An automated feeding system for the African killifish reveals effects of dietary restriction on lifespan and allows scalable assessment of associative learning

Andrew McKay[1,2], Chi-Kuo Hu[1], Sharon Chen[1], Claire Nicole Bedbrook[1], Mike Thielvoldt[4], Tony Wyss-Coray[3,5], Anne Brunet[1,5,6]

[1]Department of Genetics, Stanford University, Stanford, CA94305

[2]Biology Graduate Program, Stanford University, Stanford, CA94305

[3]Department of Neurology and Neurological Sciences, Stanford University, Stanford, CA94305

[4]Thielvoldt Engineering, Albany, CA94706

[5]Glenn Laboratories for the Biology of Aging, Stanford University, CA94305

[6]Corresponding author: abrunet1@stanford.edu

Abstract

The African turquoise killifish is an exciting new vertebrate model for aging studies. A significant challenge for any model organism is control over its diet in space and time. To address this challenge, we created an automated and networked fish feeding system. Our automated feeder is designed to be open-source, easily transferable, and built from widely available components. Compared to manual feeding, our automated system is highly precise and flexible. As a proof-of-concept for the feeding schedule flexibility of these automated feeders, we define a favorable regimen for growth and fertility for the African killifish and a dietary restriction regimen where both feeding time and quantity are reduced. We show that this dietary restriction regimen extends lifespan in males. Moreover, combining our automated feeding

system with a video camera, we establish an associative learning assay for the killifish. This learning assay provides an integrative measure of cognitive decline during aging. The ability to precisely control food delivery in the killifish opens new areas to assess lifespan and cognitive behavior dynamics and to screen for dietary interventions and drugs in a high-throughput manner previously impossible with traditional vertebrate model organisms.

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Introduction

The African turquoise killifish, *Nothobranchius furzeri*, is a new genetically tractable model organism that has been developed for the study of aging and 'suspended animation' (embryonic diapause) (Cellerino et al., 2016; Harel et al., 2015; Hu and Brunet, 2018; Poeschla and Valenzano, 2020). This fish has a naturally compressed lifespan of 4-6 months, which is 6 times shorter than the maximum lifespan of mice and 10 times shorter than zebrafish (Harel and Brunet, 2015). In its short life, the African killifish exhibit hallmarks of aging, including cognitive decline (Valenzano et al., 2006b), neurodegeneration (Matsui et al., 2019; Terzibasi et al., 2008), fertility decline (Api et al., 2018; Zak and Reichard, 2021), cellular senescence
40 (Valenzano et al., 2006a), impaired regeneration and wound healing (Wang et al., 2020; Wendler et al., 2015), defects in heart function (Ahuja et al., 2019), and an increased risk of cancer (Baumgart et al., 2015; Di Cicco et al., 2011). A genetic and genomic toolkit has been developed for the killifish, including sequencing of its genome (Reichwald et al., 2015; Valenzano et al., 2015), Tol2-based transgenesis (Hartmann and Englert, 2012; Valenzano et al., 2011), and CRISPR/Cas9-mediated genome-editing (Harel et al., 2015). These developments have allowed disease modeling in the killifish (Harel et al., 2015), identification of genes potentially involved

in lifespan differences (Reichwald et al., 2015; Valenzano et al., 2015) and sex determination (Reichwald et al., 2015), determination of enhancers involved in tissue regeneration (Wang et al., 2020), and discovery of chromatin regulators important for embryonic diapause (Hu et al., 2020).

A major challenge for any model system is the precise control of its diet. A proper diet is important for robust growth and fertility in colony maintenance and genetic manipulations. A controlled feeding regimen is also critical for lifespan studies, given the impact of dietary restriction – and more generally diet – on lifespan in a wide variety of species (Bartke et al., 2001; Fontana and Partridge, 2015; Mair and Dillin, 2008), including worms (Houthoofd and Vanfleteren, 2007), flies (Partridge et al., 2005), killifish (Terzibasi et al., 2009), mice (Mitchell et al., 2019; Weindruch et al., 1986), rats (Goodrick et al., 1983), and monkeys (Colman et al., 2009; Colman et al., 2014; Mattison et al., 2017; Mattison et al., 2012). Importantly, food is not only essential for growth, fertility, and survival, but it can also serve as a reward. Indeed, several cognitive tests rely on the ability of associating food with a task (Flagel and Robinson, 2017; Jarrard, 1993; Olton and Samuelson, 1976).

In the wild, adult African turquoise killifish primarily feed upon small crustaceans and insect larvae ('bloodworms') that are present in their ephemeral ponds in Africa (Reichard and Polacik, 2019). In laboratory settings, African killifish are often fed using bloodworms, either live (Hartmann et al., 2011; Reichwald et al., 2015; Terzibasi et al., 2009; Wendler et al., 2015), frozen (Terzibasi et al., 2008; Valenzano et al., 2006b; Zupkovitz et al., 2018), or lyophilized (Harel et al., 2015; Hu et al., 2020; Valenzano et al., 2015; Valenzano et al., 2011). However, feeding with live bloodworms might introduce pathogens in the colony and may lead to

70 variability, depending on lot or lifecycle (live bloodworm larvae continue through their lifecycle in the tank). To alleviate these issues, dry food pellets have been recently adopted for killifish, either alone (Hu et al., 2020; Zak et al., 2020) or in combination with live food (Matsui et al., 2019). But a main challenge of dry fish food – and fish food in general – is that it needs to be delivered for each feeding, otherwise it loses its appeal to fish and remains uneaten in the tanks. As feeding fish is largely based on manual feeding, food delivery is a limiting factor: it is hard to perform in a consistent manner, to scale up, and to schedule at any time of the day or night. These features hamper the testing of different feeding regimens and other interventions. Control over food delivery will help the development of the killifish as a high-throughput model for lifespan and other traits and to allow interventions.

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To address these challenges and develop the scalability of the killifish as a model system, we have created an automated feeding system for killifish feeding. We provide evidence that this system is precise and reliable, and that it allows controlled and tunable feeding throughout the day or night. Using this new flexible feeding system, we explore the parameters of diet in the killifish and present a dietary restriction regime that extend lifespan in the African killifish. Moreover, our automated feeders allow us to design a novel associative learning assay to test cognitive function in the African killifish during aging. This automated feeding system will help the development of the killifish as a high-throughput model for lifespan and will allow scalable intervention or drug screening.

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Results

A wireless networked automated feeding system

Controlling feeding automatically is a critical component for the development and scalability of a model organism. Automated feeders have been developed for fish, but they are rarely used due to their imprecision (e.g. hobbyist feeding systems) or prohibitive costs (e.g. scientific-grade feeding systems such as Tritone from Tecniplast). While a system developed for zebrafish solved some of these constraints (Doyle et al., 2017), it is not scalable to hundreds of animals simultaneously being fed (Manabe et al., 2013; Yang et al., 2019).

To address the main limitations with current feeding methods, we developed a networked automated feeding system for the African turquoise killifish. We created a system in which different components function independently to confer robustness and avoid single points of failure that affect the overall feeding scheme. This is particularly important when feeding needs to happen over a lifespan. The automated feeder we designed and built is placed on top of each animal's 2.8L tank (Figure 1A) and drops dry food (e.g. Otohime fish diet) from a small feed hopper (Figure 1B) directly into the tank (which houses one individual fish). The feeder is powered by an attached battery and the food pellets are automatically segregated from the hopper by a rotating acrylic disc, with the resulting pieces of food dropping into the water through a 3mm diameter opening cut out in the supporting acrylic plate below (See Supplementary Movie 1). The 3mm opening rotates from under the feed hopper, collects food, and travels to the drop site above the tank opening. Each rotation delivers a fixed volume of food, averaging around 5

mg in mass, and multiples of 5 mg can be programmed to increase food amount per feeding. Feedings are also fully programmable to any frequency or time of day by the user, allowing flexibility in feeding schedule (Table 1).

To determine whether food has indeed dropped into the tank, we designed the acrylic disc's opening such that it pushes the food between a photoresistor and a light-emitting diode (LED) before reaching the drop site (Figure 1C). The photoresistor and LED provide confirmation for feeding by measuring the resistance of the photoresistor when food obstructs the light from the LED (outgoing trip), and after feeding, when the empty food-receptacle allows light to pass (return trip). We also designed the feeder such that each feeder communicates feeding confirmations independently to a local server using the 802.11 wireless communication standard, which can then be aggregated across groups of feeders to a cloud-based server (Figure 1D). Thus, with our automated system, feedings can be recorded and backed up remotely, providing an automatic log for the user (Table 1).

Lastly, the wireless communication and battery-powered function allows our system to function remotely and flexibly. This is an improvement over other automated systems (such as Tritone from Tecniplast), whose monolithic design creates many single points of system-wide failure (Table 1), or over designs that are not networked or restricted to less flexible wire-based communication (Doyle et al., 2017; Manabe et al., 2013; Yang et al., 2019). Hence, our design for an automated feeding system allows controlled, tunable, and recorded feedings throughout the lifespan of the killifish.

Fidelity and precision of the automated feeding system

140 We tested the fidelity and precision of the automated feeding system. A representative feeder that was set to deliver 7 feedings a day for 30 days successfully delivered food 98.1% of the time (206 actual feedings for 210 scheduled feedings; Figure 2A). Overall, aggregating 41 feeders for 2279 cumulative days of feeding showed that most feedings were fully accounted for (Figure 2B), with only 7.89% of days deviating by one unconfirmed feeding (Figure 2C). This fidelity was confirmed independently with a separate set of automated feeders of the same design built independently by another researcher (Supplementary Figure 1).

We compared the precision of the mass of food dropped per feeding for our automated feeder versus manual feeding by individuals. Single or multiple automated feeders were more precise at
150 delivering a given amount of food by an order of magnitude compared to a group of 6 different individuals (similar to what can be done in fish rooms to offset the workload) or a single individual measuring and delivering food (Figure 2D).

Because of its flexibility, the automated feeder can deliver up to 5 mg per unit every 10 minutes, or 720 mg per 12 hours period, representing a potential 20x increase over the baseline dietary regime for the day (Table 1). The automated feeder can also feed during the night if desired, which would not be practical for manual feeding. Thus, our automatic system decreases the amount of labor and provides high precision, reproducibility, and flexibility for husbandry and for varying diet regimens (Table 1).

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Defining a daily dietary restriction feeding schedule in the killifish

We used our automated feeding system to define a variety of dietary regimens in killifish – dietary restriction and overfeeding. Dietary restriction has been shown to delay signs of aging and age-related diseases in multiple species. Dietary restriction regimens encompass restricting overall food (Colman et al., 2009; Colman et al., 2014; Mattison et al., 2017; Mattison et al., 2012; Weindruch et al., 1986) or restricting the time of feeding during the day (Mitchell et al., 2019) or over longer periods (Brandhorst et al., 2015). In killifish, dietary restriction has been done by every other day feeding because it is difficult to do otherwise with manual feeding
170 (Terzibasi et al., 2009). A dietary restriction regimen is expected to reduce growth and fertility (especially when applied early in adulthood) compared to an *ad libitum* feeding regimen. In contrast, overfeeding has negative consequences on health, and it is expected to increase growth but to reduce fertility compared to an *ad libitum* regimen (Magwere et al., 2004). We fed individually housed male and female killifish, starting in young adults (1 month of age), with different food regimens. Using our programmable automated feeders, we varied both the amount and timing of feedings throughout the day. For *ad libitum* (AL, blue), we fed 35 mg of dry food per day, in 7 feedings of 5 mg evenly spaced over 12 hours of the day, for a total of 245 mg per week (roughly similar to manual feeding) (Figure 3A). For dietary restriction (DR, orange), we fed 15 mg per day, in 3 feedings of 5 mg over 2 hours, for a total of 105 mg per week (Figure
180 3A). This DR regimen is both time- and amount-restricted (achieving amount-restriction without time restriction would not be possible in current settings because of the minimum 5 mg delivery amount). This DR regimen led to significantly smaller (Figure 3B and C) and less fertile (Figure 3D) animals than AL, consistent with what is expected under dietary restriction. Importantly, at 7 feedings of 5 mg a day (AL), animals were not overfed because they could be fed more (12

feedings of 5 mg a day) (Figure 3E), and this still increased size (Figure 3F and G) but decreased fertility (Figure 3H). Hence, a favorable feeding regimen is around 7 feedings per day and 35 mg of food per day for the African killifish. Together, these experiments define diets that optimize growth and fertility in the African killifish, and they identify a dietary restriction regimen in this species.

