universität wien

MASTERARBEIT | MASTER'S THESIS

Titel | Title

The influence of the nutritional state on stress responses and behavioural flexibility

verfasst von | submitted by

Xenia-Noel Gabrielidis BA BSc

angestrebter akademischer Grad | in partial fulfilment of the requirements for the degree of

Master of Science (MSc)

Wien | Vienna, 2025

Studienkennzahl lt. Studienblatt | Degree programme code as it appears on the student record sheet:	UA 066 878
Studienrichtung lt. Studienblatt | Degree programme as it appears on the student record sheet:	Masterstudium Cognition, Behavior and Neurobiology
Betreut von | Supervisor:	Mag. Dr. Sabine Tebbich Privatdoz.

Abstract

Um zu überleben, müssen sich Organismen an veränderte Lebensumstände anpassen, und Verhaltensflexibilität durch Lernen spielt eine wichtige Rolle bei der Anpassung im Laufe des Lebens eines Individuums. Verhaltensflexibilität ist wichtig für die Anforderungen des täglichen Lebens, wie z.B. bei der erfolgreichen Nahrungssuche oder der erfolgreichen Abwehr von Räubern. Die physiologische Stressreaktion bereitet ein Tier auf widrige Umstände vor und liefert zusätzliche Energie für eine Notfallreaktion. Kurzzeitiger Stress kann die Aufmerksamkeit und die Gedächtnisbildung verbessern, aber eine langanhaltende Störung führt zu einer chronischen Stressaktivierung, die die Flexibilität beeinträchtigen kann. Der Ernährungszustand wirkt sich auf die Fähigkeit zur Bewältigung von Stresssituationen aus, und die Theorie der allostatischen Belastung sagt voraus, dass gut ernährte Tiere unter den Folgen einer chronischen Stressreaktion leiden. Dieser Zusammenhang ist besonders relevant für Tiere, die in Gefangenschaft gezüchtet werden und mit einem übermäßigen Angebot an Futter versorgt werden. In unserer Studie untersuchten wir, ob ein Zusammenhang zwischen Ernährungszustand, Verhaltensflexibilität und Stressreaktionen bei Regenbogenforellen besteht. Wir sagten voraus, dass Fische mit einem übermäßigen Nahrungsangebot eine geringere Verhaltensflexibilität und höhere Cortisolwerte nach einem Stressor aufweisen würden als Fische mit einem reduzierten Nahrungsangebot. Wir stellten fest, dass Fische mit reduziertem Futterangebot in neuen, stressigen Situationen eine größere Verhaltensflexibilität zeigten als solche mit übermäßigem Futterangebot. In veränderten Situationen und bei der Anzahl an Fehlversuchen gab es keine Unterschiede. Die kurzfristige Stressreaktion (Plasmacortisolspiegel) war bei Fischen mit reduziertem Futterangebot höher. Die langfristige Stressreaktion (Kortisolkonzentration in Schuppen) unterschied sich nicht zwischen den Gruppen, was möglicherweise auf die geringe Intensität und die seltenen Stressoren in unserem Versuchsaufbau zurückzuführen ist. Dies deutet darauf hin, dass Fische mit reduziertem Futterangebot besser in der Lage waren, akute, aber nicht chronische Stressoren zu bewältigen als Fische mit übermäßigem Futterangebot. Zusammenfassend lässt sich sagen, dass unsere Ergebnisse die Hypothese teilweise bestätigen, dass übermäßiger Konsum an Futter die Verhaltensflexibilität und die Fähigkeit, mit neuen Stresssituationen umzugehen, beeinträchtigen kann. Unsere Ergebnisse zeigen auch, dass Vorerfahrung und Versuchsbedingungen eine entscheidende Rolle für die kognitive Leistung und die Stressphysiologie spielen.

Schlüsselwörter: Verhaltensflexibilität, Anpassung, Stress, Überernährung, Fischzucht

To survive, organisms must adapt to changing living conditions, and behavioural flexibility through learning plays an important role in adapting within an individual's lifetime. Behavioural flexibility is important for the demands of daily life, such as successfully foraging for food or fending off predators. The physiological stress response prepares an animal for adverse condition providing extra energy for an emergency response. Brief stress may enhance attention and memory formation, but long-lasting disturbance results in chronic stress activation, which may impair flexibility. The nutritional state also affects the ability to cope with stressful events, and the allostatic load theory predicts that well-fed animals suffer the consequences of a chronic stress response This relationship is particularly relevant for animals that are bred in captivity and provided with an excessive supply of food. In our study, we investigated whether a relationship between nutritional status, behavioural flexibility, and stress responses in Rainbow trout exists. We predicted that fish with an excessive food supply would have lower behavioural flexibility and higher cortisol levels following a stressor than fish with a reduced food supply. We found that fish with reduced food availability showed greater behavioural flexibility in novel, stressful situations than those with excessive food availability. There were no differences in modified situations and in the number of failed attempts. The short-term stress response (plasma cortisol level) was higher in fish with reduced food availability. The long-term stress response (cortisol concentration in scales) did not differ between the groups, possibly due to the low-intensity and infrequent stressors in our experimental setup. This indicates that fish with reduced food availability were better able to cope with acute but not chronic stressors than fish with excessive food availability. To summarize, our results partially confirm the hypothesis, that excessive food consumption may impair behavioural flexibility, and the ability to cope with stressful novel situations. Our results also highlight that prior experience, and experimental conditions also play crucial roles in cognitive performance and stress physiology.

Keywords: behavioural flexibility, adaption, stress, overfeeding, fish farming

Index

The influence of nutritional state on stress responses and behavioural flexibility

1. Introduction

In farmed fish, chronic stress is a major factor that negatively impacts well-being and overall performance, leading to reduced immune function, growth and reproduction. The effects result in economic losses, such as lower flesh quality and reduced success in stocking efforts (Dara et al., 2023; Johansson, 1981; Lewin et al., 2006; Oliva-Teles, 2012). Since fish are generally more sensitive to stressors than other vertebrates (Lemos et al., 2023), maintaining optimal rearing conditions is essential for ensuring good fish welfare (Dara et al., 2023; Johansson, 1981; Lai et al., 2021).

Although it is well-established that adequate nutrition is essential for preventing deficiency signs, maintaining optimal performance, and supporting overall health (Assan et al., 2021; Oliva-Teles, 2012), farmed fish often face inadequate artificial diets and feeding regimes (Lai et al., 2021; López-Olmeda et al., 2012).

Developing optimal feeding regimes in aquaculture has been a key focus of research, aiming to reduce feed costs, minimize environmental impact, and enhance fish welfare. Several studies have demonstrated a connection between food availability, health, and stress – also in fish (Dara et al., 2023; Kawata, 2008; Lai et al., 2023; Mohapatra et al., 2013; Oliva-Teles, 2012). However, these studies often focused on specific aspects such as feeding behaviour (Juell, 1995), optimal feeding times (López-Olmeda et al., 2012), the number of feeding rations (Flores and Vergara, 2012), or particular dietary ingredients (Oliva-Teles, 2012). For example, research has explored the use of probiotics to help fish cope with various physical, chemical, and biological stressors (Mohapatra et al., 2013) and the cost differences between high-fat and high-protein diets in Atlantic salmon (Weihe et al., 2019). Oliva-Teles (2012) reviewed the effects of dietary nutrients on health, welfare, and disease resistance in fish, while Dara et al. (2023) examined fish welfare and stress mediators, comparing conventional and organic diets. Despite these insights, there is still a knowledge gap regarding how the quantity of feed intake impacts behavioural flexibility and stress responses in fish reared in aquaculture.

Our study aims to address this gap by examining the influence of nutritional state on behavioural flexibility and the stress responses in Rainbow trout, a commercially important species also used in rehabilitation projects for declining wild population.

The stress response is an adaptive mechanism that prepares an organism for unfavourable conditions by providing additional energy and maintaining homeostasis in changing environments and life

circumstances (Herman et al., 2016; McEwen and Wingfield, 2003; Romero et al., 2009). This response is influenced by factors such as current environmental conditions, including the frequency of the stressor and the individual's nutritional state (Dara et al., 2023; McEwen and Wingfield, 2003).

In fish, the primary physiological, hormonal responses to stress involves the activation of the hypothalamic-pituitary-interrenal (HPI) axis, which is analogous to the hypothalamic-pituitary-adrenal (HPA) axis in mammals (Dara et al., 2023; Herman et al., 2016; Kennedy and Janz, 2023; Lai et al., 2021; Mommsen et al., 1999). The HPI response starts within minutes with a release of adrenocorticotropic hormone (ACTH) but lasts relatively short. Whereas, the glucocorticoid response, which involves hormones like cortisol – the main stress hormone in fish - lags in time and lasts longer (Dara et al., 2023; Herman et al., 2016; Koakoski et al., 2012; Lemos et al., 2023; Mommsen et al., 1999; Samaras et al., 2021). Glucocorticoids are released into the bloodstream to mobilize energy and tissue nitrogen needed to cope with stressful situations, weather in reaction to a real physical threat (reactive response) or in preparation for an anticipated one (anticipatory response) (Goymann and Wingfield, 2004; Herman et al., 2016; Lai et al., 2021).

Short-term elevations of glucocorticoids as consequence of acute stressors enable adaptive behavioural and physiological responses (Carbajal et al., 2019b; Goymann and Wingfield, 2004; Lai et al., 2021). Whereas, long-term or chronic elevations of glucocorticoids, as consequence of chronic stress, can lead to negative outcomes such as reproductive suppression (lower fitness), osmoregulatory issues, reduced growth rate, immune suppression and diseases, including neurodegenerative brain disorders linked to HPI axis dysregulation (Carbajal et al., 2019b, 2019a; Goymann and Wingfield, 2004; Kennedy and Janz, 2023; Lai et al., 2021; Romero et al., 2009; Samaras et al., 2021). Stress impacts also cognition through various mechanisms and over different periods (McEwen and Sapolsky, 1995). In the short term, stress enhances sensory, perceptual, and cognitive mechanisms, including memory formation, by focusing attention on a specific threat (McEwen and Sapolsky, 1995; Oliveira and Galhardo, 2009).

However, severe or prolonged stress can impair various cognitive functions, likely due to reversible changes in neuron morphology within the hippocampus, a brain region crucial for learning and memory. Prolonged exposure to stress can result in irreversible loss of hippocampal neuron, leading to cognitive deficits. Acute emotional arousal has been shown to enhance memory formation, as evidenced by studies with both animals and humans. Stress-induced glucocorticoid levels can disrupt learning and memory by affecting long-term potentiation, hippocampal excitability, and hippocampal energy metabolism (McEwen and Sapolsky, 1995). Thus, exposure to long or short-term stress may also affect behavioural flexibility via learning.

Behavioural flexibility, a form of phenotypic plasticity, enables individuals to respond appropriate to

both acute and prolonged threats, helping them to cope with stressful situations and is advantageous in variable environments (Brown and Tait, 2014; Dill, 1983; Herman et al., 2016; Kotrschal and Taborsky, 2010; Mayrand, 2017; Oliveira and Galhardo, 2009). Behavioural flexibility requires cognitive skills like inhibition control and learning abilities (Kabadayi et al., 2018; Kotrschal and Taborsky, 2010; Lucon-Xiccato et al., 2017). Therefore, inhibition control is an external function, which has a positive impact on the performance in various cognitive tasks. It allows an individual to withhold a behaviour when it is not adapted to the context, which is important not only in social interactions but also in adapting to challenging environmental changes (Kabadayi et al., 2018; Lucon-Xiccato et al., 2022). Although inhibition control is often associated with vertebrates that have complex neuronal systems, such as primates, research has shown that fish are also capable of highly flexible and specialised behaviours. Detour tests are used for measuring inhibition control and behavioural flexibility under stress in fish and other animals (Kabadayi et al., 2018). The test is based on psychological, cognitive, behavioural, ecological, neurobiological and developmental aspects. The basic setup involves a transparent or non-transparent obstacle that the individual must navigate around to reach a visible or hidden target, such as food. Thorndike first employed the detour test as early as 1911, successfully examining both fish and chickens (Lucon-Xiccato et al., 2017).

Nutrition plays a crucial role in fish health, primarily by meeting their protein and energy needs, which influences feeding behaviour, feed intake, appetite, and ultimately growth (Assan et al., 2021). Inadequate feeding conditions can cause stress, altered behaviour, reduced performance, and ultimately diseases and death (Lai et al., 2021; López-Olmeda et al., 2012).

Feeding strategies influence not only feeding behaviour but also overall behaviour, and potentially the ability to cope with environmental stressors.

Stress with an increase in glucocorticoids, requires additional energy sourced from endogenous fat, glycogen, and protein stores, as well as from the environment. This process is called an emergency response. According to the theory of allostatic load, these emergency responses are activated only when the allostatic load crosses a certain energetic threshold, which then helps to reduce the allostatic load and cortisol levels. Nutritional state plays a crucial role in an individual's ability to cope with stressful situations. Well-nourished or over-nourished animals can compensate the increased energy demands caused by stress. As a result, they do not reach the energetic threshold that triggers an emergency response and a subsequent decrease in cortisol levels. This lack of response leads to prolonged exposure of high cortisol levels and a chronic state of stress, which can impair learning and reduce behavioural flexibility (see Figure 1; adapted from McEwen and Wingfield, 2003).

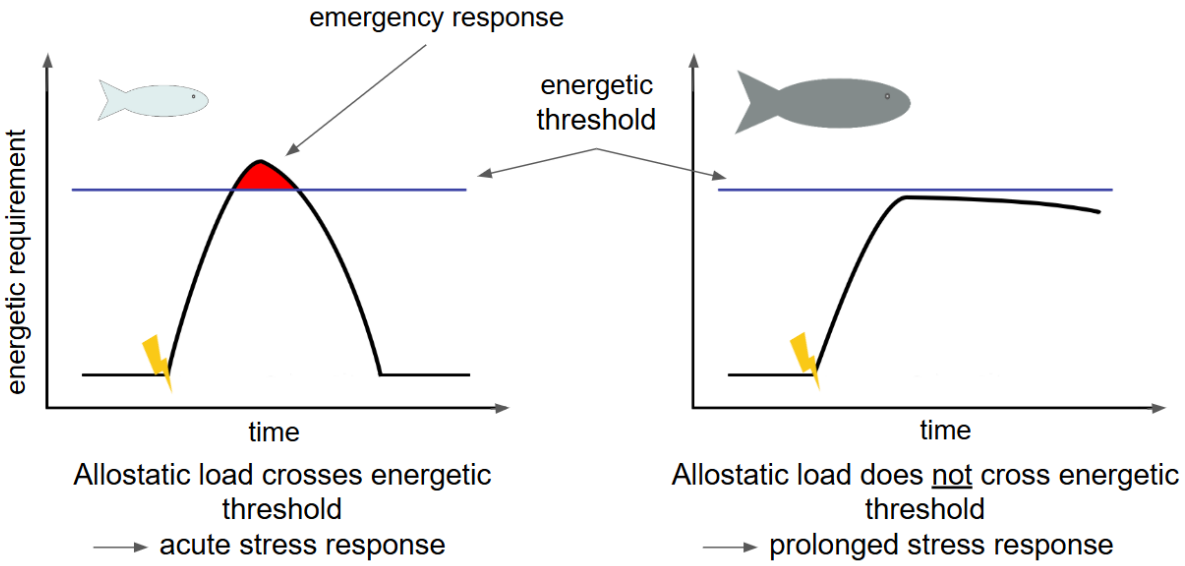

Figure 1 A scheme of the allostatic load theory. The y-axis represents the energetic requirement of an organism. When a stressor is experienced, the energetic requirements increase while coping with the stressor. If the energetic requirements cross an energetic threshold (blue line, which is determined by the energy available in the environment) then a life-history-emergency response (red area) is triggered which lowers the energetic requirements and helps the organisms to successfully cope with the stressor (yellow flash). The x-axis shows the timeline, and the black line indicates the energetic requirements before, during and after a stressor is perceived which is also indicative of cortisol concentrations. In a stressful situation, a normal or reduced nourished fish shows an increase in cortisol-level, which reaches the energetic threshold to cause an emergency response for providing energy through endogenous stores of fat, glycogen, and proteins (left). In stressful situations, a well or over-nourished fish shows an increase in cortisol-level, but does not reach the energetic threshold, because it can compensate for the additional energy needed. Subsequently, there is a prolonged high cortisol-level (right) (McEwen and Wingfield, 2003).

The unlimited availability of food has been shown to negatively impact cognition, behavioural flexibility, and the ability to cope with stress in many captive-bred species, including fish (Aquakulturinfo, 2019; Kawata, 2008; Mattson, 2019). Mattson (2019) explored why overconsumption impairs cognition, from both an evolutionary perspective and by examining physiological mechanisms. Focusing primarily on humans, rodents, and birds, Mattson (2019) found that signalling pathways, which evolved to respond adaptively to food scarcity, remain inactive when there is a constant availability and overconsumption of energy-rich food. This inactivity negatively affects cognition. Studies in rodents suggest that excessive food consumption and sedentary behaviour can reduce hippocampal volume by decreasing neurogenesis, dendritic arborization, and synaptic density. For example, genetically engineered mice with excessive food intake (due to leptin

receptor mutants) exhibited impaired hippocampus-dependent spatial learning, memory, and novel object recognition. These cognitive deficits were associated with impaired long-term potentiation at the synapses between the performant path and hippocampal dentate granule neuron, as well as reduced hippocampal neurogenesis. However, most of this research focuses primarily on mammals and birds.

We conducted trials with juvenile Rainbow trout (smolts) to test two predictions: (1) fish with excessive food supply (increased feeding regime) will exhibit lower behavioural flexibility in a detour task, as indicated by longer latency and higher number of failed attempts before solving the task, compared to fish with reduced food supply (reduced feeding regime); and (2) fish with excessive food supply will show higher physiological stress responses, evidenced by elevated cortisol levels in both blood plasma and scale samples, compared to those with reduced food supply (Goymann and Wingfield, 2004; McEwen and Wingfield, 2003).

To investigate these hypotheses, we divided the fish into two groups and assigned each to a different feeding regime treatment over the course of a month. One group received a reduced amount of food (reduced feeding regime treatment), while the other received an increased amount (increased feeding regime treatment) (see 2.3 Feeding regime). We evaluated behavioural flexibility using a detour test that included both a motivation-control task and a detour task. Each fish was tested subsequentially in both tasks, with the order of task alternated to create a stressful situation by presenting a novel or modified environment.

2. Methods

2.1. Study species - *Oncorhynchus mykiss* (Rainbow trout)

The Rainbow trout (*Oncorhynchus mykiss*) belongs to the family of Salmonidae, and its natural distribution is the Pacific Ocean in Asia and in North America. It is found in a variety of water bodies, such as marine, freshwater, brackish and bathypelagic water. They prefer clear, cold water with temperatures up to 25°C and high oxygen concentrations (Kawamura, 2008). Their habitats range from headwaters, creeks, and rivers to lakes and intertidal areas (Nelson, 1992). Rainbow trout include anadromous forms, meaning juveniles migrate after three years (until they become smolts) from freshwater to the sea and back to freshwater for spawning. However, some populations, such as the coastal Rainbow trout (*Oncorhynchus mykiss irideus*), remain in freshwater their entire life, while others, known as steelhead (anadromous form of coastal Rainbow trout), migrate to sea (Van Den Boogaart et al., 2023). The nutrition of Rainbow trout is composed of various aquatic and terrestrial invertebrates, small fish, and cephalopods (at the sea) (Kawamura, 2008). Furthermore, as a commercially important fish species it is of high economic value in the global aquaculture industry and has been introduced into at least 99 countries (FAO, 2020; Stanković et al., 2015; Verspoor et al., 2007). The Rainbow trout is one of the most reared species in aquacultures besides Atlantic salmon and the production involves commercial fish farming for food and sport fishing, but also for rehabilitation of declining fish populations (FAO, 2020; Verspoor et al., 2007). Rainbow trout are farmed in diverse environments, such as cages, tanks, and raceways, in both freshwater and salt or brackish water, which reflects the different migratory behaviour (Van Den Boogaart et al., 2023). It is cultivated in 28 European countries, with highest production in Turkey and Norway, but also in France, Italy, Denmark, Spain, Finland and the United Kingdom (Van Den Boogaart et al., 2023). In Austria, the Rainbow trout is the most frequently produced species in aquaculture (~ 1.5 million kg, (Statistik Austria, 2021). However, populations of Rainbow trout established in at least 53 countries, and can outcompete other salmonid species regarding space and food (Stanković et al., 2015).

