




# Impact of environmental complexity and stocking density on affective states of rainbow trout (*Oncorhynchus mykiss*)

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## Abstract

Environmental condition, such as environmental complexity or stocking density, can directly or indirectly influence animal emotion and ultimately, affective state. Affective states of animals can be assessed through judgement bias tests, evaluating responses to ambiguous situations. In this study, we aimed to determine whether environmental complexity and stocking density impacted rainbow trout affective state. Rainbow trout ( $n = 108$ ) were housed in recirculating aquaculture systems under commercial conditions while trained at tank-level to discriminate between a positively reinforced chamber (feed) in one location and a negative chamber (positive punishment; chase by net for 1 s) in the opposing location. Fish from successful tanks (two out of five tanks) were then housed in treatment tanks of either high- or low- environmental complexity at either high (165 fish/m<sup>3</sup>) or low (69 fish/m<sup>3</sup>) stocking density. Trained fish were tested for latencies to approach three intermediate, ambiguous chambers. Fish housed in high-density tanks were faster to enter all chambers than those housed in low-density tanks (8.5 s vs. 15.2 s;  $P = 0.001$ ), with faster entries into the positive (7.4 s vs. 15.2 s;  $P = 0.02$ ) and near-negative chambers (10.2 s vs. 17.4 s;  $P = 0.006$ ), suggesting that these fish were more optimistic to receive a feed reward. Tank complexity did not affect test outcomes. No differences between treatments were observed between body weight, length, and plasma cortisol. Overall, rainbow trout are capable of discriminating between cues during a judgement bias test and fish housed in high-density environments respond more optimistically in ambiguous situations compared to fish in low-density environments.

**Keywords** Animal welfare · Rainbow trout · Environmental complexity · Enrichment · Stocking density · Affective state

## Introduction

The welfare of terrestrial livestock has undergone much study, partly because of a push from concerned consumers. However, relatively little has been investigated about the welfare of our aquatic food sources. Rainbow trout (*Oncorhynchus mykiss*) are typically housed in a variety of barren ponds, raceways, or recirculating aquaculture systems (Woynarovich et al. 2011). These environments provide no access to biologically relevant enrichments, besides that of

interaction with conspecifics. Environmental complexity can have a positive impact on animals' biological functioning and behavior (Newberry 1995; Gerber et al. 2015). For example, rainbow trout reared in enriched environments with cobblestone tank substrate had better fin condition, and steelhead trout (*Oncorhynchus mykiss irideus*) housed in tanks with unmoved rocks exhibited more exploratory behavior than fish in a barren control environment (Bosakowski and Wagner 1995; Lee and Berejikian 2008). Stocking density is another environmental aspect to consider, but guidelines differ due to the complex interactions between density, fish behavior, fin damage, availability of feed, and type of rearing system (Ellis et al. 2002; Turnbull et al. 2008; Noble et al. 2020). High-stocking densities could indirectly affect fish welfare through reduced water quality, decreased growth rate, and competition for feed (Pitcher 1986; Ellis et al. 2002; Martins et al. 2012; Noble et al. 2020). Therefore, it is important to consider the effect of stocking density, in addition to environmental complexity, on fish health and welfare.

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Fish can experience negative emotions, such as pain, fear, and suffering, just like their terrestrial counterparts, through systems similar to the prefrontal cortex (Ashley and Sneddon 2008; Braithwaite and Boulcott 2008; Relić et al. 2010). These short-term emotions are adaptive and allow the animal to appropriately respond to changing environments (Crump et al. 2020). Short-term emotional responses can shape an animal's affective state, which are long-term states that reflect the valence, positive or negative, of emotions over time (Mendl et al. 2010). Affective states can influence the way an animal makes decisions (cognitive bias) (Kleinginna and Kleinginna 1981; Lazarus 1982; Paul et al. 2005; Millot et al. 2014; Dolcos and Denkova 2015; Roelofs et al. 2016; Cerqueira et al. 2017; Laubu et al. 2019; Rogers et al. 2020). Cognitive bias tests are a well-validated indicator of animal welfare for a variety of terrestrial species, however, there is little evidence of this type of test being employed in an aquatic setting for farmed fish (Baciadonna and McElligott 2015; Bethell 2015; Crump et al. 2020). One type of cognitive bias test measures a subject's judgement bias through responses to ambiguous cues, which is then used to determine the subject's level of optimism or pessimism. Shorter latencies to approach ambiguous cues would indicate optimism (greater expectation of a reward), whereas longer latencies to approach ambiguous cues would indicate pessimism (lower expectation of a reward) (MacLeod and Byrne 1996; Harding et al. 2004; Mendl et al. 2009, 2010; Crump et al. 2020). Ultimately, the judgement bias test has been considered the "gold standard" for evaluating affective states in animals and could be a valid tool to assess fish welfare (Bateson and Nettle 2015).

Environmental complexity has the potential to positively impact fish welfare and affective states. Providing shelter structures has been shown to decrease aggression, fin erosion, and distress in Atlantic salmon (*Salmo salar*) and rainbow trout, possibly because fish have the opportunity to escape bullies (Bosakowski and Wagner 1995; Brockmark et al. 2007; Näslund et al. 2013; Näslund and Johnson 2016). Artificial vegetation decreased the frequency of startle responses in tiger muskellunge (*Esox masquinongy* × *Esox lucius*) and reduced the habituation period for bream (*Abramis brama*) in experimental conditions (Gerasimov and Stolbunov 2007; Einfalt et al. 2013). Additionally, providing floating artificial vegetation can serve as partial visual cover, which is preferred over unshaded areas, and can increase growth rate and decrease stress of Atlantic salmon (Pickering et al. 1987; Nordgreen et al. 2013). Based on prior findings, providing environmental complexity within the tank could lead to a reduction of emotions associated with a negative affective state and induce an overall positive affective state.

Affective states of zebrafish (*Danio rerio*) housed in different environments were successfully evaluated through a

judgement bias test (Wojtas et al. 2015). Zebrafish housed in enriched tanks showed more exploratory behavior within ambiguous cues than those housed in barren tanks, suggesting affective state was manipulated by environmental conditions. Additionally, female cichlid fish (*Cichlidae*) housed with non-preferred males showed pessimistic responses during a judgement bias test (Laubu et al. 2019). Based on these studies, judgement bias tests could be a useful tool to assess affective states of rainbow trout housed under varying environmental conditions, however, this has not yet been employed.

