow the larvae providing precise positional information over long time series, with few or no identity switches in case multiple animals are tracked simultaneously. In this study, we identified Toxtrac (43) as a suitable open-sourced tracking software, we built customizable hardware and developed an automated behaviour analysis pipeline for *C. intestinalis* larvae.

Our analysis suggests that *C. intestinalis* larvae show a surprising amount of complexity in their spontaneous swimming behaviour. Notably, the larvae exhibited a large range of swimming patterns showing significant variation in path complexity (Fig 1F), and individuals used a broad range of behavioural mode sequences (e.g. Fig 3F). The advent of tracking methods has revealed the presence of multiple characteristic scales of organisation in single and multiple animal traces that can be explained only if we consider theoretical frameworks for mobility that extend beyond simple diffusion mechanisms (96, 97). In the future it would be interesting to study the temporal structure of spontaneous swimming in *C. intestinalis* larva in greater detail. Intriguing topics to investigate are

for example whether some of the swimming patterns of *C. intestinalis* exhibit a Lévy-like behaviour(49, 98) and whether they may play a role in a dispersal strategy(99, 100) . In accordance with our expectations, external sensory cues seemed to influence the swimming strategy of the larvae. Interestingly, path complexity appeared to be modulated in opposite ways by sensory arousal and crowd size (Fig 9).

The sensitivity of our measurements allowed us to quantify the activity levels of the animals in different experimental contexts. We found that larvae showed both bursts of activity and bouts in irregular intervals. Intermittent locomotion(101) is one of the various morphological (102)and behavioural strategies(103, 104), often under evolutionary pressure(105), that have been implemented by moving organisms, including marine larvae in order to conserve energy(106). An alternative hypothesis is that fluctuations in activity levels may reflect an adjustment of motility to small changes in flow taking place in the arena. Similar bouts and bursts of activity characterise zooplankton motility in turbulent flow(107).

Quantitative behavioural analysis has been moving from subjective observation, and imprecise annotation of behavioural data, towards the automated recognition and classification of behaviours amongst very large data sets (2, 4, 7, 108). Some of the breakthroughs that have permitted this progress have focused on generating low-level representations of behaviour for automated analysis and automated classification algorithms of behaviours. The ultimate goal is to break down complex behaviours into their constituent building blocks. In this study, we employed a clustering methodology, using first unsupervised clustering of a minimal feature-set to identify behavioural modes, followed by training a K-Nearest-Neighbours classifier in order to classify all of our data corresponding to approximately 1.8 million observations from 850 data-frames. We found 11 distinct clusters that were classified in an equal number of basic behavioural modes. These 11 behavioural modes provide an unbiased way to dissect the structure of behaviour and will allow us to systematically classify complex behavioural phenotypes that result from pharmacological, genetic or 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*

larvae is likely due to mechanical stimulation from transfer to the arena it would be interesting to study the contribution of mechanosensory circuits to this behavioural state. In the case of Drosophila, acute sensory arousal is more apparent when comparing between states of wakefulness and sleep(118). A first clue as to which circuits might be involved comes from the observation that arousal in *C. intestinalis* is modulated by the drug modafinil (Fig.9). Modafinil has been classified as a psychostimulant and has been extensively used in narcoleptic patients in order to address sleep related disorders. It is thought to act as a selective dopamine (119) and norepinephrine transporter inhibitor (120), thus raising the possibility that monoamine signalling plays an important role in modulate the arousal state in *C. intestinalis* larvae. In this study we show that beyond modulating the arousal state of the animals, modafinil appears to alter the activity coefficient. The larvae also showed less quiescent periods with a higher activity coefficient. This has also been observed in mice and zebrafish. In mice, modafinil results in wake-promoting action, possibly via dopamine transporters(121), while in zebrafish modafinil shortens the periods of sleep(122).

Age related changes

A large number of animals exhibit behavioural changes linked to post-embryonic development of their nervous system. For example, in *Xenopus laevis*, locomotor activity patterns are modified as the animals transition from sessile hatchlings to free-swimming larvae(76), through changes in the cell properties of neurons(123) and a nitrogen oxide signalling mechanism (75). Other examples of post-embryonic changes in behaviour include the photoresponses of the stick insect *Carausius morosus* (77) and the chemotactic responses in *C. elegans*(124). *C. intestinalis* larvae are also subject to post-embryonic developmental changes and associated behavioural modifications. It has previously been reported that light and gravity dependent behaviours change during the larval life (26, 31). Here we quantified how progression through larval life changes behaviour of the animals, and found age dependent differences in the distribution of behavioural modes (Fig 4). This puts a quantitative angle to previously reported findings in the literature(30). What may be the mechanisms that bring about

these changes in behavioural modes used in larvae of different ages? One possibility is that some neurons of the larval system fully differentiate and connect to the nervous system only after hatching. Indeed, there has been evidence for post embryonic terminal differentiation of dopaminergic cells in *C. intestinalis* larvae (125). The authors of the study postulated that dopamine might modulate the neural circuits involved in the age-dependent changes in swimming behaviour of the larva. The genetically tractable *C. intestinalis* is proving to be a powerful model for providing in-depth insight into developmental processes in the post-embryonic nervous system and the functional basis of locomotor dynamics changes throughout larval life. Notably, the use of a fast-growing larval animal with associated changes in body proportions and shape, provides us with the opportunity to understand how alterations in biometrics and sensory capacity may relate to simultaneous changes in locomotory behaviour.

Crowd size effects on larval behaviour

Many behaviours, such as mating, shoaling, schooling, aggressive encounters rely on the interaction of two or more individuals. These interactions largely depend on achieving a coordinated movement between individuals and the entire group(126).