2.2. Housing conditions

The experiment was conducted at the Linnaeus University in Kalmar, Sweden, and lasted from 2nd January to 28th February 2024 and consisted of three separate phases: (1) feeding phase, (2) behavioural assay phase and (3) blood and scale sampling phase. A total of 105 Rainbow trout were used in this study. We picked them up from a breeder for sport fishing called Hultsby fiskodling approx. 15 min from Kalmar (website: https://www.hultsby.com/index.htm). The subadult fish were smolts and lived in sex-mixed groups. We could not determine the sex due to the immature age of the

smolts. After arrival at the fish facility of the Linnaeus University, all individuals were randomly assigned to four 1500 L holding tanks (142.8 x 79.2 x 69 [L x W x H in cm]) with 26–27 individuals per tank. The substrate-free tanks were filled with filtered (two sand filters) Baltic Sea water, with a temperature of 15 °C (min 12 °C and max 16 °C) and supplied with oxygen. Fish were slowly acclimatized to the brackish water over six hours. The tanks had a free-flowing water system with a turnover rate of six hours. The PH value of the water was between 6.83 and 7.14 (Hanna instruments, HI 2210 pH Meter). For further parameters of water quality see 11.2 Additional Tables and Results Table 13. Two further tanks with the same dimension and water parameters were used as test tanks for the behavioural assay (see 2.4 Behavioural assay phase).

To avoid the stress of repeatedly catching and handling the same individual, fish were moved twice to different holding tanks: once after PIT tagging (see 2.7 PIT tagging and body measurements) and another time just before the behavioural assay phase started. After PIT tagging the fish stayed undisturbed in the same holding tank for the majority of the experimental period (~ 1 month). A couple of days before the behavioural assay started (see 2.4 Behavioural assay phase), all fish from the reduced feeding regime treatment (0.5 % food amount – see below), were transferred into the same tank, and all fish from the increased feeding regime treatment (1.5 % food amount – see below) were transferred into another separate tank. After the behavioural assay test fish were moved back to a holding tank that did not contain any untested fish. The fish stayed in this tank until blood and scale sampling took place.

The fish were maintained in a 14–10 hours light/dark photoperiod and were fed once per day in the evening. Before PIT tagging, all fish were fed with the same food amount of 5 g pellets (Inicio Plus 2.0 mm, BioMar a/s, brande, Denmark) per tank (26 to 27 individuals).

2.3. Feeding regime

One month before the behavioural assay phase started, the fish received an adapted food amount corresponding to their assigned feeding regime treatment. Fish stayed on these feeding regimes until the blood and scale sampling phase at the end of the experiment. We used two feeding regime treatments, one with a reduced food amount and one with an increased food amount. We randomly assigned two tanks to one of the feeding regimes and the remaining tanks to the other feeding regime. Individuals in tanks 1 and 4 received the reduced feeding regime with a daily food amount of 0.5 % of the average body weight in g from all the individuals in the tank (0.53 g). Individuals in tanks 2 and 5 received the increased feeding regime with a daily food amount of 1.5 % of the average body weight in g from all the individuals in the tank (1.59 g). These two feeding regimes were used to test a feeding regime that is slightly under or over the standard food amount provided in commercial fish

farms (~1 % of the average body weight in g). We could not control how much each individual fish ate, because they were kept in groups. This means that although the two feeding regimes had the predicted effect with fish receiving more food having a higher body mass than fish receiving less food (see 3.1 Validation of the feeding regime treatments) we still observed some variation in body mass within the same treatment group.

2.4. Behavioural assay phase

The conducted behavioural experiment was based on the detour paradigm, which is suitable for testing behavioural flexibility and inhibition control under a stressful situation (Kabadayi et al., 2018). In the detour task the access to a reward is blocked by a transparent obstacle requiring a detour. The motivation-control task allows us to assess if the detour task poses a challenge to the fish and if the feeding regime affects their motivation or task solving speed. Generally, we tested two fish in two test tanks simultaneously. The test tanks had the same measurements and water quality like the holding tanks (see 2.2 Housing conditions and Figure 3 (A)). We video recorded the trials for 60 min using a security camera (Avtech AVM52J) connected to a security system (Avtech AVH408P) which was placed 2 m above the test tanks. Over a period of approx. 2 weeks, a total of 93 Rainbow trout were successfully tested in 93 trials. Each trial for the Detour test was composed of two 30-min tasks: the motivation-control task and the detour task. Half of the test fish received the motivation-control task first whereas the other half received the detour task first. This created two test conditions: one where behavioural flexibility was assessed in a novel environment (detour task first) and one where behavioural flexibility was assessed in an already known environment (motivation-control task first). The number of fish from the two feeding regime treatments (reduced and increased) was the same.

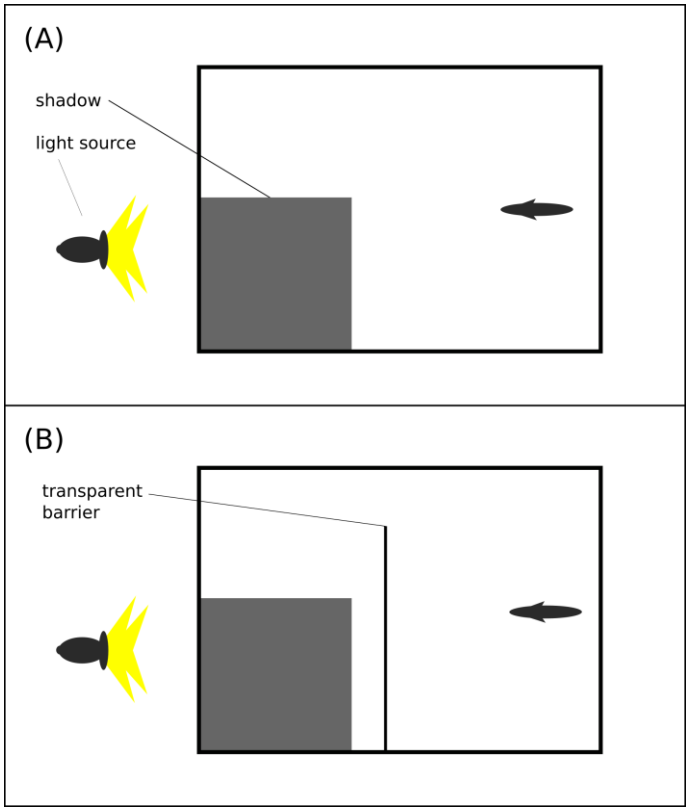

Figure 2 A schematic scheme of the (A) motivation-control and (B) detour task. The direction of the light source is indicated, which created a shadow in one corner of the test tank (indicated as grey rectangles). Fish were released on the other side of the shadow and could either (A) directly swim towards the shadow or (B) had to detour a transparent barrier.

Each day, we tested the same number of fish from each treatment. Daily, we alternated the test tanks between the two treatments. We tested the fish once in the motivation-control task and once in the detour task. For both tasks we used a brightly lit tank with a clear shadow on one side of the tank which test fish swiftly tried to reach and served as the reward. Fish were always released on the other side of the shadow and in the motivation-control task fish could swim directly to the shadow (see Figure 2 (A)). In the detour task the direct route to the shadow was blocked by a transparent barrier (see Figure 2 (B); size of barrier: see Figure 3 (B)).

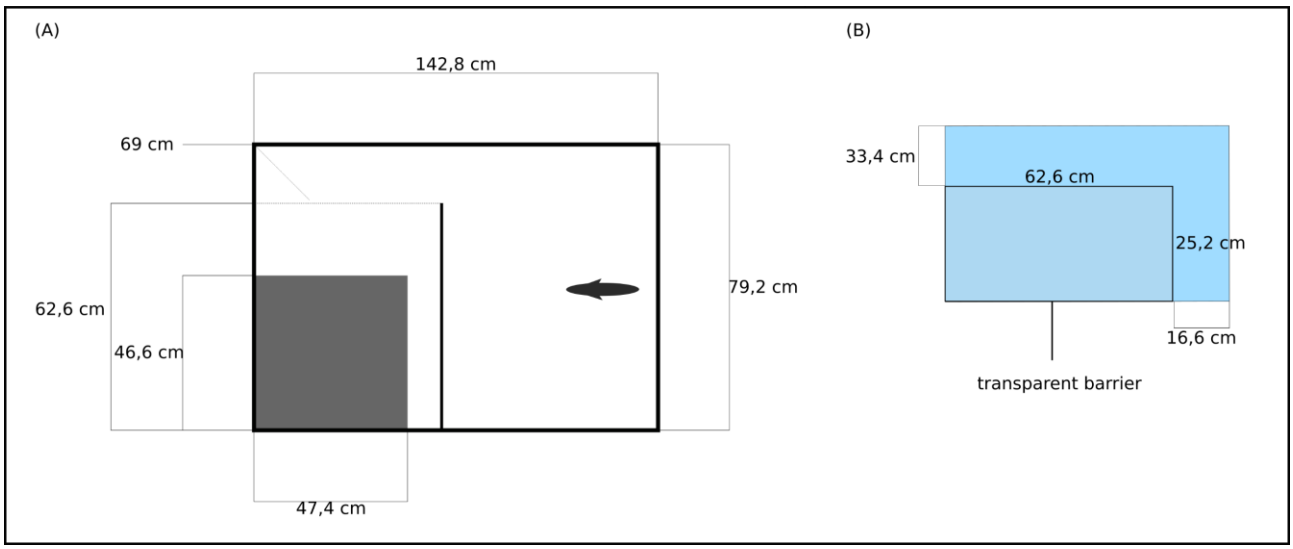

Figure 3 Size measurements of the test tanks used for the behavioural assay: (A) The test tank from above with the transparent barrier between the shadow and the fish; (B) The test tank viewed from the front showing the exact measurements of the transparent barrier.

2.4.1. Behavioural trials

We used two similarly illuminated test tanks without any substrate (see 2.2 Housing conditions and Figure 3 (A)). The supply with oxygen was interrupted during the trials. However, during the preparation for the next trials, the test tanks were supplied with oxygen. To create a shadow in the tank, we used a wooden board placed in one corner of the tank covering an area of approx. 47.4 cm x 46.6 cm. As transparent barriers, we used completely transparent plexiglass plates, fixed with two stones to the ground and with two wires to the side of the test tank. To avoid any injuries, the barrier would slightly move on a strong impact of a fish. The plexiglass plate measured 62.6 cm in length, 25.2 cm in width and had a thickness of 2 mm (see Figure 3 (B)). In each corner was a hole to fix the wire or stones. On the long lower side, we fixed the two stones with a piece of wire, and on the long upper side we fixed two wires shaped like a hook to fix the plexiglass plate onto the edge of the test

tank. The video cameras recorded the entire tank from above. For the motivation-control task, the wooden board was positioned in the previously described position, and the shadow was underneath. For the detour task, we added the transparent barrier (plexiglass plate) in front of the shadow. After preparing the test tanks for a motivation-control or detour task, two fish from different treatments were transferred to the separate test tanks from the holding tanks. To transfer, the fish caught first was placed in a transportation tank (10 L) and positioned in front of the first test tank on the opposite side of the shadow. Then we caught the second fish and positioned it in front of the second test tank. After starting the cameras, we transferred the first and then the second fish to the test tank within 30 sec. As the fish were released into the test tanks, the first task started (either motivation-control or detour task, depending on the order). After 30 min, the first task ended, and we prepared both tanks for the second task. For this we caught the fish, placed them underwater in a small fish net to be able to remove or add the transparent barrier without disturbing the fish too much. Afterwards, we released the fish in the same place as for the previous task. This was the start of the second task and lasted again 30 min. After the second task we stopped the cameras, caught the fish, and placed them into a transportation tank where their PIT tags could be easily read (using a PIT tag reader, Biomark, HPR Lite). Afterwards the fish were transferred to their respective holding tanks.

2.5. Blood and scale sampling phase

At the end of the experiment, we obtained blood and scale samples from 60 test fish (n = 30 per feeding regime treatment). The fish were starved for 17 hours before blood sampling. Before sampling, they were anesthetised by maintaining the fish in a 40 L tank filled with tank water and the anaesthetic solution of MS-222 (80 mg/L) (E10521-Ethyl 3-aminobenzoate methanesulfonate) for 5 min until the fish were unresponsive. Afterwards, the fish were culled by stunning the head with an aluminium fish knocker. Within 5 min after death, blood was collected by a caudal venous puncture using 1 mL heparinized syringes fitted with a 21G x 1” (⌀ 0.80 x 25 mm) needle and then transferred into 2 mL Microcentrifuge tubes. After collecting all blood samples, the plasma was separated using a centrifuge at 3900 rpm for 10 min and transferred in two separate 0.5 mL Microcentrifuge tubes. Lateral scales were sampled with tweezers and a spatula between the dorsal fin and the adipose fin. Afterwards, the scales were transferred to a 5 mL Microcentrifuge tube. All samples were stored at -20 °C until cortisol analysis at the University of Vienna.

2.6. Cortisol analysis in blood plasma and scales

We used scale samples for long-term (Kennedy and Janz, 2023) and blood plasma samples for short-term measurement of cortisol levels. All procedures were conducted in the Endocrinology laboratory of the Department of Behavioral & Cognitive Biology at the University of Vienna.

Serum preparation and hormone extraction

Blood plasma samples collected from individual fish (n = 60) were analysed for cortisol levels. The sample volume for each sample was approx. 0.5 mL. Prior to hormone extraction, samples were warmed to room temperature, briefly vortexed, and centrifuged for 10 sec at 3900 rpm. For the liquid–liquid extraction (LLE), 10 µL (reduced feeding regime) or 20 µL (increased feeding regime) of plasma was mixed with 200 µL of ultrapure water in a glass tube, followed by the addition of 1 mL diethyl ether (ROTIPURAN, Roth, Art. Nr. 3942.1). The sample was placed on a shaker at 1000 rpm for 10 min at room temperature. After the first LLE, samples were left for 10 min to allow the organic (diethyl ether) and the aqueous phase (water) to separate. The aqueous phase was then frozen in a dry ice bath, and the organic phase was carefully decanted into a new glass tube. The remaining aqueous phase was defrosted and frozen again to facilitate improved separation, as the two phases do not always separate cleanly after the first freezing. A second LLE was then performed by adding another 1 mL of diethyl ether to the defrosted aqueous phase, shaking again for 10 min at 1000 rpm at room temperature, and allowing it to separate for 10 min. The organic phase from this second extraction was also collected after freezing the aqueous phase in a dry ice bath and combined with the first diethyl ether extract. The pooled organic extracts were then evaporated to dryness using a dry heating block at 37 °C under a nitrogen stream. The dried samples were resuspended in 200–400 µL of assay buffer (Arbor Assays, Assay Buffer X053) provided with the ELISA (Enzymelinked Immunosorbent Assay) kit. Samples were stored overnight at 4 °C and subsequently analysed the following day. The dilution factors ranged between 10 and 90.

Scale preparation and hormone extraction

Scales collected from individual fish (n = 60) were analysed for cortisol levels. Prior to hormone extraction, fish scales were washed and ground following a modified protocol from Kennedy and Janz (2023, 2022). Each scales sample was washed four to five times with 4 mL methanol and vortexed for 150 sec per wash to remove slime and any external cortisol and ensure accurate measurement of internal cortisol concentrations. After each wash, the methanol was decanted, and fresh methanol was used for the next wash. The washed scales were then dried on filter paper in a petri-dish with off-set lid to allow air flow for approx. 24 hours at room temperature. Following drying, the scales were ground in a 10 mL stainless steel grinding jar with a 12 mm stainless steel grinding ball at a frequency of 30 Hz using a Retsch ball mill MM 400. The grinding was done in 10-sec intervals (total of 100 sec), with jars pre-cooled to -80°C for 15 min to prevent the scales from clumping and to minimize thermal damage to the scale material caused by friction. After each 10-sec interval, the jar walls were scraped to remove accumulated scales, and the sample was stirred before continuing. Grinding was complete when the sample material was a relatively homogeneous, fine powder. After the grinding procedure, each jar was cleaned with 70 % ethanol and a spatula to prevent

cross-contamination. The ground scale material was weighed with a fine scale (Satorius Entris224i-1s, readout=0.1 mg) and transferred into a 2 mL microcentrifuge tube. The amounts of scale material used for extraction ranged between 0.01 g to 0.11 g, with a mean of 0.06 g per sample.

Subsequently, 1 mL of methanol (Supleco EMSURE Methanol for analysis, Ref. No. 1.06009.1011) was added to the scale material, and the sample was vortexed for 15 sec. The tubes were then placed on an orbital shaker at ~ 1000rpm for 18 hours at room temperature. Afterwards, the samples were centrifuged for 15 min at 4500 rpm at room temperature. The methanol supernatant was transferred into a 10 mm glass tube. Then, 1 mL of fresh methanol was added to the original 2 mL Microcentrifuge tube containing the powdered scale material and vortexed for 40 sec. This extraction procedure was repeated for a total of three times. After the third extraction, the methanol was completely evaporated under a gentle nitrogen stream at 38 °C using a sample concentrator with a dry heating block (Stuart block heater SBH200D/3). After, the inner walls of the glass tube were rinsed with 1 mL methanol to wash down any cortisol residue and samples were dried again at 38 °C under a nitrogen stream. This rinsing and drying process was repeated sequentially using 0.4 mL, 0.2 mL, and finally 0.15 mL methanol, with each step followed by complete evaporation under the same conditions. The dried sample was then reconstituted in 200 µL of assay buffer (Arbor Assays, Assay Buffer X053) provided with the ELISA kit (see below). The tubes were capped, gently vortexed, and incubated for 12 hours at 4 °C. The following day, the samples were removed from the refrigerator and briefly vortexed. The entire sample was transferred into a 0.6 mL microcentrifuge tube and centrifuged for 5 min to separate any remaining finely powdered scale material. The supernatant was then transferred to a clean 0.6 mL microcentrifuge tube for further analysis.

Serum and scale hormone quantitation

Cortisol was measured in plasma and scale samples with the DetectX® Cortisol ELISA Kit (Arbor Assays Catalogue No. K003, Lot No. 24CO23b, 23CO32c) according to the instructions of the manufacturer. All the reagents and samples were warmed to room temperature before use. The standard curve was diluted according to instructions, and resulted in a curve ranging from 50 – 3200 pg/mL.