The objective of this study was to evaluate the effects of environmental complexity and stocking density on affective states of rainbow trout through a judgement bias test. Additional measurements were taken, such as individual weight and length to evaluate potential impact of housing environments on production outcomes, feeding behavior, and plasma cortisol to assess fish stress levels. We hypothesized that fish reared in high-complexity tanks of either high- or low-density would exhibit increased optimism in the judgement bias test through shorter latencies to approach ambiguous cues compared to fish reared in low-complexity tanks of either density. We also expected weight and length of fish from high-complexity, low-density tanks to be greater than fish from any other treatment group. We expected fish housed in high-density tanks of either complexity level to show the shortest latencies to begin feeding compared to all other treatment groups because of increased competition for feed. Lastly, it was hypothesized that fish housed in high-complexity, low-density tanks would show decreased stress when compared to all other treatment groups.

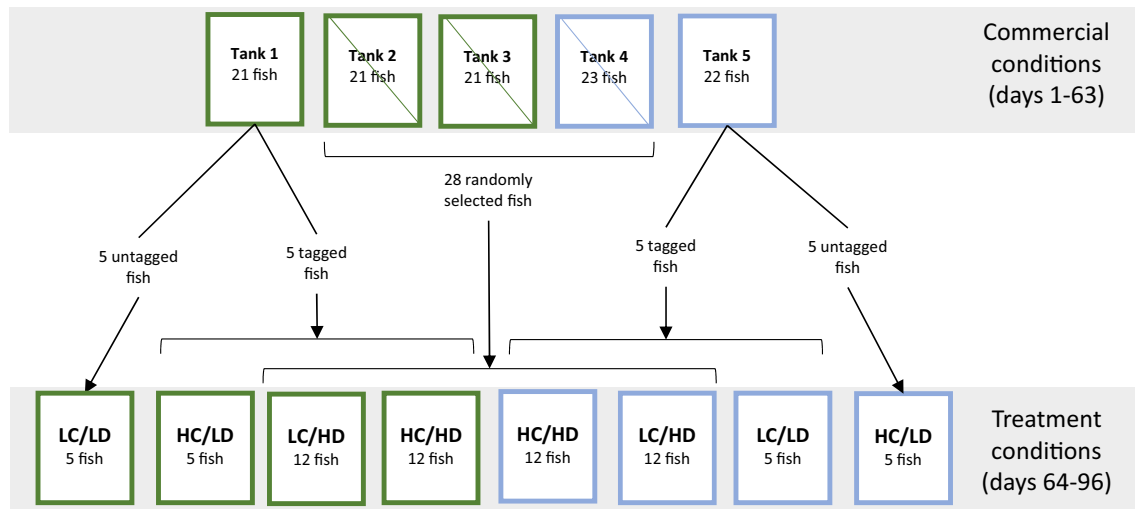
## Materials and methods

### Ethics

This experiment was approved by Virginia Tech's Institutional Animal Care and Use Committee (Protocol No. 20-074) and was conducted in the Department of Food Science and Technology's aquaculture facility at Virginia Tech's Human, Agriculture, and Biosciences Building I from August to December 2020. This experiment was performed in accordance with the Institutional Animal Care and Use Committee's relevant guidelines and regulations.

### Subjects and housing

Rainbow trout ( $n = 108$ ; F1 generation Shasta strain) were bred and hatched in January 2020 and cultured at the Wytheville State Fish Hatchery (Wytheville, VA). In August 2020, fingerlings were transported to the research



**Fig. 1** Overview of housing conditions and fish distribution throughout the experiment. Tanks in green were trained with the positive cue on the far left of the judgement bias arena, while tanks in blue were trained with the positive cue on the far right. Tanks with fish that did

not pass phase 3 learning criterion are crossed with a diagonal line. *LC* low-complexity, *HC* high-complpant were used tolexity, *LD* low-density, *HD* high-density treatments

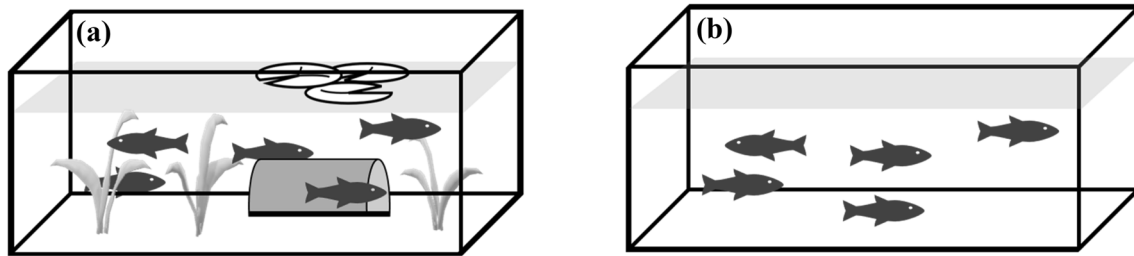
facility in two tanks of 14.1 °C water. Upon arrival, fingerlings were acclimated to the recirculating aquaculture system water conditions (13.3 °C) and facility management for 4 weeks. No mortalities were observed during or 24 h post-transportation. On day 1 of the experiment, fish were distributed across five tanks (45.7 × 73.7 × 21.6 cm; water volume = 0.0726 m<sup>3</sup>) under commercial conditions with 21, 22, or 23 fish in each tank. Temperature, dissolved oxygen level, ammonia-*N*, nitrite-*N*, nitrate-*N*, pH, and alkalinity were monitored at least once per week. All water quality parameters remained within suitable ranges (Ausseil 2013; Cline 2019), with the exception of alkalinity, which dropped below the optimal range on day 3. Sodium bicarbonate was added to the system throughout the trial to maintain optimal alkalinity values. Fish were fed a commercial trout diet (3 mm Finfish Gold, Zeigler Bros Inc., Gardner, PA, USA) once daily ad libitum. A subsample of fish ( $n = 72$ ) were tagged with T-bar tags (Floy Tag, WA, USA) after sedation with sodium bicarbonate buffered MS-222 (Syndel, Ferndale, WA, USA) on days 1–3 (AVMA 2020).

## Treatments

This experiment involved a 2 × 2 factorial design using environmental complexity and stocking density as factors, resulting in four treatment groups: high-complexity/high-density (HC/HD), high-complexity/low-density (HC/LD), low-complexity/high-density (LC/HD), and low-complexity/low-density (LC/LD). All fish ( $n = 108$ ) were kept under the same commercial conditions (five tanks) until

day 64 of the experiment. During this time, all fish were trained on a judgement bias task at tank-level. After the judgement bias training was completed and on day 64, 40 fish from the two tanks that were successfully trained were allocated to eight treatment tanks (five successfully trained fish/treatment tank). Tags of successfully trained fish were marked with black marker ( $n = 40$ ) to differentiate them from fish that were not successfully trained, most importantly in HD tanks. Three arbitrarily selected fish from the two successfully trained tanks were excluded from the experiment to achieve an even distribution of successfully trained fish across the eight treatment tanks. Twenty-eight fish from the remaining three tanks that did not meet the learning criterion were arbitrarily selected and evenly distributed across four of the eight tanks for the HD treatment ( $n = 7$  fish/HD tank). The remaining fish ( $n = 37$ ) were excluded from the experiment. Thus, from days 1–64 all fish were kept under commercial conditions in five tanks, then a subsample of fish ( $n = 68$ ) were redistributed over eight treatment tanks in which they remained until day 96 (Fig. 1).

