Report

Current Biology

A Complex Hierarchy of Avoidance Behaviors in a Single-Cell Eukaryote

Highlights

- A hierarchy of avoidance behaviors is demonstrated in the ciliate *Stentor roeseli*
- This replicates the previously disputed, century-old observations of Jennings
- Video microscopy and statistical analysis show evidence of complex decision-making
- The decision between contraction and detachment resembles a fair coin toss

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In Brief

Dexter et al. replicate the disputed, century-old observations of Jennings, confirming by video microscopy and statistical analysis that the single-cell ciliate *Stentor roeseli* exhibits a hierarchy of avoidance behaviors. They show further that each organism's decision between contracting and detaching resembles a fair coin toss.

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Report

A Complex Hierarchy of Avoidance Behaviors in a Single-Cell Eukaryote

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SUMMARY

Complex behavior is associated with animals with nervous systems, but decision-making and learning also occur in non-neural organisms [1], including singly nucleated cells [2–5] and multinucleate synctia [6–8]. Ciliates are single-cell eukaryotes, widely dispersed in aquatic habitats [9], with an extensive behavioral repertoire [10–13]. In 1906, Herbert Spencer Jennings [14, 15] described in the sessile ciliate *Stentor roeseli* a hierarchy of responses to repeated stimulation, which are among the most complex behaviors reported for a singly nucleated cell [16, 17]. These results attracted widespread interest [18, 19] and exert continuing fascination [7, 20–22] but were discredited during the behaviorist orthodoxy by claims of non-reproducibility [23]. These claims were based on experiments with the motile ciliate *Stentor coeruleus*. We acquired and maintained the correct organism in laboratory culture and used micromanipulation and video microscopy to confirm Jennings' observations. Despite significant individual variation, not addressed by Jennings, *S. roeseli* exhibits avoidance behaviors in a characteristic hierarchy of bending, ciliary alteration, contractions, and detachment, which is distinct from habituation or conditioning. Remarkably, the choice of contraction versus detachment is consistent with a fair coin toss. Such behavioral complexity may have had an evolutionary advantage in protist ecosystems, and the ciliate cortex may have provided mechanisms for implementing such behavior prior to the emergence of multicellularity. Our work resurrects Jennings' pioneering insights and adds to the list of exceptional features, including regeneration [24], genome rearrangement [25], codon reassignment [26], and cortical inheritance [27], for which the ciliate clade is renowned.

RESULTS AND DISCUSSION

Experimental Setup

Ciliates form a clade of single-cell eukaryotes characterized by their eponymous cilia, nuclear dimorphism, and sexual conjugation [9] (Figure 1A). *S. roeseli* is colorless, trumpet shaped, and visible to the naked eye (Figures S1A and 1B show the morphologically similar *S. coeruleus* [29]). Ciliary rows along the axis and ciliary spirals at the wider end generate a fluid vortex to bring food particles to the "mouth." *S. roeseli* is typically sessile, anchoring itself to algal detritus with a holdfast of secreted mucus.

We obtained *S. roeseli*, confirmed its identity, and maintained it in laboratory culture (STAR Methods). In our hands, stimulation with carmine powder suspended in pond water, as originally described by Jennings [16], rarely elicited avoidance behavior. We instead used polystyrene beads in an aqueous suspension with NaN_3 (hereafter, "beads"), which reproducibly elicited such behavior (STAR Methods). *S. roeseli* may recognize this stimulation as different to that which Jennings used. If so, its response appears very similar, which may indicate a more generalized avoidance strategy. The need to modify the original experimental protocol illustrates the subtleties of reproducibility after such a long time; we could easily have concluded that Jennings' procedure did not work.

Figure S1B shows the experimental setup (STAR Methods). Organisms were placed in a droplet on the stage of an inverted microscope equipped for video recording. Beads were delivered through a microinjection needle, which we positioned near the organism while observing through the microscope. Pulses of stimulation were generated by opening and closing a stopcock on a gravity-fed system.