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A time- and amount-restricted diet regimen extends lifespan in males

As a proof-of-concept, we asked if the time- and amount-restricted DR regimen defined above, when performed over an entire lifespan, could promote longevity in the killifish. To this end, we tested the lifespan of female and male African killifish, in *ad libitum* (AL, blue: 7 evenly spaced feedings of 5 mg per day over 12 hours of the day) or diet- and time-restricted conditions (DR, orange: food and time restricted: 3 feedings of 5 mg per day over 2 hours in the morning) (Figure 4A). We enrolled young adult animals in two independent cohorts (33 males and 26 females in cohort 1 and 43 males and 49 females in cohort 2) (Figure 4A, Supplementary Figure 3A). The dietary regimen was initiated in young adults (1 month of age) until death (Figure 4A).

200 Interestingly, males fed the dietary restricted diet (DR, orange) lived longer than those fed the ad libitum diet (AL, blue; 22.1% median lifespan extension for cohort 2, 16.6% median lifespan extension for cohort 1) (Figure 4B, Supplementary Figure 3B). In contrast, females fed with this restricted diet did not live significantly longer than those fed the AL diet in either cohort (Figure 4C, Supplementary Figure 3C). Animals fed a DR diet exhibited lifespan differences between sexes, with male living significantly longer than females in this restricted regimen (Figure 4 D and E, Supplementary Figure 3D and E). The sex-specific effect from DR on the killifish was also supported using Cox Proportional Hazards in a factorial design (Supplementary Table 1)

where the interaction term between sex and dietary regimen was found to be significant ($p = 0.0454$). Furthermore, fitting the survival data of male killifish fed either an AL or DR feeding regimen into a Gompertz distribution resulted in an estimated reduced slope for dietary restricted males, suggesting that this DR regimen reduces the 'rate of aging' in male killifish (Figure 4F), in line with the effect of intermittent feeding on lifespan (Terzibasi *et al.*, 2009). Thus, this DR regimen (restricted in time and amount) significantly extends the lifespan of males in the African killifish.

Associative learning assay for the killifish to assess cognitive decline with age

Food not only influences growth, fertility, and lifespan, but it is also a potent reward across species, including humans (Lutter and Nestler, 2009). The rewarding aspect of food has been used for developing feeding-associated learning behaviors in many model organisms, including worms (Cho et al., 2016; Kauffman et al., 2010; Lim et al., 2018; Stein and Murphy, 2014), flies (Das et al., 2014), zebrafish (Doyle et al., 2017; Sison and Gerlai, 2010), mice (Steinberg et al., 2020), and non-human primates (Rolls, 2006). We therefore used our programmable feeding platform to establish a positive associative behavior ('learning') assay for the killifish and tested whether this platform could measure cognitive decline during aging. We developed a classical conditioning paradigm built on our automated feeders. In this paradigm, a red LED light above the tank serves as the conditioned stimulus, which is activated 3 seconds prior to the automatic dropping of food in the tank (Figure 5A). This setup is integrated into our automated feeding system and connects to an individual camera facing the front of each killifish's 2.8 L tank. Killifish initially do not react in a positive manner to the red light, only going to the top of the tank after the food is dropped (Figure 5B, Supplementary Video 2). But after a sequence of

training sessions (i.e. instances of red light followed by automatic feeding), killifish individuals start associating red light to food, and they rapidly move to the top of the tank when the light turns on in anticipation, before the food is dropped (Figure 5B, Supplementary Video 3).

We processed training videos of male and female killifish at different ages (between 36 days and 130 days, raised by manual feeding) and tracked individual fish trajectories using DeepLabCut (Mathis et al., 2018) (Figure 5C). We verified that the initial locations for all animals in all videos did not show difference between the young and old animals (Supplementary Figure 4A, B). Compass plot analysis of individual trajectories for young fish showed that in early sessions, individual fish displayed neutral types of trajectories when the red light turned on (Figure 5D). In contrast, in later training sessions, individual fish exhibited directed trajectories towards the top of the tank when the red light turned on, and even before the food was delivered (Figure 5D). We verified that old fish could still see the red light and were still motivated toward food. Both older fish (110 days and older) and younger fish (70 days and younger) exhibited a sudden 'startle' movement as the red light turned on, indicating they both saw this red light (Figure 5F, Supplementary Figure 4A). Moreover, both young and old fish also moved towards the food after it had dropped (Figure 5G, Supplementary Figure 4B), indicating no overt changes in food perception and motivation with age.

We developed a pipeline to automatically score the recorded trajectories of young and old fish based on the distance covered between the animal's starting location when the light turns on and the top of the tank during the 3 seconds before the food drops. Successful training was defined as two consecutive movements to the top that passed a pre-determined threshold (see Materials and

Methods). We calculated a learning index for each animal as the inverse of the number of training sessions needed for successful training. Automatic scoring showed that young individuals had significantly higher learning index scores than old individuals (Figure 5E). This was confirmed by manual scoring (Supplementary Figure 4E and F). Together, these observations suggest that a deficiency in learning in old animals. Thus, our automated feeding system allows scalable measurements of associative conditioning, providing a measure of cognitive fitness during aging.

Discussion

The automated feeding system presented here provides a valuable resource to the killifish community to consistently and reproducibly control the critical factor of feeding. Our system also provides an increased resolution on diet's impact on lifespan in a vertebrate model of aging. The automated feeders improve the precision of food dropped compared to manual feeding, which could reduce variability in experiments (e.g. lifespan studies) and deliver a well-defined diet. Compared to feeding approaches that risk introducing infection (live food) or are labor intensive (manual feeding), our feeding system is a safe and modular solution to feeding not only killifish and other teleost model organisms such as medaka, stickleback, or zebrafish. Furthermore, the tunable frequency and quantity of feedings that can be programmed opens up an experimental space previously infeasible with conventional feeding approaches. Our design also allows each tank to have its own food hopper and allows straightforward nutrient and drug testing, such as high-fat diets, ketogenic diets (Newman et al., 2017), or dosing of animals with drug-encapsulated food (Valenzano et al., 2006b). While there are still areas for optimization (e.g. improved battery life and a simplified user interface), the performance of our automated

feeding system is beyond that of manual feeding or other automated feeding systems (Doyle et al., 2017; Manabe et al., 2013; Yang et al., 2019) in terms of combining precise amount of food dropped, modularity, and scalability.

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Using the automated feeding system, we explore the dietary parameter space and identify a feeding regimen close to an *ad libitum* diet as well as a daily dietary restriction diet for the African killifish. We show that this dietary restriction diet extends the lifespan of male, but not female killifish. Gompertz analysis confirms that dietary restriction reduces the rate of aging in males only. This sex difference in lifespan extension is likely independent of killifish social dynamics, as all animals were housed individually. Lifespan differences between sexes in response to dietary restriction is also observed in other species. In mice, extreme dietary restriction (40%) extends lifespan in males, but not in females (Mitchell et al., 2016), though milder dietary restriction leads to a greater lifespan extension in females than males (Bonkowski

290 et al., 2006; Kane et al., 2018; Mitchell et al., 2016). In addition, other strains of mice exhibit more lifespan extension in response to dietary restriction in males than in females (Liao et al., 2010). In flies, dietary restriction has also been shown to impact lifespan differently between the sexes, with female flies enjoying the maximum lifespan benefits from DR at higher calorie levels than male flies (Magwere et al., 2004). These observations underline the importance of performing experiments in both male and female animals (Miller et al., 2005; Miller et al., 2014). It is possible that our DR regimen needs to be optimized differently in males and females, due to the different metabolic requirements for reproduction in the two sexes. The DR regimen defined here may in fact represent a form of starvation for females and might be too severe to provide lifespan benefits to females. The median and maximum lifespan of the killifish in these

300 experiments *in ad libitum* conditions are shorter than some previous studies (Hu et al., 2020) but longer than others (Terzibasi et al., 2008; Valenzano et al., 2006b). These differences might be linked to amount or food supply differences or other husbandry differences. Regardless of differences across experiments, feeding killifish more times than the traditional twice a day feeding, even with the same total amount of food, might better reflect the situation in nature in ephemeral ponds where African turquoise killifish feed more continuously (Reichard and Polacik, 2019).

Building upon the killifish feeding platform, we show a proof-of-concept positive reinforcement classical conditioning assay that uses automated feeders to couple food with a red light.
310 Combining video cameras with feeders allows many animals to be conditioned simultaneously, at any time, and with minimal investigator-induced disturbance. As scoring of behavioral videos is a significant obstacle to large scale behavioral experiments, we developed an automatic scoring pipeline that aligned well with our manually scored results. Using both manual and automatic scoring, we find that animals showed an age-dependent decline in their ability to learn, agreeing with previously performed negative reinforcement assays in the African killifish (Valenzano et al., 2006b). The advantage of positive association learning over a negative one is that it is less stressful for the animal and could be done in conjunction with a lifespan assay without inducing additional stress perturbations. However, in a case where this positive association assay is done in conjunction with lifespan, food amounts dispensed for the assay
320 itself would need to be carefully accounted for in the total amount, to avoid inadvertently affecting the overall diet and the lifespan itself. Together, our automated feeding system and

scalable tools will help develop the killifish as a high-throughput model to screen for genes or compounds that counter aging and age-related decline.

Contributions

A.M. created the killifish feeding system and conducted and analyzed the validation, fertility, lifespan, and conditioning assay experiments with the guidance of A.B.. C-K.H. had intellectual input and provided earlier figure iterations. S.C. assisted in manual scoring early iterations of the killifish conditioning assay. C.N.B. validated killifish feeding system by generating independent feeders. M.T. provided consultation on aspects of the electrical design. C-K.H. and C.N.B. helped with independent code validation. T.W.C. provided intellectual input. A.M. wrote the manuscript with the help of A.B., and all authors commented on the manuscript.

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Table 1

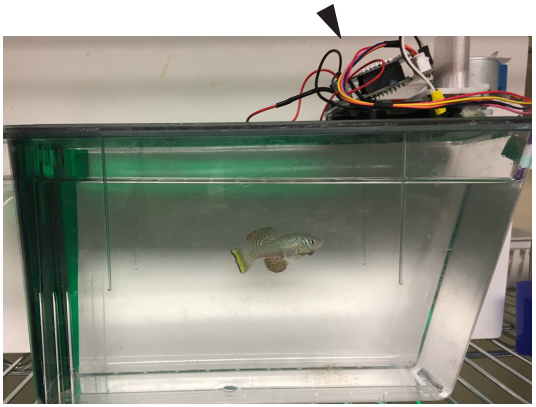
	Manual Feeding	Automated Feeding
Schedule Flexibility	Limited to weekday working hours	24/7
Maximum Frequency	2-3 times a day	144 times a day
Labor Required	Daily, linearly increasing with tanks	Initial setup and biweekly checks
Precision	Low, with batch effects for different individuals (0.016)	Higher (0.512)

Table 1

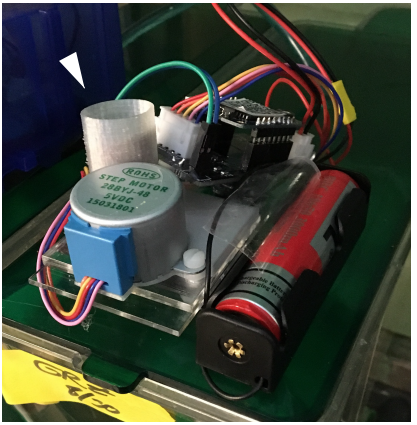
Automated feeding allows for more flexible and frequent feedings as compared to manual feeding.

Figure 1

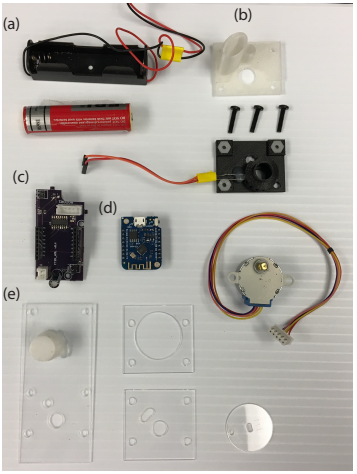
A Automated feeder (Side view)



B Automated feeder (Top view)



C Automated feeder hardware



D Software schematic of information flow

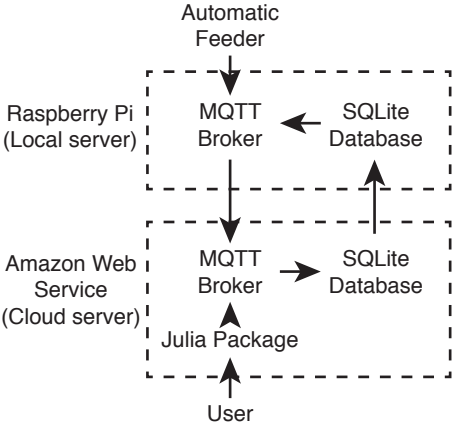


Figure 1. An automated 3D printed feeding system for the African turquoise killifish

350 (A) Side view of an individual automated feeding unit placed on the top front of 2.8 L fish tanks supplied by Aquaneering™. Dark arrowhead: feeding unit on the lid of a tank.