Four tanks provided a complex environment (HC; Fig. 2a), while the other four tanks provided a simple environment similar to commercial standards (LC; Fig. 2b). HC tanks contained one PVC shelter structure (cut in half, 10.2 cm diameter, 15.2 cm long), which was placed at the bottom of the tank, two artificial floating lily pads (17.5 cm × 17.0 cm, Amazon.com, Inc., WA, USA), and two artificial Cabomba plants (17.8 cm, AquaTop, CA, USA). Enrichment objects were removed and disinfected daily. LC tanks contained no enrichment objects.



**Fig. 2** **a** Schematic of high-complexity (HC) tanks, which included three artificial floating lily pads, three artificial Cabomba plants, and a PVC shelter structure. **b** Schematic of low-complexity (LC) tanks without enrichment items

Based on the mean fish weight of 287 g at day 96 and the Food and Agriculture Organization of the United Nations guidelines (Woynarovich et al. 2011), four tanks (HC = 2; LC = 2) were stocked at a high-density (HD) of 12 fish (165 fish/m<sup>3</sup>). The other four tanks (HC = 2; LC = 2) were stocked at a low-density (LD) of 5 fish (69 fish/m<sup>3</sup>).

### Judgement bias test

The judgement bias process followed a seven-step approach (habituation; training: phase 1, phase 1A, phase 2, phase 3; reminder training; and testing; Table 1) and took place in the home tanks, at tank-level. All steps of the judgement bias test were performed using a removable plexiglass arena with five equally distanced opaque blue chambers and sliding doors (Fig. 3). Sliding doors

allowed for the experimenter to provide or deny access to each of the five chambers in the arena.

Habituation to the judgement bias arena without sliding doors was performed with all fish within a tank ( $n = 21\text{--}23$  fish/tank; Table 1). A single experimenter gently placed the arena in the tanks from days 13–31 for nine habituation sessions. For the first four sessions, the arena was placed into each tank for 5 min with the experimenter out of sight. For the following three sessions, the arena was placed and the experimenter remained in line of sight for 15 min per session. During the last two sessions, the arena was placed and the experimenter sprinkled feed into the tank, remaining in line of sight for 15 min per session.

Training phases 1–3, reminder training, and testing involved placing the arena into each tank with the sliding doors closed for 3 min to allow fish to acclimate. Afterwards, if a fish entered the POS chamber within any 15 s

**Table 1** Overview of judgement bias training and testing approach for rainbow trout during days 13–95. Fish ( $n = 5$  tanks; 108 fish) were trained to associate a positive reward (handful of feed) with the POS chamber and a negative punishment (1 s chase by net) with the NEG chamber

Judgement bias phase	Objective	Day <sup>1</sup>	Maximum duration/ session (min)	Attempts/ session (n)	Measurements
Habituation	Acclimate fish to arena in groups of ~21	13–31	Sessions 1–4 5 Sessions 5–9 15	1 1	n/a
1	Associate POS chamber with reward in groups of ~21	32–58	5	12	Fish that entered chamber (n), fish feeding (y/n)
1A	Autoshaping in groups of ~21	39–57	5	12	Fish that oriented towards or entered chamber (n), fish feeding (n)
2	Enter POS chamber while in groups of ~10	49–59	5	12	Fish that entered the chamber (n), fish feeding (n), latency of first fish to enter (s)
3	Associate NEG chamber with punishment	59–61	2.5	6	Latency for the first fish to enter (s)
Reminder training <sup>2</sup>	Reinforce NEG and POS chamber responses	71, 79, 88	2.5	6	Latency for the first fish to enter (s)
Testing <sup>2</sup>	Record responses to ambiguous cues	92, 93, 95	4	7	Latency for the first fish to enter (s)

A subsample of fish ( $n = 2$  tanks; 38 fish) moved on to the reminder training and testing phases

<sup>1</sup>From start of the phase until the last tank met the learning criterion for that phase

<sup>2</sup>Occurred after fish were allocated to their treatment groups

**Table 2** Mean latency for the first fish to enter ( $s \pm$  standard error) each chamber during the judgement bias test for trout in either high- or low-complexity tanks ( $n=8$  tanks; 38 fish)

Complexity level	Mean latency (s) for the first fish to enter (LSM $\pm$ SEM)				
	POS	NP	MID	NN	NEG
High	11.56 $\pm$ 1.69	11.17 $\pm$ 2.75	10.41 $\pm$ 2.76	12.09 $\pm$ 2.33	13.39 $\pm$ 2.45
Low	11.01 $\pm$ 1.69	9.87 $\pm$ 2.75	8.78 $\pm$ 2.76	15.43 $\pm$ 2.33	14.60 $\pm$ 2.45
<i>P</i> value	0.891	0.752	0.692	0.350	0.739

attempt during phases 1–3, feed was immediately placed into the chamber and fish were allowed 10 s to feed. After every attempt, the chamber door was closed and remaining feed was removed. Habituation and phases 1–3 were performed while fish were housed in commercial conditions, while reminder training and testing took place while fish were housed under treatment conditions (Table 1).

Phase 1 of training was performed at tank-level (Table 1). Fish in each tank ( $n=21$ – $23$  fish/tank) were trained to associate a chamber on either the far left (three tanks) or far right (two tanks) side of the arena with a reward (POS; approximately 30 feed pellets). If no fish entered the chamber within 15 s, feed was placed into the chamber and 10 s was allowed for fish to enter and feed. The learning criterion for phase 1 was met when at least 1 fish entered the POS chamber within 15 s for 9 out of 12 attempts during two consecutive phase 1 training sessions. Two tanks passed the learning criterion for phase 1 of training between days 38 and 58, the other three tanks moved on to phase 1A of training, as the fish were not close to meeting the learning criterion after five sessions. Training phase 1A was similar to phase 1, however, fish were rewarded with feed if they oriented towards or swam within 15 cm of the POS chamber opening. Tanks returned to phase 1 of training once at least 1 fish entered the POS chamber within 15 s during two consecutive phase 1A training sessions. Only one tank met the learning criterion for phase 1A and returned to phase 1 on day 46, meeting the phase 1 learning criterion on day 47. The other two tanks remained in phase 1A until day 57, when they were excluded from the judgement bias task due to unresponsiveness.