Jennings acquired facility with his experimental procedure over many years, and it may have had advantages over the one used here. His descriptions suggest that he could position the pipette flexibly and accurately in three dimensions to point at an organism's oral cavity. In contrast, our pulses could only be delivered in the three-dimensional vicinity of the organism, making it harder to tell whether it was the arrival of the pulse, its duration, or even the accumulation of NaN_3 over several pulses to which the organism was reacting.

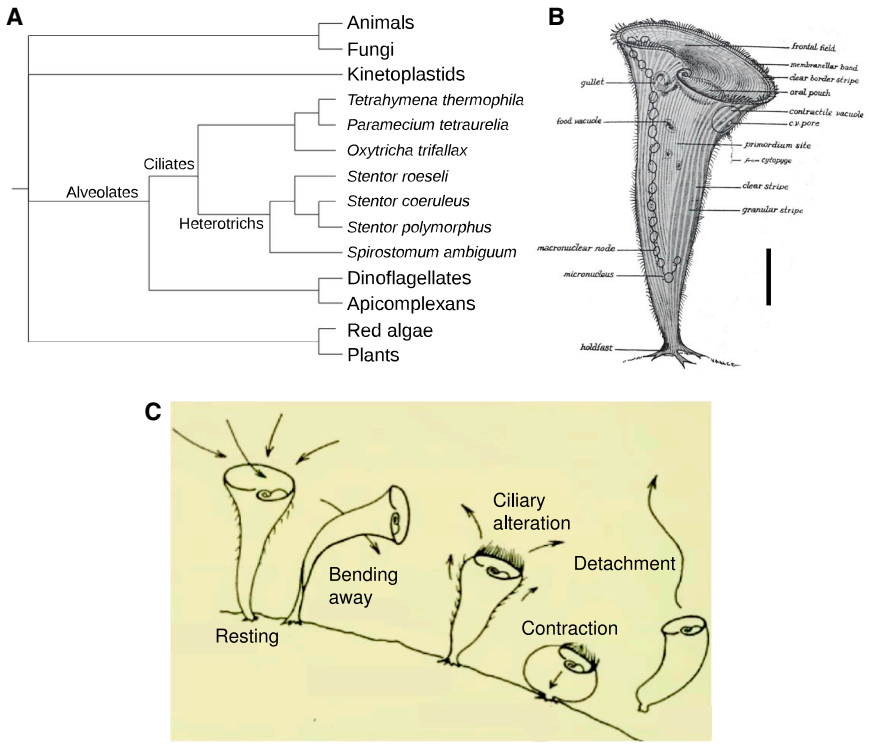

Figure 1. Ciliate Evolution, Structure, and Behavior

(A) Simplified phylogeny based on [9, 28] with ciliate species italicized.

(B) Drawing of *S. coeruleus* showing principal features [24], largely shared with *S. roeseli*, except for the beaded macronucleus (Figure S1A). Scale bar, ~100 μm.

(C) Sketch of avoidance hierarchy in *S. roeseli* based on Jennings' original descriptions [24].

See also Figures S1 and S2.

Behavior Identification

Jennings reported a hierarchy of behaviors—resting (R), bending away (B), ciliary alteration (A), contraction (C), and detachment from the holdfast (D)—in response to repeated stimulation (Figure 1C). Figure 2A illustrates for an individual organism, in response to the pulse stimulation in Figure 2B, each of these reported behaviors (see STAR Methods for further characterization). We found these same behaviors repeatedly in experiments conducted over several months. The videos of each experiment are freely available on Mendeley (Table S1), and this archive provides the raw data from which our conclusions are drawn. Data S1 lists, for each experiment, the sequence of pulses and behaviors and their estimated times. Experiments are referred to by the identifier NL, where N is the day number, from 1 to 18, and L is a letter, from A to I, for each organism observed on that day.

The sequence of behaviors observed in each experiment is summarized as a sequence of symbols, such as RpCpAC2AC2-pABCD for experiment 15B (Table 1). Here, "p" denotes a pulse; the other letters are as given above. Contractions sometimes took place repeatedly after a pulse, perhaps because of the continued presence of beads in the vicinity, and a numeral after C gives the number of contractions without intervening pulses or behaviors. The behaviors A and B often occurred together (Figure 2A, frame 2), making their relative order difficult to determine.