(B) Top view of an individual automated feeding unit. This is a compact, self-contained unit with individual power supply, food hopper, stepper motor for food delivery, and microcontroller for control and communication. White arrowhead: hopper where the dry food is placed.

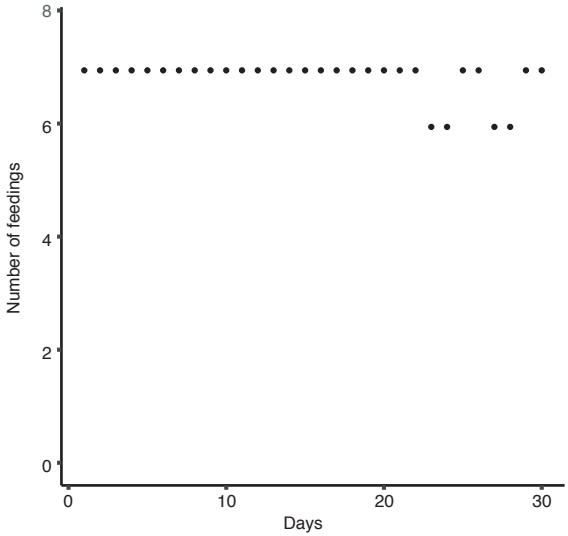
(C) Components of an individual automated feeder. Each feeder is composed of a lithium ion battery and holder (a), 3D printed parts (b) coupled with a custom printed circuit board (c), a Wemos D1 mini ESP8266-based microcontroller board (d), and laser cut acrylic parts (e).

(D) Users control the automated feeding system by interacting with a cloud-based server. This server communicates with small local servers on the premise, which then communicate with the

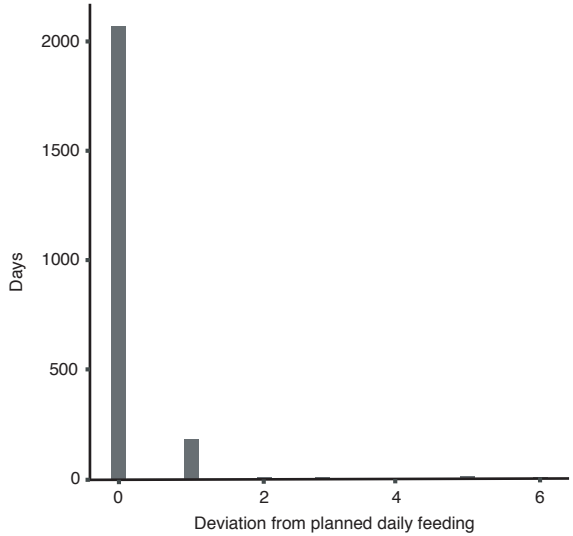
360 individual automated feeders. Changes to feeding schedules are provided by users and filter down to the appropriate feeders, while status updates such as feeding confirmations, make the return trip back to the users.

Figure 2

A Fidelity of one automated feeder



B Fidelity of multiple automated feeders



C Feeding number and frequency

Deviation from planned daily feeding	Days	Frequency
0	2069	90.7%
1	180	7.89%
2	5	0.22%
3	6	0.26%
4	4	0.17%
5	10	0.44%
6	5	0.22%

D Precision comparison between automated and manual feeding

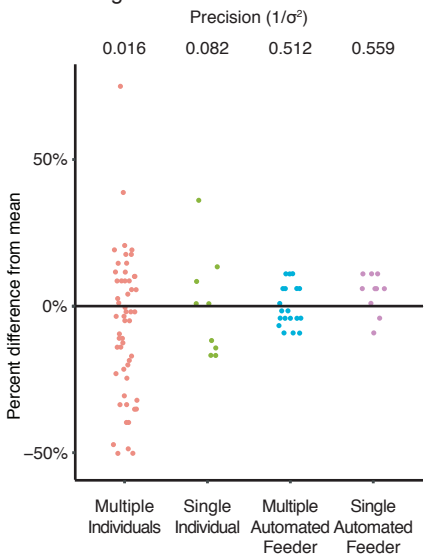


Figure 2: Fidelity and precision of the automated feeding system

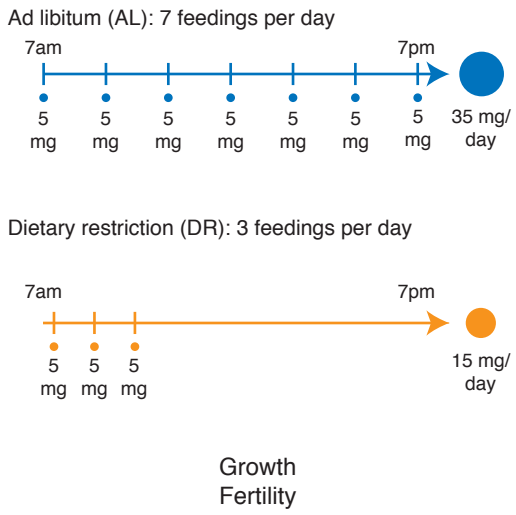
(A) Logged feedings per day from a representative feeder over a 30-day period, with 7 feedings of 5 mg programmed for each day. Note that recording of six feedings instead of seven does not necessarily mean that the feeding was missed and could also be due to an unlogged feeding due to inability to connect to main server. Source data: Source Data 1

(B-C) Histogram (B) of deviations from scheduled feedings for 2279 days of feedings over 41 feeders with different assigned amounts tabulated in (C). The vast majority (>98%) of feedings have 0 or 1 missed feeding. Source data: Source Data 2

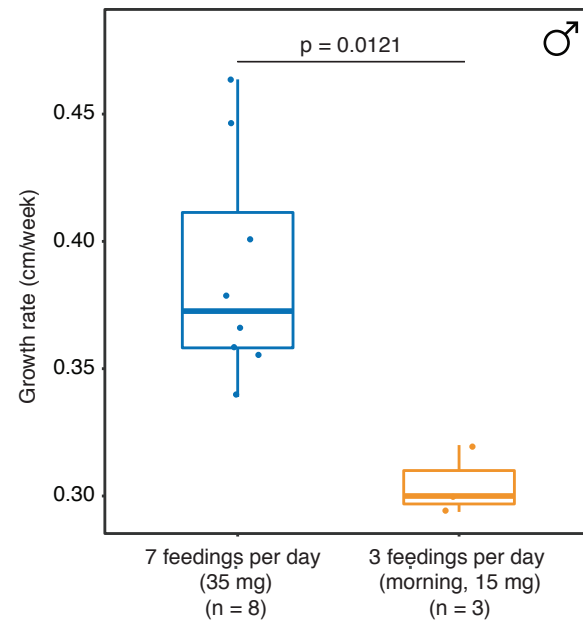
(D) Automated feeding provides higher feeding precision than manual feeding as seen from the dispersion of feeding volumes as a percentage of the mean. When compared to manual feeding by multiple individuals or a single individual, automated feeders are more precise in the amount of food delivered. Precision is defined as the reciprocal of the estimated variance given either six individuals (n = 54, precision = 0.016), one individual (n = 9, precision = 0.082), four feeders (n = 19, precision = 0.512), or one single feeder (n = 10, precision = 0.559). Precision derived from bootstrapped estimates of the standard deviation for each group. Source data: Source Data 3

Figure 3

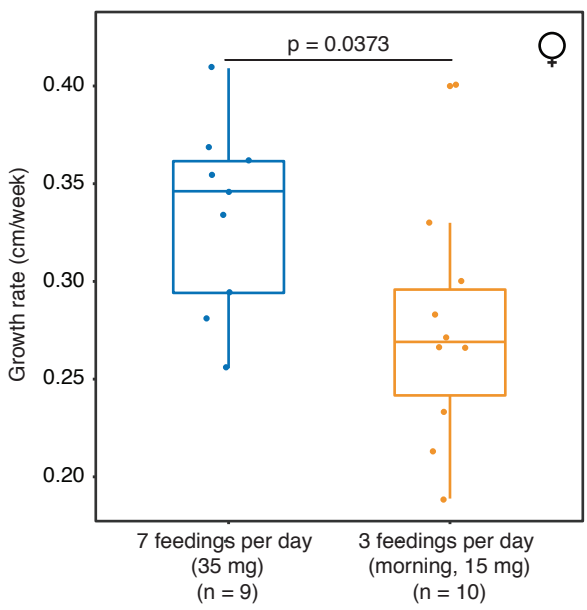
A Experimental scheme for dietary restriction



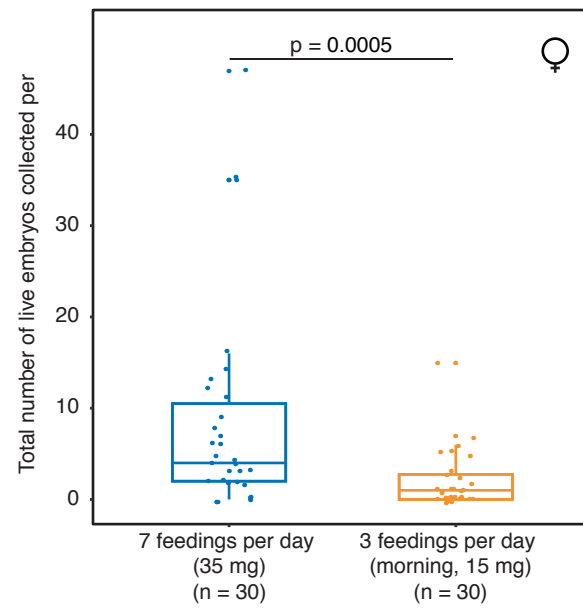
B Male growth rate upon dietary restriction



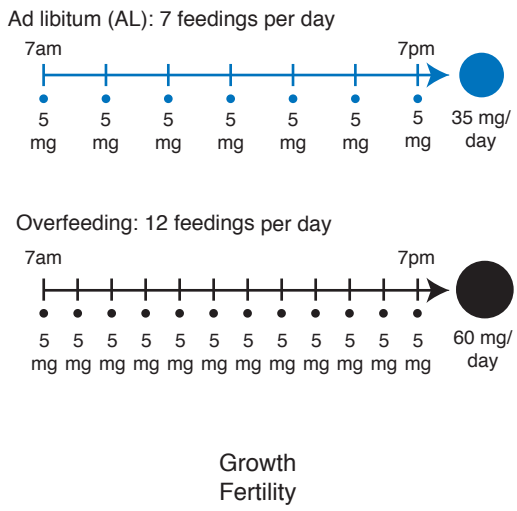
C Female growth rate upon dietary restriction



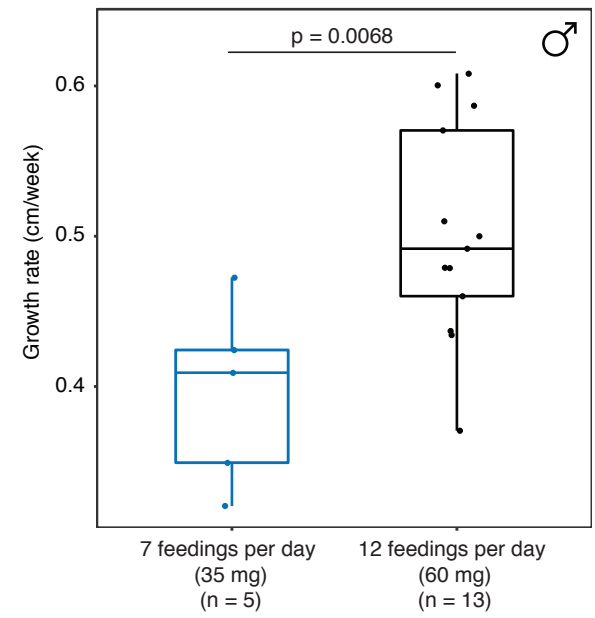
D Fertilized embryos produced upon dietary restric-