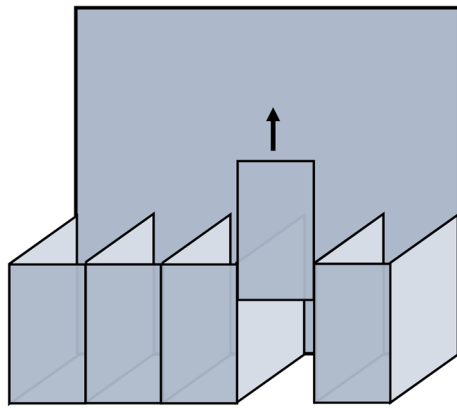
Phase 2 of training with the remaining three tanks was aimed to habituate fish to being in a smaller group within their tank (approximately 10 fish/group; 2 groups/tank; Table 1). Half of the fish (group 1) were gently herded to the side of the tank containing the arena and separated from their conspecifics (group 2) by placing a blue opaque plexiglass separator into the middle of the tank. Then, the POS chamber door was opened. If no fish entered the chamber within the first 15 s attempt, feed was placed into the chamber and fish were allowed 10 s to enter and feed. If no fish entered during any of the following 15 s attempts, the chamber door was immediately closed and the next attempt began. If a fish entered the chamber within any of the 15 s attempts,

feed was immediately placed into the chamber and fish were allowed 10 s to feed. After group 1, group 2 underwent the same training session. Phase 2 learning criterion was met when at least one fish in both groups 1 and 2 entered the POS chamber within 15 s and consumed feed in 9 out of 12 attempts during two consecutive phase 2 sessions. All three tanks passed the learning criterion for phase 2 of training between days 45 and 59.

In training phase 3, the negative chamber was introduced (NEG; net placed in water for 1 s; Table 1). NEG and POS cue presentations were pseudorandomized according to a pre-determined order, never allowing more than two consecutive presentations of the NEG or POS cue, and began and ended with the POS cue. Half of the fish (group 1; approximately 10 fish) were gently herded to the side of the tank containing the arena and separated from their conspecifics (group 2; approximately 10 fish) by placing the blue opaque plexiglass separator into the middle of the tank. Then, the POS chamber door was opened. If a fish did not enter within 15 s, the door was immediately closed. When the NEG chamber was opened, fish were allotted 15 s to enter the chamber. If a fish entered within 15 s, a green net was placed into the water for approximately 1 s, then the chamber door was closed. If no fish entered within 15 s, the chamber door was immediately closed. After six attempts (1 session) for group 1, group 2 was trained. Phase 3 learning criterion was met when at least one fish from both groups enter the POS chamber 100% of the time it was accessible and neglected to enter the NEG chamber 100% of the time it was accessible during two consecutive phase 3 training sessions. Two tanks passed the learning criterion for phase 3 on day 61. The third tank was excluded from the judgement bias task due to time constraints.

The trained subsample of fish (HD) or all fish (LD) underwent weekly reminder training sessions identical to phase 3 of training (Table 1). In HC tanks, enrichment objects were removed prior to a session.

Each of the eight tanks ( $n=38$  fish) were tested for judgement bias three times on days 92, 93, and 95 (Table 1). In addition to the POS and NEG cues, three ambiguous cues (near positive, NP; middle, MID; near negative, NN) were individually presented at intermediate locations within the arena (near left, middle, and near right; Fig. 3). Each



**Fig. 3** Diagram of the judgement bias arena. Positive (POS) and negative (NEG) chambers were balanced across tanks ( $n=8$ ). The middle three ambiguous chambers were introduced during testing. Entrance into each chamber was allowed by opening a sliding plexiglass door

ambiguous cue was presented once per session according to a pre-determined order. Fish were given 20 s to enter an opened chamber. Testing always began and ended with the POS cue, which was presented 8 times with fish receiving a feed reward if they entered the chamber. The NEG cue was presented 4 times and if a fish entered the chamber, it was chased by a net for 1 s. All ambiguous cues were neither rewarded nor punished. For HC tanks, enrichment objects were removed prior to testing. In HD tanks, the tagged subsample of fish was separated from the other seven fish with a blue plexiglass separator throughout testing. Fish were allowed 3 min to acclimate. Latency for the first fish to enter each chamber (s) was recorded. A maximum latency score of 20 s was appointed to attempts during which no fish entered the chamber.

### Body weight and standard length

On days 64 ( $n=68$ ) and 96 ( $n=66$ ), individual weights and lengths were recorded. On day 96, only 66 fish were weighed and measured due to two mortalities. Fish were individually netted and placed into a buffered MS-222 water bath (sedation strength of 75–100 mg/L) for approximately 5 min or until sedated (AVMA 2020). After sedation, the fish was placed on a scale (PTS3000, 0.1 g precision, PESOLA, CH, USA) and then beside a ruler to measure standard length (from the tip of the nose to the base of the caudal fin). Then, the fish was immediately placed in a fresh water recovery tank where it was monitored until normal swimming behavior resumed. At this point, the fish was placed back into the home tank.

### Feeding behavior

Latency to begin feeding was assessed at tank-level on days 79–95 (observation  $n=128$ ). During daily feeding, the observer sprinkled a handful of feed into the tank and immediately started a timer. Latency until the first fish began feeding(s) was recorded for each tank once daily.

### Plasma cortisol

On day 96, blood was collected from the 38 fish trained on the judgement bias task ( $n=4-5$ /tank). Fish ( $n=38$ ) were individually netted and placed into a buffered MS-222 water bath (sedation strength of 75–100 mg/L) for approximately 5 min or until sedated (AVMA 2020). After sedation, the fish was placed on a wetted work table and bled from the caudal tail vessels using a 23-gauge needle and syringe. Approximately 0.5–1.0 mL of blood was obtained, then the fish was immediately euthanized by an overdose of buffered MS-222 (euthanasia strength of 250 mg/L). Blood was placed in heparinized tubes and kept on ice until centrifugation. Samples were centrifuged at  $3000\times g$  for 10 min at 12 °C, then plasma was stored at  $-80$  °C until ELISA analysis using a commercial cortisol express ELISA kit (Cayman Chemical, Ann Arbor, MI, USA). The ELISA was performed following the manufacturer protocol. Four of 38 samples were excluded from the statistical analysis due to unreliable assay results.