For all standards and samples, 50 µL were pipetted into each well of the coated 96 well-plate except for 75 µL of 1X Assay Buffer for the NSB (non-specific binding) well. Then, 25 µL of DetectX® Cortisol Conjugate (Arbor Assays Cat. No. C039) and 25 µL of DetectX® Cortisol Antibody (Arbor Assays Cat. No. C041) were added to each well with an electronic multipipette. No cortisol antibody was added to the NSB wells. All standards and samples were measured in duplicate to ensure reliability and reproducibility of the results. The plate was then incubated for 1 hour on an orbital shaker at ~700rpm. After the first incubation, the plate was washed four times with 300 µL of wash

buffer (Arbor Assays Cat. No. X007, 1:20 dilution). Then 100 µL TMB (3,3',5,5'-Tetramethylbenzidine, Arbor Assays Cat.No. X019) was added to each well with an electronic multipipette and the plate was incubated in darkness for 30 mins. To stop the reaction, 50 µL of Stop Solution (1M hydrochloric acid, Arbor Assays Cat. No. X020) was added to each well, resulting in a colour change from blue to yellow. Absorption was measured at 450 nm within 10 min of stopping the reaction with a spectrophotometer (Thermo Fisher Multiskan Go, Ref. No. 51119200).

The performance of the ELISA kit was evaluated by calculating inter- and intra-assay variation using the coefficient of variation (% CV, SD/mean). The CV between duplicates was below 5 % for plasma samples and below 8 % for scales samples. Mean concentrations were corrected for the respective dilution factors used during sample preparation. Inter-assay variation (IAV), which reflects variability between plates, was determined across all assays runs and was 7 %, based on two control samples per plate (assay buffer containing 600 pg/mL cortisol provided by the manufacturer). Intra-assay variation, reflecting variability within the same plate, was calculated from duplicate control samples and was 1.35 % for plasma samples and 1.8 % for scale samples.

2.7. PIT tagging and body measurements

On arrival to the fish facility all individuals were PIT tagged, and body measures were taken. We measured weight in g and size (standard and total length) in mm before the feeding regime phase as well as at the end of the experiment after the blood sampling phase. The standard length (SL) was defined as the length between the most anterior tip of the body and the midlateral posterior edge of the hypural plate. The total length (TL) was defined as the length between the most anterior tip of the body and the longest extension of the caudal fin. For PIT tagging and the body measures taken before the feeding phase started, the fish were anesthetised by transferring and maintaining 4 to 6 individuals in a 40 L tank filled with tank water and an anaesthetic solution of MS-222 (60 mg/L) (E10521-Ethyl 3-aminobenzoate methanesulfonate) for 5 min until fish were unresponsive. The anesthetised fish were transferred to a water-filled tray, where the PIT tagging, and the size measurement was done. We inserted the PIT tags into the stomach cavity right in the middle between anal fin and pelvic fin while the head of the fish was still under water. Afterwards and while the fish fully emerged in the water, we used a vernier calliper to measure SL and TL. For the weight measurement, fish were transferred to a wet bowl placed on top of a standard scale.

2.8. Video analysis

To analyse the videos, we used the Behavioural Observation Research Interactive Software BORIS v. 8.21.8 – 2023-10-05 (Friard and Gamba, 2016). In the motivation-control task and in the detour task, we recorded the latency until test fish reached the shadow and hid under the wooden board. The

latency was calculated as the time interval (in seconds) between the start of the trial (when the test fish was placed in the test tank and when the fish entered inside the shadow (when both pectoral fins disappeared underneath the wooden board). Additionally, in the detour task, we recorded the direction of the detour (over or around the barrier) and the number of failed attempts (bumping against the wall).

2.9. Statistical analysis

We used R-Studio 2024.04.1 Build 748 to analyse the data. The significance threshold was set at $p < 0.05$ unless stated otherwise. We tested for normality using a 'Shapiro-Wilk test' and subsequently applied 'Student-t-tests', 'Mann-Whitney-U-tests', 'Spearman's Rho Correlations', 'linear mixed effects model (LMM), or 'generalized linear mixed effect models (GLMM)' depending on the structure of the data. To create the box plot- and line-diagrams, we used the R package ggplot2. To obtain a measure for the nutritional state of individuals, we calculated a body condition index, also called the Fulton's condition factor, calculated used the following formula: (weight (g) / standard length^3 (cm)) x 100 g (Sutton et al., 2000).

For the tests, or models that investigate the differences between the two tasks we always used a factor 'type of task' with two levels (detour task, motivation-control task) as the independent variable. For the tests, or models that investigate the differences between the two feeding regimes we always used a factor 'feeding regime' with two levels (reduced diet, increased diet) as the independent variable. LMMs and GLMMs were fitted with the 'lmer' or 'glmer' function in the lme4 R package (Bates et al., 2015), respectively. When significant interactions were identified, we fitted post hoc tests using Mann-Whitney-U-Tests with an adjusted significance threshold (α-value) using a Bonferroni correction for multiple testing. In all LMMs and GLMMs applied to behavioural assay phase we also always included the order of the task (i.e. whether the detour was the first or the second task) and the two-way interaction with the independent variable 'type of task' or 'feeding regime', depending on the question. In the LMM applied to the blood sampling phase we included the sampling time (early, mid-time, late), the standard length and the two-way interaction with the independent variable 'feeding regime'. We included 'holding tank ID' as a random effect in all LMMs and GLMMs to account for a potential influence of the holding tank (i.e. individuals that are were kept in the same group and in the same holding tank before the behavioural assay).

3. Results

3.1. Validation of the feeding regime treatments

(1) Is there a weight or body size difference between the fish before they were assigned to the two treatment groups?

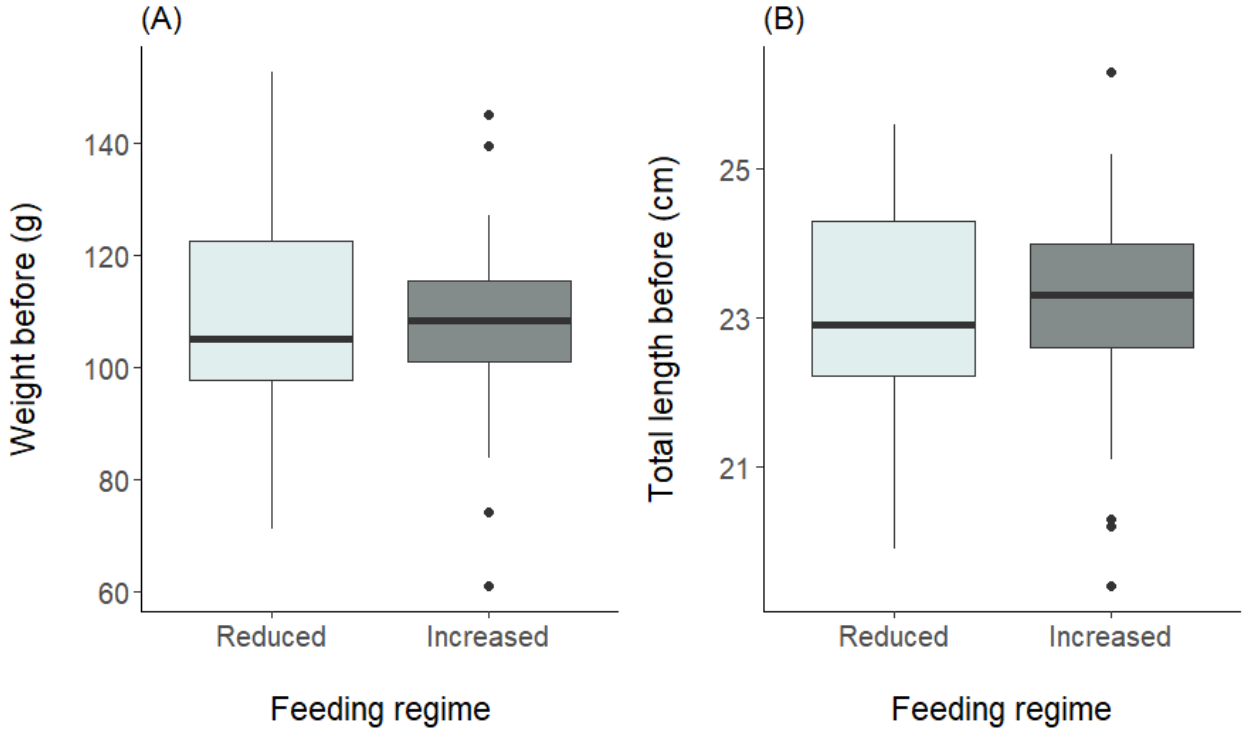

Figure 4 Body measures of test fish before the feeding phase started did not differ. (A) The weight in g and (B) the total length in cm of test fish that were later assigned to the reduced (n = 51 light boxes) or increased (n = 51, dark boxes) feeding regime.

Table 1 Statistical tests and results for the body measurements taken before the feeding phase started. Test fish that were later assigned to the two feeding regimes did not differ in size or weight.

Dependent variable	**Test**					**Figure**
	Shapiro-Wilk-Test		**W**	-	**p-value**	
weight before		weight before	0.99	-	0.372	-
size before		total length before	0.98	-	0.3	-
	T-Test		**t-value**	**df**	**p-value**	

weight before	weight before ~ treatment	0.01	89.43	0.991	Figure 4 (A)
size before	total length before ~ treatment	-0.34	90.44	0.732	Figure 4 (B)

There was no statistically significant difference in body weight and size before the feeding phase started (weight before: (Student-t-test; weight before: t(89.43) = 0.01, p = 0.991; size before: t(90.44) = -0.34, p = 0.732); see Table 1, Figure 4).

(2) Did the feeding regimes lead to a pronounced difference in body condition, weight, or growth between the two treatment groups?

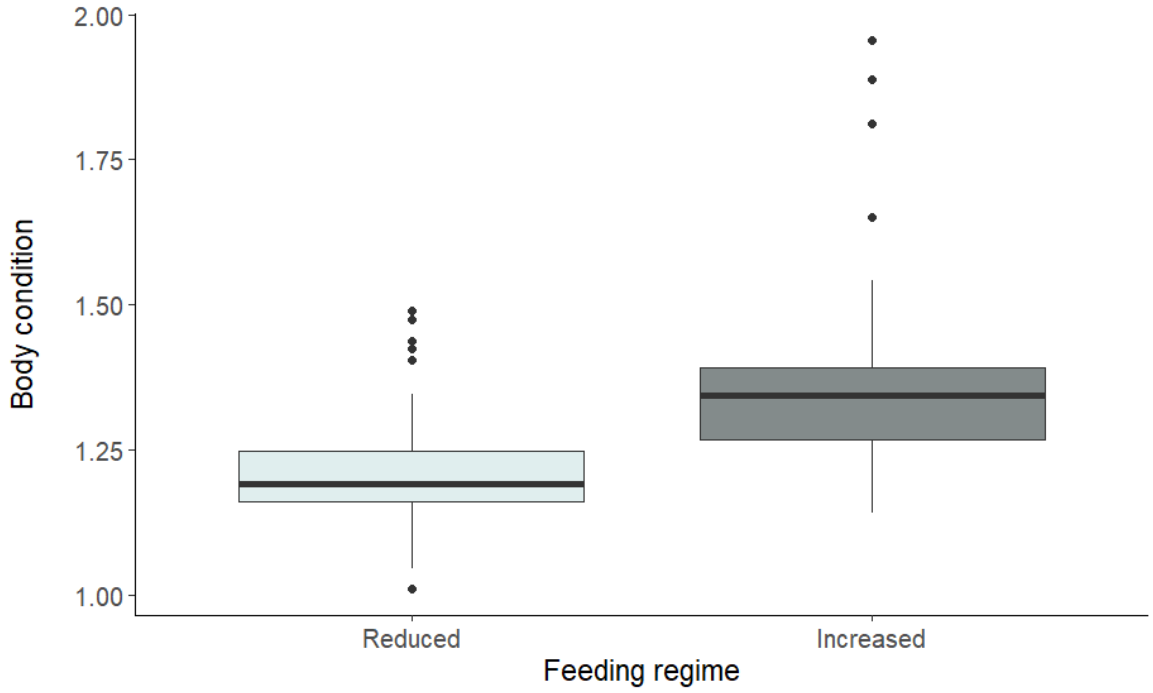

Figure 5: Body condition (for calculation see Statistical analysis) of fish at the end of the experiment significantly differed depending on their feeding regime. After receiving either a reduced (n = 46, light box) or increased (n = 47, dark box) feeding regime.

Dependent variable	Test		W	p-value	Figure
body condition	**Shapiro-Wilk-Test**	body condition after	0.87	< 0.001	-
	Mann-Whitney-U-Test	body condition after ~ treatment	326	< 0.001	Figure 5

Table 2 Statistical tests and results for body condition of fish calculated at the end of the experiment

after receiving either an increased or reduced feeding regime. Fish on the increased feeding regime had a higher body condition than fish on the reduced feeding regime.

There was a statistically significant difference in body condition after the feeding phase (Mann-Whitney-U-Test: W = 326, p < 0.001; see
Table 2, Figure 5).

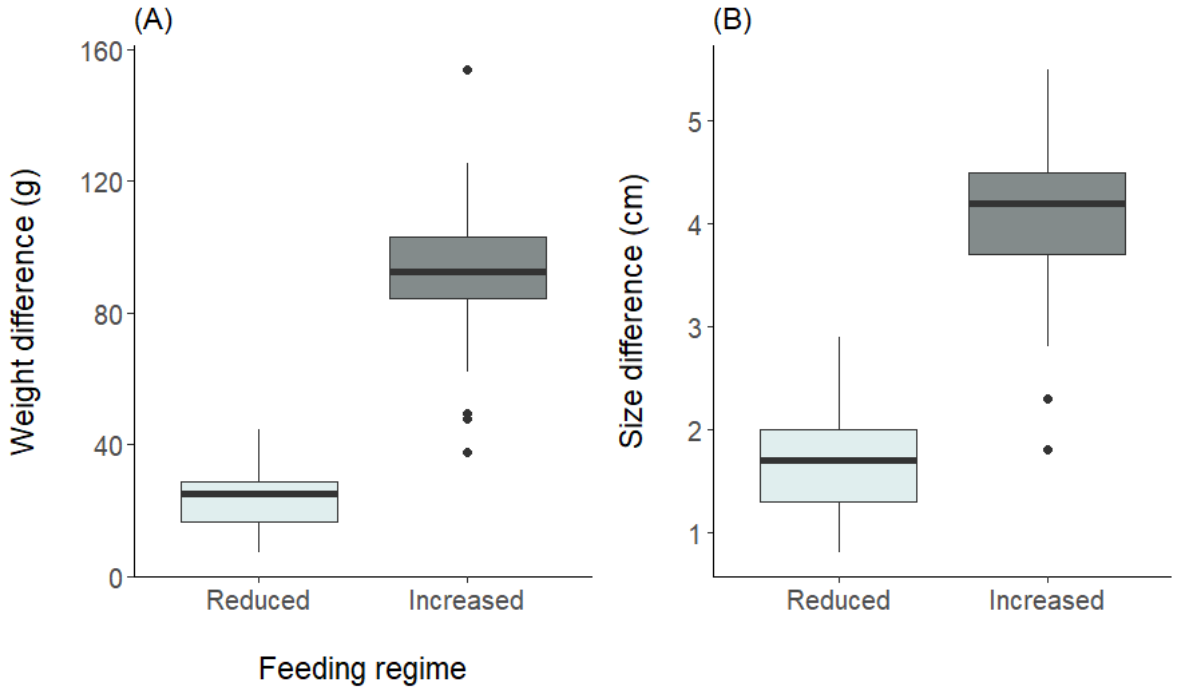

Figure 6: Body size measurements of fish at the end of the experiment significantly changed depending on their feeding regime. (A) the weight in g, and (B) the total size in cm of after receiving either a reduced (n = 46, light box) or increased (n = 47, dark box) feeding regime.

Table 3 Statistical tests and results for body measures of fish taken at the end of the experiment after receiving either an increased or reduced feeding regime. Fish on the increased feeding regime gained more weight and grew the difference in body condition, weight, or growth between the two treatment

Dependent variable	**Test**		**W**	**p-value**	**Figure**
body condition	**Shapiro-Wilk-Test**	body condition after	0.87	< 0.001	-
	Mann-Whitney-U-Test	body condition after ~ treatment	326	< 0.001	Figure 5

groups: reduced and increased feeding regime.

Dependent variable	**Test**				**Figure**
	Shapiro-Wilk-Test		**W**	**p-value**	
weight gain		weight difference	0.89	< 0.001	-
growth		total length difference	0.91	< 0.001	-
	Mann-Whitney-U-Test				
weight gain		weight difference ~ treatment	3	< 0.001	Figure 6 (A)
growth		total length difference ~ treatment	22.5	< 0.001	Figure 6 (B)

There was a statistically significant difference in weight gain and growth after the feeding phase (weight gain: $W = 3$, $p < 0.001$; growth: $W = 22.5$, $p < 0.001$; see Table 3, Figure 6).

3.2. Validation of the behavioural assay

(3) Is the Detour task cognitively more challenging than the motivation-control task?

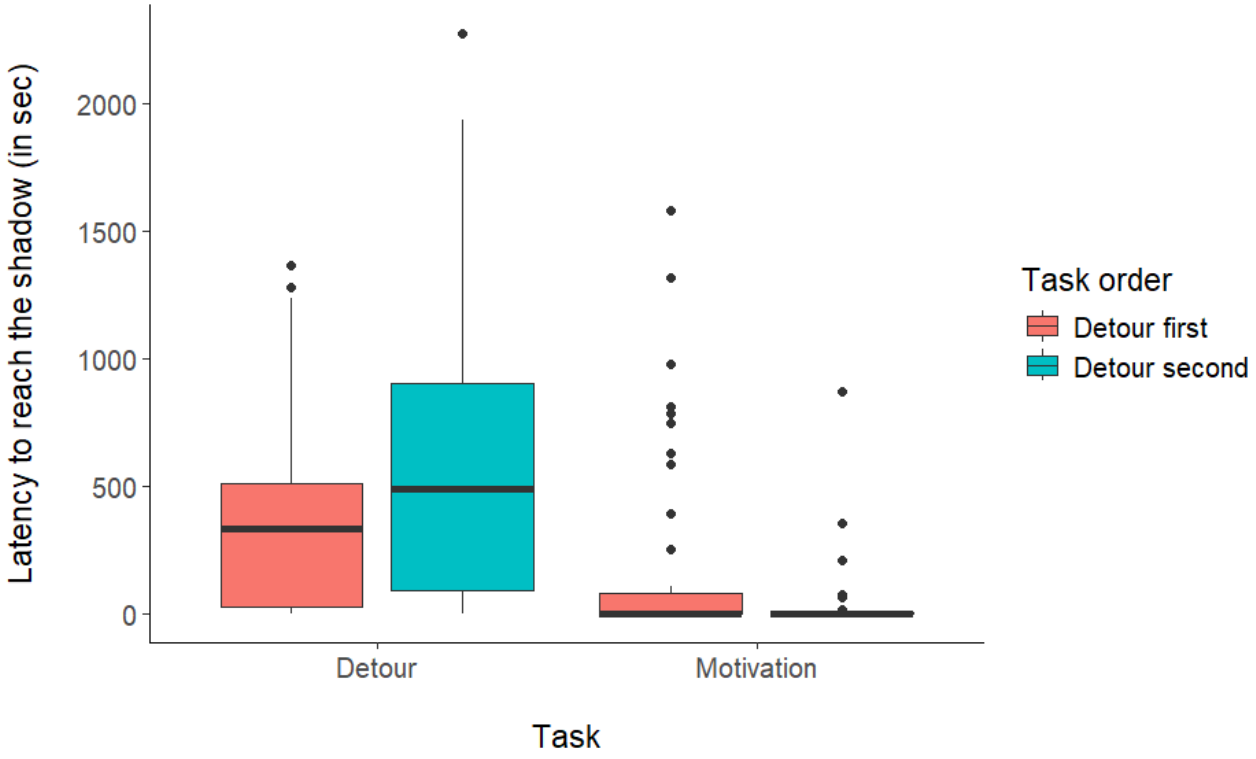

Figure 7 Latency of fish to reach the shadow (in seconds) in the detour task and motivation-control task depending on whether the detour task was the first (red) or the second task (blue). Irrespective of the order of the tasks, test fish took longer to reach the shadow in the detour task than in the motivation-control task.