Previous work in *C. intestinalis* has shown that larvae can aggregate into a column when placed in a three dimensional chamber and that they can form swarms, especially upon agitation of the water(29). Also it has been shown that larval distribution can change in the presence or absence of light(30). Notably, it has been reported that ascidian behaviours prior to settlement are largely influenced by conspecifics, while the larvae exhibit a form gregariousness(35, 127). However, these are largely qualitative observations that were made in the course of experiments that were not designed to specifically address the interactions between conspecifics. Further difficulties for providing a quantitative description of larval interactions stem from the lack (until recently) of automated tracking software that faithfully maintained the identity of each tracked animal(5). Taking advantage of the ability of ToxTrac to maintain the identity of multiple animals in the same arena we

attempted to determine whether there are differences when single vs multiple individuals are placed in the arena. We find that the presence of two or three animals in the arena can already result in a few changes in the measured behavioural parameters (Fig 9). Notably, a significant change in one of the thigmotactic indices is also observed (Fig 3D). Interestingly, an enhancement of thigmotaxis in individual versus group context has been observed in the case of ants(128). Given the past literature it will be interesting to determine if and how *C. intestinalis* larvae achieve coordinated movement. It is believed that the type of distributed sensing required to generate robust collective behaviour is rather simple, requires rudimentary circuits and thus it may be widespread across different animal taxa(129). *C. intestinalis* has a small nervous system and thus is ideal to study the neural circuits controlling pairwise and group-level behaviours. We note that a limitation of our method is that our crowd size experiments were conducted in an extremely small volume of sea water compared to the ethologically relevant volumes that these animals would encounter in the sea. Future experiments should be conducted in larger arenas that may be ethologically more relevant for crowd size experiments.

Behavioural robustness to altered rearing conditions

Temperature is a known modulator of key physiological processes and behaviours in numerous animals(130). For example, thermal rearing conditions can affect the dispersal of adult spiders(131), the host-seeking behaviour of parasitic nematodes(132), mating behaviour in Drosophila(133), and the feeding behaviour in the mud snail *Heleobla australis*(134). To contextualise our data we compared our main wild type group reared and recorded at 14°C to animals at 18°C, since behaviour of *C. intestinalis* larvae has previously often been studied at 18°C e.g. (27, 31, 32) or even at room temperature (29) . We describe the distinct difference in the speed of post hatching development at different rearing temperatures (Fig 4, S2) At 18°C the period of higher spontaneous locomotor activity coinciding with the lack of significant responses to light-off stimuli lasts for ca. 4 h post 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

Thigmotaxis and modafinil effects

Using an open-arena to monitor our animals, we noticed that a large fraction of larvae exhibited strong thigmotactic behaviour. This appears to be an adaptive behaviour that has been observed in numerous organisms, where the circular wall of the arena allows the animals to exhibit a defensive response (i.e. to hide from potential predators) and facilitates their orientation in space(142). Therefore, it is not unlikely that thigmotaxis presents an evolutionarily conserved behaviour. One may wonder what role thigmotaxis plays in the larvae of *C. intestinalis*. In fact, almost thirty years ago it was hypothesised that thigmotaxis, amongst other behaviours, may be involved in the selection of habitats for larval settlement(35). Interestingly, we have been able to demonstrate that physiological (rearing temperature) and morphological changes (dechorionation) can affect the animal's ability to perform thigmotaxis. Moreover, we found that modafinil increased thigmotaxis levels in *C. intestinalis* larvae. This is interesting in light of the fact that the effects of modafinil treatment in both humans and other animals has shown variable effects. In some cases, it acts as an anxiogenic drug like in one study in humans (143)and in others as an anxiolytic drug such as in marmosets(144). Notably, one study showed that modafinil increased the exploratory behaviour of mice in a dose dependent manner (145). It has also been shown that Modafinil can reduce thigmotaxis levels in zebrafish, (95). The strong effect that the anxiotropic drug modafinil has on *C. intestinalis* larval thigmotaxis is evidence that a common mechanism might mediate thigmotaxis across taxa(63, 95, 146). Future work should explore the molecular and cellular underpinnings of thigmotactic behaviour in *C. intestinalis* larvae and aim to understand the ecological context in which it may be used.

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

For years, cutting edge functional imaging and automated behavioural analysis was the privilege of a few model organisms. However, there has been a turn of tide in recent years. For one, the 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