### Statistical analysis

All data were analyzed in JMP pro 15 (SAS Institute Inc., Cary, NC, USA). Judgement bias data residuals were deemed normally distributed based on visual examination of normal quantile plots. Mixed models were used with complexity, stocking density, and test session as fixed factors, tank as a random factor, and latency to enter each chamber as the response variable. There was an effect of stocking density on latencies, so we blocked the analysis by chamber type (POS, NP, MID, NN, NEG) to assess the effect of density on latencies for each separate chamber. Tukey HSD post hoc comparisons were used to evaluate pairwise differences. Length and weight data residuals were normally distributed and analyzed using mixed models, with environmental complexity and stocking density as fixed factors and tank as a random factor. Latency to begin feeding data were log<sub>10</sub> transformed to obtain a normal distribution of data residuals and are presented as raw means. Then, mixed models were used with environmental complexity and stocking density as fixed factors and tank as a random factor. Data are presented as least squares means  $\pm$  standard error unless otherwise noted.

## Results

### Judgement bias test

After 32 days under treatment conditions (Fig. 1), fish kept in HC/HD, HC/LD, LC/HD, and LC/LD treatments (eight tanks total) were tested for judgement bias, measuring latencies to enter the positive (POS), negative (NEG), and three ambiguous chambers (near positive, NP; middle, MID; near negative, NN; Fig. 3). Mean latencies to enter all chambers were  $10.91 \pm 1.59$  s for test session 1,  $11.94 \pm 1.59$  s for test session 2, and  $12.55 \pm 1.59$  s for test session 3. Testing session did not impact responses of fish during the judgement bias test ( $F_{1,2} = 0.973$ ;  $P = 0.380$ ).

Environmental complexity did not affect latencies for fish to enter all chambers during the judgement bias test (HC =  $11.76 \pm 0.95$  s; LC =  $11.84 \pm 0.95$  s;  $P = 0.955$ ). Furthermore, environmental complexity did not impact latencies to enter the POS ( $F_{1,6} = 0.02$ ;  $P = 0.891$ ), NP ( $F_{1,6} = 0.11$ ;  $P = 0.752$ ), MID ( $F_{1,6} = 0.173$ ;  $P = 0.692$ ), NN ( $F_{1,6} = 1.026$ ;  $P = 0.350$ ), or NEG ( $F_{1,6} = 0.121$ ;  $P = 0.739$ ) chambers (Table 2). There was no interaction effect of chamber type and complexity level on latencies to enter all chamber cues ( $F_{1,148} = 0.699$ ;  $P = 0.594$ ).

Fish housed in HD tanks were faster to enter the test chambers than fish housed in LD tanks (HD =  $8.45 \pm 0.91$  s; LD =  $15.2 \pm 0.91$  s;  $P = 0.001$ ). Fish from HD tanks were faster to enter the NN ( $F_{1,6} = 20.3$ ;  $P = 0.006$ ) and POS ( $F_{1,6} = 10.797$ ;  $P = 0.022$ ) chambers compared to fish from LD tanks (Fig. 4). Additionally, fish from HD tanks tended to enter the MID ( $F_{1,6} = 4.68$ ;  $P = 0.083$ ) and NP ( $F_{1,6} = 5.075$ ;  $P = 0.074$ ) chambers faster than fish from LD tanks. There were no pairwise differences in latency to enter the NEG chamber ( $F_{1,6} = 3.118$ ;  $P = 0.138$ ; Fig. 4). Mean latencies to enter each chamber for density treatments are presented in Table 3.

### Body weight and standard length

There was no effect of environmental complexity or stocking density on body weight (Fig. 5) and length of fish on day 64 or 96 ( $P > 0.1$ ; Fig. 6).

### Feeding behavior

Environmental complexity did not impact latencies to begin feeding during daily feedings ( $F_{1,15} = 0.161$ ;  $P = 0.709$ ; Fig. 7a). However, fish housed in high-density tanks had shorter latencies to begin feeding than fish housed in low-density tanks ( $F_{1,15} = 13.9$ ;  $P = 0.0203$ ; Fig. 7b).

### Plasma cortisol

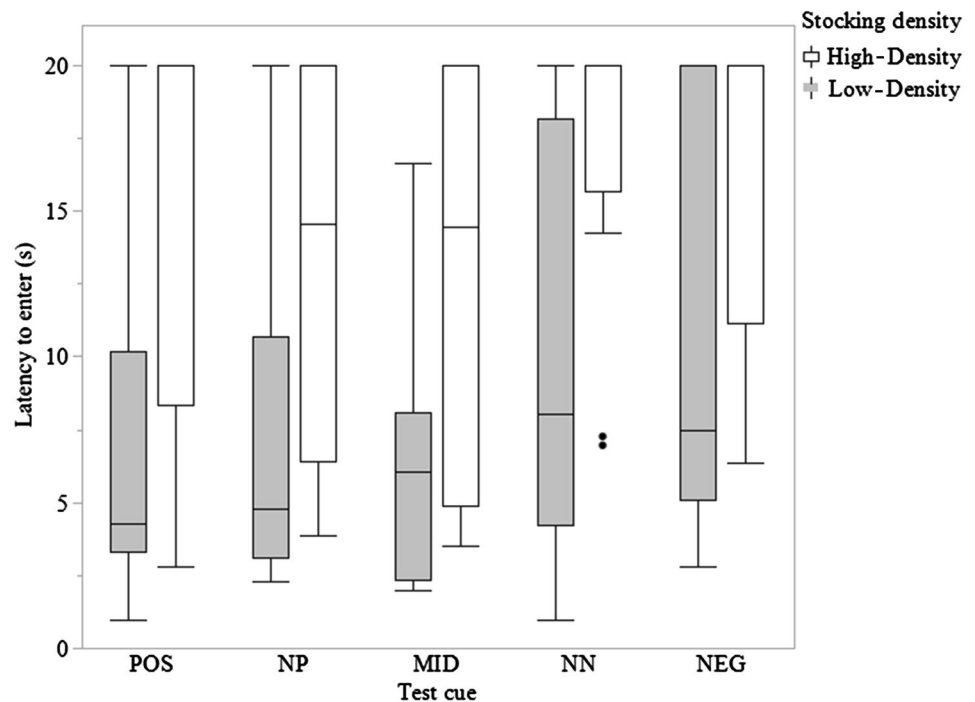
Cortisol concentrations did not differ between treatment groups ( $P > 0.1$ ). Mean cortisol concentrations were 1.16 ng/mL for HD, 3.68 ng/mL for LD, 3.09 ng/mL for HC, and 1.74 ng/mL for LC fish (standard error = 1.10;  $n = 34$ ).