Behavior Hierarchy

Pulses of stimulation were not administered in a fixed sequence. Instead, pulsing was adapted to each organism during observation. With this protocol, it is difficult to attribute an individual behavior to an individual pulse. The decision to administer a pulse depended on whether the organism appeared to have returned to a resting state; had we waited longer, we cannot rule out that it might have responded again. Accordingly, we interpret the behavior sequences as arising from a generalized "stimulation" and focus on the pattern of observed behaviors, A, B, C, and D, and not on the pattern of pulses, p.

A further point with our experimental design is that we did not set aside time for control observation of each organism. We only appreciated the significance of this once we began quantitatively analyzing the data. In partial compensation, Table S2 lists the behaviors seen before each organism was stimulated. We found one of the four behaviors 10 out of 70 times (14%), and this was typically either A or B (9/10). The one contraction (experiment 12A) may have been due to an accidental pulse. A sequence of behaviors was observed only once (AB in experiment 5A); in the analysis below, behaviors A and B are treated together. Although these control durations varied between organisms, they provide a baseline for each individual organism's behavior in the absence of stimulation. On this basis, we consider the behavior sequences in Table 1 to be a specific response to stimulation.

Jennings emphasized that *S. roeseli* exhibited a behavior hierarchy (Figure 1C). However, the data in Table 1 reveal substantial heterogeneity. We found few instances of the full hierarchy (Figure 2A) but many partial instances with varying orders of occurrence of individual behaviors.

To test whether there is a hierarchy, we first asked whether, among those organisms that detach (D), which is always the last behavior exhibited, there is a tendency for behavior X to occur somewhere in the sequence before D. If there is no such tendency, we would expect X to occur as often as not in repeated experiments. The probability of X occurring k times in N experiments is then given by the binomial distribution, $\binom{N}{k}0.5^N$. We determined a Z score as $|o - m|/s$, where o is the number of times in which X was observed at least once among N trials and m and s are the mean and standard deviation of the appropriate binomial distribution, $N/2$ and $\sqrt{N}/2$, respectively. We excluded the second behavior sequences in experiments 12B, 14C, and 14E, in which the same organism was followed after detachment, as no pulse was administered. From Table 1, we see that D is always preceded by C (44/44, Z score = 6.6). For reasons noted above, we consider A and B

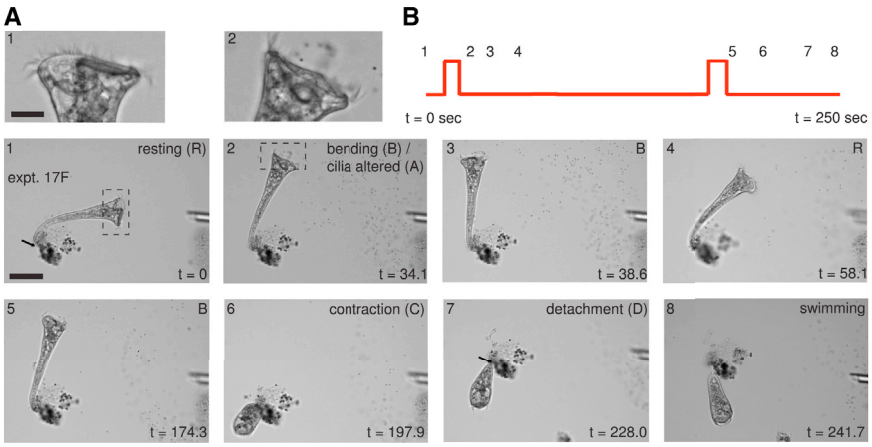

Figure 2. Behavior Identification and Hierarchy

(A) Frames numbered 1–8 (top left corner of each panel) show each classified behavior, as annotated (top right); scale bar in frame 1, 100 μm. Pipette tip on the right. Top two panels show enlarged views of ciliary alteration from the dashed boxes in frames 1 and 2; scale bar, 50 μm.

(B) Timeline of pulse stimulation for behavior in (A), with approximate time point of each numbered frame.