Table 4 Statistical tests and results for the latency of fish to reach the shadow in the detour task and the motivation-control task depending on whether the detour task was the first or second task. The linear mixed model shows a significant interaction between 'task order' and the 'type of task'. Estimates are shown as difference to the reference level 'detour' and 'detour second' for factor 'task' and 'order'

Linear Mixed Model: sqrt(latency) ~ type of task * task order + (1|holding tank ID)					
Factor	**Estimate ± SE**	**Num. df**	**Den. Df**	**t-value**	**p-value**
Intercept	20.73 ± 1.66	-	-	-	-
Type of task	-17.68 ± 2.22	1	172	-7.95	< 0.001
Task order	-4.17 ± 2.26	1	172	-1.85	0.067
Interaction	8.98 ± 3.10	1	172	2.90	0.004
Mann-Whitney-U-Test				**W**	**p-value**

(Bonferroni adjusted significance threshold: 0.012): latency ~ task		
Detour as first task vs motivation-control as second task	1522	< 0.001
Detour as second task vs motivation-control as first task	1694.5	< 0.001
Detour task as first task vs. detour as second task	1039	0.094
Motivation-control as first task vs motivation-control as second task	663.5	0.001

The linear mixed effect model revealed a significant interaction between type of task and the order of the task (LMM, $F_{1,172} = 8.396$, $p = 0.004$, N = 93, see Table 4, Figure 7). The post hoc tests showed that fish took longer to reach the shadow in the detour task compared to the motivation-control task, irrespective of whether the detour was the first ($W = 1522$, $p < 0.001$; see Table 4, Figure 7) or the second task ($W = 1694.5$, $p < 0.001$; see Table 4, Figure 7). Furthermore, in the detour task, fish did not have a different latency when it was the first or the second task ($W = 1039$, $p = 0.094$; see Table 4, Figure 7). In contrast, in the motivation-control task, fish were faster to reach the shadow when it was the first task compared to when it was the second task ($W = 663.5$, $p = 0.001$; see Table 4, Figure 7).

3.3. The effects of nutritional state on behavioural flexibility

(4) Did fish on the reduced diet solve the motivation task faster than fish on the increased diet?

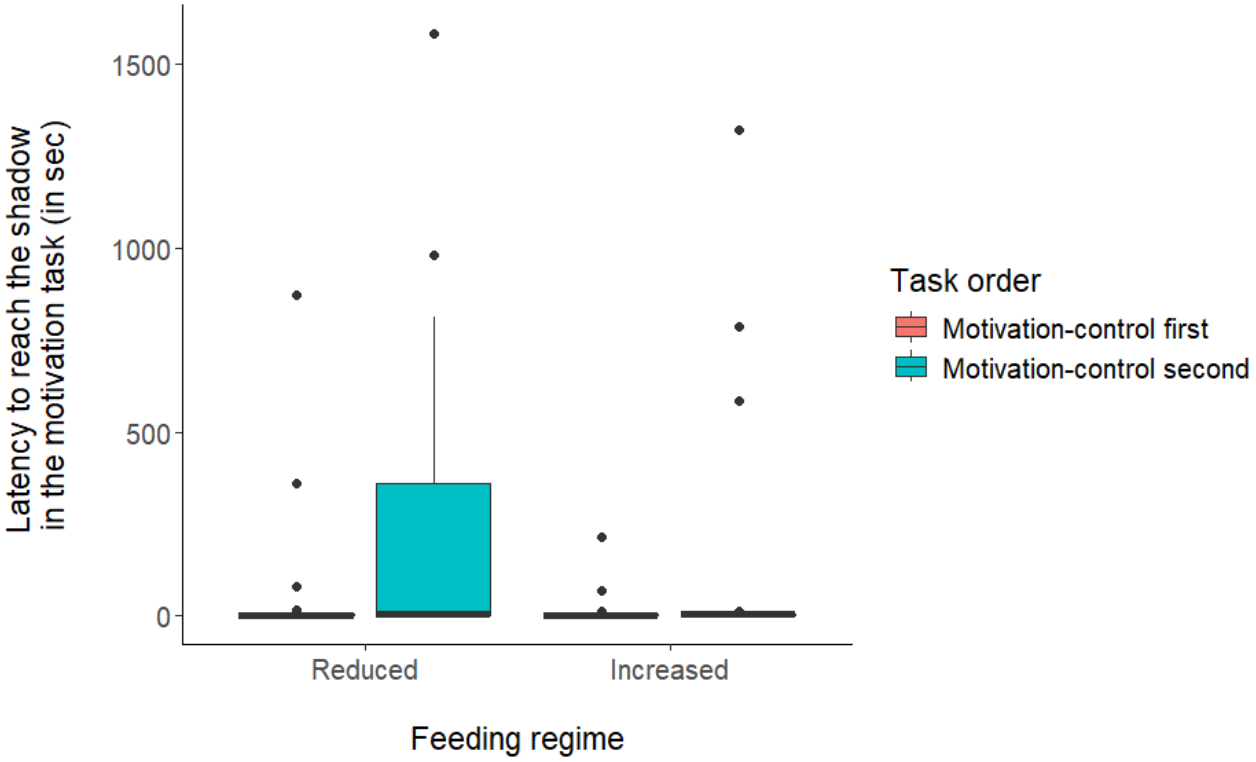

Figure 8 Latency to reach the shadow (in seconds) in the motivation-control task of fish on a reduced (n = 46) or increased (n = 47) diet, depending on whether the motivation-control task was the first (red boxes) or the second task (blue boxes).

Table 5 Statistical tests and results for the latency to reach the shadow in the motivation-control task of fish on a reduced or increased diet depending on whether the motivation-control task was the first or the second task. The linear mixed model showed no significant interaction between 'feeding regime' and 'task order'. Estimates are shown as difference to the reference level 'reduced feeding regime' and 'motivation first' for factor 'feeding regime' and 'task order'.

Linear Mixed Model: sqrt(latency motivation) ~ feeding regime * task order + (1|holding tank ID)					
Factor	**Estimate ± SE**	**Num. df**	**Den. df**	**t-value**	**p-value**
Intercept	3.72 ± 1.76	-	-	-	-
Feeding regime	-1.36 ± 2.48	1	89	-0.55	0.586
Order	6.73 ± 2.54	1	89	2.65	0.009
Interaction	-3.72 ± 3.57	1	89	-1.04	0.301

We found no significant interaction between feeding regime and order of the task (LMM, $F_{1,89} = -1.084$, $p = 0.301$, $N = 93$, see Table 5, Figure 8).

(5) Did fish on the reduced diet solve the detour task faster than fish on the increased diet?

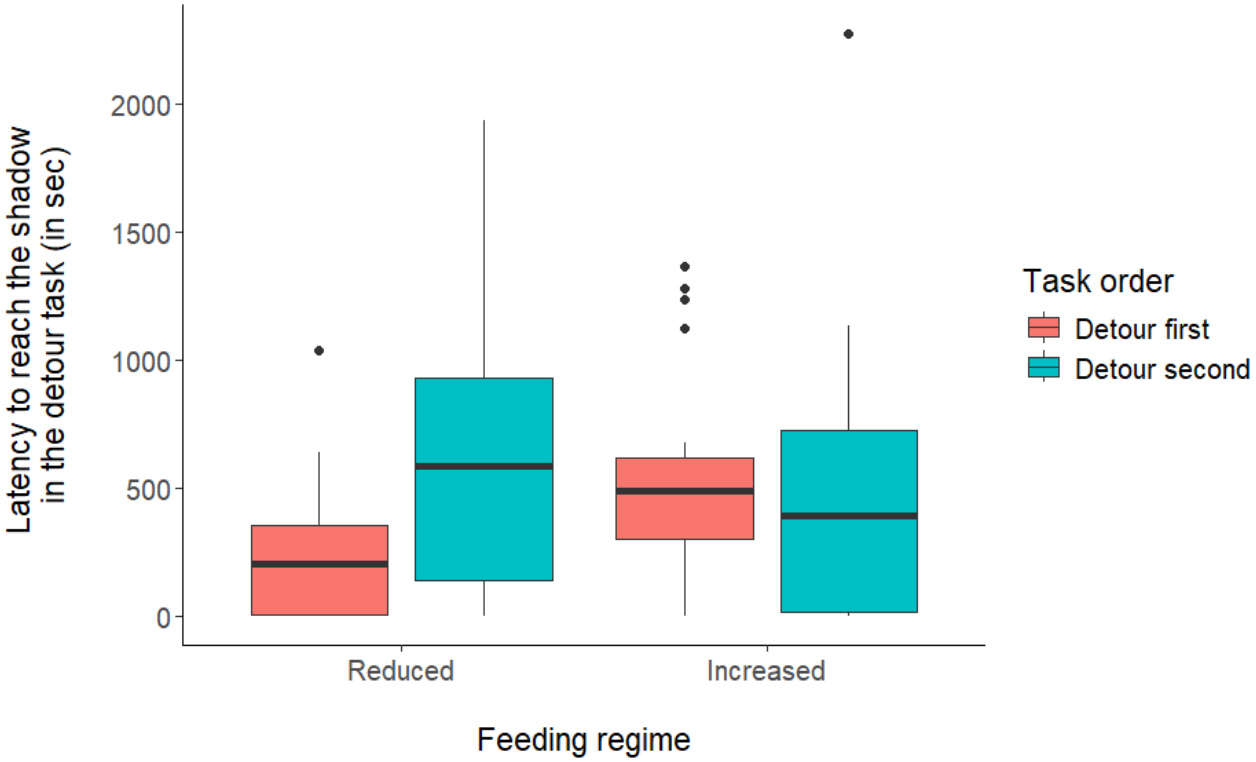

Figure 9 Latency to reach the shadow (in seconds) in the detour task of fish on a reduced (n = 40) or increased (n = 43) diet, depending on whether the detour was the first (red boxes) or the second task (blue boxes).

Table 6 Statistical tests and results for the latency to reach the shadow in the detour task of fish on a reduced or increased diet depending on whether the detour task was the first or the second task. The linear mixed model showed a significant interaction between 'feeding regime' and 'task order'. Estimates are shown as difference to the reference level 'reduced feeding regime' and 'detour second' for factor 'feeding regime' and 'task order'.

Linear Mixed Model: sqrt(latency detour) ~ feeding regime * task order + (1|holding tank ID)					
Factor	**Estimate ± SE**	**Num. df**	**Den. df**	**t-value**	**p-value**
Intercept	22.75 ± 2.68	-	-	-	-
Feeding regime	-3.84 ± 3.70	1	79	-1.04	0.302
Order	-10.38 ± 3.62	1	79	-2.87	0.005
Interaction	12.04 ± 5.02	1	79	2.40	0.019
Mann-Whitney-U-Test: Bonferroni adjusted significance threshold: 0.0125) latency detour ~ treatment				**W**	**p-value**
Detour is the first task				139	0.010

Detour is the second task	215	0.317
Detour task as first task vs detour as second task (reduced)	297	0.007
Detour task as first task vs detour as second task (increased)	216	0.745

We found a significant interaction between feeding regime and order of the task (LMM, $F_{1,79} = 5.75$, $p = 0.019$, $N = 83$, see Table 6, Figure 9). The post hoc tests showed that fish on the reduced diet had a shorter latency to reach the shadow than fish on the increased diet when the detour task was the first task ($W = 139$, $p = 0.010$; see Table 6, Figure 9). There was no difference in the latency to reach the shadow for fish on the two diets when the detour task was the second task ($W = 215$, $p = 0.317$; see Table 6, Figure 9). Fish on the reduced diet had a shorter latency to reach the shadow when the detour task was the first task than the second ($W = 297$, $p = 0.007$; see Table 6, Figure 9). Fish on the increased diet did not differ in the latency to reach the shadow when the detour task was the first or the second task ($W = 216$, $p = 0.745$; see Table 6, Figure 9).

(6) Did fish on the reduced diet have less failed attempts before solving the detour task than fish on the increased diet?

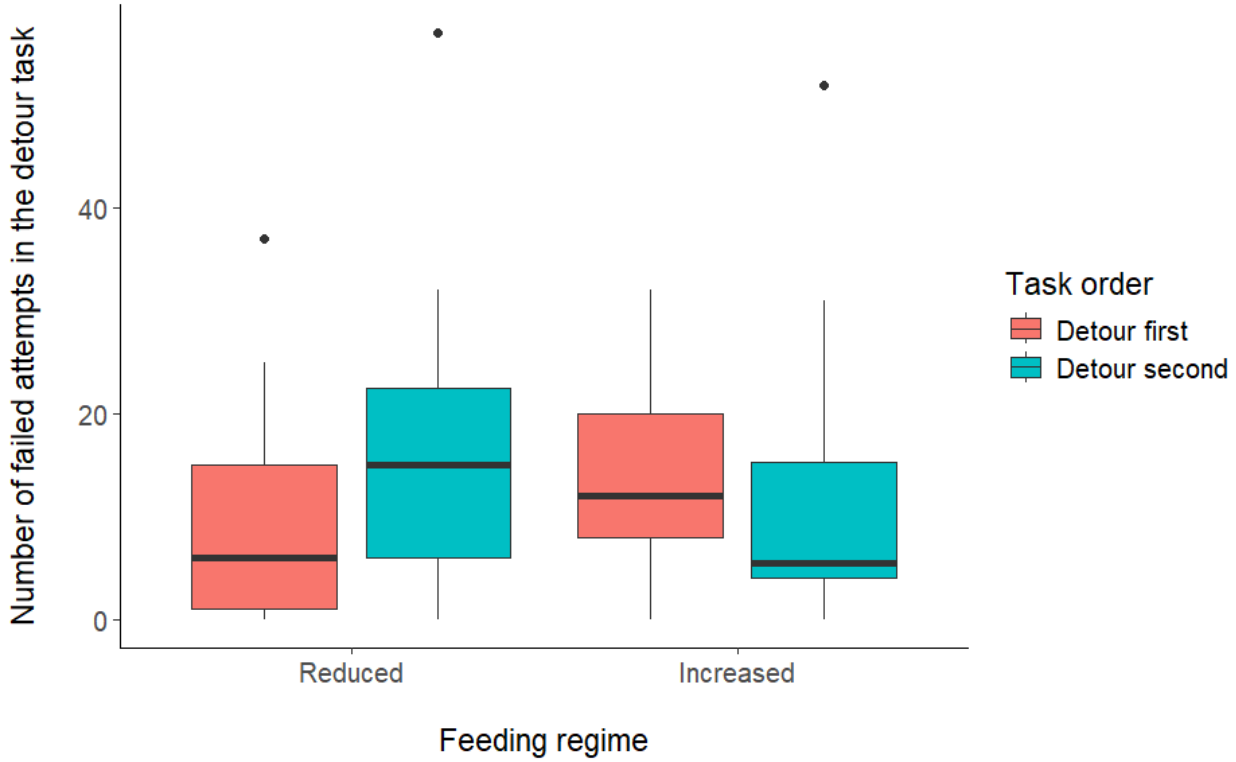

Figure 10 Number of failed attempts in the detour task of fish on a reduced (n = 46) or increased (n = 47) diet, depending on whether the detour was the first (red boxes) or the second task (blue boxes).

Table 7 Statistical tests and results for the number of failed attempts before solving the detour task of fish on a reduced (n = 46) or increased (n = 47) diet, depending on whether the detour was the first or the second task.

Generalised Linear Mixed Model: failed attempts ~ feeding regime * task order + (1|holding tank ID)				
Factor	**Estimate ± SE**	**Num. df**	**z-value**	**p-value**
Intercept	2.77 ± 0.14	-	-	-
Feeding regime	-0.40 ± 0.21	1	-1.93	0.053
Task order	-0.55 ± 0.09	1	-6.33	< 0.001
Interaction	0.77 ± 0.12	1	6.41	< 0.001
Mann-Whitney-U-Test: Bonferroni adjusted significance threshold: 0.0125) failed attempts ~ treatment			**W**	**p-value**
Detour is the first task			169	0.057
Detour is the second task			363.5	0.121
Detour task as first task vs detour as second task (reduced)			351	0.057
Detour task as first task vs detour as second task (increased)			203	0.122

We found a significant interaction between feeding regime and the order of the task (GLMM, $z = 6.41$, $p < 0.001$, $N = 93$, see Table 7, Figure 10). Although the interaction was significant, post hoc test showed that fish on the increased diet did not have more failed attempts than fish on the reduced died before solving the detour task irrespective of whether the detour task was the first ($W = 169$ $p = 0.057$; see Table 7, Figure 10) or the second task ($W = 363.5$, $p = 0.121$; see Table 7, Figure 10). Fish within the feeding regimes had no significant difference in the number of failed attempts in the detour task between the two task orders (reduced: $W = 351$, $p = 0.057$; increased: $W = 203$, $p = 0.122$; see Table 7, Figure 10).

3.4. The effects of nutritional state on physiological stress response

(7) Did fish on the increased diet have higher plasma cortisol levels than fish on the reduced diet?

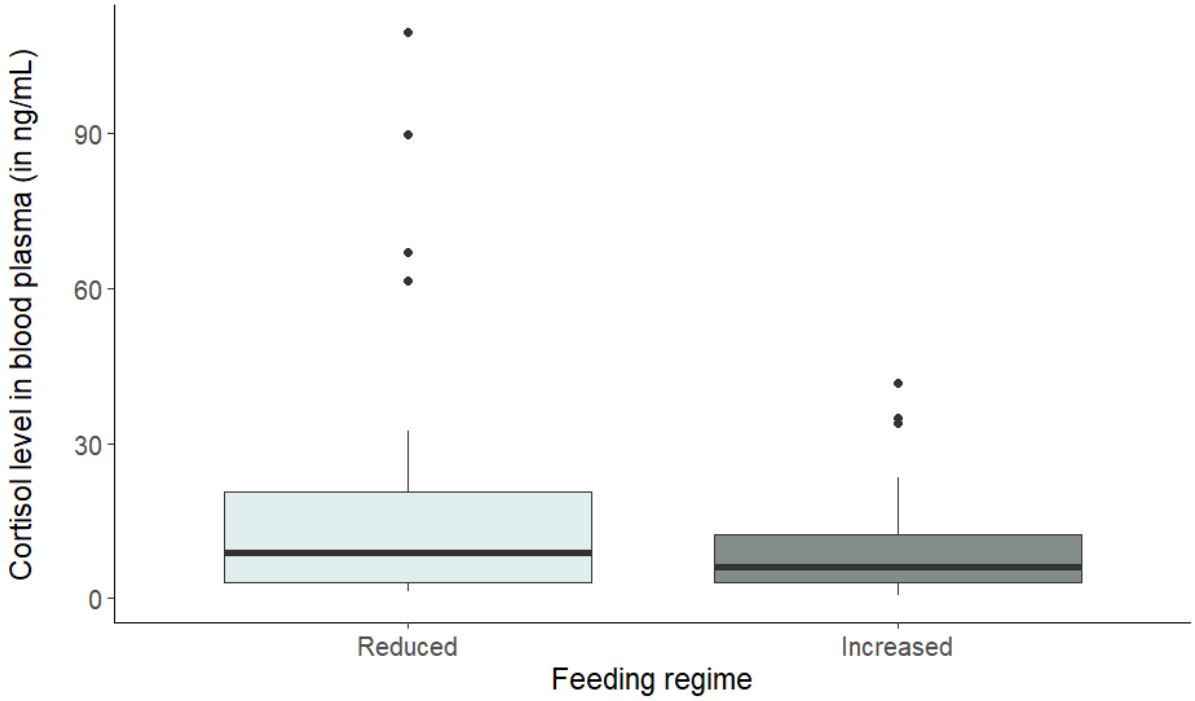

Figure 11 Plasma cortisol levels are reported in ng/mL, for the two feeding regime treatments (reduced: n = 30, light box and increased: n = 30, dark box). Plasma cortisol levels of fish after the experimental phase did not significantly differ depending on their feeding regime.