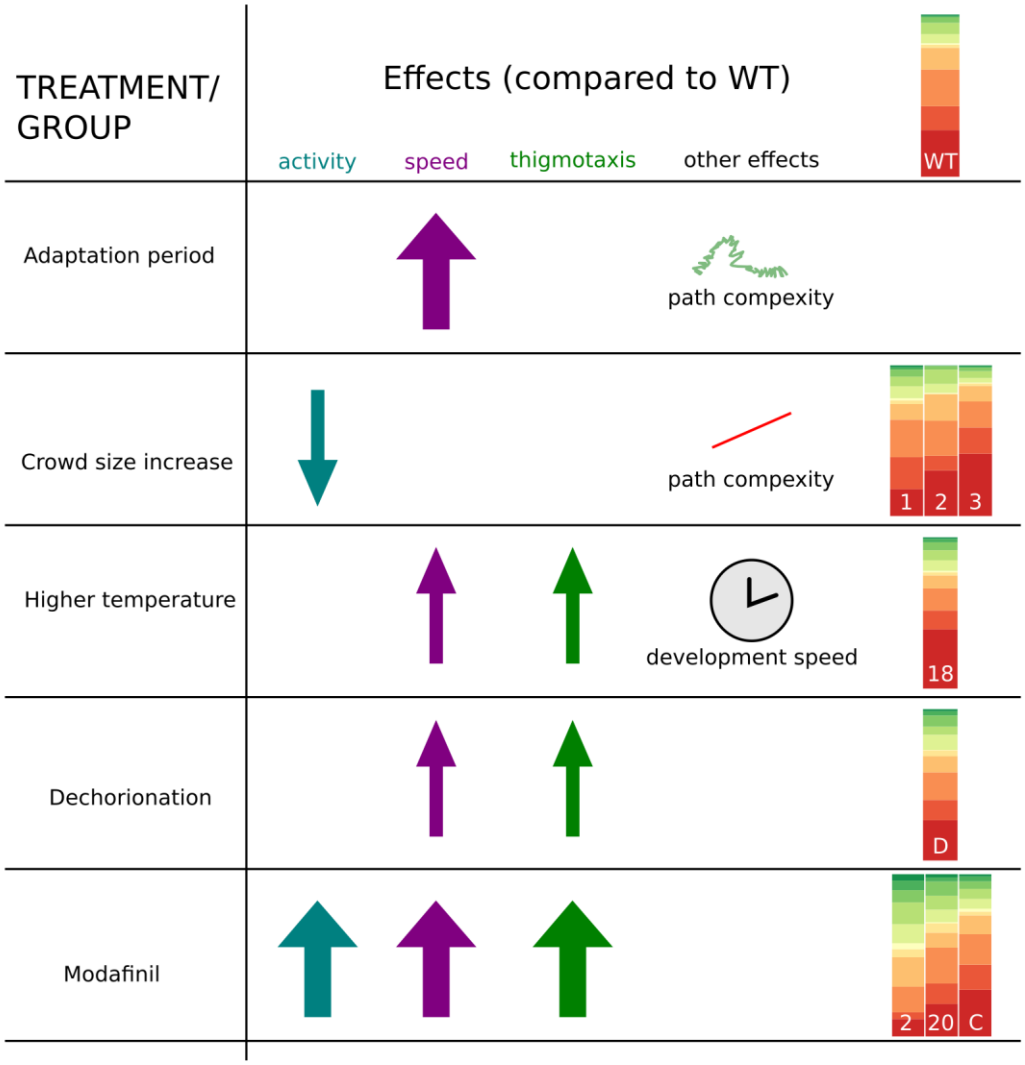

Fig 9. Summary

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

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
709 **Acknowledgments**
710 We would like to thank Felicia Keulder-Stenevik for animal collection, Mie Wong and Kushal Kolar for
711 comments on the manuscript. M.C. would like to thank Lionel Christiaen, Alberto Stolfi and Florian
712 Razy-Krajka for introducing him to *Ciona intestinalis* experimental methods.

713
714 **References**
715
716 1. Sjulson L, Cassataro D, DasGupta S, Miesenbock G. Cell-Specific Targeting of Genetically
717 Encoded Tools for Neuroscience. Annu Rev Genet. 2016;50:571-94.
718 2. Anderson DJ, Perona P. Toward a science of computational ethology. Neuron. 2014;84(1):18-
719 31.
720 3. Krakauer JW, Ghazanfar AA, Gomez-Marin A, MacIver MA, Poeppel D. Neuroscience Needs
721 Behavior: Correcting a Reductionist Bias. Neuron. 2017;93(3):480-90.
722 4. Egnor SE, Branson K. Computational Analysis of Behavior. Annu Rev Neurosci. 2016;39:217-
723 36.

5. Dell AI, Bender JA, Branson K, Couzin ID, de Polavieja GG, Noldus LP, et al. Automated image-based tracking and its application in ecology. Trends Ecol Evol. 2014;29(7):417-28.
6. Geissmann Q, Garcia Rodriguez L, Beckwith EJ, French AS, Jamasb AR, Gilestro GF. Ethoscopes: An open platform for high-throughput ethomics. PLoS Biol. 2017;15(10):e2003026.
7. Berman GJ. Measuring behavior across scales. BMC Biol. 2018;16(1):23.
8. Brown AE, Yemini EI, Grundy LJ, Jucikas T, Schafer WR. A dictionary of behavioral motifs reveals clusters of genes affecting Caenorhabditis elegans locomotion. Proc Natl Acad Sci U S A. 2013;110(2):791-6.
9. Stephens GJ, Johnson-Kerner B, Bialek W, Ryu WS. Dimensionality and dynamics in the behavior of C. elegans. PLoS Comput Biol. 2008;4(4):e1000028.
10. Berman GJ, Choi DM, Bialek W, Shaevitz JW. Mapping the stereotyped behaviour of freely moving fruit flies. Journal of The Royal Society Interface. 2014;11(99):20140672-.
11. Marques JC, Lackner S, Félix R, Orger MB. Structure of the Zebrafish Locomotor Repertoire Revealed with Unsupervised Behavioral Clustering. Current Biology. 2018.
12. Orger MB, de Polavieja GG. Zebrafish Behavior: Opportunities and Challenges. Annu Rev Neurosci. 2017;40:125-47.
13. Wiltschko AB, Johnson MJ, Iurilli G, Peterson RE, Katon JM, Pashkovski SL, et al. Mapping Sub-Second Structure in Mouse Behavior. Neuron. 2015;88(6):1121-35.
14. Katz PS, Harris-Warrick RM. The evolution of neuronal circuits underlying species-specific behavior. Current Opinion in Neurobiology. 1999;9(5):628-33.
15. Tosches MA. Developmental and genetic mechanisms of neural circuit evolution. Dev Biol. 2017;431(1):16-25.
16. Perez-Escudero A, Vicente-Page J, Hinz RC, Arganda S, de Polavieja GG. idTracker: tracking individuals in a group by automatic identification of unmarked animals. Nature Methods. 2014;11(7):743-U193.
17. Kabra M, Robie AA, Rivera-Alba M, Branson S, Branson K. JAABA: interactive machine learning for automatic annotation of animal behavior. Nat Methods. 2013;10(1):64-7.
18. Han S, Taralova E, Dupre C, Yuste R. Comprehensive machine learning analysis of Hydra behavior reveals a stable basal behavioral repertoire. Elife. 2018;7.
19. Lemaire P. Evolutionary crossroads in developmental biology: the tunicates. Development. 2011;138(11):2143-52.
20. Passamaneck YJ, Di Gregorio A. Ciona intestinalis: chordate development made simple. Dev Dyn. 2005;233(1):1-19.
21. Sasakura Y, Mita K, Ogura Y, Horie T. Ascidians as excellent chordate models for studying the development of the nervous system during embryogenesis and metamorphosis. Dev Growth Differ. 2012;54(3):420-37.
22. Ryan K, Lu Z, Meinertzhagen IA. The CNS connectome of a tadpole larva of Ciona intestinalis (L.) highlights sidedness in the brain of a chordate sibling. Elife. 2016;5.
23. Ryan K, Lu Z, Meinertzhagen IA. Circuit Homology between Decussating Pathways in the Ciona Larval CNS and the Vertebrate Startle-Response Pathway. Curr Biol. 2017;27(5):721-8.
24. Ryan K, Lu Z, Meinertzhagen IA. The peripheral nervous system of the ascidian tadpole larva: Types of neurons and their synaptic networks. J Comp Neurol. 2017.
25. White JG, Southgate E, Thomson JN, Brenner S. The Structure of the Nervous-System of the Nematode Caenorhabditis-Elegans. Philos T Roy Soc B. 1986;314(1165):1-340.
26. Tsuda M. Direct evidence for the role of pigment cells in the brain of ascidian larvae by laser ablation. Journal of Experimental Biology. 2003;206(8):1409-17.
27. Jiang D, Tresser JW, Horie T, Tsuda M, Smith WC. Pigmentation in the sensory organs of the ascidian larva is essential for normal behavior. J Exp Biol. 2005;208(Pt 3):433-8.
28. Nakagawa M, Miyamoto T, Ohkuma M, Tsuda M. Action Spectrum for the Photophobic Response of Ciona intestinalis (Ascidieacea, Urochordata) Larvae Implicates Retinal Protein. Photochemistry and Photobiology. 1999;70(3):359-62.