See also Figure S2.

together as "A or B." This amounts to setting both symbols to X and counting how many times X occurs at least once. We find that D is typically preceded by A or B (30/44, Z score = 2.4).

As a second test, we asked whether, among those organisms that show both behaviors X and Y, there is a preferred order of appearance. If there is no preferred order, we would expect to see the first occurrence of X in the sequence as often before the first occurrence of Y as in the opposite order. The probability of seeing X before Y in this way k times in N experiments is given by the same binomial distribution as above, so we adopted the same Z score. From Table 1, we find that A or B, considered together as above, is far more likely to appear before C than after C (40/44 occurrences, Z score = 5.4).

We conclude that a behavior hierarchy is strongly supported statistically.

Evidence for Complex Decision-Making

We consider the behavior hierarchy as a form of sequential decision-making [7], in the sense that, when given similar stimulation repeatedly, the organism "changes its mind" about which response to give, thereby following the observed hierarchy. Cellular decision-making has been widely discussed, but this form of it is simpler than heritable phenotypic change [30] or adaptive choice when confronting multiple stimulations [31].

An alternative possibility to decision-making is the "Clever Hans" effect [32], in which the organism picks up distinguishing cues, unwittingly or invisibly provided by the experimenter (Figure 3A). Evidence against this comes from a rare instance in which we stimulated two organisms with the same pulse and elicited distinct behaviors from each (Figure S3). More compelling evidence is the very existence of the behavior hierarchy. This strongly argues against a Clever Hans effect, for otherwise it would imply that we, the experimenters, had subliminally learned how to elicit the complex behaviors we were seeking. We consider this implausible.

While a Clever Hans effect may be ruled out, it is possible that organisms are picking up cues other than the pulse stimulation, which affect their behavior. If such cues exist, they seem most likely to arise from the experimental setup, which remains the same for different organisms during one day of experiments but varies from day to day. We therefore considered the day-to-day variation in the distribution of the total number of C's exhibited by each organism (Figure 3B). We excluded as an outlier experiment 18B, in which the organism contracted 20 times after a single pulse (Table 1). We used the non-parametric Kruskal-Wallis test to ask if the remaining samples came from the same distribution. The p value for this being so was 0.11; if experiment 18B was included the p value declined to 0.07. We conclude that the experimental context may be influencing an organism's behavior beyond the effect of stimulation, but the statistical support for this is borderline and hard to disentangle from behavioral heterogeneity.

If the organism is making internal decisions, the heterogeneity makes it difficult to determine its overall decision strategy. Staddon has examined several potential strategies to explain Jennings' observations but without replicating the experiments or addressing the heterogeneity ([22], Chapter 4). We considered the proportion of organisms that remain attached after a given number of contractions (Figure 3C). The resulting curve is well fitted ($R^2 = 0.98$) to an exponential decline with rate 0.689. An exponential decline is consistent with each individual organism following the memory-less (Markov) process shown in Figure 3C, in which an organism transitions between resting and contraction, with the possibility of detachment after contraction (as noted above, no organism detached without contracting first). Detachment is represented as an absorbing exit state. With the transition rates shown, the probability of detaching after contraction is $p = d/(r + d)$. Assuming organisms make decisions independently, the proportion remaining after k contractions is $(1-p)^k$, for which the data imply that $p = 1 - \exp(-0.689) = 0.50$. Hence, in so far as the decision between contracting and detaching is concerned, the data are consistent with each organism independently flipping an unbiased coin at each decision, irrespective of previous decisions.

Summary and Conclusions

We hope to have resolved in this paper the strange fate of Herbert Spencer Jennings' results on *Stentor roeseli*. They played a key role in the early debates between Jennings and Jacques Loeb on animal behavior [15, 18] but have been discredited among those who work on ciliates: "Jennings' account of behavioral modification in *Stentor* makes good reading, but the sequence of events he described has not proven to be reproducible" (Reynierse, Psychological Record, 1967) (D. Wood, personal communication).