Table 8 Statistical tests and results for the blood plasma cortisol value after the experimental phase. The blood plasma cortisol value did not differ depending on feeding regime (reduced and increased).

Dependent variable	**Test**					**Figure**
Plasma cortisol value	**Shapiro-Wilk-Test**		**W**	-	**p-value**	
		plasma cortisol value	0.63	-	< 0.001	-
		log(plasma cortisol value)	0.98	-	0.60	-
	Kolmogorov-Smirnov-Test		**D**	-	**p-value**	
		plasma cortisol value	0.25	-	< 0.001	-
		log(plasma cortisol value)	0.09	-	0.33	-

T-Test		t-value	df	p-value	
	log(plasma cortisol value) ~ treatment	1.35	56.62	0.18	Figure 11

The Shapiro-Wilk-Test (W = 0.98, p = 0.60) and a Kolmogorov-Smirnov-Tests (D = 0.09, p = 0.33) confirmed that a log transformation is required to obtain a normal distribution (see

Table 8). There was no statistically significant difference in plasma cortisol level between the two groups (t(1.35) = 56.62, p = 0.18); see Table 8, Figure 11).

Figure 12 Plasma cortisol levels are reported in ng/mL during sampling time periods (early, middle, late) for the two feeding regime treatments (reduced and increased).

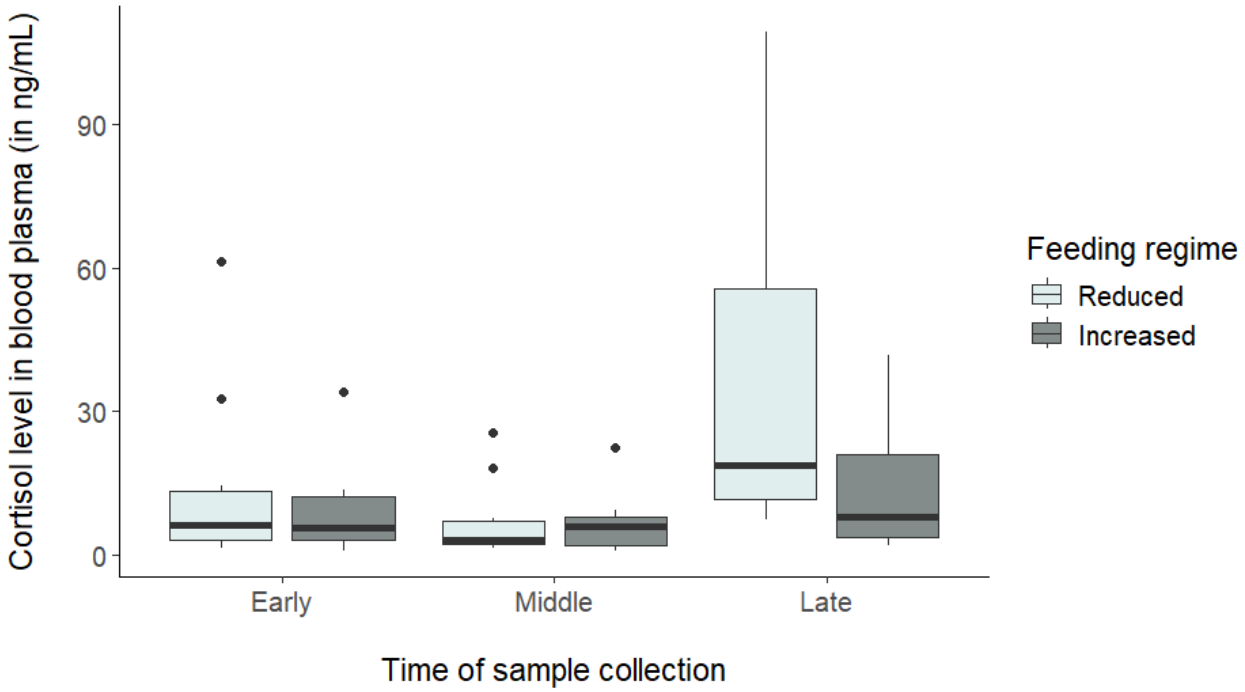

Table 9 Statistical tests and results for the cortisol concentration in blood plasma depending on the feeding regime treatment and the sampling time. The linear mixed model showed no significant interaction between the feeding regime treatment and sampling time was thus dropped from the final model. Estimates are shown as difference to the reference level 'reduced feeding regime' and 'early' for factor 'feeding regime' and 'sampling time'.

Linear Mixed Model: log(plasma cortisol value) ~ feeding regime + sampling time + standard length + (1|holding tank ID)					
Factor	**Estimate ± SE**	**Num. df**	**Den. df**	**t-value**	**p-value**
Intercept	2.96 ± 2.78	-	-	-	-
Feeding regime	-0.31 ± 0.42	1	55	-0.75	0.46

Sampling time (mid-time)	-0.36 ± 0.35	2	55	-1.04	0.30
Sampling time (late)	0.83 ± 0.35	2	55	2.37	0.02
Standard length	-0.04 ± 0.12	1	55	-0.34	0.73
T-Test (Bonferroni adjusted significance threshold: 0.25): log(plasma cortisol value) ~ sampling time			t	df	p-value
Sampling time early vs late			-2.28	38.00	0.028
Sampling mid-time vs late			3.50	37.35	0.001

The linear mixed effect model revealed no significant interaction between feeding regime treatment and sampling time (LMM, $F_{2,53}$ = 1.187, p = 0.313, N = 60). However, timing of the sampling significantly influenced cortisol concentrations (LMM, $F_{2,55}$ = 6.107, p = 0.004, N = 60). The post hoc tests showed that fish sampled late had higher plasma cortisol levels compared to samples collected early (t(38.00) = -2.28, p = 0.028) or mid-way (t(37.35) = 3.50, p = 0.001) (see Table 9).

Dependent variable	**Test**		**t**	**df**	**p-value**	**Figure**
plasma cortisol value (only late)	**T-test**	log(plasma cortisol value) ~ treatment	2.22	17.66	0.04	Figure 12

Table 10 Statistical test and results for the blood plasma cortisol value after the experimental phase on the sample time point late. The blood plasma cortisol value significantly differed depending on feeding regime (reduced and increased).

We focused our further analysis only on the sample time point late, after confirming that all samples collected later, irrespective of the feeding regime, had higher cortisol concentrations than samples collected earlier. We found a statistically significant difference in plasma cortisol level (Student-t-test, t(2.22) = 17.66, p = 0.04), indicating that fish sampled late on the reduced diet had higher cortisol concentrations than fish on the increased diet (see Table 10).

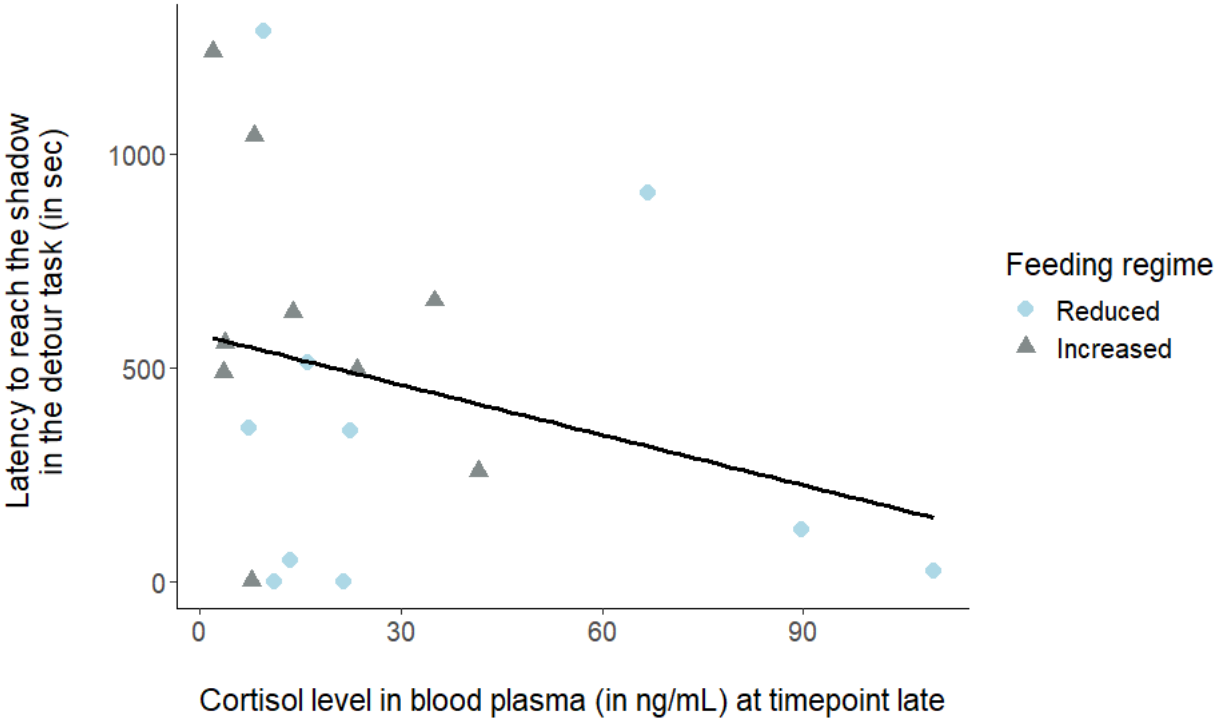

Figure 13 Relationship between plasma cortisol levels, reported in ng/mL, at timepoint late and the latency to reach the shadow in the detour task in fish of both reduced (n = 30, light points) and increased (n = 30, dark triangle) feeding regime treatment groups. A black trend line indicates a weak negative correlation between plasma cortisol levels and the latency to reach the shadow in the detour task.

We conducted three Spearman's Rho Correlations with log transformed data to determine the relationship between the latency to solve the detour task and the plasma cortisol level (a) in all individuals sampled at timepoint late, (b) individuals of the reduced treatment group, and (c) individuals of the increased treatment group. There was no significant relationship between the latency to solve the detour task and the plasma cortisol value (a) in all individuals ($r_s = -0.24$; $p = 0.314$), (b) in fish of the reduced treatment group ($r_s = -0.21$; $p = 0.567$) and (c) in fish of the increased treatment group ($r_s = -0.22$; $p = 0.581$) (see Figure 13).

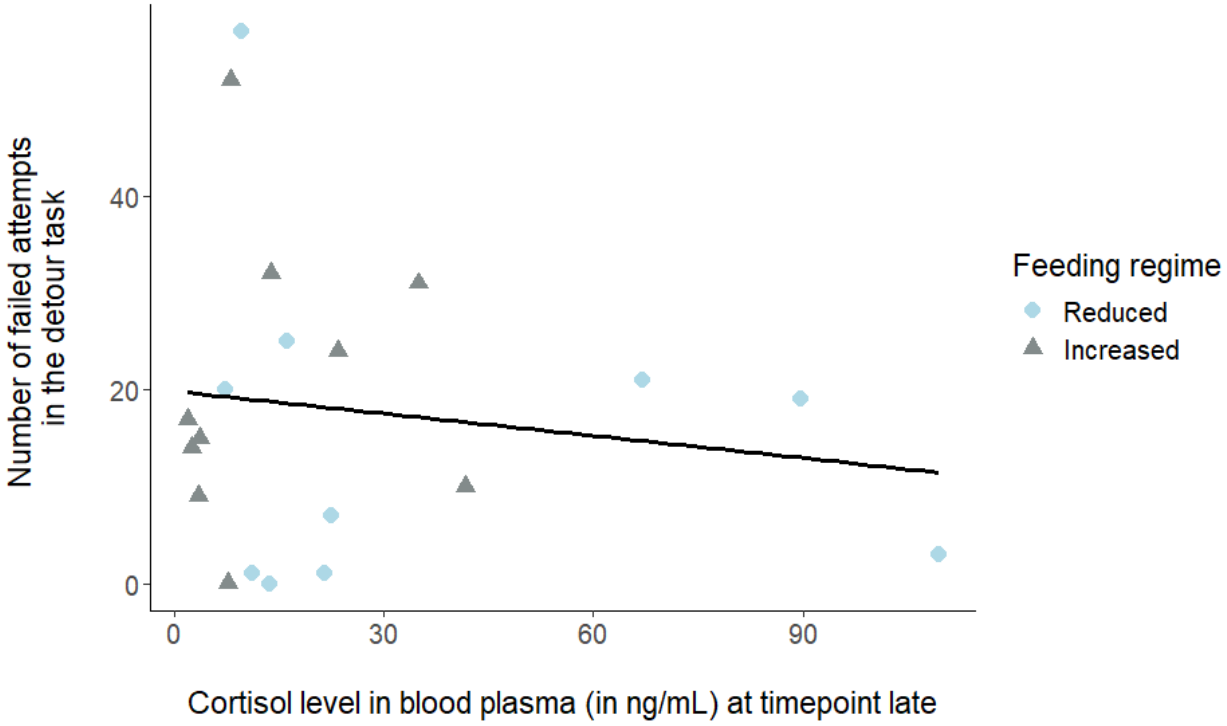

Figure 14 Relationship between plasma cortisol levels, reported in ng/mL, at timepoint late and the number of failed attempts in the detour task in fish of both reduced (n = 30, light points) and increased (n = 30, dark triangle) feeding regime treatment groups. A black trend line indicates a weak negative correlation between plasma cortisol levels and the number of failed attempts in the detour task.

We conducted three Spearman's Rho Correlations with log transformed data to determine the relationship between the number of failed attempts before solving the detour task and the plasma cortisol level (a) in all individuals sampled at timepoint late, (b) individuals of the reduced treatment group, and (c) individuals of the increased treatment group. There was no significant relationship between the number of failed attempts before solving the detour task and the plasma cortisol value (a) in all individuals ($r_s = 0.05$; $p = 0.845$), (b) in fish of the reduced treatment group ($r_s = -0.13$; $p = 0.713$) and (c) in fish of the increased treatment group ($r_s = 0.28$; $p = 0.427$) (see Figure 14).

(8) Did fish on the increased diet have higher scale cortisol levels than fish on the reduced diet?

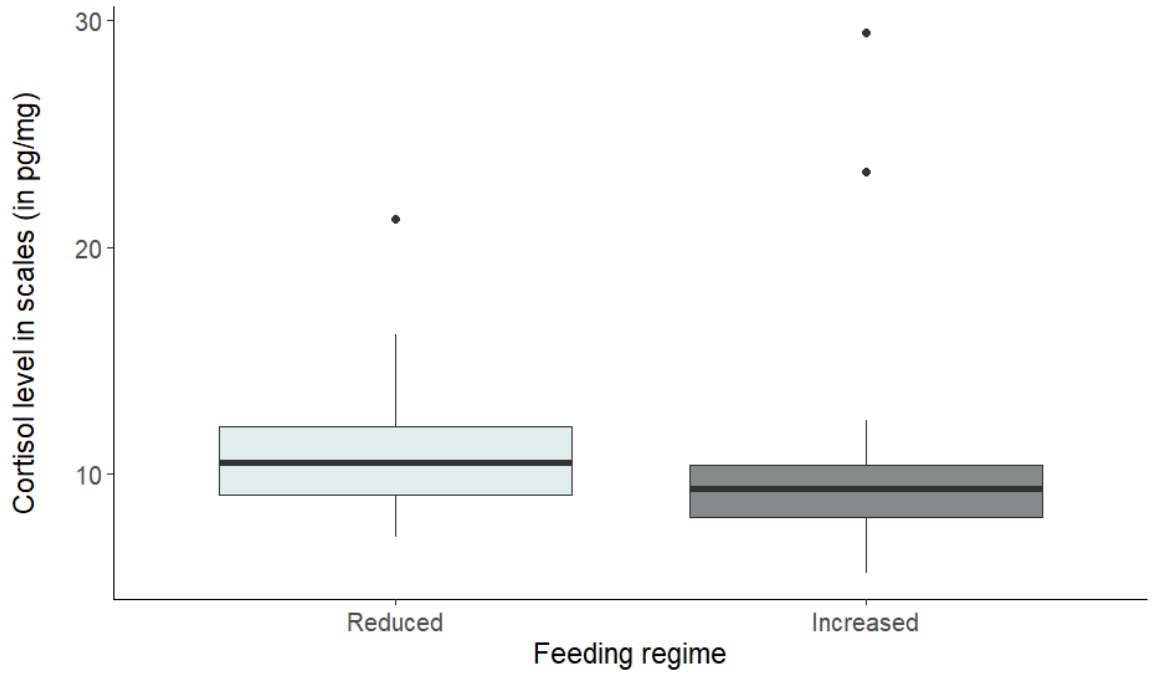

Figure 15 Scale cortisol levels are reported in pg/mg, for the two feeding regime treatments (reduced: n = 28, light box and increased: n = 25, dark box). Scale cortisol levels of fish after the experimental phase did not significantly differ depending on their feeding regime.

Table 11 Statistical tests and results for the scale cortisol levels after the experimental phase. The scale cortisol value did not differ depending on feeding regime (reduced and increased).

Dependent variable	**Test**			**Figure**
Scale cortisol value	**Shapiro-Wilk-Test**	**W**	**p-value**	
	scale cortisol value	0.74	< 0.001	-
	Kolmogorov-Smirnov-Test	**D**	**p-value**	
	scale cortisol value	0.21	< 0.001	-
	Mann-Whitney-U-Test	**W**	**p-value**	
	scale cortisol value ~ treatment	430	0.092	Figure 15

The Shapiro-Wilk and Kolmogorov-Smirnov tests showed that the variable (scale cortisol value) did not follow a normal distribution ($W = 0.74$, $p < 0.001$; $D = 0.21$, $p < 0.001$; see Table 11). There was no statistically significant difference in scale cortisol level after the experimental phase ($W = 430$, $p = 0.092$; see Table 11, Figure 15).

4. Discussion

In the current study, we used a commercially important fish species, the Rainbow trout to assess the influence of nutrition on behavioural flexibility and stress responses.

We found that fish on the increased diet need more time solved the detour task than those on the reduced diet, but only in a novel situation, when the detour task was performed first. Further, fish on the increased diet showed significantly lower cortisol concentrations in blood plasma, while there was no difference in scale cortisol levels. This suggests that nutritional state influenced the behavioural flexibility and the acute physiological stress response.

Validation of the feeding regimes

The potential effects of differences in feeding prior to the start of the feeding regimes were investigated to control for the fact of individual variability in growth and weight among the fish. This step was important to ensure that observed differences during the experimental phase can only be explained by the feeding regime treatments rather than by pre-existing differences. Initial measurements showed no statistically significant differences in body weight or size among the fish before they were assigned to the two treatment groups. This finding indicates that both treatment groups started the feeding phase from a comparable baseline in terms of body weight or size.

After one month on the respective feeding regimes, the fish had pronounced differences in body condition. Fish assigned to the increased feeding regime exhibited notable weight gain and enhanced growth compared to those on the reduced feeding regime. These differences in body condition were statistically significant and reflect significant differences in weight gain and growth rate. These results confirm the effectiveness of the feeding regimes in creating two distinct groups with different body conditions: one with a lower (reduced feeding regime treatment) and one with a higher body condition (increased feeding regime treatment).

The successful creation of these two groups provided a strong foundation for the subsequent behavioural assay phase and sampling phase to examine differences in behavioural flexibility and stress response between fish with lower and higher body condition.