## Discussion

This study is the first to apply a judgement bias test to evaluate affective states of rainbow trout. While housed in commercial conditions, fish were trained at tank-level to discriminate between two opposing locations of an arena, with one location associated with a feed reward and the other location associated with being chased by a net. Fish underwent habituation and six training steps prior to testing, with each training step completed after fish met the learning criterion (Table 1). After 64 days of training, two of the five tanks successfully passed the learning criterion to be tested. Then, we attempted to manipulate affective state by placing fish in either high- or low-complexity tanks under either high- or low-density. Fish from HD tanks were faster to enter all chambers than fish from LD tanks, and had shorter latencies to enter the NN and POS chambers, suggesting optimism for a reward in the fish from HD tanks. Environmental complexity did not affect latencies to enter chambers during the judgement bias test, suggesting environmental complexity had no impact on fish affective state or optimism in this study.

The lack of impact of the environmental complexity treatment was opposite to our hypothesis. We provided environmental enrichments that were biologically relevant and ultimately improved trout affective state through rearing them in an environment closest to that of their natural living conditions (Newberry 1995). In their natural habitat, rainbow trout will seek cover in the form of overhanging vegetation, undercut banks, aquatic vegetation, logs, or debris piles to rest and avoid predation (Bernstein and Montgomery 2008). We used artificial floating lily pads as visual cover because it has been shown that rainbow trout prefer darker areas of a tank environment over exposure to bright lights, and seek safety in these areas when in the presence of a threatening stimulus (Kwain and MacCrimmon 1969; Becket and Barnes 2015). Additionally, a PVC shelter structure was provided, because the presence, not necessarily the utilization, of shelters reduced basal cortisol and metabolic rates in Atlantic salmon (Millidine et al. 2006; Näslund et al. 2013). Finally, artificial trout in small groups abomba plants were used to simulate aquatic vegetation, a form of shelter used by wild rainbow trout fry (Bernstein and Montgomery 2008). Similar environmental enrichments (shelter and gentle light) for zebrafish resulted in more optimistic responses during

**Fig. 4** Boxplot of latencies for the first fish to enter (seconds) each chamber (with outliers; dots) during the judgement bias test for trout housed under high- or low- stocking density ( $n = 8$  tanks; 38 fish).  $**P < 0.01$ ;  $*P < 0.05$ ;  $\dagger P < 0.1$



the judgement bias test compared to the control, with more exploratory behavior within the ambiguous cues (28% of observed time compared to 7.5%) (Wojtas et al. 2015). Social enrichment for cod (*Gadus morhua*) affected their cognitive bias; cod housed with a larger, more aggressive conspecific for 24 h were 12 times less likely to enter ambiguous chambers during a judgement bias test compared to fish housed in social isolation for 24 h (Rogers et al. 2020). Similarly, female cichlids housed with an unpreferred male showed longer latencies to respond to the ambiguous signal than females housed with a preferred male (approximately 600 s versus 300 s) (Laubu et al. 2019). These studies show that judgement bias can be influenced by environmental conditions, however, it is possible that fish in our study were not exposed to the enrichments for long enough to observe any effect on affective state, with 3 weeks of exposure prior to judgement bias testing in our study compared to 7–18 weeks in previous work. Alternatively, the effect of density might have overshadowed any potential effect of environmental complexity. Perhaps the LD treatment was too great of a stressor that environmental complexity could not alleviate that stress. Similarly, the HD environment may have provided such a welfare benefit that environmental complexity could not contribute further to trout responses. As the fish used in the current study were of an F1 generation, the lack of domestication may be an influencing factor. Domestication can impact fish behavior, including swimming and foraging behavior, with aquaculture-raised fish generally showing slower swim speed and velocity, and foraging at the water surface rather than the water depth, compared to

the same species from the wild (reviewed by Pasquet 2018). This may impact how later generations of trout respond to environmental complexity and stocking density treatments. In future studies, later generations should be tested to confirm the lack of impact of environmental complexity on affective state of rainbow trout.

To our knowledge, there are no published studies investigating the effects of stocking density on affective states of trout. During testing, fish from HD tanks were overall more optimistic than fish in LD tanks, and specifically more optimistic to receive a reward in the NN chamber. Fish from HD tanks also tended to be more optimistic to receive a reward in the MID and NP chambers than fish from LD tanks. We hypothesized that access to more space was preferable over large group sizes, yet our results indicate the contrary. Little is known about the preferred group sizes of rainbow trout in a semi-natural setting (Jenkins 1969; Johnsson 2003). In the wild, however, low population densities often result in territorial defense and dominant fish driving out subordinate fish from a preferred area, while higher population densities lead to the formation of fish aggregates (Bernstein and Montgomery 2008). Therefore, it is possible that fewer territorial interactions occurred in the high-density treatment, improving their affective state. It could be hypothesized that the faster entry into intermediate chambers seen in HD tanks compared to LD tanks is due to increased motivation to find food. However, the intermediate chambers presented during testing were novel, so fish would not have known whether entering the chambers resulted in a food reward or a punishment (being chased by a net). During the experiment fish

**Table 3** Mean latency for the first fish to enter ( $s \pm$  standard error) each chamber during the judgement bias test for trout in either high- or low-density tanks ( $n=8$  tanks; 38 fish)

Density level	Mean latency (s) for the first fish to enter (LSM $\pm$ SEM)				
	POS	NP	MID	NN	NEG
High	7.35 $\pm$ 1.69	7.13 $\pm$ 1.98	6.28 $\pm$ 2.04	10.15 $\pm$ 1.41	11.36 $\pm$ 1.95
Low	15.21 $\pm$ 1.69	13.91 $\pm$ 1.98	12.91 $\pm$ 2.04	17.37 $\pm$ 1.41	16.62 $\pm$ 1.95
<i>P</i> value	0.022	0.074	0.083	0.006	0.138

were fed ad libitum daily, suggesting that levels of hunger would not differ between density treatments. Additionally, a measure to avoid density effects during actual testing was to test fish at the same group size. During the test, fish from both LD and HD tanks were tested in groups of 5, so the pressure to obtain food should be similar between treatment. Therefore, we do not expect the shorter latency to enter intermediate chambers to be due to a greater motivation to feed, but due to an optimistic outlook that by entering the chamber, they will be rewarded with food. Our findings suggest that housing rainbow trout in small groups at high-densities results in fish that are optimistic in novel situations, therefore, in a more positive affective state than fish housed in small groups at low densities.

One limitation of our study was the time-intensive judgement bias training process. To be practically useful, judgement bias measures need to be easily attainable. We recommend further study into the modification of the judgement bias cues to be more biologically relevant. Perhaps utilizing access to conspecifics and social isolation as reward and punishment cues may allow for quicker training and a larger sample size for testing, however, this has not yet been investigated.