Table 1. *S. roeseli* Behaviors

M/D/Y	Experiment #	Behavior	M/D/Y	Experiment #	Behavior
11/3/2014	1A	RpACD	12/5/2014	11A	RpA
11/3/2014	1B	RpABpCD, RpApCD	12/5/2014	11B	RpABCD
11/3/2014	1C	RpBC2ACpACD	12/5/2014	11C	RpC2
11/3/2014	1D	RpACD, RpCD, RpC2D	12/5/2014	11D	RpCD
11/3/2014	1E	RpABCpBCD	12/8/2014	12A	RpACpCpCD
11/3/2014	1F	RpABpCD	12/8/2014	12B	RpCpBCBpBCpCpBCpCD → RpC
11/5/2014	2A	RpCpAC2pACpC2D	12/9/2014	13A	RpABC2pACAC
11/7/2014	3A	RpCD, RpABC, RpABC3	12/10/2014	14A	RpAC, RpCD
11/7/2014	3B	RpACpCpC	12/10/2014	14B	RpC2D
11/7/2014	3C	RpABCD	12/10/2014	14C	RpACpCD → RC2
11/7/2014	3D	RpACpCD, RpCD	12/10/2014	14D	RpABCpCpCD
11/10/2014	4A	RppCp, RppCp, RppCp	12/10/2014	14E	RpBACpAC2BC7D → RC2D
11/10/2014	4B	RpABC	12/15/2014	15A	RpACpABCD
11/10/2014	4C	RpAC	12/15/2014	15B	RpCpAC2AC2pABCD
11/10/2014	4D	RpApABCpCD	12/15/2014	15C	RpCD, RpCD, RpC
11/10/2014	4E	RpAC2pD	12/15/2014	15D	RpCD
11/10/2014	4F	RpABCD	1/21/2015	16A	RpABC4pCD
11/12/2014	5A	RpCpABCpCpCACAC	1/22/2015	17A	RpCD, RpAC2D
11/14/2014	6A	RpACpC	1/22/2015	17B	RpACD
11/14/2014	6B	RpCD	1/22/2015	17C	RpACpACpCpACpCpC
11/14/2014	6C	RpCpC	1/22/2015	17D	RpAC
11/14/2014	6D	RpACD, RpC2	1/22/2015	17E	RpABCD
11/15/2014	7A	RpC	1/22/2015	17F	RpBApBACD
11/25/2014	8A	RpC2	1/22/2015	17G	RpACD
11/25/2014	8B	RpACp	1/22/2015	17H	RpACD
11/26/2014	9A	RpACpACpCpCpCpCpC pACpACpACpACpCpC	1/22/2015	17I	RpABC4D
12/3/2014	10A	RpC3	1/30/2015	18A	RpC3D
12/3/2014	10B	RpC2	1/30/2015	18B	RpAC20
12/3/2014	10C	RpCD			

M/D/Y, month, day, year. Behaviors are summarized in a symbol sequence, as described in the Results. Commas separate behaviors of different organisms in the same experiment; arrows (→) separate behaviors of the same organism, followed after detachment. Videos for each experiment are available on Mendeley; see Table S1. See also Table S2 and Data S1.

The historical context for this judgement is instructive. Non-associative learning, such as habituation, is well established in single cells [2–5]. Suggestions that ciliates also exhibited associative learning, either classical or instrumental [22], encountered repeated failures of reproducibility [33–35], leading to a consensus against such behavior. Reynierse and Walsh, working within the behaviorist paradigm, tried to interpret Jenning's observations as classical conditioning, using a prod from a dissecting needle as the conditional stimulus and carmine dye pipetting as the unconditional stimulus [23]. Unable to obtain *S. roeseli*, they used *S. coeruleus* instead. Behaviorism was strongly environmentalist, eschewing innate as well as cognitive capabilities, so perhaps one species of *Stentor* seemed as good as another. But *S. coeruleus* strongly prefers to be motile. As Reynierse and Walsh reported, "Stentor became free-swimming quickly whenever the carmine US was presented, regardless of experimental conditions" [23]. On that basis, Jennings' careful observations were discredited.