775 29. Kajiwara S, Yoshida M. Changes in Behavior and Ocellar Structure during the Larval Life of
776 Solitary Ascidians. Biol Bull. 1985;169(3):565-77.
777 30. Zega G, Thorndyke MC, Brown ER. Development of swimming behaviour in the larva of the
778 ascidian Ciona intestinalis. J Exp Biol. 2006;209(Pt 17):3405-12.
779 31. Tsuda M, Kawakami I, Shiraishi S. Sensitization and habituation of the swimming behavior in
780 ascidian larvae to light. Zoolog Sci. 2003;20(1):13-22.
781 32. Kawakami I, Shiraishi S, Tsuda M. Photoresponse and learning behavior of ascidian larvae, a
782 primitive chordate, to repeated stimuli of step-up and step-down of light. J Biol Phys. 2002;28(3):549-
783 59.
784 33. Salas P, Vinaithirthan V, Newman-Smith E, Kourakis MJ, Smith WC. Photoreceptor
785 specialization and the visuomotor repertoire of the primitive chordate Ciona. J Exp Biol. 2018.
786 34. Inada K, Horie T, Kusakabe T, Tsuda M. Targeted knockdown of an opsin gene inhibits the
787 swimming behaviour photoresponse of ascidian larvae. Neurosci Lett. 2003;347(3):167-70.
788 35. Svane I, YOUNG CM. The Ecology and Behaviour of Ascidian Larvae. Oceanography and
789 Marine Biology: an Annual Review. 1989(27):45-90.
790 36. Bone Q. On the Locomotion of Ascidian Tadpole Larvae. Journal of the Marine Biological
791 Association of the United Kingdom. 1992;72(1):161-86.
792 37. Mackie GO, Burighel P, Caicci F, Manni L. Innervation of ascidian siphons and their responses
793 to stimulation. Canadian Journal of Zoology. 2006;84(8):1146-62.
794 38. Torrence SA, Cloney RA. Nervous-System of Ascidian Larvae - Caudal Primary Sensory
795 Neurons. Zoomorphology. 1982;99(2):103-15.
796 39. Mackie GO, Bone Q. Skin Impulses and Locomotion in an Ascidian Tadpole. Journal of the
797 Marine Biological Association of the United Kingdom. 1976;56(3):751-&.
798 40. Bone Q, Ryan KP. Cupular Sense-Organs in Ciona (Tunicata-Ascidiacea). J Zool.
799 1978;186(Nov):417-29.
800 41. Stolfi A, Christiaen L. Genetic and genomic toolbox of the chordate Ciona intestinalis.
801 Genetics. 2012;192(1):55-66.
802 42. Mei Y, Zhang F. Molecular tools and approaches for optogenetics. Biol Psychiatry.
803 2012;71(12):1033-8.
804 43. Rodriguez A, Zhang H, Klaminder J, Brodin T, Andersson PL, Andersson M, et al. ToxTrac
805 : A fast and robust software for tracking organisms. Methods in Ecology and Evolution. 2017.
806 44. McHenry MJ, Patek SN. The evolution of larval morphology and swimming performance in
807 ascidians. Evolution. 2004;58(6):1209-24.
808 45. Chia FS, Bucklandnicks J, Young CM. Locomotion of Marine Invertebrate Larvae - a Review.
809 Canadian Journal of Zoology. 1984;62(7):1205-22.
810 46. McHenry MJ. Mechanisms of helical swimming: asymmetries in the morphology, movement
811 and mechanics of larvae of the ascidian Distaplia occidentalis. J Exp Biol. 2001;204(Pt 17):2959-73.
812 47. McHenry MJ, Azizi E, Strother JA. The hydrodynamics of locomotion at intermediate Reynolds
813 numbers: undulatory swimming in ascidian larvae (Botrylloides sp.). J Exp Biol. 2003;206(Pt 2):327-
814 43.
815 48. McHenry M, Strother J. The kinematics of phototaxis in larvae of the ascidian Aplidium
816 constellatum. Marine Biology. 2003;142(1):173-84.
817 49. Bartumeus F, Peters F, Pueyo S, Marrase C, Catalan J. Helical Levy walks: Adjusting searching
818 statistics to resource availability in microzooplankton. P Natl Acad Sci USA. 2003;100(22):12771-5.
819 50. Chan KY. Biomechanics of larval morphology affect swimming: insights from the sand dollars
820 Dendraster excentricus. Integr Comp Biol. 2012;52(4):458-69.
821 51. Roberts S, Guilford T, Rezek I, Biro D. Positional entropy during pigeon homing I: application
822 of Bayesian latent state modelling. Journal of Theoretical Biology. 2004;227(1):39-50.
823 52. Inoue T, Hoshino H, Yamashita T, Shimoyama S, Agata K. Planarian shows decision-making
824 behavior in response to multiple stimuli by integrative brain function. Zoological Lett. 2015;1:7.

