Research Topics

Current state of fish behaviour & welfare research: Honoring Victoria Braithwaite

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Current state of fish behaviour & welfare research: Honoring Victoria Braithwaite

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Editorial: Current state of fish behaviour & welfare research: Honoring Victoria Braithwaite

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Editorial on the Research Topic
Current state of fish behaviour & welfare research: Honoring Victoria Braithwaite

The aim of this special Golden Research Topic collection is to review the current status of research into two related areas of behavioral biology: the cognitive capacities of fish and their welfare status. An important additional aim is to honor the work of Victoria Braithwaite (1967–2019), a brilliant scientist whose lasting contributions include seminal work in both these fields. Victoria died on 30th of September 2019, after a long fight against cancer.

Several papers in this collection are written by researchers who collaborated directly with Braithwaite in various capacities; others have been influenced by her published work. In both cases, her scientific influence can be clearly identified. The diversity of subject matter and approaches reflects Braithwaite's own breadth of vision and her sustained interest in both fundamental understanding of cognitive process and practical concern for improving fish welfare.

Studies of fish cognition

As discussed by Healy and Patton (*It began in ponds and rivers: charting the beginning of the ecology of fish cognition*), Braithwaite's research career started at the University of Oxford, UK, with her doctorate (1993) on the use of learned visual landmarks by pigeons [e.g., (1)]. This marked the beginning of an enduring interest in spatial learning in animals and what this can tell us about their cognitive capacities. Having gained her doctorate, Braithwaite moved to the University of Glasgow UK, where she worked on the use of visual and olfactory landmarks in juvenile Atlantic salmon [e.g., (2)]. In 1994, Braithwaite took up a lectureship at the University of Edinburgh UK, where she initiated several new lines of work on cognitive variability among fishes. In addition to Healy and Patton's analysis of Braithwaite's contribution to current understanding of cognitive ecology, this research theme is represented in this special issue by Droege et al., completing a project initiated by Braithwaite (*Fishnition: Developing models from cognition toward consciousness)* and Franks et al., investigating curiosity in zebrafish and its link to welfare (*Curiosity in zebrafish (Danio rerio)? Behavioral responses to 30 novel objects*).

Studies of fish welfare

In 1999 Braithwaite started the line of research for which she is best known. Together with her colleague Mike Gentle, she developed a multi-disciplinary study into pain perception in fishes, funded by the UK's Biotechnology and Biological Sciences Research Council. The subsequent recruitment of a skilled post-doctoral researcher, Lynne Sneddon, completed an impressive research team, showing that rainbow trout possess some of the kinds of nociceptors found in mammals, responding in similar ways to standard nociceptive cues (3). They also showed that nociceptive stimuli induce physiological stress and shifts in motivation in trout, so their responses are more complex than simple reflexes (4). The article in the special issue by Elwood (*Potential pain in fish and decapods: similar experimental approaches and similar results*) explains just how important this work was in stimulating and guiding his own studies. Braithwaite was well aware of the difficulty of demonstrating that non-human animals experience the emotion of pain, as expounded in her excellent monograph on this topic, *Do Fish Feel Pain*? (5). She would have appreciated the challenging discussion that continues in these pages, for example by Mason and Lavery (*What is it like to be a bass? Red herrings, sentience and the study of fish pain*), also part of a programme developed by Braithwaite. She would also have appreciated the account by Jarvis et al. of the first use of quantitative behavioral assessment in fish, which showed how experienced fish farmers are willing and able to assign affective states to the fish they farm (*Qualitative Behavioral Assessment in juvenile farmed Atlantic Salmon: potential for on-farm welfare assessment).*

A strand of Braithwaite's research that is not represented in this special issue but is of clear relevance to fish health concerned the relationship between behavior and parasitic infection. Thus, her team showed that sticklebacks with brightly-colored fathers grow less quickly than half–siblings with dully-colored fathers, but are more resistant to parasitic challenge. It seems that highly ornamented males confer inherited disease resistance on their offspring, but at a cost (6).

Practical steps to identify and improve fish welfare

After moving to Penn State University, USA (2007), Braithwaite continued research into behavior, cognition and welfare in fishes, including studies into the effects of environmental deprivation and enrichment on brain structure and cognition. Articles here developing this theme include: Alnes et al. (*Ontogenetic change in behavioral responses to structural enrichment from fry to parr in juvenile Atlantic salmon*); DePasquale et al. (*The influence of an enriched environment in enhancing recognition memory in zebrafish*); and Delaval et al. (*Does vaterite otolith deformation affect post-release survival and predation susceptibility of hatchery-reared juvenile Atlantic Salmon*?).

From a different perspective but with the same objectives, Turnbull uses human behavioral theory to explore what makes welfare interventions in aquaculture successful (*The complex influences on how we care for farmed fish*). Finally, Gaffney and Lavery summarize research recommendations from the Canadian Code of Practice for Farmed Salmonids (*Research before policy: identifying gaps in salmonid welfare research that require further study to inform evidence-based aquaculture guidelines in Canada*). As an early contributor to this project, Braihtwaite helped to set the future research agenda for understanding and protection of the welfare of captive fishes.