The results presented here confirm that Jennings was right. In response to stimulation, *S. roeseli* exhibits each of the individual avoidance behaviors he identified (Figure 2A). We find substantial heterogeneity in behavior (Table 1), which Jennings did not address, but by following a quantitative approach, in contrast to his descriptive methods, we provide compelling evidence for Jennings' behavior hierarchy. Remarkably, the choice between contraction and detachment is consistent with a fair coin toss (Figure 3C), raising the intriguing question as to how *S. roeseli* implements this so accurately at a molecular level.

We can only speculate on the evolutionary forces that led to the emergence of such complex behavior. Several ciliate species have multiple mating types [36], suggesting the need for powerful social recognition mechanisms [37]. *S. coeruleus* has binary mating [11], but the mating behavior of *S. roeseli* is not known. It is, however, a voracious predator, able to devour unwary rotifers, which have a thousand cells and a nervous system. A

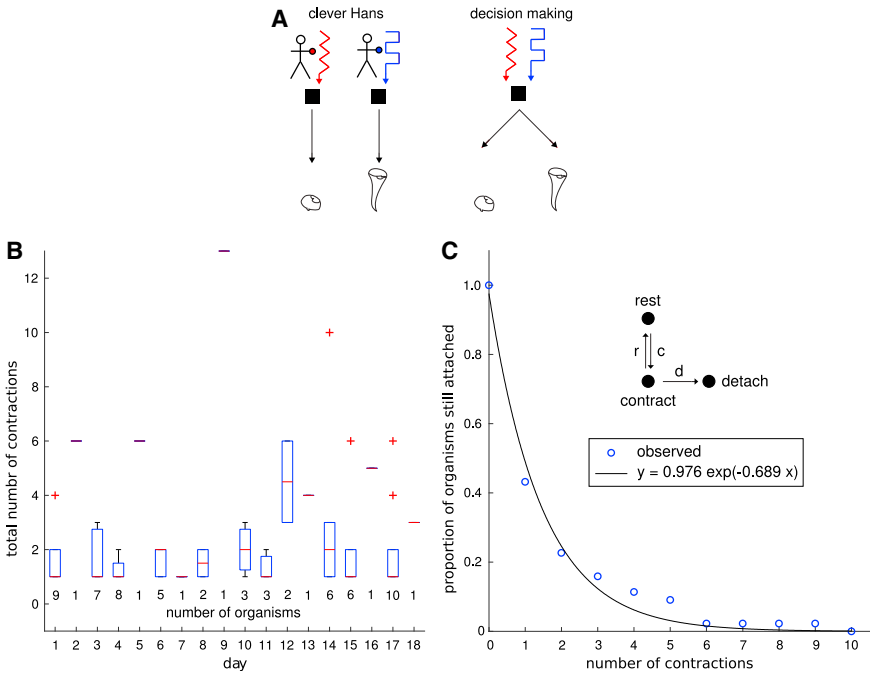

Figure 3. Evidence for Complex Decision-Making

(A) Schematic of Clever Hans effect compared to decision-making.

(B) Boxplots with distributions of total numbers of contractions for each organism on each numbered day. Blue box shows inter-quartile range (IQR = Q1 to Q3); red bar shows median; whiskers extend to the furthest non-outlier; red crosses show outliers, defined as $< Q1 - 1.5 \times IQR$ or $> Q3 + 1.5 \times IQR$. Numbers of organisms for each day are listed above the day number; data for 68 organisms. Experiment 18B was excluded as an outlier; see the text.

(C) Plot of proportion of organisms not detached against number of contractions, showing a good fit to an exponential decline; data for 44 organisms. The data are consistent with the Markov process shown (see text), where r, c, and d denote the instantaneous transition rates, with dimensions of $(\text{time})^{-1}$.

See also Figure S3.

behavior hierarchy could have been an efficient strategy to avoid the costly process of detachment and relocation, once a rich hunting ground had been located. As to why the decision between contraction and detachment appears to be perfectly random (Figure 3C), perhaps the answer lies in some form of game-theoretic optimization arising from this ecological context.