825 53. Sharma S, Coombs S, Patton P, Burt de Perera T. The function of wall-following behaviors in
826 the Mexican blind cavefish and a sighted relative, the Mexican tetra (Astyanax). J Comp Physiol A
827 Neuroethol Sens Neural Behav Physiol. 2009;195(3):225-40.
828 54. Treit D, Fundytus M. Thigmotaxis as a Test for Anxiolytic Activity in Rats. Pharmacol Biochem
829 Be. 1988;31(4):959-62.
830 55. Kallai J, Makany T, Karadi K, Jacobs WJ. Spatial orientation strategies in Morris-type virtual
831 water task for humans. Behav Brain Res. 2005;159(2):187-96.
832 56. Walz N, Muhlberger A, Pauli P. A Human Open Field Test Reveals Thigmotaxis Related to
833 Agoraphobic Fear. Biol Psychiatry. 2016;80(5):390-7.
834 57. Kallai J, Makany T, Csatho A, Karadi K, Horvath D, Kovacs-Labadi B, et al. Cognitive and
835 affective aspects of thigmotaxis strategy in humans. Behav Neurosci. 2007;121(1):21-30.
836 58. Schnorr SJ, Steenbergen PJ, Richardson MK, Champagne DL. Measuring thigmotaxis in larval
837 zebrafish. Behav Brain Res. 2012;228(2):367-74.
838 59. Calderon DP, Kilinc M, Maritan A, Banavar JR, Pfaff D. Generalized CNS arousal: An
839 elementary force within the vertebrate nervous system. Neurosci Biobehav Rev. 2016;68:167-76.
840 60. Pfaff D, Ribeiro A, Matthews J, Kow LM. Concepts and mechanisms of generalized central
841 nervous system arousal. Ann N Y Acad Sci. 2008;1129:11-25.
842 61. Benjamini Y, Fonio E, Galili T, Havkin GZ, Golani I. Quantifying the buildup in extent and
843 complexity of free exploration in mice. Proc Natl Acad Sci U S A. 2011;108 Suppl 3:15580-7.
844 62. Cohen E, Yemini E, Schafer W, Feitelson DG, Treinin M. Locomotion analysis identifies roles of
845 mechanosensory neurons in governing locomotion dynamics of C. elegans. J Exp Biol. 2012;215(Pt
846 20):3639-48.
847 63. Simon P, Dupuis R, Costentin J. Thigmotaxis as an Index of Anxiety in Mice - Influence of
848 Dopaminergic Transmissions. Behavioural Brain Research. 1994;61(1):59-64.
849 64. Fiore VG, Dolan RJ, Strausfeld NJ, Hirth F. Evolutionarily conserved mechanisms for the
850 selection and maintenance of behavioural activity. Philos Trans R Soc Lond B Biol Sci.
851 2015;370(1684).
852 65. Guilford T, Roberts S, Biro D, Rezek I. Positional entropy during pigeon homing II: navigational
853 interpretation of Bayesian latent state models. J Theor Biol. 2004;227(1):25-38.
854 66. Sumpter D, Buhl J, Biro D, Couzin I. Information transfer in moving animal groups. Theory
855 Biosci. 2008;127(2):177-86.
856 67. Couzin ID. Collective cognition in animal groups. Trends Cogn Sci. 2009;13(1):36-43.
857 68. Dahanukar A, Ray A. Courtship, aggression and avoidance: Pheromones, receptors and
858 neurons for social behaviors in Drosophila. Fly. 2014;5(1):58-63.
859 69. Ugelvig LV, Cremer S. Social prophylaxis: group interaction promotes collective immunity in
860 ant colonies. Curr Biol. 2007;17(22):1967-71.
861 70. Rosenthal SB, Twomey CR, Hartnett AT, Wu HS, Couzin ID. Revealing the hidden networks of
862 interaction in mobile animal groups allows prediction of complex behavioral contagion. Proc Natl
863 Acad Sci U S A. 2015;112(15):4690-5.
864 71. Ioannou CC, Guttal V, Couzin ID. Predatory Fish Select for Coordinated Collective Motion in
865 Virtual Prey. Science. 2012;337(6099):1212-5.
866 72. Rogers SM. Mechanosensory-induced behavioural gregarization in the desert locust
867 Schistocerca gregaria. Journal of Experimental Biology. 2003;206(22):3991-4002.
868 73. Ramdya P, Lichocki P, Cruchet S, Frisch L, Tse W, Floreano D, et al. Mechanosensory
869 interactions drive collective behaviour in Drosophila. Nature. 2015;519(7542):233-6.
870 74. Saint-Amant L, Drapeau P. Time course of the development of motor behaviors in the
871 zebrafish embryo. J Neurobiol. 1998;37(4):622-32.
872 75. Currie SP, Combes D, Scott NW, Simmers J, Sillar KT. A behaviorally related developmental
873 switch in nitrergic modulation of locomotor rhythmogenesis in larval Xenopus tadpoles. J
874 Neurophysiol. 2016;115(3):1446-57.
875 76. Sillar KT, Wedderburn JF, Simmers AJ. The development of swimming rhythmicity in post-
876 embryonic Xenopus laevis. Proc Biol Sci. 1991;246(1316):147-53.