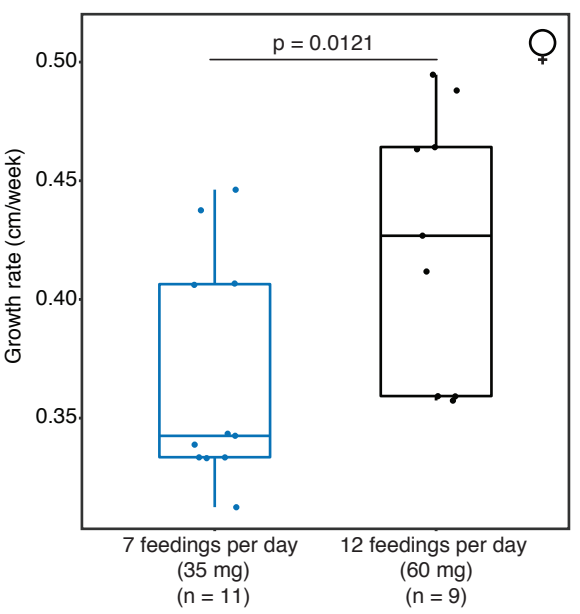
E Experimental scheme for overfeeding



F Male growth rate upon overfeeding



G Female growth rate upon overfeeding



H Fertilized embryos produced upon overfeeding

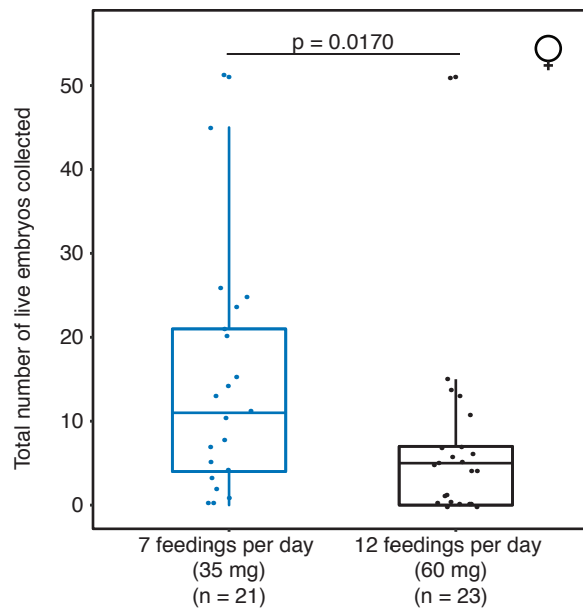


Figure 3: Automated feeding enables the definition of different diets for the African killifish

(A) Experimental scheme to compare two automated feeding schedules for killifish: feeding 7 times a day evenly through 12 hours (5mg Otohime fish diet per feeding, 35 mg total per day) and feeding 3 times a day within a two-hour period in the morning (5mg Otohime fish diet per feeding, 15 mg total per day). These regimens were applied from 1 month of age to death (for growth measurements) and from 1 month of age until death of one individual in the pair (for fertility measurements). For fertility measurements, each individual in the pair was fed individually while single-housed and then crossed for 24 hours once per week to assess fertility.

(B) The average growth rate (cm/week) of male killifish from 1 month of age to death fed either 7 times a day (blue, median = 0.3726 cm/week, n = 8) is significantly greater than those fed 3 times a day in the morning (orange, median = 0.3 cm/week, n = 3). Each dot represents a single animal's growth averaged over its lifespan. Animals that lived longer than 4 months were not considered due to concerns with non-linear growth rate. Significance determined by Wilcoxon sum rank test (p-value = 0.0121). Animals were from the same cohort as Supplementary Figure 3 (Cohort 1). Source data: Source Data 11

(C) The average growth rate (cm/week) of female killifish from 1 month of age to death fed 7 times a day (blue, median = 0.3461 cm/week, n = 9) is significantly greater than those fed 3 times a day in the morning (orange, median = 0.2690 cm/week, n = 10). Each dot represents a single animal's growth averaged over their lifespan. Animals that lived longer than 4 months were not considered due to concerns with non-linear growth rate. Significance determined by Wilcoxon sum rank test (p-value = 0.0373). Animals were from the same cohort as Supplementary Figure 3 (Cohort 1). Source data: Source Data 11

(D) Killifish mating pairs (one male and one female) fed 3 times a day are significantly less fertile (median = 1 fertilized embryo per mating, n = 30 matings across 5 pairs) than mating pairs fed 7 times a day (median = 4 fertilized embryos per mating, n = 30 matings across 5 pairs). Each dot represents fertilized embryos collected from one pair crossing overnight, and pairs were mated from 8 weeks of age until one individual in the pair died. Significance determined by Wilcoxon sum rank test (p-value = 0.0005). Source data: Source Data 5 and Source Data 6

(E) Experimental scheme to compare two automated feeding schedules for killifish: feeding 12 times a day (5 mg Otohime fish diet per feeding, 60 mg total per day) compared to feeding 7 times a day (5 mg Otohime fish diet per feeding, 35 mg total per day). These regimens were applied from 1 month of age to death (for growth measurements) and from 2 months of age until death of one individual in the pair (for fertility measurements). For fertility measurements, each individual in the pair was fed individually while single-housed and then crossed for 24 hours once per week to assess fertility.

(F) The average growth rate (cm/week) of male killifish from 1 month of age to death is significantly higher for animals fed 12 times a day (black, median = 0.4917 cm/week, n = 13 males) than for animals fed 7 times a day (blue, median = 0.4092 cm/week, n = 5 males). Each dot represents a single animal's growth averaged over its lifespan. Animals that lived longer than 4 months were not considered due to concerns with non-linear growth rate. Significance determined by Wilcoxon sum rank test (p-value = 0.0068). Source data: Source Data 7

(G) The average growth rate (cm/week) of female killifish from 1 month of age to death is significantly higher for animals fed 12 times a day (black, median = 0.4268 cm/week, n = 11 females) than for animals fed 7 times a day (blue, median = 0.3425 cm/week, n = 9 females). Each dot represents a single animal's growth averaged over its lifespan. Animals that lived

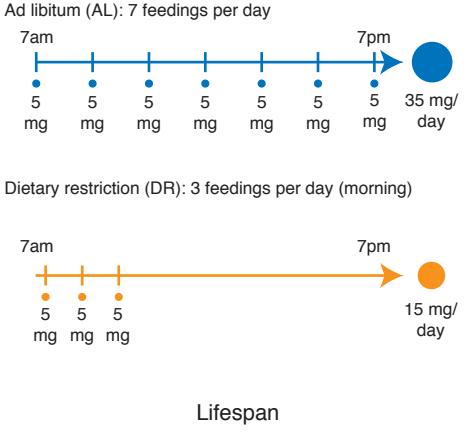
longer than 4 months were not considered due to concerns with non-linear growth rate.

430 Significance determined by Wilcoxon sum rank test (p-value = 0.0121). Source data: Source Data 7

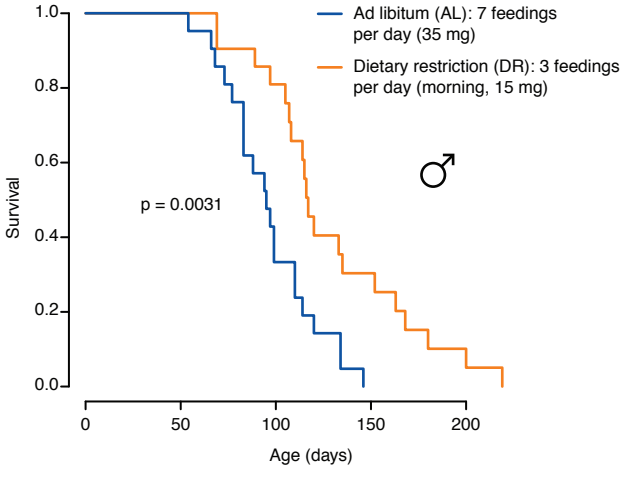
(H) Killifish mating pair fertility is significantly lower when overfed (black feeding schedule, median = 5 fertilized embryos per mating, n = 23 matings) than for those fed 7 times a day (blue feeding schedule, median = 11 fertilized embryos per mating, n = 21 matings). Each dot represents fertilized embryos collected from one pair upon crossing for 24 hours and pairs were mated from 2 months of age until one individual in the pair died. Significance determined by Wilcoxon sum rank test (p-value = 0.0170). Source data: Source Data 8 and Source Data 9

Figure 4

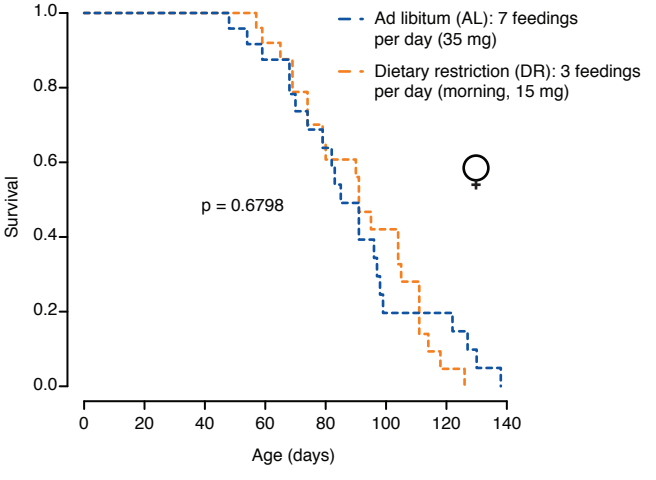
A Feeding regimens: Dietary restriction



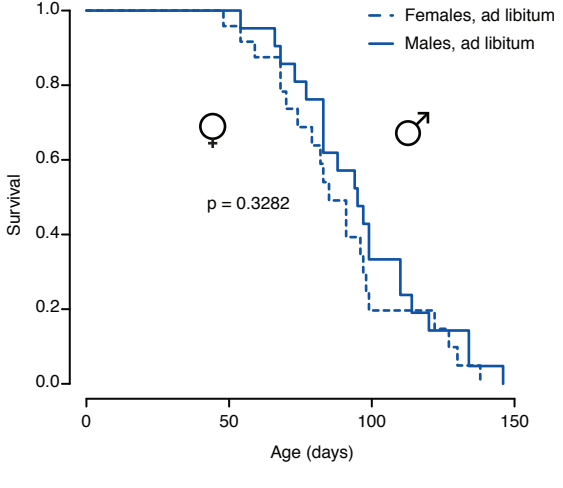
B Lifespan of male killifish in response to dietary restriction



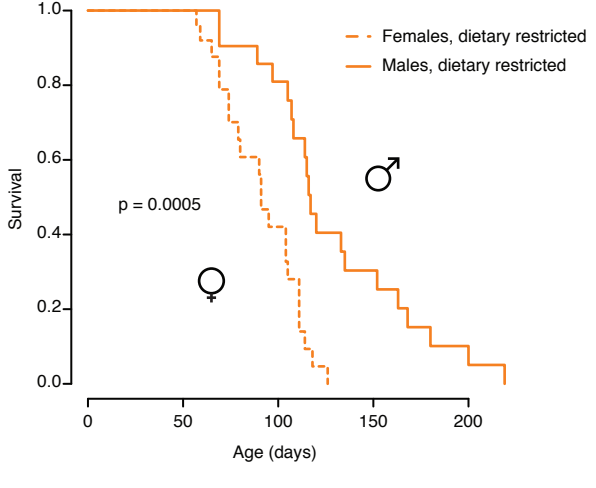
C Lifespan of female killifish in response to dietary restriction



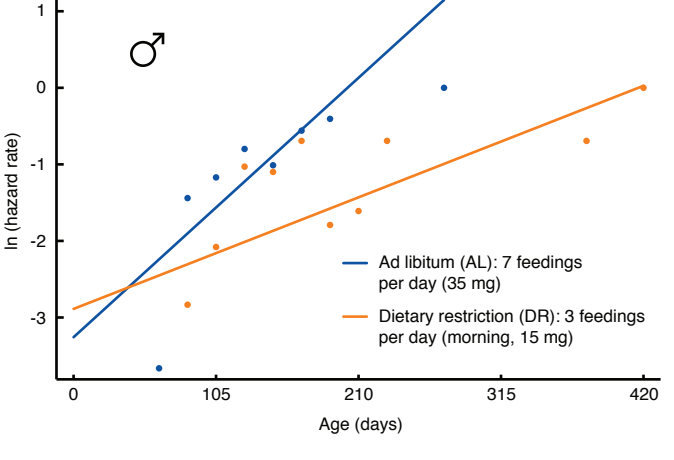
D Lifespan of male and female killifish in ad libitum conditions



E Lifespan of male and female killifish in dietary restricted conditions



F Gompertz curve fit for male killifish in response to dietary restriction



440 **Figure 4: An amount- and time-restricted dietary regimen robustly extends lifespan in male African turquoise killifish**

(A) Experimental scheme comparing two automated feeding schedules for killifish: an *ad libitum* (AL) regimen (7 times a day, 35 mg Otohime fish diet per day, blue) or a dietary restricted (DR) regimen (3 times in the morning, 15 mg Otohime fish diet per day, orange). Automated feeding regimens were started after sexual maturity, at 1 month of age.