The ciliate membrane and cytoskeletal cortex are the most likely candidates for mechanistically implementing the behaviors observed here. They underlie each of the individual behaviors shown in Figure 1C. The ciliate membrane is excitable. It harbors voltage-dependent and mechanosensitive ion channels that generate action potentials, analogous to those in neurons, and these channels play a key role in habituation [3]. The cortex can propagate to daughter cells, in a non-genetic and Lamarckian manner, micro-surgical alterations to ciliary geometry, giving rise thereby to "cortical inheritance" [27]. This phenomenon, discovered by Beisson and Jennings' student, Sonneborn, rests on far more solid ground than Jennings' avoidance behaviors [24, 38, 39] but inspires rather similar incredulity, outside the few who have struggled to understand it [40]. The cortex also plays the central role in regeneration: excised fragments of a ciliate, provided they contain appropriate parts of the cortex, will reconstruct themselves into smaller, whole organisms [24]. With such extravagant capabilities for self-organization at its disposal, *S. roeseli*'s avoidance hierarchy may begin to seem less extraordinary.

Ciliate exceptionalism is not limited to cortical inheritance, regeneration, and now, behavior. Ciliates are known to molecular biologists for reassigning stop codons [26] and especially for their wizardry in RNA-directed genome rearrangement [25, 41]. In these respects, ciliates have illuminated central aspects of molecular biology. Perhaps the very strength of that molecular spotlight has cast a deeper shadow over those other features of ciliates, which do not fit so comfortably, as yet, into a modern perspective.

Jennings' experiments on *Stentor* were evidence for agency—the capacity for cellular decision-making—in contrast to Loeb's insistence that life was merely physical chemistry [15, 18]. Loeb is celebrated for inspiring behaviorism and anticipating the success of molecular reductionism. Jennings is sometimes unfairly associated with the wooly holism of some of his admirers [19, 21]. Yet his ciliates continue to haunt the same debate, now couched in different language. Kirschner, Gerhart, and Mitchison mischievously refer to it as the problem of "molecular vitalism" and remind us of the challenge to molecular understanding presented by ciliate cortical inheritance and regeneration [42]. There has been important progress here: the genome of *S. coeruleus* has been sequenced [43] and molecular insights acquired into ciliary patterning and regeneration [44, 45]. Jennings' avoidance hierarchy presents the same challenge as self-organization. It reveals unexpected depths in the cognitive capabilities of singly nucleated cells [7]. We should explore these more broadly in their natural context and unravel their molecular underpinnings. Nobody would be more delighted by such molecular vitalism than Jennings himself [46].

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- LEAD CONTACT AND MATERIALS AVAILABILITY
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
  - Stentor roeseli
- METHOD DETAILS
  - Beads
  - Needle construction
  - Stimulation apparatus and protocol

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.cub.2019.10.059.

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AUTHOR CONTRIBUTIONS

J.P.D. and S.P. designed and undertook the experiments; all authors undertook analysis and interpretation of the data; and J.G. conceived the project and wrote the paper with the assistance of J.P.D. and S.P.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, Peptides, and Recombinant Proteins		
Latex beads, carboxylate-modified polystyrene, fluorescent red	Sigma-Aldrich	Cat#L3030
Carmine	Sigma-Aldrich	Cat#C1022
Deposited Data		
Videos of *Stentor* behavior sequences	This paper; Mendeley Data	See Table S1
Experimental Models: Organisms/Strains		
Stentor roeseli	Sciento	Cat#P370

LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Jeremy Gunawardena (jeremy@hms.harvard.edu). This study did not generate new unique reagents.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Stentor roeseli

S. roeseli was purchased from Sciento (Manchester, UK) who harvested the organisms from a pond on the property of Whitefield Golf Club (83 Higher Lane, Whitefield, Manchester, UK). We confirmed their identification based on shape, vermiform macronucleus, colorless cortical granules and absence of symbiotic algae, as specified in the taxonomic classification of heterotrich ciliates (Figure S1A). We maintained *S. roeseli* in pond water (Carolina Biological Supply Company, Burlington, NC) supplemented with soil-water (Carolina) and wheat grains (to promote bacterial growth) in well-aerated glass flasks. Flasks were kept at room temperature under partial sunlight. Organisms were fed 1 mL of dense cultures of *Chilomonas* sp. and *Chlamydomonas* sp. (Carolina) twice per week. Although healthy cultures could be maintained and passaged for several weeks, all experiments reported here were performed on organisms purchased no more than two weeks prior.