77. Frolov R, Immonen EV, Vahasoyrinki M, Weckstrom M. Postembryonic developmental changes in photoreceptors of the stick insect Carausius morosus enhance the shift to an adult nocturnal life-style. J Neurosci. 2012;32(47):16821-31.
78. Yoshida M, Matsuura K, Uematsu K. Developmental Changes in the Swimming Behavior and Underlying Motoneuron Activity in the Larval Angelfish, Pterophyllum scalare. Zool Sci. 1996;13(2):229-34.
79. Hanzi S, Straka H. Developmental changes in head movement kinematics during swimming in Xenopus laevis tadpoles. J Exp Biol. 2017;220(Pt 2):227-36.
80. Sato A, Kawashima T, Fujie M, Hughes S, Satoh N, Shimeld SM. Molecular basis of canalization in an ascidian species complex adapted to different thermal conditions. Sci Rep. 2015;5:16717.
81. Siegal ML, Bergman A. Waddington's canalization revisited: Developmental stability and evolution. P Natl Acad Sci USA. 2002;99(16):10528-32.
82. Gottlieb G. Experiential Canalization of Behavioral-Development - Theory. Dev Psychol. 1991;27(1):4-13.
83. Careau V, Buttemer WA, Buchanan KL. Early-developmental stress, repeatability, and canalization in a suite of physiological and behavioral traits in female zebra finches. Integr Comp Biol. 2014;54(4):539-54.
84. Bell AM, Stamps JA. Development of behavioural differences between individuals and populations of sticklebacks, Gasterosteus aculeatus. Animal Behaviour. 2004;68(6):1339-48.
85. Sugi T, Nishida Y, Mori I. Regulation of behavioral plasticity by systemic temperature signaling in Caenorhabditis elegans. Nat Neurosci. 2011;14(8):984-92.
86. Debat V, David P. Mapping phenotypes: canalization, plasticity and developmental stability. Trends in Ecology & Evolution. 2001;16(10):555-61.
87. Gillooly JF, Charnov EL, West GB, Savage VM, Brown JH. Effects of size and temperature on developmental time. Nature. 2002;417(6884):70-3.
88. Goldstein JA, Hoff KVS, Hillyard SD. The effect of temperature on development and behaviour of relict leopard frog tadpoles. Conserv Physiol. 2017;5(1):cow075.
89. Ramler D, Mitteroecker P, Shama LN, Wegner KM, Ahnelt H. Nonlinear effects of temperature on body form and developmental canalization in the threespine stickleback. J Evol Biol. 2014;27(3):497-507.
90. Jones JC, Helliwell P, Beekman M, Maleszka R, Oldroyd BP. The effects of rearing temperature on developmental stability and learning and memory in the honey bee, Apis mellifera. J Comp Physiol A Neuroethol Sens Neural Behav Physiol. 2005;191(12):1121-9.
91. Tautz J, Maier S, Groh C, Rossler W, Brockmann A. Behavioral performance in adult honey bees is influenced by the temperature experienced during their pupal development. P Natl Acad Sci USA. 2003;100(12):7343-7.
92. Brunetti R, Gissi C, Pennati R, Caicci F, Gasparini F, Manni L. Morphological evidence that the molecularly determinedCiona intestinalistype A and type B are different species:Ciona robustaandCiona intestinalis. Journal of Zoological Systematics and Evolutionary Research. 2015;53(3):186-93.
93. Bouchemousse S, Bishop JD, Viard F. Contrasting global genetic patterns in two biologically similar, widespread and invasive Ciona species (Tunicata, Ascidiacea). Sci Rep. 2016;6:24875.
94. Shimeld SM, Levin M. Evidence for the regulation of left-right asymmetry in Ciona intestinalis by ion flux. Dev Dyn. 2006;235(6):1543-53.
95. Johnson A, Hamilton TJ. Modafinil decreases anxiety-like behaviour in zebrafish. PeerJ. 2017;5:e2994.
96. Codling EA, Plank MJ, Benhamou S. Random walk models in biology. J R Soc Interface. 2008;5(25):813-34.
97. Benhamou S. Of scales and stationarity in animal movements. Ecol Lett. 2014;17(3):261-72.
98. Reynolds AM. Current status and future directions of Levy walk research. Biol Open. 2018;7(1).