Validation of the behavioural assay

Since the detour test included both a motivation-control task and a detour task, we predicted that the detour task would be cognitively more challenging, leading to a longer latency to solve this task due to the fact that fish had to detour a transparent barrier, compared to the motivation-control task without a barrier. Our results support this expectation, as fish took longer to solve the detour task compared to the motivation-control task, irrespective of the order of the tasks. Nevertheless, the order of the tasks did affect the latency to reach the shadow in the motivation-control task, but not in the detour

task. Fish reached the shadow more quickly when the motivation-control task was performed first, compared to when it was performed second. This result is in line with previous studies suggesting that prior experience with obstacles or (frightening) stimuli can influence subsequent navigation strategies (Odling-Smee and Braithwaite, 2003; Yue et al., 2004). The longer latencies when the motivation-control task was second could be explained by a negative conditioned learning effect. The prior experience of a barrier likely caused them to hesitate more during the subsequent motivation-control task compared to fish tested in the motivation-control task fist, that had no prior experience with a barrier. This hesitation may also indicate a carryover effect from the initial detour task, where fish had to navigate around a barrier (Braithwaite and Girvan, 2003; Odling-Smee and Braithwaite, 2003; Yue et al., 2004).

One may argue, that fish exhibit spatial learning and have the ability to learn and navigate within their environment efficiently by memorizing landmarks and routes (Bshary and Brown, 2014; Oliveira and Galhardo, 2009). Especially, anadromous species, such as the Rainbow trout or the Atlantic salmon can successfully travel across oceans (Rodríguez et al., 2021). In this study, we used green wires and two grey stones to fix the barrier to the ground under water, but the barrier itself remained fully transparent with no visible framing. This made it impossible for the fish to precisely determine the barrier's location without detailed exploration over a longer time. Thus, our findings are consistent with those reported in other studies, which highlight the learning abilities in fish and their capacity to use local and directional information, combined with memory, to orient and navigate through environmental routes (Bshary and Brown, 2014; Oliveira and Galhardo, 2009; Rodríguez et al., 2021).

Moreover, the fact that all individual fish successfully solved the motivation-control task provides strong evidence that the shadow is a suitable motivational factor for Rainbow trout. This suggests that shadows or shaded areas can act as attractive refuges for Rainbow trout, likely due to their association with shelter and predator avoidance (Kelley and Magurran, 2003; Laland et al., 2003).

The effects of nutritional state on behavioural flexibility

Latency to reach the shadow in the motivation-control task

In the motivation-control task, we did not observe any significant differences in the speed of solving the task between fish on the reduced or increased diet, regardless of the order of the task. Besides the fact that fish in both groups just simply were equally fast in solving the task, this result may be due to the generally lower latency to reach the shadow in the motivation-control task compared to the detour task. As the speed to solve the task was higher in the motivation-control task, it may not have been possible to make a meaningful distinction between the two treatment groups.

The primary functions of the motivation-control task were not to assess behavioural flexibility, but to determine whether fish perceive the shadow as a motivating factor and to rule out potential differences

in motivation between the two groups. By using a shadow as motivation factor, we ensured that both groups were equally motivated, since a food reward would have been highly problematic in the context of different feeding regimes and learning assays. Additionally, this task facilitated the differentiation between two conditions: a novel situation (detour task first / motivation-control task second) and a modified situation (motivation-control task first / detour task second). Thus, we did not expect to find significant differences in the motivation-control task between the two groups.

Additionally, when the motivation-control task was the second task, fish were able to adapt to the modified situation without a barrier, but with longer latencies to reach the shadow compared to when the motivation-control task was performed first with no difference between the two groups.

Latency to reach the shadow in the detour task

In our study we found that fish on the reduced diet solved the detour task faster than those on the increased diet, but only when the detour task was performed first. When the detour task was performed second, we observed no significant differences in the latency to solve the task. Within the reduced diet group, fish reached the shadow more quickly when the detour task was presented first, while the order of the task had no effect on the performance within the increased diet group. This suggests that, particularly in the reduced died group, task order had an effect on speed in the performance of the detour task.

The improved performance of fish on the reduced diet group may be explained by a heightened attentional state, driven by a stronger physiological stress response to novel situations (McEwen and Sapolsky, 1995; Oliveira and Galhardo, 2009). This corresponds to the theory of allostatic load, where acute stress activates an emergency response requiring additional energy from endogenous stores (e.g., fat, glycogen, and proteins) through short-term elevations in glucocorticoids like cortisol (McEwen and Wingfield, 2003). This helps the individual to respond adequately in stressful situations. First, by a focused response to the stressful situation that enhances sensory, perceptual, and cognitive mechanisms, including memory formation for learning (McEwen and Sapolsky, 1995; Oliveira and Galhardo, 2009); and second, by a subsequent decrease in cortisol levels. However, emergency responses are only activated when the allostatic load (i.e. the energy required to overcome a stressor) crosses a certain threshold (McEwen and Wingfield, 2003).

Fish on the increased diet, being better nourished, are likely to compensate more effectively for the additional energy requirements in stressful situations. Consequently, they experience fewer short-term cortisol elevations and do not reach the energetic threshold required for an emergency response. This damped physiological stress response (*cortisol levels in blood plasma* see below) could result in reduced attentional focus, impairing their performance in novel situations. Additionally, prolonged exposure to high cortisol-levels can lead to chronic states of stress, which may impair behavioural

flexibility and learning abilities (McEwen and Wingfield, 2003). This could explain, why fish on the increased diet performed worse when confronted with stressful novel situations. Conversely, fish on the reduced diet appeared to exhibit enhanced behavioural flexibility and learning abilities in stressful novel situations due to their stronger acute stress response.

When the detour task was performed second, there was no significant difference in the latency to reach the shadow between the feeding regime groups. This also suggests that task order played an important role in influencing behavioural flexibility in the detour task. For fish on the reduced diet, prior task experience with the motivation-control task may have diminished their performance. However, a lower performance could also be explained by a drop in concentration (Adriaenssens and Johnsson, 2011) or by a lower stress response due to familiarity with the task environment when the detour task was performed second.

Fish performing the detour task second may have associated the way how they reach the shadow with a direct, unobstructed path which they learned in the motivation-control task. When later confronted with the transparent barrier (in a modified situation), this prior expectation likely caused greater surprise, resulting in more hesitation or exploratory behaviour after bumping into the barrier, especially within the reduced group. In contrast, fish encountering the detour task first lacked such prior expectations and adapted more rapidly to this novel situation. It could be argued that individuals with more behavioural flexibility would adapt more quickly to the modified situation with a barrier. The transparency of the barrier presented an additional challenge, as unexpected and unpleasant collisions with it may provoke stronger hesitation (Yue et al., 2004). Since the shadow, which is also a visible stimulus, was crucial for motivating the fish, the barrier had to be transparent, which enforced a careful exploration of the environment to identify an alternative route.

Additionally, each task lasted 30 min, with a total of 60 min of testing and only a brief 7-min break in a holding net between tasks. This extended testing period may have contributed to a reduced focus during the second task, compared to other studies where longer intervals between tasks (e.g., 4 – 5 hours) allowed for more recovery (Adriaenssens and Johnsson, 2011; Johnsson and Fredrik Sundström, 2007). Adriaenssens and Johnsson (2011) and Johnsson and Fredrik Sundström (2007) used maze tasks to examine learning abilities in fish (Brown trout or Minnows). However, also, when the task in our study lasted in total 60 min, the continuous sampling lasted only 30 min. This could indicate a higher concentration and activity of fish in the first 30 min, followed by a decline in performance. This diminished concentration could also be linked to a decline in cortisol levels after an initial peak (after around 45 min) (Lai et al., 2021). Additionally, the handling stress before the first task, which involved catching and transportation of fish, was likely greater than before the second task, where fish were only briefly contained in a net underwater after the catching event. This

difference in handling stress may have resulted in lower physiological stress responses, reflected in reduced cortisol levels and subsequently reduced focused state during the second task.

One might argue that the cortisol peak at around 45 min could challenge the idea of lower cortisol concentrations during the second task, which began approx. 40 min after the first task started. However, the stressful situation does not begin with the first task itself but with the catching event when the fish were removed from the holding tanks, approx. 7 to 10 min before the first task started. This means that the stress response was already initiated before the task began. Moreover, for survival, an organism must exhibit adaptive behaviour immediately following a stressor and not delayed. Stress triggers not only the activation of the HPI axis with a release of glucocorticoids, but also the rapid release of adrenocorticotropic hormone (ACTH) (Dara et al., 2023; Herman et al., 2016; Kennedy and Janz, 2023; Koakoski et al., 2012; Lai et al., 2021; Lemos et al., 2023; Mommsen et al., 1999; Samaras et al., 2021) and catecholamines such as dopamine, noradrenaline, and adrenaline into the bloodstream, which peak within a few minutes (Reid et al., 1998). This supports the idea that fish, especially on the reduced diet, are more alert and responsive during the first task as they actively seek a solution.

Moreover, the familiar setting of the second task may have reduced stress (prior experience and less anticipation of unknown elements), even with the unexpected transparent barrier. Further, fish are not only familiar with the environment and its conditions, but also with the shadow, which they have already identified as a safe place where they feel comfortable (Kelley and Magurran, 2003; Laland et al., 2003). This prior experience likely reduced the novelty and the stress in the second task, further explaining the differences in performance between the two subsequent tasks.

Number of failed attempts

Regarding the number of failed attempts before successfully solving the detour task, we did not observe any significant differences between fish on the reduced diet and those on the increased diet, regardless of task order. Additionally, within each feeding regimes, there was no significant difference in the number of failed attempts dependent on the order of the task. This suggests that the feeding regime treatment did not influence the number of failed attempts in this context.

The number of failed attempts as a measurement of task-solving ability may not be as reliable as latency, as different motivations could lead an individual fish bumping against the transparent barrier. One possibility is that repeated bumping against the barrier represent persistent behaviour, which would align with the classical definition of a failed attempt due to a lack of behavioural flexibility (Lucon-Xiccato et al., 2022). In this case, fish that struggle to solve the detour task effectively (not successful) or efficiently (longer latency) might exhibit repeated failed attempts before giving up or succeeding. However, an alternative explanation is that the number of failed attempts reflect

exploratory behaviour, where individuals are actively investigating their environment – in this case, the transparent barrier – by touching it. This exploratory strategy could be beneficial, allowing fish to estimate the barrier's size and determine an optional way into the shadow.

If exploratory behaviour contributes to the number of failed attempts, fish with higher behavioural flexibility might exhibit more failed attempts, rather than fewer. Conversely, fish with lower behavioural flexibility may show fewer failed attempts, as they initially perform persistent behaviour before ultimately giving up. This discrepancy could be an indication of a weakness in this measurement, which raises concerns about the reliability of using failed attempts as an adequate measure of behavioural flexibility in this context. Therefore, to investigate this issue, we analysed our video recordings with an additional focus on differentiating between exploratory and persistent behaviour. Our results did not reveal a significant interaction between feeding regime and the type of behaviour exhibited in the detour task.

Furthermore, we found that approx. 80 % of the fish displayed exploratory behaviour, both across all individuals and within the respective treatment groups. This finding suggests that there is no significant difference in behavioural type (of failed attempts) between the two treatment groups, further highlighting the limitations of using the number of failed attempts as a measurement for behavioural flexibility. Specifically, the validity of this measure is questionable, if a higher number of failed attempts is interpreted as indication of lower behavioural flexibility and a lower number as an indication of higher behavioural flexibility.

Given these findings, further research is needed to assess whether the number of failed attempts is an adequate and comprehensive measurement of behavioural flexibility. While failed attempts may indeed reflect the accuracy with which fish solve a task, they capture a different aspect of learning than speed-related measurement, such as latency. Our study does not directly address the relationship between these two measurements. However, our results suggest that latency to reach the shadow might provide additional insights, particularly regarding the efficiency or speed of task solving ability.

In summary, fish from different treatment groups performed equally well in the detour task when considering the number of failed attempts, indicating similar levels of accuracy. However, differences in latency suggest that while fish may have been equally accurate in solving the task, they differed in how quickly they did so. These findings highlight the value of using multiple measurements, such as accuracy and speed, to obtain a more nuanced understanding of behavioural flexibility.

The effects of nutritional state on physiological stress responses

Cortisol levels in blood plasma

Fish on the reduced diet showed significantly higher cortisol concentrations in blood plasma than those on the increased diet. This suggests that nutritional state influenced the acute physiological

stress response. Sampling time significantly influenced cortisol concentrations, as fish sampled 45 min after a stressor exhibited higher plasma cortisol levels than those sampled right after experiencing the stressor. Focusing specifically on the fish that were sampled with a longer interval after experiencing the first stressor, we identified a significant difference in plasma cortisol levels between fish assigned to the reduced and increased feeding treatments. In contrast, there were no overall significant differences between the two treatment groups in cortisol levels in blood plasma as a measure for an acute physiological stress response (Kennedy and Janz, 2023; Lemos et al., 2023; Mommsen et al., 1999), and there was no significant interaction between feeding regime and sampling time.

One possible explanation for the elevated cortisol levels in fish is the physiological stress response. However, cortisol, which is released into the bloodstream via the HPI axis, has a delayed peak, typically within around 45 min after the initial stressor occurs (Lai et al., 2021). This aligns with our findings, as we only found differences in fish which were sampled 45 min after experiencing the stressor. Another contributing factor could be that it is increasingly more difficult to catch individual fish when the number of remaining fish in the tank decrease. If only a few fish remain (1 to 10 individuals), catching them becomes more challenging, likely leads to greater stress during handling for those fish. As a result, fish sampled last may have experienced an elevated level of stress, which resulted in a higher plasma cortisol level compared to those sampled earlier. However, this does still not explain the differences between the feeding treatments.

The differences in cortisol levels between the feeding treatments support the theory of allostatic load (McEwen and Wingfield, 2003). Fish on the increased diet showed a more blunted stress response indicated by lower cortisol levels (McEwen and Sapolsky, 1995; McEwen and Wingfield, 2003; Oliveira and Galhardo, 2009). These findings are consistent with our behavioural results, where fish on the reduced diet solved the detour task more quickly than those on the increased diet when it was the first task. This may be explained by their higher attentional state driven by an acute physiological stress response, which enhances sensory perception, cognition, and memory formation, leading to more efficient problem-solving (McEwen and Sapolsky, 1995; Oliveira and Galhardo, 2009). Conversely, fish on the increased diet may have experienced lower stress levels, resulting in reduced attentional focus and impaired performance in the novel detour task.

However, we found no significant relationship between the latency to solve the detour task and plasma cortisol levels across all individuals and within the reduced and increased feeding regime treatment groups. Additionally, we could not observe any significant correlation between the number of failed attempts before solving the detour task and plasma cortisol levels across all individuals and within the reduced and increased feeding regime treatment groups. The lack of a significant correlation

between the speed to solve the task or number of failed attempts and blood plasma cortisol level suggests that individual cortisol level does not directly explain task performance. Importantly, fish were not sampled immediately after the detour task, but at a later point in time. Therefore, the measured cortisol levels may not accurately reflect acute stress response experienced during the task itself. This time gap between 1 to 8 days limits our ability to draw conclusions about how stress experienced during task performance may have influenced behavioural flexibility. Ideally, cortisol should have been measured directly after task completion to better capture its relationship to individual performance.

Although we initially hypothesized that fish with a higher nutritional state would exhibit a stronger physiological stress response, based on the theory of allostatic load (Goymann and Wingfield, 2004; McEwen and Wingfield, 2003), our plasma cortisol data supports this theory. It is important to distinguish between acute and chronic stress responses. In a stressful situation, fish with a normal or reduced nutritional state exhibit an acute increase in cortisol levels, reaching the energetic threshold required to trigger an emergency response. This facilitates adaptive behaviours by mobilizing energy from endogenous stores such as fat, glycogen, and proteins. In contrast, a well-nourished or over-nourished fish, although experiencing an increase in cortisol level, does not reach this energetic threshold, because they can compensate for additional energy requirements without activating an emergency response. Consequently, they exhibit prolonged, moderate elevations in cortisol level rather than acute peaks (McEwen and Wingfield, 2003).

This prolonged stress response in overnourished fish may explain their impaired performance in the novel, challenging detour task. Without the acute physiological stress response that enhances cognitive function and attentional focus, they may be less efficient in adapting to new environments or problem-solving.

Cortisol levels in scales

Since cortisol levels in scales have been shown to reliably indicate chronic stress in Rainbow trout (Carbajal et al., 2019a; Lemos et al., 2023), we used cortisol in scales as a measure of long-term stress. However, we found no significant differences between the two treatment groups.

Aerts et al. (2015) found increased scale cortisol levels in common carp that were stressed daily, while the control group showed no such increase. Their findings validate cortisol in scales as an indicator of chronic stress, suggesting that cortisol accumulates in scales in a time- and stressor-dependent manner. Similarly, Samaras et al. (2021) reported significantly higher scale cortisol levels in chronically stressed European sea bass, compared to non-stressed individuals. Based on these findings and the theory of allostatic load (McEwen and Wingfield, 2003), we hypothesised that fish on the increased diet would exhibit higher cortisol levels in scales due to chronic stress. However, we

could not find these differences between the two treatment groups.

One possible explanation for this absence is that fish were not exposed to repeated stressors like in the mentioned studies. Chronic stress typically results from prolonged or repeated exposure to stressors, whereas in our study, stressors such as the detour task and handling (catching, transportation) may have been too brief for an accumulation of cortisol in scales. During the feeding phase disturbances were minimal, limited to daily cleaning and feeding, while fish were also kept at a relatively low density (16 to 17 individuals per tank).

One may argue that if chronic stress leads to a downregulation of the HPI axis, this should also be reflected in differences in scale cortisol level between the treatment groups (Aerts et al., 2015; Opinion et al., 2023; Samaras et al., 2021). However, in our study, neither group was exposed to more stress than the other. While other studies often contrast clearly defined "stressed" and "non-stressed" groups, both of our treatment groups were exposed to the same general conditions, with the only difference being the feeding regime. It is therefore possible that the differences in diet alone were not sufficient to induce measurable effects detectable in scale cortisol.

It is also possible that the intensity of stressors in our experiment was not comparable to the chronic stressors experienced during routine management in aquacultures, where fish are confronted with higher stocking densities, frequent handling, transportation, and increased environmental disturbances such as noise and water quality fluctuations. These conditions are known to contribute to chronic stress in farmed fish and may explain why scale cortisol differences were not detected in our experiment (Assan et al., 2021; Dara et al., 2023; Kawata, 2008; Kleiber et al., 2023; Lai et al., 2021; López-Olmeda et al., 2012; Mohapatra et al., 2013; Oliva-Teles, 2012). Furthermore, the frequency of stress exposure required for cortisol to accumulate in fish scales remain unclear, as measurable elevations are typically observed only after prolonged or repeated high-intensity stress.

An additional explanation why we did not find any differences in scale cortisol level between the groups could be the specific sampling region of fish body. Scale cortisol concentrations have been shown to vary across body regions. Laberge et al., 2019 found higher cortisol concentrations in scales sampled along the midline of goldfish (Carassius auratus) compared to rostral and caudal extremities. Although their study also showed higher values in the dorsal area, differences in cortisol distribution across body regions might still have affected our results, because our samples included a mix of dorsal, midline, and caudal scales (regarding the definition of Laberge et al., 2019).

Finally, our findings raise the question of whether scale cortisol is a suitable biomarker for detecting chronic stress differences in this specific experimental setup. While the method has been validated for more frequent and stronger stress conditions, it may not be sensitive enough to detect subtle differences between the two treatment groups exposed to relatively low or infrequent stressors.