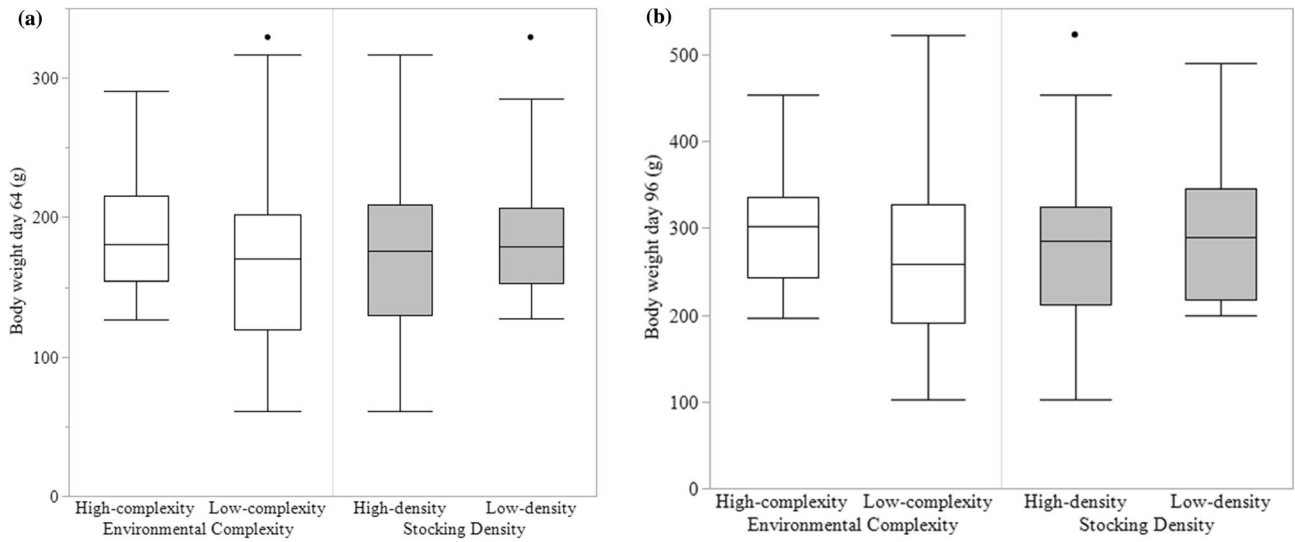
Previous studies have found positive effects of tank complexity on performance parameters of rainbow trout (Kientz et al. 2018; Krebs et al. 2018; Huysman et al. 2019; White et al. 2019). In the present study, neither environmental complexity nor stocking density impacted body weight or length of fish. The lack of effect of the former could be due to the length of exposure to these enrichments. Vertically suspended aluminum angles or rods with varying enrichment exposure duration (51, 61, 110, or 141 days) were associated with better trout weights, lengths, tank weight gain, and feed conversion ratio compared to trout housed in barren tanks (Kientz and Barnes 2016; Krebs et al. 2018; Huysman et al. 2019; White et al. 2019). Similarly, tanks with hanging colored plastic balls (for 70 or 127 days) showed improved tank weight gain, feed conversion ratio, and individual weight and lengths compared to trout housed without enrichments (Kientz et al. 2018; Crank et al. 2019). The previous work shows longer exposure times than the current study, which could be the reason for the lack of impact of complexity on production parameters in the current study.

The lack of effect of stocking density on production outcomes is somewhat in line with earlier findings (Kebus

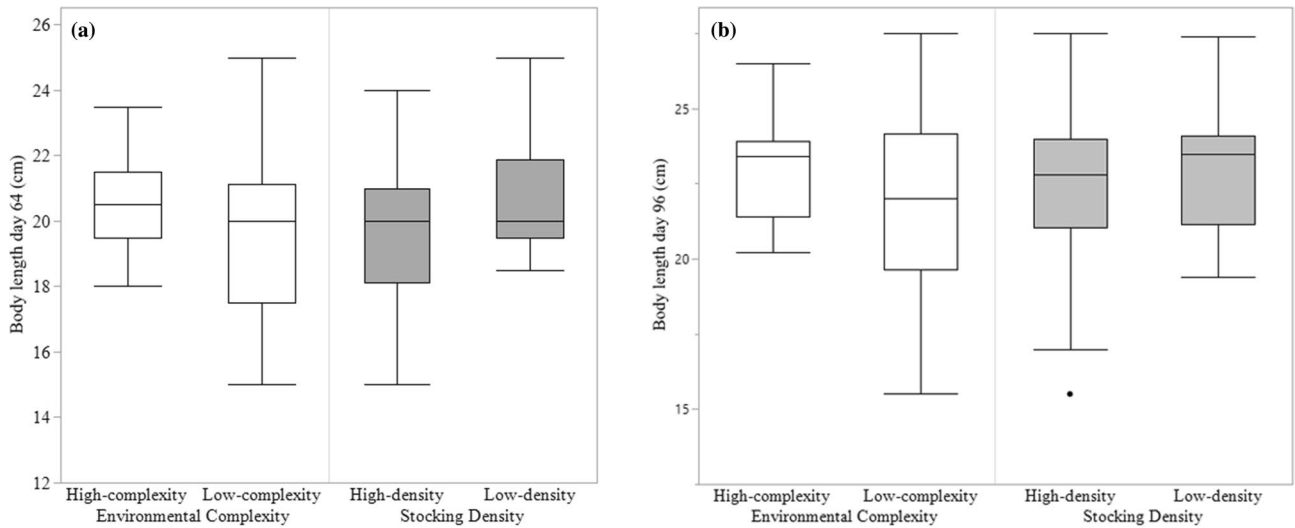
et al. 1992; Wagner et al. 1996). For instance, rainbow trout reared at either 10, 40, or 80 kg/m<sup>3</sup> did not differ in growth rate (North et al. 2006). Contrary to our findings, 70% of reviewed publications reported adverse effects of high-densities (similar or higher than the density in the current study) on trout growth (see review by Ellis et al. 2002). For example, rainbow trout housed at 312 fish/m<sup>3</sup> had the worst growth rate compared to trout housed at low densities of either 31, 94, 156, or 250 fish/m<sup>3</sup> (compared to 165 fish/m<sup>3</sup> versus 69 fish/m<sup>3</sup> in the present study), with no differences in growth rate between the lower density levels (Papoutsoglou et al. 1987). Based on the judgement bias test responses in HD tanks and the lack of effects of stocking density on production outcomes, we can conclude that the high stocking density in this study was not detrimental to trout welfare or production up to day 96.