929 99. Niebuhr BB, Wosniack ME, Santos MC, Raposo EP, Viswanathan GM, da Luz MG, et al.
930 Survival in patchy landscapes: the interplay between dispersal, habitat loss and fragmentation. Sci
931 Rep. 2015;5:11898.
932 100. Dannemann T, Boyer D, Miramontes O. Levy flight movements prevent extinctions and
933 maximize population abundances in fragile Lotka-Volterra systems. Proc Natl Acad Sci U S A.
934 2018;115(15):3794-9.
935 101. Kramer DL, McLaughlin RL. The Behavioral Ecology of Intermittent Locomotion1. Am Zool.
936 2001;41(2):137-53.
937 102. Alexander RM, Bennetclark HC. Storage of Elastic Strain-Energy in Muscle and Other Tissues.
938 Nature. 1977;265(5590):114-7.
939 103. Weimerskirch H, Martin J, Clerquin Y, Alexandre P, Jiraskova S. Energy saving in flight
940 formation - Pelicans flying in a 'V' can glide for extended periods using the other birds' air streams.
941 Nature. 2001;413(6857):697-8.
942 104. Herskin J, Steffensen JF. Energy savings in sea bass swimming in a school: measurements of
943 tail beat frequency and oxygen consumption at different swimming speeds. J Fish Biol.
944 1998;53(2):366-76.
945 105. Richardson JML. A comparative study of activity levels in larval anurans and response to the
946 presence of different predators. Behav Ecol. 2001;12(1):51-8.
947 106. Schmidtnielsen K. Locomotion - Energy Cost of Swimming, Flying, and Running. Science.
948 1972;177(4045):222-+.
949 107. Michalec FG, Fouxon I, Souissi S, Holzner M. Zooplankton can actively adjust their motility to
950 turbulent flow. Proc Natl Acad Sci U S A. 2017;114(52):E11199-E207.
951 108. Gomez-Marin A, Paton JJ, Kampff AR, Costa RM, Mainen ZF. Big behavioral data: psychology,
952 ethology and the foundations of neuroscience. Nat Neurosci. 2014;17(11):1455-62.
953 109. Rihel J, Prober DA, Arvanites A, Lam K, Zimmerman S, Jang S, et al. Zebrafish Behavioral
954 Profiling Links Drugs to Biological Targets and Rest/Wake Regulation. Science. 2010;327(5963):348-
955 51.
956 110. Yemini E, Jucikas T, Grundy LJ, Brown AE, Schafer WR. A database of Caenorhabditis elegans
957 behavioral phenotypes. Nat Methods. 2013;10(9):877-9.
958 111. Vogelstein JT, Park Y, Ohyama T, Kerr RA, Truman JW, Priebe CE, et al. Discovery of brainwide
959 neural-behavioral maps via multiscale unsupervised structure learning. Science. 2014;344(6182):386-
960 92.
961 112. Robie AA, Hirokawa J, Edwards AW, Umayam LA, Lee A, Phillips ML, et al. Mapping the
962 Neural Substrates of Behavior. Cell. 2017;170(2):393-406 e28.
963 113. Jing J, Gillette R, Weiss KR. Evolving Concepts of Arousal: Insights from Simple Model
964 Systems. Rev Neuroscience. 2009;20(5-6):405-27.
965 114. Weiss KR, Chiel HJ, Koch U, Kupfermann I. Activity of an Identified Histaminergic Neuron, and
966 Its Possible Role in Arousal of Feeding-Behavior in Semi-Intact Aplysia. Journal of Neuroscience.
967 1986;6(8):2403-15.
968 115. Choi S, Taylor KP, Chatzigeorgiou M, Hu Z, Schafer WR, Kaplan JM. Sensory Neurons Arouse
969 C. elegans Locomotion via Both Glutamate and Neuropeptide Release. PLoS Genet.
970 2015;11(7):e1005359.
971 116. Cao W, Edery I. A novel pathway for sensory-mediated arousal involves splicing of an intron
972 in the period clock gene. Sleep. 2015;38(1):41-51.
973 117. Lebestky T, Chang JS, Dankert H, Zelnik L, Kim YC, Han KA, et al. Two different forms of
974 arousal in Drosophila are oppositely regulated by the dopamine D1 receptor ortholog DopR via
975 distinct neural circuits. Neuron. 2009;64(4):522-36.
976 118. Kumar S, Chen D, Sehgal A. Dopamine acts through Cryptochrome to promote acute arousal
977 in Drosophila. Genes Dev. 2012;26(11):1224-34.
978 119. Volkow ND, Fowler JS, Logan J, Alexoff D, Zhu W, Telang F, et al. Effects of Modafinil on
979 Dopamine and Dopamine Transporters in the Male Human Brain Clinical Implications. Jama-J Am
980 Med Assoc. 2009;301(11):1148-54.