(B) Male killifish fed a dietary restricted regimen (solid orange, median lifespan = 116 days, n = 21) lived significantly longer than male killifish fed an *ad libitum* regimen (solid blue, median lifespan = 95 days, n = 21) (p = 0.003111, logrank test). Source data: Source Data 4

(C) Female killifish fed a dietary restricted regimen (dashed orange, median lifespan = 90 days, n
450 = 25) did not live significantly longer than female killifish fed an *ad libitum* regimen (dashed blue, median lifespan = 82.5 days, n = 24) (p = 0.6798, logrank test). Source data: Source Data 4

(D) In *ad libitum* conditions, male killifish (solid blue, median lifespan = 95 days, n = 21) did not exhibit lifespan difference with female killifish (dashed blue, median lifespan = 82.5 days, n = 24) (p = 0.3282, logrank test). Source data: Source Data 4

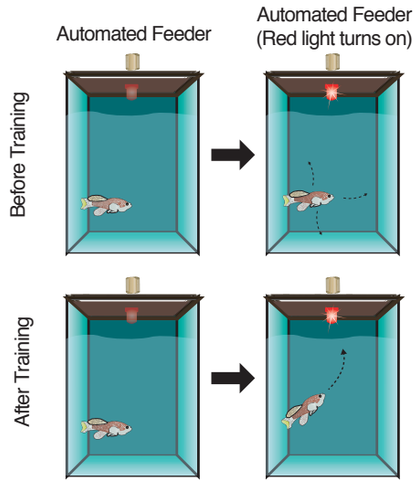
(E) In dietary restricted conditions, male killifish (solid orange, median lifespan = 116 days, n = 21) lived significantly longer than female killifish (dashed orange, median lifespan = 90 days, n = 25) (p = 0.0005, logrank test). Source data: Source Data 4

(F) Fitted curve of the binned 27-day hazard rate of male killifish from both cohorts to a Gompertz distribution and then transformed into the natural log of the hazard rate. The estimated
460 "rate of aging" (slope) of killifish on the AL feeding regimen is 0.3388 (95% confidence interval = 0.2437 to 0.434) and is significantly different from the rate of aging for DR, which is 0.1457 (95% confidence interval = 0.0846 to 0.2069). The estimated "frailty" (intercept) for killifish on

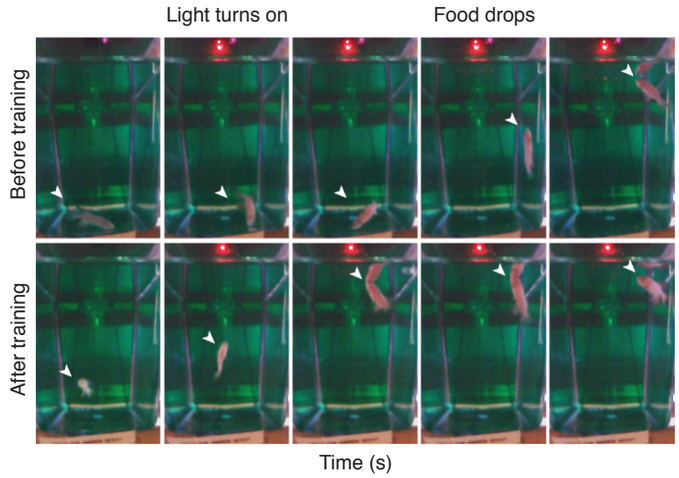
the AL feeding regimen is 0.0385 (95% confidence interval = 0.0203 to 0.073) compared to 0.0557 (95% confidence interval = 0.0315 to 0.0986) for killifish on DR (overlapping confidence intervals for intercept parameter indicate that these parameters are not significant). Source data: Source Data 4 and Source Data 11

Figure 5

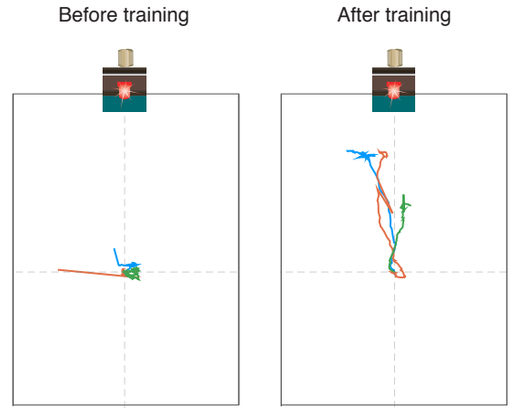
A Scheme for associative learning behavior



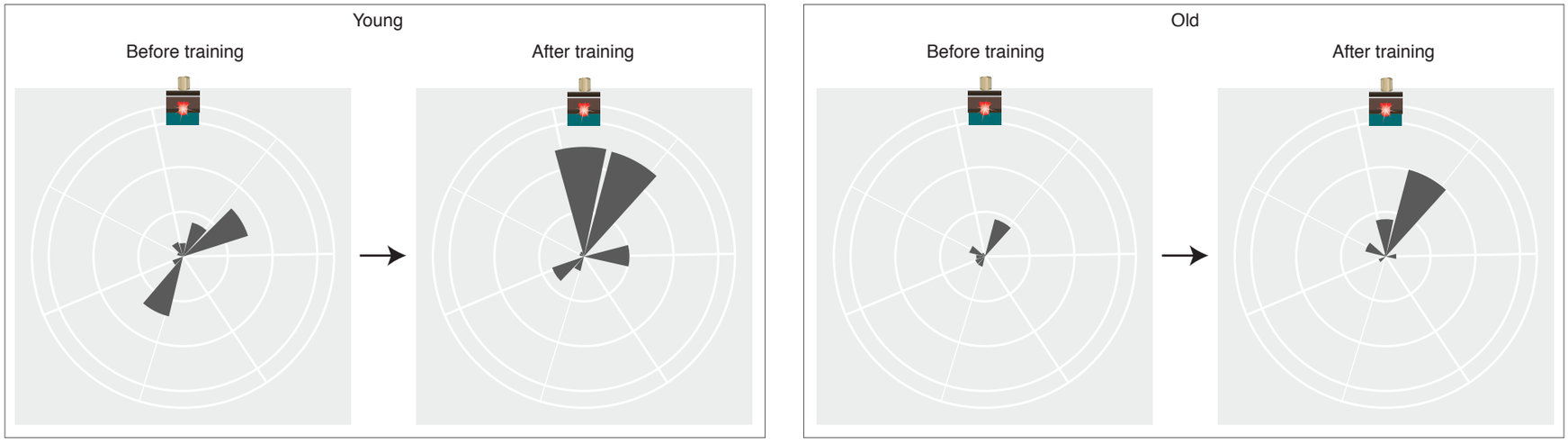
B Video frames



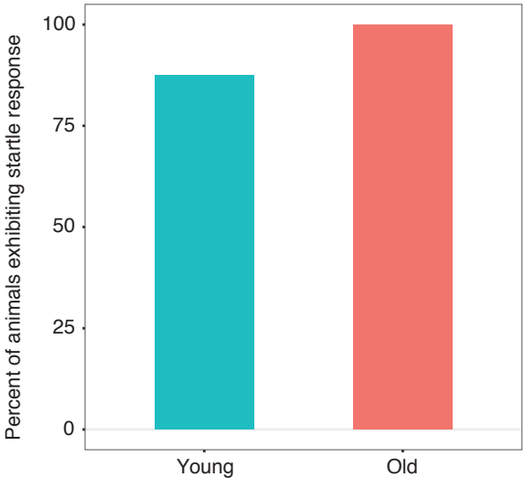
C Trajectories (using DeepLabCut)



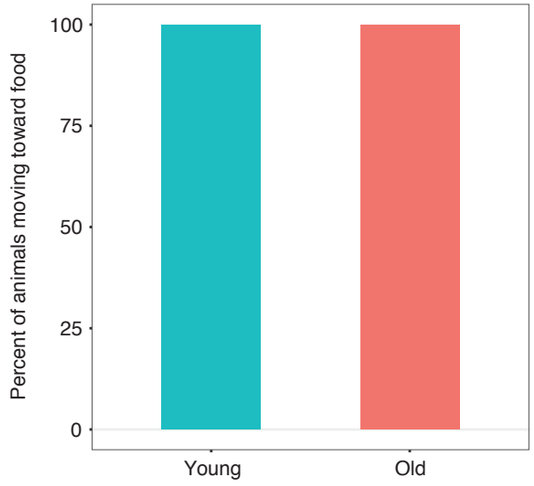
D Compass plot of trajectories



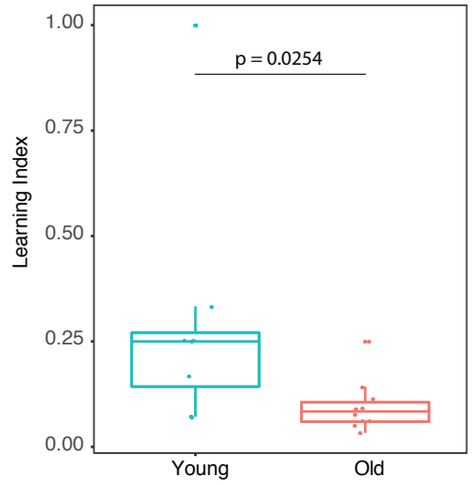
E Ability to see red light ('startle' response)



F Ability to move toward food



G Associative learning index (automated scoring)



470 **Figure 5: Use of automated feeder for high-throughput associative learning behavior to test cognitive decline with aging**

(A) Scheme for associative learning behavior assay the killifish. The red light switches on 3 seconds before the food drops in the tank. With training sessions, animals are conditioned to associate the preceding red light and anticipate feeding by moving to the top of the tank before feeding begins.

(B) Recording video frames from the front of the 2.8 L tank. Before training (top row), naïve killifish fail to anticipate food after the red light turns on. After training (bottom row), killifish move to the top of the tank in anticipation of food being dropped.

(C) Example trajectories of animal movements were extracted from training videos, providing
480 visualization of animal movements before and after training as well as data for automatic scoring of conditioning. Source data: Source Data 12 and Training Trajectories

(D) Compass plots of binned movement over the course of training. Automatically generated trajectories for all animals were binned by polar orientation of movement to the top of the tank. Young animal (less than 70 days of age) and old animal (more than 110 days of age) trajectories were compared either for a subset of trainings either before most young animals had been trained (training sessions 2 through 4) or after (training sessions 4 through 6). Source data: Source Data 12 and Training Trajectories

(E) Young and old animals were assessed for a startle response (i.e. sudden movement by the animal) upon the turning on of the red light before food dropped. A startle response is an
490 indication that animals can see the red light. Animals were scored on a binary scale for either a recorded startle response or no detected response, with percentage showing responses reported for each group. Source data: Source Data 12

(F) Young and old animals were assessed for movement to food as a proxy for motivation. Animals were scored on a binary scale for either a recorded movement towards food during the training sessions or no movement, with percentage showing movement reported for each group. Source data: Source Data 12

(G) Automatic scoring of recorded training sessions showed a significant decline in old animal (more than 110 days of age) learning index scores as compared to young animals (less than 70 days of age). Significance determined by Wilcoxon sum rank test (p-value = 0.0254, n = 18).