In generals, chronic stress is complex because the individual response depends on factors like intensity, duration, repeatability, predictability, and controllability of the stressors. Moreover, individual fish may respond differently to the same stressor depending on their personality traits or stress-coping strategies (Adriaenssens and Johnsson, 2011; Koolhaas et al., 1999; Samaras et al., 2021; Sneddon, 2003; White et al., 2017). This shows that more research is needed to improve methods for measuring long-term stress in different environments and experiments.

Conclusion

Our findings highlight the complex relationship between nutritional state, behavioural flexibility, and stress responses in fish. Fish on a reduced diet showed improved behavioural flexibility during a detour task had higher cortisol concentrations in blood samples after experiencing a stressor. Thus, an improved behavioural flexibility may have been the consequence of a heightened attentional state driven by a stronger physiological stress response, as indicated by elevated plasma cortisol levels. Conversely, fish on an increased diet showed impaired behavioural flexibility, probably because they had more flattened acute stress responses, which may have reduced their cognitive focus and adaptability. However, task order and prior experience played a significant role, as differences between the treatment groups disappeared when confronted with a modified situation (detour task second), suggesting that learning effects and familiarity influenced outcomes of the behavioural assay. This aligns with the theory of allostatic load.

While we initially hypothesized that fish on an increased diet would be less capable of coping with (chronic) stressors and therefore exhibit higher chronic stress responses, we found no significant differences in scale cortisol levels between the feeding treatments, suggesting that the stressors applied were insufficient to induce detectable chronic stress. This could be due to the relatively low-intensity and infrequent stressors in our experimental setup, as chronic stress typically results from prolonged or repeated stress exposure found, for example, in fish farms.

Additionally, the number of failed attempts in the detour task did not differ significantly between the feeding regimes, which could reflect a similar accuracy in task solving between the two groups.

In summary, our study partially supports the theory of allostatic load and provides evidence that nutritional state influences acute stress responses and behavioural flexibility, particularly in response to novel challenges. However, prior experience and experimental conditions also play crucial roles in cognitive performance and stress physiology. Further research is needed to explore the long-term effects of nutritional state and chronic stress exposure on behavioural flexibility in fish, particularly under conditions found in aquaculture.

Cognitive functions and in particular behavioural flexibility are vital for fish survival and welfare, not only in the wild, but also in aquaculture and in the success rates of rehabilitation releases (Bayne

et al., 2019; Bshary and Brown, 2014; Kleiber et al., 2023; Shettleworth, 2009). Reducing stress and appropriate feeding regimes are essential for optimizing fish health and cognitive performance (Assan et al., 2021; Lai et al., 2021; López-Olmeda et al., 2012; Oliva-Teles, 2012). Understanding the connection between nutrition, stress and behavioural flexibility can lead to better practices in fish farming. Therefore, it can enhance fish welfare and productivity, as well as improve the success of restoring declining natural fish populations.

5. Ethical note

All applicable international, national, and institutional guidelines for the care and use of animals were strictly followed. The laboratory was approved as a research facility (Dnr 5.2.18-17988/18), and the study received ethical approval from the Ethical Committee on Animal Experiments of the Swedish Board of Agriculture in Linköping and Stockholm (Dnr 168677-2018; Dnr 19359-2019).

Each animal was as short as possible exposed to an unfamiliar, stress-inducing situation, which included catching, air exposure (for transfer, PIT-tagging, and measurements), and separation. During PIT tagging or the behavioural assays, no individual got injured or died due to handing or experimental setup. However, during the feeding phase six individuals died due to unpredicted diseases: Three individuals were euthanized due to pop-eye disease. Another individual was euthanized, and two others died from severe gill problems.

6. Funding

This master thesis was part of a WWTF-Project (Wiener Wissenschafts-, und Forschungs- und Technologiefond) and was additionally financed by the University of Vienna through a short-term grant abroad (KWA).

7. Acknowledgements

We thank Stefan Fischer from the University of Veterinary Medicine Vienna, Austria, the leader of the WWTF-project, for his co-supervision and support in realizing this research. We are also grateful to Sabine Tebbich from the University of Vienna, Austria, for her supervision, for the opportunity to be part of her research group, and for her valuable input during discussions. Furthermore, we greatly appreciate the excellent collaboration with the Linnaeus University in Kalmar, Sweden, and we thank the team of the fish group. In particular, we thank Petter Tibblin for providing the lab and resources and Adrian Berge for technical assistance. We would also like to thank Caroline Deimel from the University of Vienna, Austria, for technical assistance. Finally, we thank Gerhard Bauer for his support during the preparations.

8. References

Adriaenssens, B., Johnsson, J.I., 2011. Learning and context-specific exploration behaviour in hatchery and wild brown trout. Appl. Anim. Behav. Sci. 132, 90–99. https://doi.org/10.1016/j.applanim.2011.03.005

Aerts, J., Metz, J.R., Ampe, B., Decostere, A., Flik, G., De Saeger, S., 2015. Scales Tell a Story on the Stress History of Fish. PLOS ONE 10, e0123411.

https://doi.org/10.1371/journal.pone.0123411

Aquakulturinfo, 2019. Stressmanagement [WWW Document]. Aquakulturinfo. URL https://www.aquakulturinfo.de/stressmanagement (accessed 7.11.24).

Assan, D., Huang, Y., Mustapha, U.F., Addah, M.N., Li, G., Chen, H., 2021. Fish Feed Intake, Feeding Behavior, and the Physiological Response of Apelin to Fasting and Refeeding. Front. Endocrinol. 12, 798903. https://doi.org/10.3389/fendo.2021.798903

Attia, J., Millot, S., Di-Poï, C., Bégout, M.-L., Noble, C., Sanchez-Vazquez, F.J., Terova, G., Saroglia, M., Damsgård, B., 2012. Demand feeding and welfare in farmed fish. Fish Physiol. Biochem. 38, 107–118. https://doi.org/10.1007/s10695-011-9538-4

Bates, D., Mächler, M., Bolker, B., Walker, S., 2015. Fitting Linear Mixed-Effects Models Using lme4. J. Stat. Softw. 67. https://doi.org/10.18637/jss.v067.i01

Bayne, T., Brainard, D., Byrne, R.W., Chittka, L., Clayton, N., Heyes, C., Mather, J., Ölveczky, B., Shadlen, M., Suddendorf, T., Webb, B., 2019. What is cognition? Curr. Biol. 29, R608–R615. https://doi.org/10.1016/j.cub.2019.05.044

Braithwaite, V.A., Girvan, J.R., 2003. Use of water flow direction to provide spatial information in a small-scale orientation task. J. Fish Biol. 63, 74–83. https://doi.org/10.1111/j.1095-8649.2003.00218.x

Brown, V.J., Tait, D.S., 2014. Behavioral Flexibility: Attentional Shifting, Rule Switching, and Response Reversal, in: Stolerman, I.P., Price, L.H. (Eds.), Encyclopedia of Psychopharmacology. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 1–7. https://doi.org/10.1007/978-3-642-27772-6_340-2

Bshary, R., Brown, C., 2014. Fish cognition. Curr. Biol. 24, R947–R950. https://doi.org/10.1016/j.cub.2014.08.043

Cañon Jones, H.A., Hansen, L.A., Noble, C., Damsgård, B., Broom, D.M., Pearce, G.P., 2010. Social network analysis of behavioural interactions influencing fin damage development in Atlantic salmon (Salmo salar) during feed-restriction. Appl. Anim. Behav. Sci. 127, 139–151. https://doi.org/10.1016/j.applanim.2010.09.004

Carbajal, A., Reyes-López, F.E., Tallo-Parra, O., Lopez-Bejar, M., Tort, L., 2019a. Comparative assessment of cortisol in plasma, skin mucus and scales as a measure of the hypothalamic-pituitary-interrenal axis activity in fish. Aquaculture 506, 410–416. https://doi.org/10.1016/j.aquaculture.2019.04.005

Carbajal, A., Tallo-Parra, O., Monclús, L., Vinyoles, D., Solé, M., Lacorte, S., Lopez-Bejar, M., 2019b. Variation in scale cortisol concentrations of a wild freshwater fish: Habitat quality or seasonal influences? Gen. Comp. Endocrinol. 275, 44–50. https://doi.org/10.1016/j.ygcen.2019.01.015

Cherubini, E., Miles, R., 2015. The CA3 region of the hippocampus: how is it? What is it for? How does it do it? Front. Cell. Neurosci. 9. https://doi.org/10.3389/fncel.2015.00019

Chiszar, D., Murphy, J.B., Smith, H.M., 1993. In Search of Zoo-Academic Collaborations: A Research Agenda for the 1990's, Herpetologica. The Herpetologists' League, Inc.

Dara, M., Carbonara, P., La Corte, C., Parrinello, D., Cammarata, M., Parisi, M.G., 2023. Fish Welfare in Aquaculture: Physiological and Immunological Activities for Diets, Social and Spatial Stress on Mediterranean Aqua Cultured Species. Fishes 8, 414. https://doi.org/10.3390/fishes8080414

Dill, L.M., 1983. Adaptive Flexibility in the Foraging Behavior of Fishes. Can. J. Fish. Aquat. Sci. 40, 398–408. https://doi.org/10.1139/f83-058

FAO, 2020. The State of World Fisheries and Aquaculture 2020. Food and Agriculture Organization of the United Nations (FAO), Rome. https://doi.org/10.4060/ca9229en

Flores, H., Vergara, A., 2012. Efecto de reducir la frecuencia de alimentacion en la supervivencia, crecimiento, conversion y conducta alimenticia en juveniles de salmon del Atlantico Salmo salar (Linnaeus, 1758): experiencia a nivel productivo. Lat. Am. J. Aquat. Res. 40, 536–544. https://doi.org/10.3856/vol40-issue3-fulltext-3

Friard, O., Gamba, M., 2016. BORIS: a free, versatile open-source event-logging software for video/audio coding and live observations. Methods Ecol. Evol. 7, 1325–1330. https://doi.org/10.1111/2041-210X.12584

Goymann, W., Wingfield, J.C., 2004. Allostatic load, social status and stress hormones: the costs of social status matter. Anim. Behav. 67, 591–602. https://doi.org/10.1016/j.anbehav.2003.08.007

Herman, J.P., McKlveen, J.M., Ghosal, S., Kopp, B., Wulsin, A., Makinson, R., Scheimann, J., Myers, B., 2016. Regulation of the Hypothalamic-Pituitary-Adrenocortical Stress Response, in: Prakash, Y.S. (Ed.), Comprehensive Physiology. Wiley, pp. 603–621. https://doi.org/10.1002/cphy.c150015

Jiang, X., Dong, S., Liu, R., Huang, M., Dong, K., Ge, J., Gao, Q., Zhou, Y., 2021. Effects of temperature, dissolved oxygen, and their interaction on the growth performance and condition of rainbow trout (*Oncorhynchus mykiss*). J. Therm. Biol. 98, 102928. https://doi.org/10.1016/j.jtherbio.2021.102928

Johansson, N., 1981. General problems in Atlantic salmon rearing in sweden. Oikos Editor. Off., Fish Gene Pools: Preservation of Genetic Resources in Relation to Wild Fish Stocks 75–83.

Johnsson, J.I., Fredrik Sundström, L., 2007. Social Transfer of Predation Risk Information Reduces Food Locating Ability in European Minnows (*Phoxinus phoxinus*). Ethology 113, 166–173. https://doi.org/10.1111/j.1439-0310.2006.01296.x

Juell, J.-E., 1995. The behaviour of Atlantic salmon in relation to efficient cage-rearing. Rev. Fish Biol. Fish. 5, 320–335. https://doi.org/10.1007/BF00043005

Kabadayi, C., Bobrowicz, K., Osvath, M., 2018. The detour paradigm in animal cognition. Anim. Cogn. 21, 21–35. https://doi.org/10.1007/s10071-017-1152-0

Kawamura, K., 2008. “Handbook of European freshwater fishes” by M. Kottelat and J. Freyhof (2007). Ichthyol. Res. 55, 99–99. https://doi.org/10.1007/s10228-007-0012-3

Kawata, K., 2008. Zoo Animal Feeding: A Natural History Viewpoint. Zool. Gart. 78, 17–42. https://doi.org/10.1016/j.zoolgart.2008.09.004

Kelley, J.L., Magurran, A.E., 2003. Learned predator recognition and antipredator responses in fishes. Fish Fish. 4, 216–226. https://doi.org/10.1046/j.1467-2979.2003.00126.x

Kennedy, E.K.C., Janz, D.M., 2023. Chronic stress causes cortisol, cortisone and DHEA elevations in scales but not serum in rainbow trout. Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 276, 111352. https://doi.org/10.1016/j.cbpa.2022.111352

Kennedy, E.K.C., Janz, D.M., 2022. First Look into the Use of Fish Scales as a Medium for Multi-Hormone Stress Analyses. Fishes 7, 145. https://doi.org/10.3390/fishes7040145

Kleiber, A., Stomp, M., Rouby, M., Ferreira, V.H.B., Bégout, M.-L., Benhaïm, D., Labbé, L., Tocqueville, A., Levadoux, M., Calandreau, L., Guesdon, V., Colson, V., 2023. Cognitive enrichment to increase fish welfare in aquaculture: A review. Aquaculture 575, 739654. https://doi.org/10.1016/j.aquaculture.2023.739654

Koakoski, G., Oliveira, T.A., Da Rosa, J.G.S., Fagundes, M., Kreutz, L.C., Barcellos, L.J.G., 2012. Divergent time course of cortisol response to stress in fish of different ages. Physiol. Behav. 106, 129–132. https://doi.org/10.1016/j.physbeh.2012.01.013

Koolhaas, J.M., Korte, S.M., De Boer, S.F., Van Der Vegt, B.J., Van Reenen, C.G., Hopster, H., De Jong, I.C., Ruis, M.A.W., Blokhuis, H.J., 1999. Coping styles in animals: current status in behavior and stress-physiology. Neurosci. Biobehav. Rev. 23, 925–935. https://doi.org/10.1016/S0149-7634(99)00026-3

Kotrschal, A., Taborsky, B., 2010. Environmental Change Enhances Cognitive Abilities in Fish. PLoS Biol. 8, e1000351. https://doi.org/10.1371/journal.pbio.1000351

Laberge, F., Yin-Liao, I., Bernier, N.J., 2019. Temporal profiles of cortisol accumulation and clearance support scale cortisol content as an indicator of chronic stress in fish. Conserv. Physiol. 7, coz052. https://doi.org/10.1093/conphys/coz052

Lai, F., Comesaña, S., Gomes, A.S., Flatejord, D., Tolås, I., Espe, M., De Santis, C., Hartviksen, M.B., Verri, T., Soengas, J.L., Rønnestad, I., 2023. Anorectic role of high dietary leucine in farmed Atlantic salmon (Salmo salar L.): Effects on feed intake, growth, amino acid transporters and appetite-control neuropeptides. Aquaculture 566, 739204.

https://doi.org/10.1016/j.aquaculture.2022.739204

Lai, F., Royan, M.R., Gomes, A.S., Espe, M., Aksnes, A., Norberg, B., Gelebart, V., Rønnestad, I., 2021. The stress response in Atlantic salmon (Salmo salar L.): identification and functional characterization of the corticotropin-releasing factor (crf) paralogs. Gen. Comp. Endocrinol. 313, 113894. https://doi.org/10.1016/j.ygcen.2021.113894

Laland, K.N., Brown, C., Krause, J., 2003. Learning in fishes: from three-second memory to culture. Fish Fish. 4, 199–202. https://doi.org/10.1046/j.1467-2979.2003.00124.x

Larsson, S., Linnansaari, T., Vatanen, S., Serrano, I., Haikonen, A., 2011. Feeding of wild and hatchery reared Atlantic salmon (Salmo salar L.) smolts during downstream migration. Environ. Biol. Fishes 92, 361–369. https://doi.org/10.1007/s10641-011-9846-7

Lemos, L., Angarica, L., Hauser-Davis, R., Quinete, N., 2023. Cortisol as a Stress Indicator in Fish: Sampling Methods, Analytical Techniques, and Organic Pollutant Exposure Assessments. Int. J. Environ. Res. Public. Health 20, 6237. https://doi.org/10.3390/ijerph20136237

Lewin, W.-C., Arlinghaus, R., Mehner, T., 2006. Documented and Potential Biological Impacts of Recreational Fishing: Insights for Management and Conservation. Rev. Fish. Sci. 14, 305–367. https://doi.org/10.1080/10641260600886455

Loboiko, Y., Senechyn, V., Pukalo, P., Kychun, I., 2024. Efficiency of using experimental feeds with different levels of nutrition in feeding rainbow trout. Sci. Messenger LNU Vet. Med. Biotechnol. 26, 121–125. https://doi.org/10.32718/nvlvet-a10018

López-Olmeda, J.F., Noble, C., Sánchez-Vázquez, F.J., 2012. Does feeding time affect fish welfare? Fish Physiol. Biochem. 38, 143–152. https://doi.org/10.1007/s10695-011-9523-y

Lucon-Xiccato, T., Gatto, E., Bisazza, A., 2017. Fish perform like mammals and birds in inhibitory motor control tasks. Sci. Rep. 7, 13144. https://doi.org/10.1038/s41598-017-13447-4

Lucon-Xiccato, T., Montalbano, G., Reddon, A.R., Bertolucci, C., 2022. Social environment affects inhibitory control via developmental plasticity in a fish. Anim. Behav. 183, 69–76. https://doi.org/10.1016/j.anbehav.2021.11.001

Mattson, M.P., 2019. An Evolutionary Perspective on Why Food Overconsumption Impairs Cognition. Trends Cogn. Sci. 23, 200–212. https://doi.org/10.1016/j.tics.2019.01.003

Mayrand, J.L., 2017. Investigating effects of behavioural flexibility and neuroplasticity on acclimation success of outcrossed Chinook salmon (Oncorhynchus tshawytscha): applications in aquaculture.

McEwen, B.S., Sapolsky, R.M., 1995. Stress and cognitive function. Curr. Opin. Neurobiol. 5, 205–216. https://doi.org/10.1016/0959-4388(95)80028-X

McEwen, B.S., Wingfield, J.C., 2003. The concept of allostasis in biology and biomedicine. Horm. Behav. 43, 2–15. https://doi.org/10.1016/S0018-506X(02)00024-7

Mohapatra, S., Chakraborty, T., Kumar, V., DeBoeck, G., Mohanta, K.N., 2013. Aquaculture and stress management: a review of probiotic intervention. J. Anim. Physiol. Anim. Nutr. 97, 405–430. https://doi.org/10.1111/j.1439-0396.2012.01301.x

Mommsen, T.P., Vijayan, M.M., Moon, T.W., 1999. Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. Kluwer Acad. Publ., Reviews in Fish Biology and Fisheries 9, 211–268.