120. Madras BK, Xie Z, Lin Z, Jassen A, Panas H, Lynch L, et al. Modafinil occupies dopamine and norepinephrine transporters in vivo and modulates the transporters and trace amine activity in vitro. J Pharmacol Exp Ther. 2006;319(2):561-9.
121. Wisor JP, Nishino S, Sora I, Uhl GH, Mignot E, Edgar DM. Dopaminergic role in stimulant-induced wakefulness. Journal of Neuroscience. 2001;21(5):1787-94.
122. Sigurgeirsson B, Thorsteinsson H, Arnardottir H, Johannesdottir IT, Karlsson KA. Effects of modafinil on sleep-wake cycles in larval zebrafish. Zebrafish. 2011;8(3):133-40.
123. Sillar KT, Simmers AJ, Wedderburn JF. The post-embryonic development of cell properties and synaptic drive underlying locomotor rhythm generation in Xenopus larvae. Proc Biol Sci. 1992;249(1324):65-70.
124. Matsuura T, Endo S, Iwamoto R, Takahashi H, Ichinose M. Developmental changes in chemotactic response and choice of two attractants, sodium acetate and diacetyl, in the nematode Caenorhabditis elegans. Comp Biochem Physiol A Mol Integr Physiol. 2007;147(4):920-7.
125. Moret F, Christiaen L, Deyts C, Blin M, Joly JS, Vernier P. The dopamine-synthesizing cells in the swimming larva of the tunicate Ciona intestinalis are located only in the hypothalamus-related domain of the sensory vesicle. Eur J Neurosci. 2005;21(11):3043-55.
126. Herbert-Read JE. Understanding how animal groups achieve coordinated movement. J Exp Biol. 2016;219(Pt 19):2971-83.
127. Havenhand JN, Svane I. Roles of hydrodynamics and larval behaviour in determining spatial aggregation in the tunicate Ciona intestinalis. Marine Ecology Progress Series. 1990;68:271-6.
128. Dussutour A, Deneubourg JL, Fourcassie V. Amplification of individual preferences in a social context: the case of wall-following in ants. Proc Biol Sci. 2005;272(1564):705-14.
129. Berdahl A, Torney CJ, Ioannou CC, Faria JJ, Couzin ID. Emergent Sensing of Complex Environments by Mobile Animal Groups. Science. 2013;339(6119):574-6.
130. Bennett AF. Thermal-Dependence of Locomotor Capacity. Am J Physiol. 1990;259(2):R253-R8.
131. Bonte D, Travis JMJ, De Clercq N, Zwertvaegher I, Lens L. Thermal conditions during juvenile development affect adult dispersal in a spider. P Natl Acad Sci USA. 2008;105(44):17000-5.
132. Lee JH, Dillman AR, Hallem EA. Temperature-dependent changes in the host-seeking behaviors of parasitic nematodes. BMC Biol. 2016;14:36.
133. Schnebel EM, Grossfield J. Mating-Temperature Range in Drosophila. Evolution. 1984;38(6):1296-307.
134. MagalhÃEs TRF, Neves RAF, Valentin JL, Figueiredo GM. Do the changes in temperature and light affect the functional response of the benthic mud snail Heleobia australis (Mollusca: Gastropoda)? Anais da Academia Brasileira de Ciências. 2014;86(3):1197-206.
135. Linn CE, Jr., Campbell MG, Roelofs WL. Pheromone components and active spaces: what do moths smell and where do they smell it? Science. 1987;237(4815):650-2.
136. Hanada T, Kashiwayanagi M, Kurihara K. Temperature increase abolishes ability of turtle olfactory receptors to discriminate similar odorant. Am J Physiol. 1994;266(6 Pt 2):R1816-23.
137. Riveron J, Boto T, Alcorta E. The effect of environmental temperature on olfactory perception in Drosophila melanogaster. J Insect Physiol. 2009;55(10):943-51.
138. Martin F, Riveron J, Alcorta E. Environmental temperature modulates olfactory reception in Drosophila melanogaster. J Insect Physiol. 2011;57(12):1631-42.
139. Kodama-Namba E, Fenk LA, Bretscher AJ, Gross E, Busch KE, de Bono M. Cross-modulation of homeostatic responses to temperature, oxygen and carbon dioxide in C. elegans. PLoS Genet. 2013;9(12):e1004011.
140. Boorman CJ, Shimeld SM. Pitx homeobox genes in Ciona and amphioxus show left-right asymmetry is a conserved chordate character and define the ascidian adenohypophysis. Evolution & Development. 2002;4(5):354-65.
141. Frasnelli E, Vallortigara G, Rogers LJ. Left-right asymmetries of behaviour and nervous system in invertebrates. Neurosci Biobehav Rev. 2012;36(4):1273-91.

142. Besson M, Martin JR. Centrophobism/thigmotaxis, a new role for the mushroom bodies in Drosophila. J Neurobiol. 2005;62(3):386-96.
143. Randall DC, Shneerson JM, Plaha KK, File SE. Modafinil affects mood, but not cognitive function, in healthy young volunteers. Hum Psychopharmacol. 2003;18(3):163-73.
144. van Vliet SA, Jongsma MJ, Vanwersch RA, Olivier B, Philippens IH. Behavioral effects of modafinil in marmoset monkeys. Psychopharmacology (Berl). 2006;185(4):433-40.
145. Young JW, Kooistra K, Geyer MA. Dopamine receptor mediation of the exploratory/hyperactivity effects of modafinil. Neuropsychopharmacology. 2011;36(7):1385-96.
146. Minzenberg MJ, Carter CS. Modafinil: a review of neurochemical actions and effects on cognition. Neuropsychopharmacology. 2008;33(7):1477-502.
147. Dupre C, Yuste R. Non-overlapping Neural Networks in Hydra vulgaris. Curr Biol. 2017.
148. Nath RD, Bedbrook CN, Abrams MJ, Basinger T, Bois JS, Prober DA, et al. The Jellyfish Cassiopea Exhibits a Sleep-like State. Curr Biol. 2017;27(19):2984-90 e3.
149. Delsuc F, Brinkmann H, Chourrout D, Philippe H. Tunicates and not cephalochordates are the closest living relatives of vertebrates. Nature. 2006;439(7079):965-8.
150. Hudson C. The central nervous system of ascidian larvae. Wiley Interdiscip Rev Dev Biol. 2016;5(5):538-61.
151. Stolfi A, Gandhi S, Salek F, Christiaen L. Tissue-specific genome editing in Ciona embryos by CRISPR/Cas9. Development. 2014;141(21):4115-20.
152. Abdul-Wajid S, Morales-Diaz H, Khairallah SM, Smith WC. T-type Calcium Channel Regulation of Neural Tube Closure and EphrinA/EPHA Expression. Cell Rep. 2015;13(4):829-39.
153. Akahoshi T, Hotta K, Oka K. Characterization of calcium transients during early embryogenesis in ascidians Ciona robusta (Ciona intestinalis type A) and Ciona savignyi. Dev Biol. 2017;431(2):205-14.
154. Hackley C, Mulholland E, Kim GJ, Newman-Smith E, Smith WC. A transiently expressed connexin is essential for anterior neural plate development in Ciona intestinalis. Development. 2013;140(1):147-55.
155. Christiaen L, Wagner E, Shi W, Levine M. Isolation of sea squirt (Ciona) gametes, fertilization, dechorionation, and development. Cold Spring Harb Protoc. 2009;2009(12):pdb prot5344.