500 Source data: Source Data 12 and Training Trajectories

Supplementary Table 1

Results from Cox proportional hazards model

Survival ~ FeedingScheme + HatchDate + Sex + Sex:FeedingScheme

	coef	exp(coef)	se(coef)	z	Pr(>|z|)
DR Feeding	-1.0012	0.3674	0.3551	-2.819	0.00482**
Female	0.3591	1.4320	0.3253	1.104	0.26969
DR Feeding & Female	0.9736	2.6474	0.4867	2.000	0.04545*

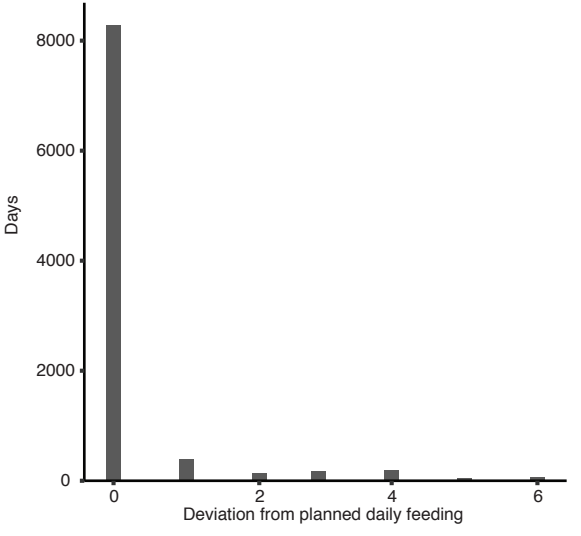
Supplementary Table 1: Result from proportional hazards model

Analyzing both lifespan cohorts using the Cox Proportional Hazards model finds a significant sex-specific effect from the time- and amount-restricted dietary restriction (DR) on killifish lifespan. DR independently and significantly reduces the hazard rate. Significance determined at an alpha of 0.05. Source data: Source Data 4 and Source Data 11

510

Supplementary Figure 1

A Fidelity of multiple automated feeders



B Feeding number and frequency

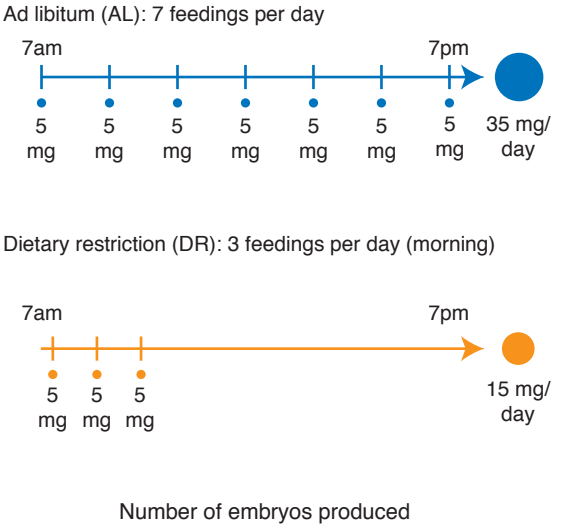
Deviation from planned daily feeding	Days	Frequency
0	8275	89.13%
1	388	4.17%
2	130	1.40%
3	180	1.94%
4	197	2.12%
5	53	0.57%
6	61	0.66%

Supplementary Figure 1: Fidelity and precision of the automated feeding system validated by additional researcher

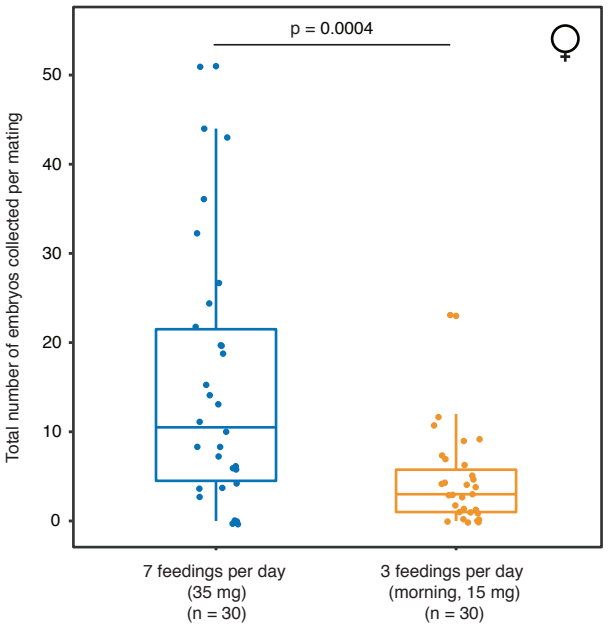
(A-B) Histogram (A) of deviations from scheduled feedings for 9284 days of feedings over 90 feeders with different assigned amounts tabulated in (B). The vast majority (>94%) of feedings have 0 or 1 missed feeding. Source data: Source Data 10

Supplementary Figure 2

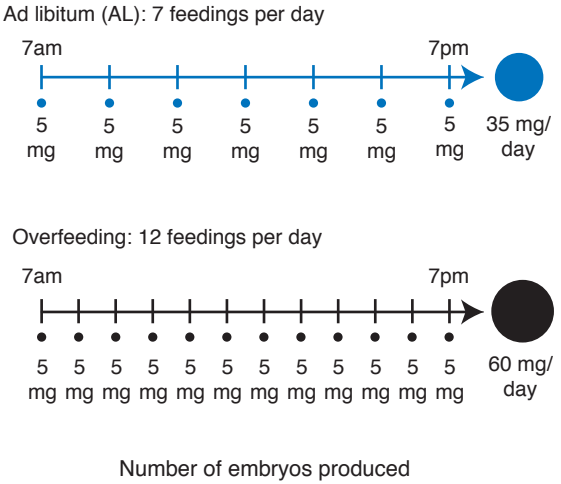
A Experimental scheme for dietary restriction



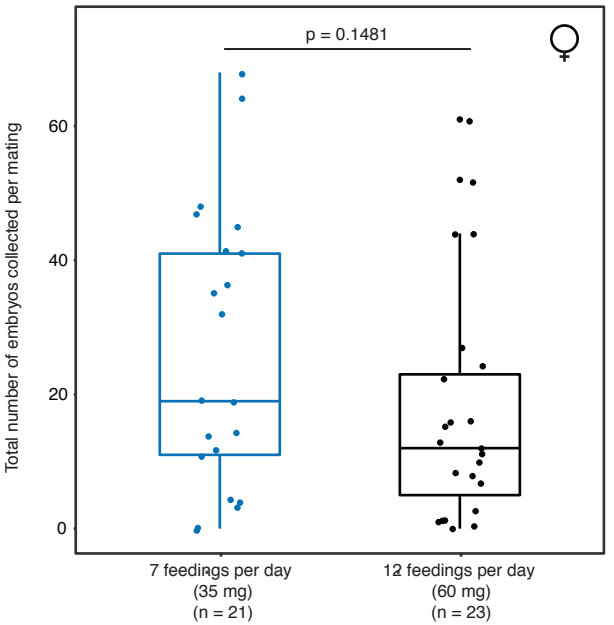
C Total embryos produced upon dietary restriction



C Experimental scheme for overfeeding



D Total embryos produced upon overfeeding



520 **Supplementary Figure 2: Total number of embryos produced in response to different diets**

(A) Experimental scheme to compare two automated feeding schedules for killifish: feeding 7 times a day evenly through 12 hours (5mg Otohime fish diet per feeding, 35 mg total per day) and feeding 3 times a day within a two-hour period in the morning (5mg Otohime fish diet per feeding, 15 mg total per day). These regimens were applied from 1 month of age until death of one individual in the pair. Each individual in the pair was fed individually while single-housed and then crossed for 24 hours once per week to assess number of embryos produced.

(B) Killifish mating pairs fed 3 times a day produce significantly fewer total embryos (median = 3 fertilized embryo per mating, n = 30 matings across 5 pairs) than mating pairs fed 7 times a day (median = 10.5 fertilized embryos per mating, n = 30 matings across 5 pairs). Each dot
530 represents total embryos collected from one pair crossing overnight. Significance determined by Wilcoxon sum rank test (p-value = 0.0004). Source data: Source Data 5 and Source Data 6

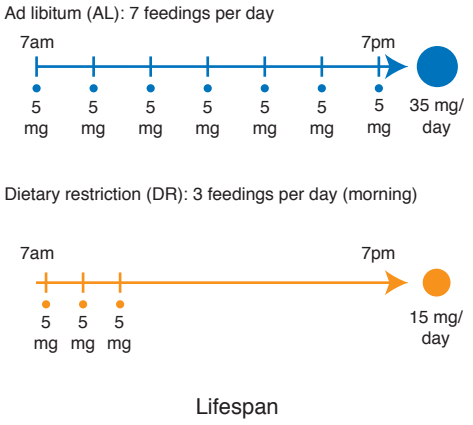
(C) Experimental scheme to compare two automated feeding schedules for killifish: feeding 12 times a day (5 mg Otohime fish diet per feeding, 60 mg total per day) compared to feeding 7 times a day (5 mg Otohime fish diet per feeding, 35 mg total per day). These regimens were applied from 2 months of age until death of one individual in the pair. Each individual in the pair was fed individually while single-housed and then crossed for 24 hours once per week to assess number of embryos produced.

(D) Killifish mating pairs fed 12 times a day do not produce significantly fewer total embryos (black feeding schedule, median = 12 total embryos per mating, n = 23 matings, across 3 pairs)
540 than for those fed 7 times a day (blue feeding schedule, median = 19 total embryos per mating, n = 21 matings across 3 pairs). Each dot represents total embryos collected from one pair crossing

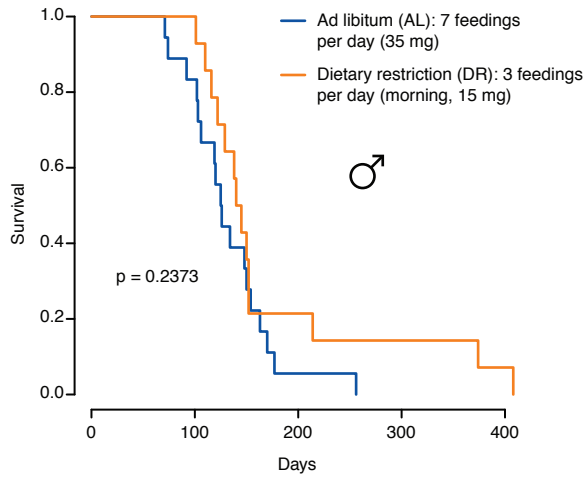
overnight. Significance determined by Wilcoxon sum rank test (p-value = 0.1481). Source data: Source Data 8 and Source Data 9

Supplementary Figure 3

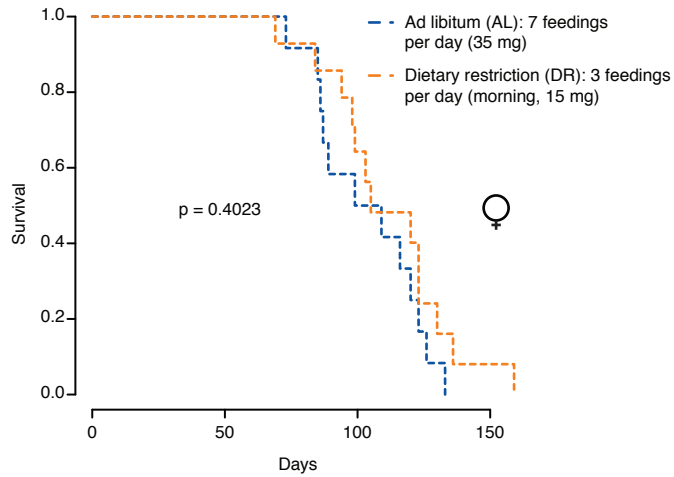
A Feeding regimens: Dietary restriction



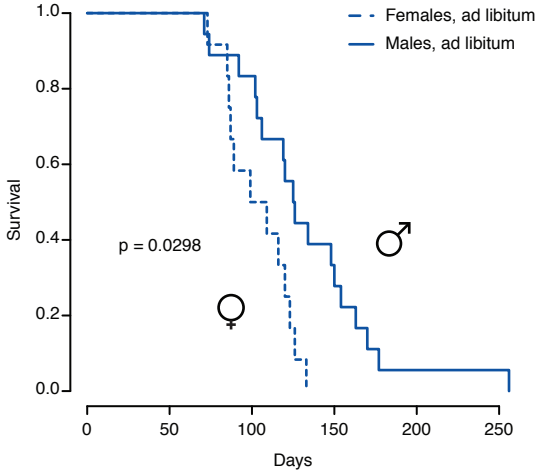
B Lifespan of male killifish in response to dietary restriction (Cohort 1)



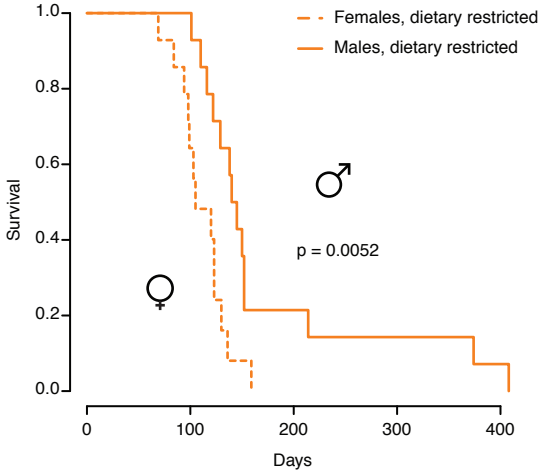
C Lifespan of female killifish in response to dietary restriction (Cohort 1)



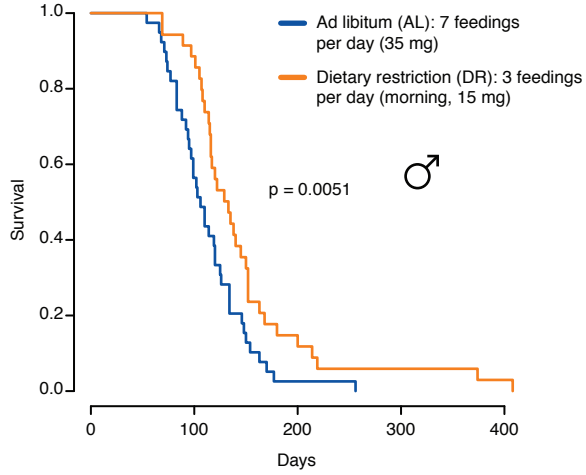
D Lifespan of male and female killifish in ad libitum conditions (Cohort 1)



E Lifespan of male and female killifish in dietary restricted conditions (Cohort 1)



F Combined lifespan for both cohorts of male killifish in ad libitum or dietary restriction conditions



Supplementary Figure 3: A second cohort undergoing amount- and time-restricted dietary regimen extends lifespan in male African turquoise killifish

550 (A) Experimental scheme comparing two automated feeding schedules for killifish: an *ad libitum* (AL) regimen (7 times a day, 35 mg Otohime fish diet per day, blue) or a dietary restricted (DR) regimen (3 times in the morning, 15 mg Otohime fish diet per day, orange). Feeding was started after sexual maturity at 1 month of age.