Feeding activity has been used widely as an observational indicator of fish welfare, as stressors can reduce feed intake and motivation to feed (Martins et al. 2012). For instance, too low stocking densities decreased feed intake in rainbow trout (Ellis et al. 2002). We predicted that fish from HD tanks would have an increased motivation to feed, as increasing group size has shown to increase food-seeking behavior (Johnsson 2003). Our results conform to our predictions, as fish from HD tanks began feeding faster than fish from LD tanks (0.25 s compared to 0.79 s). This suggests that fish from HD tanks either had an increased motivation to feed or that fish from HD tanks were less stressed compared to fish from LD tanks. Although plasma cortisol concentrations did not significantly differ between stocking density treatments, numeric values do show a similar response compared to feeding behavior latencies, with fish from HD tanks having lower plasma cortisol than fish from LD tanks. This could imply that the fish from HD tanks were less stressed (lower cortisol) and more motivated to feed (shorter latencies to begin feeding) than fish from LD tanks, suggesting improved welfare.

Previous work on impacts of stocking density on cortisol levels show varying outcomes within and between studies. We hypothesized that fish from HC/HD tanks would have the lowest levels of plasma cortisol, as environmental complexity has been shown to reduce the impact of environmental stressors, while too low of stocking densities can result in higher stress levels (Leatherland and Cho 1985; Pounder et al. 2016). Opposite to our predictions and to

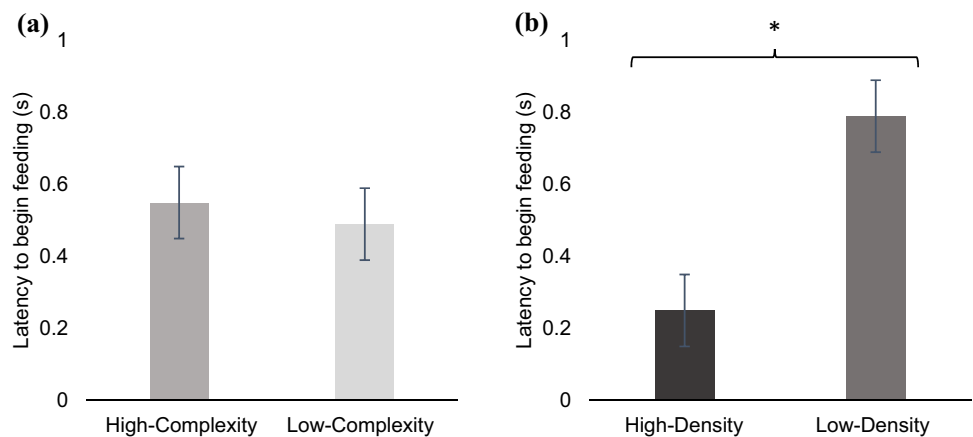


**Fig. 5** Boxplots of trout live body weight (g), with outliers (dots), in each treatment group, measured on **a** days 64 ( $n=68$ ) and **b** 96 ( $n=66$ )



**Fig. 6** Boxplots of trout body length (cm), with outliers (dots), in each treatment group, measured on **a** days 64 ( $n=68$ ) and **b** 96 ( $n=66$ )

**Fig. 7** Mean latencies for the first fish to begin feeding (seconds) during daily feedings on days 79–95 for fish housed in **a** high- or low-complexity tanks or **b** high- or low- stocking density tanks (observation  $n=128$ ).  $*P < 0.05$



some previous work, treatments did not impact plasma cortisol levels. Atlantic salmon reared with plastic tubes or shredded black plastic bag enrichments had lower basal plasma cortisol levels than salmon reared in a barren environment (approximately 35 ng/mL compared to 10–15 ng/mL) (Näslund et al. 2013). Rainbow trout reared at a low-density of 134 g/L (compared to 73.5 g/L in the present study) showed higher plasma cortisol levels compared to fish reared at a high-density of 277 g/L (approximately 18 ng/mL versus 5 ng/mL) (Leatherland and Cho 1985). Similarly, rainbow trout reared at 10 kg/m<sup>3</sup> showed higher plasma cortisol concentrations than trout reared at 80 kg/m<sup>3</sup> during five of the nine sample timepoints (North et al. 2006). Unstressed (control) brown trout (*Salmo trutta*) exhibited variable plasma cortisol concentrations, from approximately 20–70 ng/mL over the span of 8 h (Pickering et al. 1982). In contrast, basal levels of plasma cortisol in unstressed salmonid fish have been reported to remain between 0 and 5 ng/mL (Pickering and Pottinger 1989). This variation may, in addition to species and strain differences, be caused by the inconsistent nature of cortisol responses (Bry 1982; Rance et al. 1982; Woodward and Strange 1987; Pickering and Pottinger 1989), suggesting it may not be a reliable indicator for animal welfare.

This study is the first to establish the effect of environmental complexity and stocking density on judgement bias (optimism) in rainbow trout. Our results indicate that housing rainbow trout in relatively small groups at high-densities from day 64 through 96 results in improved welfare status without any negative effects on performance parameters. The high stocking density level (165 fish/m<sup>3</sup>) used in this study resulted in more optimistic responses during the judgement bias test compared to fish in low-density environments, therefore, suggesting a positive affective state in the former. Further confirmation of the beneficial effect of high-density was the increased motivation to feed compared to fish housed at low densities. Therefore, trout feeding behavior shows potential as a feasible animal welfare indicator in a production setting, as it can be easily measured by aquaculture personnel. Monitoring changes in feeding behavior could be a useful indicator of a health or welfare issue. By housing rainbow trout in the density conditions described in this study and monitoring feeding behavior regularly, producers have the opportunity to rear fish under high welfare standards.

This study showed that a group approach to judgement bias training and testing resulted in differences between density treatments suggestive of a positive affective state for trout in high stocking densities. More research is needed on effective environmental enrichment and duration of exposure to enrichments for rainbow trout. With further investigation and modification of the test approach, judgement bias tests can be a valid indicator of affective state in rainbow trout.

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**Author contributions** All authors contributed to the study conception, design, and data collection. Material preparation was performed by MGA, LJ, SAS, and DDK. MGA, LJ, and AMC performed formal data analysis. The original draft was prepared by MGA and LJ. Manuscript review and editing was performed by SAS and DDK. All authors have read and approved of the final manuscript.

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**Data availability** The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request.

## Declarations

**Conflict of interest** The authors declare no competing interests.

**Ethical approval** All procedures in this study were approved by and in accordance with Virginia Tech's Institutional Animal Care and Use Committee (Protocol No. 20-074).

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