Nelson, J.S., 1992. A field guide to freshwater fishes: North America North of Mexico: Peterson Field Guide Series Lawrence M. Page and Brooks M. Burr Houghton Mifflin Co., Boston, MA, 1991 ISBN 0-395-35307-6 (cloth, $24.95 US), 0-395-53933-1 (paper, $15.95 US) pp. xii + 432, acid-free paper, 466 figures, 33 colour plates Available from Roger Tory Peterson Institute, 110 Marvin Parkway, Jamestown, NY 14701, USA. Rev. Fish Biol. Fish. 2, 279–280. https://doi.org/10.1007/BF00045047

Odling-Smee, L., Braithwaite, V.A., 2003. The role of learning in fish orientation. Fish Fish. 4, 235–246. https://doi.org/10.1046/j.1467-2979.2003.00127.x

Oliva-Teles, A., 2012. Nutrition and health of aquaculture fish. J. Fish Dis. 35, 83–108. https://doi.org/10.1111/j.1365-2761.2011.01333.x

Oliveira, R., Galhardo, L., 2009. Psychological Stress and Welfare in Fish. Annu. Rev. Biomed. Sci. 11, 1–20. https://doi.org/10.5016/1806-8774.2009v11p1

Opinion, A.G.R., Vanhomwegen, M., De Boeck, G., Aerts, J., 2023. Long-term stress induced cortisol downregulation, growth reduction and cardiac remodeling in Atlantic salmon. J. Exp. Biol. 226, jeb246504. https://doi.org/10.1242/jeb.246504

Persson, L., Leonardsson, K., Alanärä, A., 2018. Manipulation of the energetic state of Atlantic salmon *SALMO SALAR* juveniles and the effect on migration speed. J. Fish Biol. 92, 961–978. https://doi.org/10.1111/jfb.13555

Reid, S.G., Bernier, N.J., Perry, S.F., 1998. The adrenergic stress response in fish: control of catecholamine storage and release. Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol. 120, 1–27. https://doi.org/10.1016/S0742-8413(98)00037-1

Rodríguez, F., Quintero, B., Amores, L., Madrid, D., Salas-Peña, C., Salas, C., 2021. Spatial Cognition in Teleost Fish: Strategies and Mechanisms. Animals 11, 2271. https://doi.org/10.3390/ani11082271

Romero, L.M., Dickens, M.J., Cyr, N.E., 2009. The reactive scope model — A new model integrating homeostasis, allostasis, and stress. Horm. Behav. 55, 375–389. https://doi.org/10.1016/j.yhbeh.2008.12.009

Samaras, A., Dimitroglou, A., Kollias, S., Skouradakis, G., Papadakis, I.E., Pavlidis, M., 2021. Cortisol concentration in scales is a valid indicator for the assessment of chronic stress in

European sea bass, Dicentrarchus labrax L. Aquaculture 545, 737257. https://doi.org/10.1016/j.aquaculture.2021.737257

Shettleworth, S.J., 2009. Cognition, Evolution and Behavior. Oxford University Press.

Sneddon, L.U., 2003. The bold and the shy: individual differences in rainbow trout. J. Fish Biol. 62, 971–975. https://doi.org/10.1046/j.1095-8649.2003.00084.x

Stanković, D., Crivelli, A.J., Snoj, A., 2015. Rainbow Trout in Europe: Introduction, Naturalization, and Impacts. Rev. Fish. Sci. Aquac. 23, 39–71. https://doi.org/10.1080/23308249.2015.1024825

Statistik Austria, 2021. Aquakulturen. URL https://www.statistik.at/statistiken/land-und-forstwirtschaft/tiere-tierische-erzeugung/aquakulturen

Stephens, M.A., Wand, G., 2012. Stress and the HPA axis: role of glucocorticoids in alcohol dependence. Alcohol Research 468–83.

Sutton, S.G., Bult, T.P., Haedrich, R.L., 2000. Relationships among Fat Weight, Body Weight, Water Weight, and Condition Factors in Wild Atlantic Salmon Parr. Trans. Am. Fish. Soc. 129, 527–538. https://doi.org/10.1577/1548-8659(2000)129<0527:RAFWBW>2.0.CO;2

Van Den Boogaart, L., Slabbekoorn, H., Scherer, L., 2023. Prioritization of fish welfare issues in European salmonid aquaculture using the Delphi method. Aquaculture 572, 739557. https://doi.org/10.1016/j.aquaculture.2023.739557

Venegas, F., Montiel, E., Forno, P., Rojas, M., 2003. HISTOLOGY OF THE JAW DEFORMATION IN SALMON OF SOUTHERN CHILE. Int. J. Morphol. 21. https://doi.org/10.4067/S0717-95022003000300005

Verbeek, M.E.M., Drent, P.J., Wiepkema, P.R., 1994. Consistent individual differences in early exploratory behaviour of male great tits. Anim. Behav. 48, 1113–1121. https://doi.org/10.1006/anbe.1994.1344

Verspoor, E., Stradmeyer, L., Nielsen, J.L., Atlantic Salmon Trust (Eds.), 2007. The Atlantic salmon: genetics, conservation and management. Blackwell Pub, Oxford ; Ames, Iowa.

Weihe, R., Rørvik, K.-A., Thomassen, M.S., Asche, F., 2019. A model system to evaluate the economic performance of two different dietary feeding strategies in farmed Atlantic salmon (Salmo salar L.). Aquaculture 512, 734335. https://doi.org/10.1016/j.aquaculture.2019.734335

White, S.L., Wagner, T., Gowan, C., Braithwaite, V.A., 2017. Can personality predict individual differences in brook trout spatial learning ability? Behav. Processes 141, 220–228. https://doi.org/10.1016/j.beproc.2016.08.009

Yue, S., Moccia, R.D., Duncan, I.J.H., 2004. Investigating fear in domestic rainbow trout, Oncorhynchus mykiss, using an avoidance learning task. Appl. Anim. Behav. Sci. 87, 343–

354. https://doi.org/10.1016/j.applanim.2004.01.004

9. Figures

10. Tables

11. Appendix

11.1.Definitions

Table 12 Definitions of the variables used in this study for data collection and data analysis.

Variable Information / Code Book			
Variable name	**Variable and value labels**		**Level**
date	date the experiment was conducted		chr
trial_nr	number of the trial recorded		chr
treatment	feeding regime used before the experiment starts		factor
	0.5	0.5% of the average of the body weight in g	
	1.5	1.5% of the average of the body weight in g	
original_tank_1	tank ID, where the treatment started until 11/02/24 (4 tanks, 2 treatments)		int
original_tank_2	tank ID, where the treatment continued since 12/02/24 (2 tanks, 2 treatments)		int
after_tank_1	tank ID, where the treatment continued after the Y-maze test		int
after_tank_2	tank ID, where the treatment continued after the Detour Test		int
test_maze	ID of the Y-maze, where the Y-maze test took place		int
test_tank	tank ID, where the Detour Test took place		int
channel_maze	camera ID used for filming the Y-maze test (1,3,4,6,7,8)		int
channel_detour	camera ID used for filming the Detour-Test (1,3,4,6,7,8)		int
PIT-ID	unique ID-number for each fish trough chip tagging		int
start_time	time the trial started (Central European Time)		chr
end_time	time the trial ended (Central European Time)		chr
start_timestamp	time of the camera when the trial started		chr
end_timestamp	time of the camera when the trial ended		chr
start_time_mo	time of the camera when the motivation-control task started		chr
end_time_mo	time of the camera when the motivation-control task ended		chr
start_time_de	time of the camera when the detour task started		chr
end_time_de	time of the camera when the detour task ended		chr
order	order of the tasks in one trial of the Detour Test		factor
	1	motivation-control task first, detour task second	
	2	detour task first, motivation-control task second	

active_experiments	number of active experiments at the same time		int
last feeding	time (CET) and date when the fish were fed before the experiment started		chr
last cleaning	time (CET) and date when the tanks were cleaned before the experiment started		chr
le_before_total	total length in cm before the feeding regimes started (08/01/24)		num
le_after_total	total length in cm after the experiment ended (27/02/24)		num
le_after_stand	standard-length in cm after the experiment ended (27/02/24)		num
le_diff_total	difference in total length in cm from before and after the experiment (le_after_total – le_before_total)		num
we_before	weight in g before the feeding regimes started (08/01/24)		num
we_after	weight in g after the experiment ended (27/02/24)		num
we_diff	difference in weight in g from before and after the experiment (we_after – we_before)		num
bc_before	body condition before the feeding regime started (Fulton's factor: ((weight in g) / (total length in cm ^{3})) x 100) (08/01/24)		num
bc_after	body condition after the feeding regime started (Fulton's factor: ((weight in g) / (standard length in cm ^{3})) x 100) (27/02/24)		num
mo_test	0	the fish didn't reach the shadow at least with its pectoral fins in the motivation-control task	factor
	1	the fish reached the shadow at least with its pectoral fins in the motivation-control task	factor
de_test	0	the fish didn't reach the shadow at least with its pectoral fins in the detour task	factor
	1	the fish reached the shadow at least with its pectoral fins in the detour task	factor
failed_at	number of failed attempts (bumping against the transparent barrier) in the detour task		int
task	tested task in the Detour Test		factor
	M	motivation-control task	
	D	detour task	

test_order	combination of the task in the Detour Test and the order of the task		factor
	M1	motivation-control task as first task	
	D1	detour task as first task	
	M2	motivation-control task as second task	
	D2	detour task as second task	
latency	duration in sec from the beginning of the task until the fish reached the shadow at least with its pectoral fins		num
latency_mo	duration in sec from the beginning of the task until the fish reached the shadow at least with its pectoral fins in the motivation-control task		num
latency_de	duration in sec from the beginning of the task until the fish reached the shadow at least with its pectoral fins in the detour task		num
direction	the way, the fish made the detour around the transparent barrier		factor
	0	no way	
	1	from the right side of the barrier	
	2	from above the barrier	
	3	from the left side of the barrier (break through)	
sam1_ce_ID	sample ID 1 of blood cells in 2 mL Eppendorf tube (Nr. cap - FT - Nr. tube)		chr
sam2_pl_ID	sample ID 2 of blood plasma in 0.5 mL Eppendorf tube (Nr. cap - FT - Nr. tube)		chr
sam2_pl_res	results of the cortisol value from sample ID 2 of blood plasma (using cELISA)		num
sam3_pl_ID	sample ID 3 of blood plasma in 0.5 mL Eppendorf tube (Nr. cap - FT - Nr. tube)		chr
sam3_pl_res	results of the cortisol value from sample ID 3 of blood plasma (using cELISA)		num
sam4_sc_ID	sample ID 4 of scales in 5 mL Eppendorf tube (Nr. cap - FT - Nr. tube)		chr
sam4_sc_res	results of the cortisol value from sample ID 4 of scales (using cELISA)		num

comments	comments for events during the trial or experimental phase (e.g. death of an individual, sickness, injuries)	

11.2. Additional Tables and Results

*Table 13 Parameters of Water quality after *Loboiko et al., 2024 and **Jiang et al., 2021.*

parameter	**value**
Free ammonia (NH3) *	0.05 mgN/dm^3
Total hardness*	4.0 mg-eq/dm^3
Hydrocarbonates (HCO3-) *	150.0 mg/dm^3
Conductivity at 20 °C **	120 -135 °µS/cm
Oxygen content **	8-9 mg L^{-1} mg/l
Permanganate oxidisability **	2.07-4.38 10.0 mgO/dm^3
Bichromate oxidisability **	5.73-7.48 30.0 mgO/dm^3
Ammonium nitrogen (NH4+) **	0.24-0.39 0.5 mgN/dm^3
Nitrite (N02-) **	0.04-0.06 0.1 mgN/dm^3
Nitrates (N03-) **	0.27-0.60 1.0 mgN/dm^3
Phosphates (PO43-) **	0.13-0.29 0.3 mgP/dm^3
Total iron, mg **	0.25-0.54 0.5 Fe/dm^3
Calcium (Ca2+) **	51.4-62.3 40.0 mg/dm^3
Magnesium (Mg2+) **	7.4-10.6 15.0 mg/dm^3
Sodium + potassium (Na+ + K+) **	15.7-21.2 20.0 mg/dm^3
Hydrocarbonates, (HCO3-) **	123.9-134.7 150.0 mg/dm^3
Chlorides (Cl-) **	16.3-34.9 50.0 mg/dm^3
Sulphates (SO42-) **	19.6-37.8 40.0 mg/dm^3
Mineralisation **	284.6-298.3 300.0 mg/dm^3

Table 14 Treatment - order ratio.

treatment	**order**	**count**		**percent %**	**percent % within treatment group**
reduced	**detour 2nd**	46	24	49	52
	detour 1st		22		48

increased	**detour 2nd**	47	24	51	51
	detour 1st		23		49

Table 15 Treatment - order ratio in the detour task.

Successful detour task					
treatment	**order**	**count**		**percent %**	**percent % within treatment group and order**
reduced	**detour 2nd**	83	18	89	75
	detour 1st		22		100
increased	**detour 2nd**		20		83
	detour 1st		23		100
Failed detour task					
reduced	**detour 2nd**	10	6	11	25
	detour 1st		0		0
increased	**detour 2nd**		4		17
	detour 1st		0		0

Table 16 Treatment - order ratio in the motivation-control task.

Successful motivation-control task					
treatment	**order**	**count**		**percent %**	**percent % within treatment group and order**
reduced	**detour 2nd**	93	24	100	100
	detour 1st		22		
increased	**detour 2nd**		24		
	detour 1st		23		

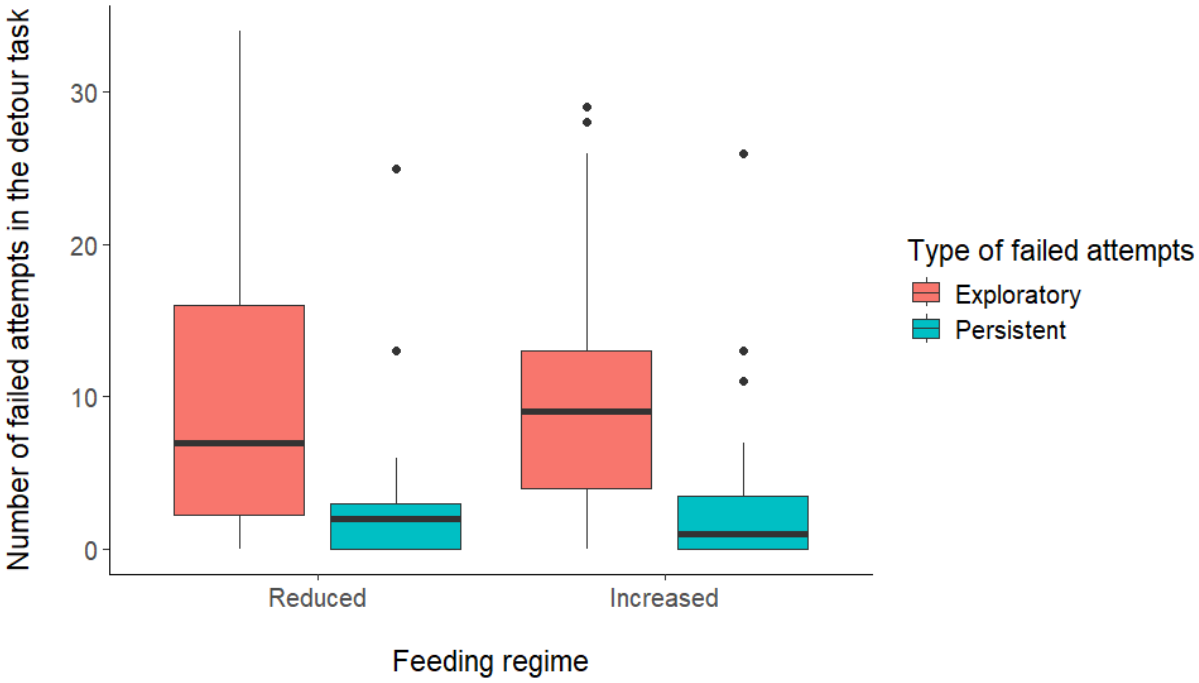

Figure 16 Number of failed attempts in the detour task of fish on a reduced (n = 46) or increased (n = 47) diet, depending on weather the fish showed an exploratory (red boxes) or persistent (blue boxes) behaviour. First failed attempt was accounted for exploratory behaviour.

Table 17 Statistical tests and results for the number of failed attempts (FA) before solving the detour task of fish on a reduced (n = 46) or increased (n = 47) diet, depending on weather the fish showed an exploratory or persistent behaviour (type of FA). The generalised linear mixed model showed no significant interaction between 'feeding regime' and 'type of failed attempts'. Results of post hoc Mann-Whitney-U-tests with a Bonferroni correction for four tests are shown to further investigate the significant interaction. Estimates are shown as difference to the reference level 'reduced feeding regime' and 'exploratory' for factor 'feeding regime' and 'type of FA'.

Generalised Linear Mixed Model: failed attempts ~ feeding regime * type of FA + (1|holding tank ID)				
Factor	**Estimate ± SE**	**Num. df**	**z-value**	**p-value**
Intercept	2.32 ± 0.14	-	-	-
Feeding regime	-0.05 ± 0.20	1	-0.28	0.78
Type of FA	-1.35 ± 0.10	1	-13.27	$< 2e^{-16}$
Interaction	-0.02 ± 0.14	1	-0.12	0.91

(GLMM, z = -0.12, p = 0.91, N = 93)

Table 18 Type of failed attempts (FA) - ratio in the detour task.

type of FA	count	percent %
exploratory	936	79.6
persistent	240	20.4

Table 19 Treatment - type of failed attempts (FA) ratio in the detour task

treatment	type of FA	count	percent % within treatment group
reduced	**exploratory**	468	79.5
	persistent	121	20.5
increased	**exploratory**	468	79.7
	persistent	119	20.3

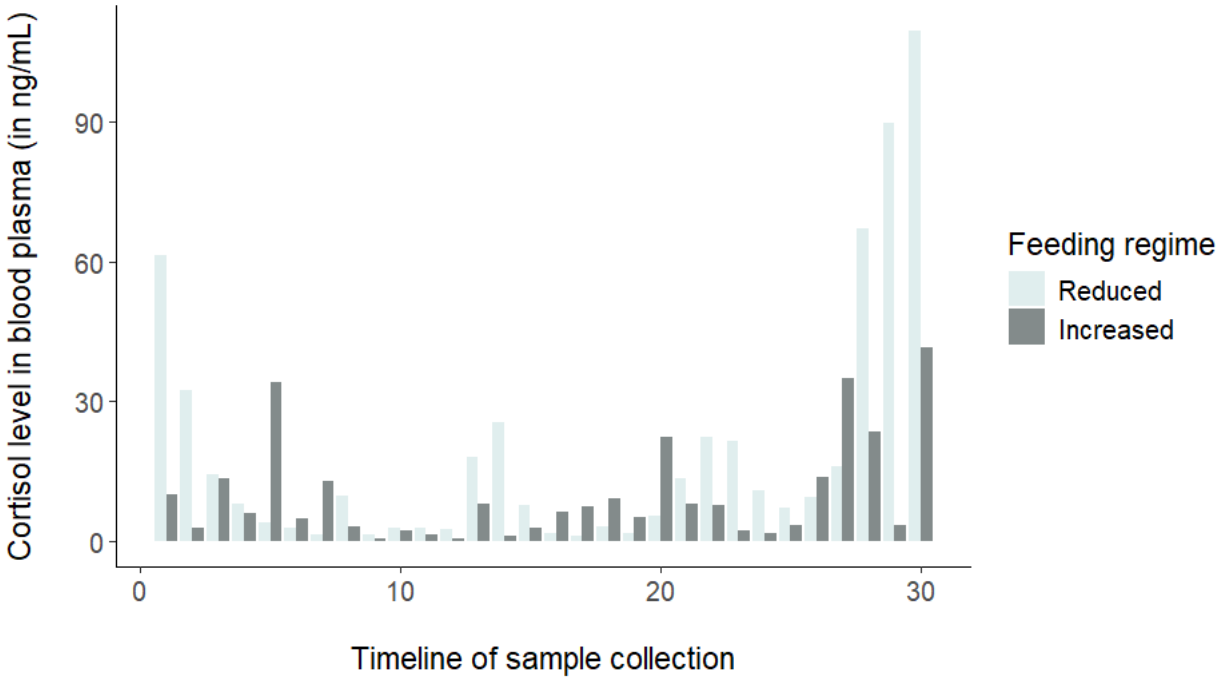

Figure 17 Plasma cortisol levels are reported in ng/mL for each individual over the sampling period (0-30). 1 refers to the first individual tested in the assigned group, and 30 refers to the last.