(B) In a second cohort, male killifish fed a dietary restricted regimen (solid orange, median lifespan = 142.5 days, n = 14) did not live significantly longer than male killifish fed an *ad libitum* regimen (solid blue, median lifespan = 125.5 days, n = 18) (p = 0.2373, logrank test). Source data: Source Data 11

(C) In a second cohort, female killifish fed a dietary restricted regimen (dashed orange, median lifespan = 104 days, n = 14) did not live significantly longer than female killifish fed an *ad*
560 *libitum* regimen (dashed blue, median lifespan = 104 days, n = 12) (p = 0.4023, logrank test). Source data: Source Data 11

(D) In a second cohort with *ad libitum* conditions, male killifish (solid blue, median lifespan = 125.5 days, n = 18) did exhibit a lifespan difference from female killifish (dashed blue, median lifespan = 104 days, n = 12) (p = 0.0298, logrank test). Source data: Source Data 11

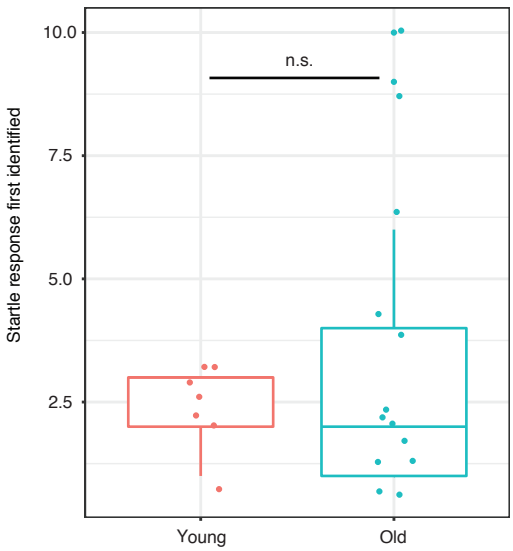
(E) In a second cohort with dietary restricted conditions, male killifish (solid orange, median lifespan = 142.5 days, n = 14) lived significantly longer than female killifish (dashed orange, median lifespan = 104 days, n = 14) (p = 0.0052, logrank test). Source data: Source Data 11

(F) Combining the lifespans of the first and second cohorts of male killifish, we found the additional animals did not change the conclusion from the second cohort that male killifish on an
570 *ad libitum* diet (solid blue, median lifespan = 106 days, n = 39) live significantly shorter than

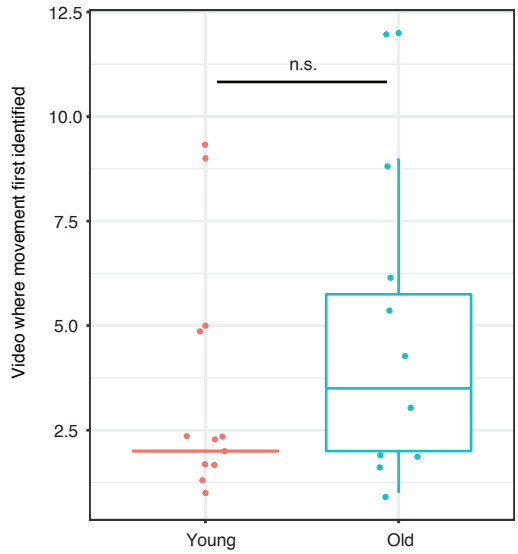
dietary restriction animals (dashed orange, median lifespan = 129 days, n = 35) ($p = 0.0051$, logrank test). Source data: Source Data 4 and Source Data 11

Supplementary Figure 4

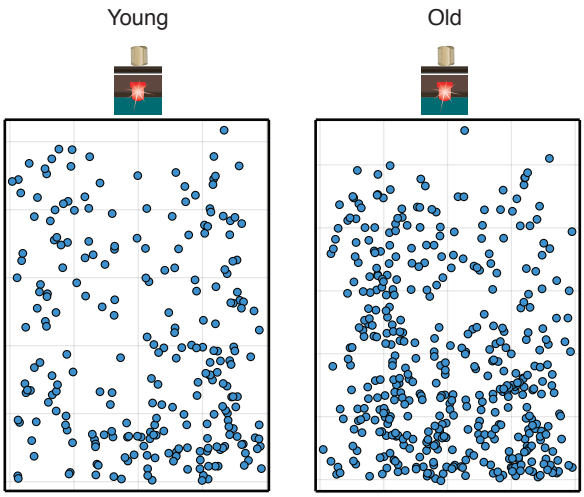
A Video where startle response was first identified



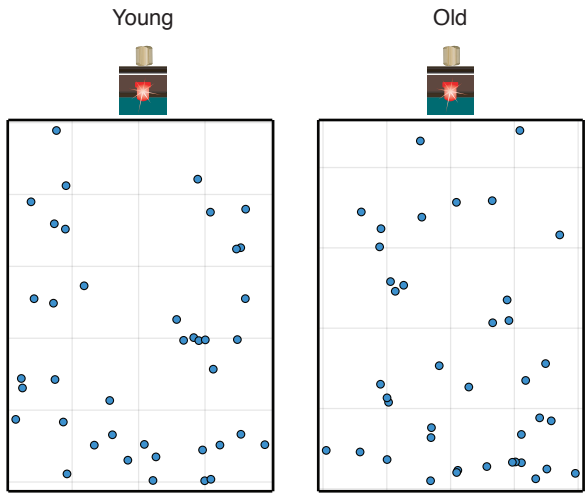
B Video where movement to food was first identified



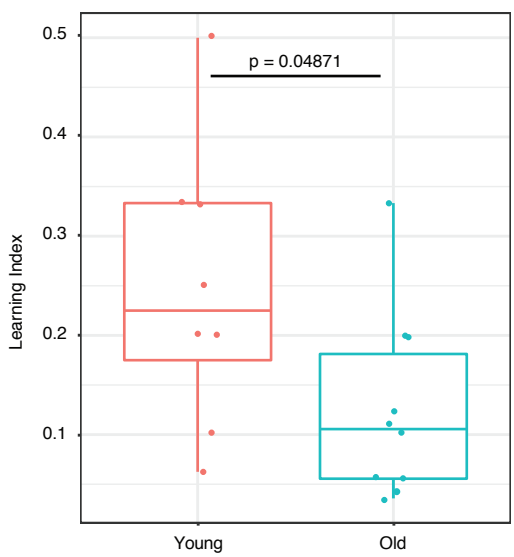
C Initial location young and old animals



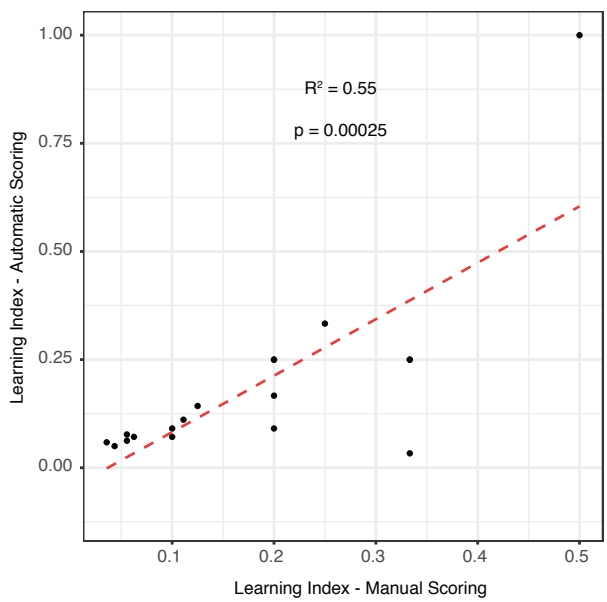
D Initial location of young and old animals downsampled to 40 each



E Associative learning index (manual scoring)



F Comparison between manual and automated scoring



Supplementary Figure 4: Control metrics for learning assay and comparison of automated scoring with manual scoring

(A) Each animal manually assessed for the earliest evidence of a startle response and plotted by binned age (young: mean = 1.4286, n= 8, old: mean = 2.4615, n = 13). Young animals are defined as less than 70 days of age and old animals are defined as more than 110 days of age. Each dot represents the earliest video at which an individual animal showed evidence of a startle response. Not significant as determined by Wilcoxon sum rank test (p-value = 1). Source data: Source Data 12

(B) Each animal manually assessed for the earliest evidence of movement to food and plotted by binned age (young: mean = 2, n = 9, old: mean = 3.6, n = 10). Young animals are defined as less than 70 days of age and old animals are defined as more than 110 days of age. Each dot represents the earliest video at which an individual animal showed evidence of movement to food. Not significant as determined by Wilcoxon sum rank test (p-value = 0.2262). Source data: Source Data 12

(C) Initial location of young and old animals in the tank when video begins recording (all videos represented). Source data: Source Data 12 and Training Trajectories

(D) Initial location of young and old animals in the tank when video begins recording (an equal random subsampling of 40 the full number of videos as shown in C). Source data: Source Data 12 and Training Trajectories

(E) Manual scoring of trained animals shows a significant reduction in learning as assessed by a Learning Index (the inverse of the number of trainings before learning). Significance determined by Wilcoxon sum rank test (p-value = 0.04871, n = 18, N = 1). Source data: Source Data 12

(F) Comparison between manual and automatic scoring approaches shows high degree of agreement with an adjusted R^2 of 0.55 (p-value = 0.00025). Source data: Source Data 12 and Training Trajectories

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Materials and Methods:

Automated fish feeding system:

We designed and built an automated fish feeding system that comprises individual battery-powered wireless automated feeders ("feeders"), a co-located server ("local server"), and a cloud-based server ("cloud server").

We designed and built the feeders so that they can be placed on individual 2.8 liter tanks supplied by Aquaneering, Inc. (San Diego, CA), which are commonly used for killifish and zebrafish husbandry, and fit into the tank lid's two foremost holes. We designed the feeders so that they can precisely deliver a fixed amount of dry fish food (Otohime fish diet, Reed Mariculture, Otohime C1) repeatedly each day on a highly flexible schedule. The feeders are composed of 3D printed parts (Hatchbox PLA, 1.75 mm), transparent 1/16 inch thick laser-cut acrylic (Amazon.com), nylon screws and nuts (Amazon.com), a 28BYJ-48 stepper motor (Amazon.com), a Wemos D1 mini ESP8266-based development board (Amazon.com), and a custom printed circuit board (PCB) of a design ordered through OSH Park (Portland, Oregon). The design incorporates a green light emitting diod (LED) and corresponding photoresistor that measures food dropped and allows for automatic calibration. The 3D printed and laser cut components were designed in Autocad (Autodesk), and PCBs were designed using Eagle software (Autodesk). We assembled feeders in house and programmed the ESP8266 microcontrollers using MicroPython language.

Feeders running MicroPython use the Message Queuing Telemetry Transport (MQTT) communication protocol to connect to the local server acting as the MQTT broker and obtain the current time and feeding regime. Feeders remain in deep sleep power-saving mode until their designated feeding, at which point they rotate their acrylic feeding disc using the onboard stepper motor from under the food hopper, in between the green LED and photoresistor array, and over the drop site, before returning to the food hopper. This releases 5 mg of food per feeding while measuring the resistance of the photoresistor, providing a reportable confirmation of food delivery.