METHOD DETAILS

Beads

Jennings used carmine powder in his original experiments, which did not work in our hands. Carmine is a natural product of the cochineal beetle, so its composition may have changed since his day. We explored a variety of particulate suspensions, including alumina, glass, sand and polystyrene beads. We found that fluorescent-red, carboxylate-modified polystyrene beads, having a mean diameter of 2 μm in aqueous suspension with 0.1% NaN_3 (Sigma Aldrich Milipore L3030) yielded reproducible avoidance behavior and used these in all experiments reported here.

Needle construction

Borosilicate glass capillaries with I.D. = 1.10 mm and O.D. = 1.5 mm (Sutter Instrument, Novato, CA) were pulled into microinjection needles using a P-1000 Flaming/Brown micropipette puller (Sutter). The following parameters were used for pulling: Heat 850, Pull 50, Velocity 80, Time 200, Pressure 500. The pulled needle was then broken manually so that the tip diameter was approximately 50% smaller than the mouth of an average *S. roeseli*.

Stimulation apparatus and protocol

We designed a custom-built apparatus to stimulate organisms (Figure S1B). A Signatone S-931 micropositioner (Gilroy, CA) was placed on a lab jack next to the stage of an inverted microscope. The microinjection glass needle was loaded with a suspension of beads and connected to an elevated reservoir of pond water using Tygon tubing (United States Plastics Corporation, Lima, OH). The needle was then taped to the end of the micropositioner. Organisms were removed from the master culture using a pipette, along with some algae, and a few drops were placed on a glass slide on the microscope stage. The droplet was allowed to settle down for a few minutes. The microinjection needle was positioned next to the mouth of the organism by hand, and its position was adjusted as needed throughout the experiment using the micropositioner. Pulses of beads were generated as a gravity flow by opening and closing a two-way stopcock (Bio-Rad Industries, Hercules, CA) connected to the base of the reservoir. As it was challenging to control both the microscope focus and the needle tip, we estimated the timing of pulses from the recorded video.

Microscopy

Images were acquired using a Nikon TE2000-U inverted microscope (Melville, NY) equipped with a 10x Plan Fluor objective lens of N.A. 0.3 attached to a Hamamatsu ORCA-100 CCD camera (Hamamatsu City, Japan). An objective with low magnification and long working distance (16 mm) was required to capture the response of the whole organism. The camera was controlled by MetaMorph 7 software (Molecular Devices, Sunnyvale, CA). Images were collected at a rate of 7 frames per second for timelapse experiments, using an exposure time of 5 ms and 1x1 binning. Organisms were kept at room temperature during all microscopy experiments.

Behavior identification

We determined the observed behaviors as follows. Ciliary alteration (A) is identified by observing individual video frames (Figure 2A, frames 1 and 2; Figure S2B). Bending (B) is the most ambiguous behavior, as the organism may be bent while resting (Figure 2A, frame 1). We defined it as a non-contractile change in three-dimensional position or orientation relative to the pipette following stimulation (Figure S2A). Contraction (C) is defined, typically, as an extremely rapid collapse of the organism onto its holdfast (Figure 2A, frame 6). If the organism does not then detach, collapse is eventually followed by a slower enlargement back to normal size. In some instances, collapse was slow, which we took as part of the organism's broader heterogeneity. Detachment (D) is obvious: the organism pulls up its holdfast and swims away (Figure 2A, frame 8). Resting (R) is also obvious, as none of the preceding behaviors occur (Figure 2A, frame 1).

QUANTIFICATION AND STATISTICAL ANALYSIS

All statistical analysis was done using MATLAB R2017b. Details about the analysis are provided in the Results and Discussion. A p value < 0.05 was taken to indicate statistical significance. The fitting in Figure 3C was undertaken using the built-in MATLAB function fitdist.

DATA AND CODE AVAILABILITY

Source data for Table 1 (57 video recordings of *S. roeseli* behaviors) are available through Mendeley. DOIs are listed in Table S1.