After feeding, a confirmation is relayed from the feeder to the local server, using the MQTT protocol, and the confirmation is uploaded to the cloud server via an MQTT bridge. The cloud server acts as the orchestrating database, receiving commands from the user via a Julia language-based command line interface that communicates with the underlying MQTT protocol and the SQLite database of feeder orders and the logs of feeding confirmations and checked in feeders. This cloud server exists as an Amazon Web Service EC2 instance and relays changes from users back through the MQTT bridge to the local server and back to individual feeders, while running maintenance scripts that provide status updates on all feeders.

An overview of the system and links to the components is available at the github repository: https://github.com/amckay1/KilliFeeder

African turquoise killifish husbandry

All experiments were performed using the GRZ strain of the African turquoise killifish species *Nothobranchius furzeri*. Animals were housed in a 26°C circulating water system kept at a conductivity between 3500 and 4500 µS/cm and a pH between 6 and 7.5, with a daily exchange
840 of 10% with water treated by reverse osmosis. All animals were kept on a 12-hour day/night cycle and housed within the Stanford Research Animal Facility in accordance with protocols approved by the Stanford Administrative Panel on Laboratory Animal Care (protocol # APLAC-13645).

Unless otherwise noted, animals were raised as follows: pairs of single GRZ males and single GRZ females between 1 month and 3 months of age were placed in a 2.8 L tank and allowed to breed over a 24-hour period in sand trays placed for embryo collection. After 24 hours, the trays were collected, and embryos were separated from the sand by sieving. Collected embryos were placed in Ringer's solution (Sigma-Aldrich, 96724) with 0.01% methylene blue at 26°C in 60
850 mm x 15 mm petri dishes (E and K Scientific, EK-36161) at a density between 10 and 50 embryos per plate. After two weeks of monitoring, embryos were transferred to moist autoclaved coconut fiber (Zoo Med Eco Earth Loose Coconut Fiber) lightly packed in petri dishes (E and K Scientific, EK-36161) at the same density as per the previous two weeks and then incubated for another two weeks at 26°C. After two weeks on moist coconut fiber, hatching was induced by placing embryos in chilled (4°C) 1 g/L humic acid solution (Sigma-Aldrich, 53680) and incubating them in that solution overnight at room temperature. The fry was transferred to 0.8 L tanks at 2 fry per tank for the first two weeks then 1 fry per tank for the final two weeks on the circulating system (26°C). Fry were fed freshly hatched brine shrimp (Brine Shrimp Direct,

BSEP6LB) twice per day for the duration of the first four weeks post-hatching. After four weeks, adult animals who had inflated swim bladders (identified by ability to float) were individually housed in 2.8 L tanks where they were fed Otohime fish diet (Reed Mariculture, Otohime C1). Animals were sexed at four weeks by visual inspection: males harbor vivid tail fin colors whereas females do not. Animals were placed on the automated feeding system starting at four weeks of age, with the exception of feeder-naïve animals used for the behavioral assays. The animals on feeders were fed for the entirety of the experiments using different regimens including *ad libitum* (7 feedings of 5 mg per feeding spread throughout the day), dietary restricted (3 feedings of 5 mg per feeding in the morning), and over-feeding (12 feedings of 5 mg per feeding spread throughout the day). For behavior experiments, feeder-naïve animals were fed twice a day 20 mg between 8am and 11am in the morning and between 2pm and 5pm in the afternoon during the week and once 20 mg per day between 8am and 5pm during weekends until they were used for the behavioral experiments.

Comparison between automated and manual feeding:

To compare automated feeding with manual feeding, automated feeders were programmed to drop a total of 40 mg Otohime fish diet (eight rotations of the feeding mechanism with 5 mg per rotation). In parallel, individual lab members were instructed to measure out a total of 40 mg of Otohime fish diet (two feeding spoons with 20 mg per spoon). The mass of each manual feeding was measured using a scale to 1 mg precision. This was repeated nine times per individual and nine times across three different automated feeding devices. Estimates of the standard deviation were calculated by bootstrap using the Bootstrap.jl package in Julia and then converted to precision by taking the reciprocal of the square.

Growth rate experiments and analysis

All fish enrolled in a lifespan experiment were measured for size at two time points: i) young adult (28 days post-hatching, which is the time of enrollment on the automatic feeders), and ii) death. To avoid concerns that non-linear growth rates with older aged animals would confound the analysis, only animals that lived 4 months or less were considered. To this end, fish were placed in a clear plastic crossing tank (Aquaneering, ZHCT100T) with a water depth of 3 cm and a reference ruler underneath. Images were taken using a fixed position digital camera. Analysis of size was carried out by measuring the length of the animals in pixels and converting to length based upon the reference ruler's size in pixels. After animals had died (the day of or the next day), dead animals were placed on a ruler and their length measured directly using a digital camera. The difference in length divided by the time between the first measurement and death was then reported as the fish growth rate. To accurately compare animal growth rates despite different times of death, only animals that died before 4 months were compared.

Growth rates were compared between conditions using the two-sided Wilcoxon sum rank test with a significance threshold alpha of 0.05.

Fertility experiments and analysis

Fish from the same hatching date at 4 weeks of age (AL & DR comparison) or 8 weeks of age (AL & OE comparison) were paired in 2.8 L tanks, with 1 male and 1 female. Each animal was given the same feeding regime prior to enrollment: brine shrimp as described above for the first 28 days post-hatching then manual feeding of Otohime C1 fish diet up until the beginning of the

enrollment on automated feeders. At the beginning of the enrollment, pairs of males and females were crossed in the afternoon with sand trays and uncrossed ~24 hours later. Pairs were crossed and uncrossed weekly on the same day and crossed until one of the pairs died. Sand trays with embryos were removed at that time and sieved as described above to isolate embryos, which were then rinsed and counted. The total number of embryos produced and the number of fertilized embryos (indicated by separation of the chorion from the yolk membrane) were recorded and reported for each pair for that week.

Fertility was analyzed between feeding conditions by treating each pairing as an independent data point and using two-sided Wilcoxon sum rank test with a significance threshold alpha of 0.05. Animals used for fertility assessment were part of an independent cohort from that used for the lifespan and growth rate evaluation.

Lifespan experiments and analysis, including Gompertz curve estimation

Embryos for lifespan experiments were produced as described above. Briefly, GRZ males and females were bred overnight, with embryos placed in Ringer's solution for two weeks at 26°C and on coconut fiber for another two weeks at 26°C before being hatched in humic acid. Any animals that did not hatch were censored and removed from the experiment. Fry that hatched were transferred to 0.8 L tanks were split on the strict timescale previously described: two fry per 0.8 L tank for the first 2 weeks post-hatching, and then one fry per 0.8 L tank for the subsequent 2 weeks post-hatching. After 4 weeks post-hatching, adult individuals were individually placed in 2.8 L tanks for the remainder of their lives. When placed in 2.8 L tanks, animals were transferred to feeding with Otohime C1 fish diet using an automated feeder. Both males and

females were used for lifespan experiments unless otherwise stated, sex determined by the bright coloring of the male tail fins. Animals were checked daily and animals that died were recorded as dying on the day found. Two cohorts in total were aged, animals from each cohort being from the same breeding pairs and enrolled into the cohort based upon hatch date. Any animals whose automated feeders stopped performing due to damage to the feeder were censored.

Statistical analysis of lifespan experiments was conducted using the Logrank test with a significance threshold alpha of 0.05. We also analyzed lifespan data with the Cox proportional hazard model controlling for hatch date (with a significance threshold of 0.05). Gompertz curves were calculated using the combined males from both cohorts 1 and 2. Briefly, males on dietary restriction or *ad libitum* feeding regimes were pooled and a Gompertz distribution fitted to the two groups using the R *flexsurv* library and the *flexsurvreg* function with a "Gompertz" distribution. The resulting estimated parameters for the intercept and slope of the Gompertz curve were then plotted in Julia using a custom script. Estimates for the hazard rate were assessed by calculating the hazard function based upon 3-week binning of mortality rates for the two groups, dietary restricted males or *ad libitum* males. Gompertz curve parameter estimates reported with estimated 95% confidence levels.

Associative learning assay

Animals were raised as described above, with the exception that GRZ pairs were crossed for as long as one week before collection of embryos and were raised for adulthood with less stringent density requirements: 4-5 fry were placed per 0.8 L tank for 2-3 days before being split to 2 fry per tank for the remainder of the four weeks before sexual maturity. After sexual maturity, they

were individually housed in 2.8 L tanks. For these experiments, fish were manually fed as previously described ("feeder-naïve"). Animals used were a mixture of males (n = 17) and females (n = 9) between the ages of 36 days post-hatching and 130 days post-hatching. Animals were trained with a conditioned stimulus (red light) delivered 3 seconds before the unconditioned stimulus (food, 5 mg dropped at a time). The red light stayed on for the duration of the feeding (~2 additional seconds). Videos were recorded using an ESP32-CAM module from the anterior of the tank and started before the red light turned on and ended shortly after the food was dropped. Videos were analyzed by manual scoring and automated tracked trajectory. Videos were censored from analysis if there were too few videos or there were technical difficulties with the camera or feeder.

Manual scoring was performed by viewing repeated training recordings until animals moved directly to the top of the tank after the red light turned on but before food dropped. Manual scores were either 1 for direct movement in anticipation or 0 for neutral or ambiguous movement. The learning threshold was determined as two consecutive training sessions scored as "1" in a row. A learning index was then calculated as the inverse of the learning threshold determined and plotted for young and old animals. Old animals (males and females) were binned as animals over 110 days old at the start of training, young were binned as 70 days old or below. Scoring was compared using the two-sided Wilcoxon sum rank test with a significance threshold alpha of 0.05.

Automated tracking was performed by DeepLabCut 2.2 (Mathis et al., 2018), an open-source deep-learning tracking software and custom Julia scripts. Briefly, the DeepLabCut software was

trained on representative training video stills using a transfer learning approach in which superficial layers of the neural network were updated while the core ResNet-50 layers were left untouched, resulting in relatively rapid training of the network. Using this trained network, all killifish training videos were processed, resulting in tracked videos and corresponding coordinates with likelihood values for both fish snouts and the red training light. Thresholding these likelihoods at 0.999 and interpolating the resulting trajectories allowed for the plotting of traces and distances closed between the fish's initial position and the red light.

Traces were then processed in an algorithmic approach similar to the manual scoring: a threshold upward velocity was manually determined to be 0.5, and an animal was given a 1 if this threshold was passed and a 0 otherwise for each training session. A learning threshold was used as described above with manual scoring, with two consecutive successful trainings (scored as "1" twice in a row) being the threshold. The learning index was then calculated as the inverse of this learning threshold as for manual scoring. Binning and comparison between young and old performed identically to manual scoring using the Wilcoxon sum rank test with a significance threshold alpha of 0.05. Correlation between scoring and automated scoring was performed using R's linear regression function *lm* and plotting in R.

Compass graphs were generated by converting the automatically tracked trajectory of the animal from the initial location after the red light turned on into polar coordinates. These coordinates were then binned and combined for older animals (110 days and older) or younger animals (70 days and younger), pretraining (sessions 1 and 2 where the animals are naïve) and post-trainings

(sessions 3 and 4 where young animals have begun to learn but older animals are still unable). All plots were performed with ggplot2 in R.

To determine whether young and old animals could see the red light, we used the videos to check if individuals exhibit a startle response when the red light turns on. Startle responses were manually scored by observing each animal's training videos sequentially and determining the first indication of a startle response (sudden movement by the animal) upon the red light turning on. This allowed for a binary classification of the startle response (whether the animal ever demonstrated a startle response in a video) and a quantification of the startle response in terms of videos observed before startle response. Significance was determined using the two-sided Wilcoxon sum rank test with a significance threshold alpha of 0.05.

To test for motivation, we assessed whether young and old animals could see the food dropping and go toward it. To this end, we examined the videos after food was dropped. Motivation for food was determined by sequentially scoring videos manually and looking for evidence the animals have moved to the top of the tank at some point to begin feeding. This allowed for a binary classification of movement towards food for each animal and a quantification of the number of videos before movement towards food was detected. Significance was determined using the two-sided Wilcoxon sum rank test with a significance threshold alpha of 0.05.

Due to the stochastic nature of the animal's location in the tank, both startle responses and motivation scoring are conservative estimates as the animals may sometimes be far enough from the red light or the food to not be startled or not detect the food during the video's duration.

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Code is available in Github repository (https://github.com/brunetlab/McKay_etal_2021).