Overview

Braithwaite displayed remarkable qualities as a researcher, with a flair for identifying areas where important questions were waiting to be asked: *How do juvenile salmon navigate? Do fish feel pain? Can social learning be used to enhance life skills in cultured fish?* She embraced an interdisciplinary approach and was never deterred by the challenge of learning about unfamiliar topics, however complex and initially unfamiliar. The number and geographic range of her co-authors speaks to her special qualities as a stimulating and considerate colleague and collaborator. Her many publications with young researchers as first author speak to her excellence as guide and mentor. These qualities are all reflected in the papers presented in this collection, which we hope will serve as a fitting memorial to this highly gifted and generous scientist.

Author contributions

FH wrote the editorial. BF and SH wrote sections and provided comments. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
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Potential Pain in Fish and Decapods: Similar Experimental Approaches and Similar Results

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I review studies that examined the possibility of pain experience in fish and note how they provided guidance on general methods that could be applied to other animals such as decapod crustaceans. The fish studies initially reported the occurrence of prolonged rocking movements in trout and rubbing of their lips if they were injected with acetic acid. Subsequent studies examined the role of morphine in reducing these activities and examined shifts in attention when responding to noxious stimuli. Various studies take up these themes in decapods. The results reported for the two taxonomic groups are remarkably similar and indicate that responses of both go beyond those expected of mere nociceptive reflex. Thus, the idea of pain cannot be dismissed by the argument that fish and decapods respond only by reflex. The responses of both clearly involve central processing, and pain experience, although not proven for either, is a distinct possibility. These studies have been the subjects of highly critical opinion pieces and these are examined and rebutted. The conclusion is that both fish and decapods should be awarded consideration for their welfare.

Keywords: pain, fish, decapod, nociception, reflex

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INTRODUCTION

The seminal paper on rainbow trout (*Oncorhynchus mykiss*) by Sneddon et al. (1) established general methods for investigating pain in animals. It used two main approaches. First, two types of neurons that detected potentially painful stimuli were demonstrated, and their neural responses to specific noxious stimuli were reported. Second, the paper described various behavioral responses to a potentially painful event that could not be simple reflexes. It was this latter approach that provided the greater guidance for work on possible pain in decapod crustaceans. Various receptors had already been found on the antennae of spiny lobsters (*Panulirus argus*), some of which are chemosensory, whereas others responded to mechanical and chemical stimulation (2). Subsequent studies demonstrated receptors in crayfish (*Procambarus clarki*) that respond to high but not low temperatures and appear to function as nociceptors (3). Thus, in my laboratory, we chose not to examine the neurons but, rather, concentrated on asking if the responses to noxious stimuli were reflexive or not. This was a priority because the idea of invertebrates being able to experience pain had long been dismissed because their responses were said to be pure reflexes (4). A reflex is defined here as a short-term reaction to a stimulus without integrating information about other motivational requirements. Responses that are influenced by other sources of information and motivational requirements result from central processing, and swift avoidance learning, and long-term behavioral changes that are likely to enhance future avoidance of tissue damage are not reflexes.

ABNORMAL BEHAVIOR AND EFFECTS OF ANALGESICS AND LOCAL ANESTHETICS

To examine behavioral responses to potentially painful stimuli, Sneddon et al. (1) injected acetic acid, bee venom or saline control into the upper and lower lips, or simply handled rainbow trout in a further control group. Acetic acid and bee venom were selected because they cause pain in humans and are used in pain research in mammals. All groups of fish showed an increase in opercular ventilation rate, but the acetic acid and bee venom produced a greater response, and the elevated rate lasted longer than in the two control groups. This possibly reflects a greater physiological stress response that demands a high oxygen consumption with the potentially painful stimuli. All groups of fish stopped feeding but those of the venom and acetic acid groups avoided ingesting food for longer than did the control groups. That is the noxious stimuli interrupted normal behavior, but the use of a covered shelter in the tank was not affected and neither was the general swimming activity. Another finding was the performance of apparently anomalous activities following noxious treatments. Trout injected with venom or acid performed a rocking movement, where they moved from side to side, balancing on either pectoral fin, while resting on the gravel substrate. Further, the acid group rubbed their lips into the gravel and against the side of the tank. It was this final observation that had the largest impact on my own thoughts on potential pain because it showed a prolonged activity directed at the point at which the noxious stimulus had been applied. Similar directed rubbing is seen in humans and other mammals and it is thought to be a key indicator of pain (5–7). Trout injected with both morphine and acetic acid show much decreased rocking and rubbing compared to those injected with just the acid (8). Injection with a local anesthetic (lidocaine) has a similar effect (9).

This work on rainbow trout formed the basis of my laboratory's first experiment on potential pain in decapod crustaceans (10). We sought to examine the behavioral responses of glass prawns (*Palaemon elegans*) that had acetic acid, sodium hydroxide or seawater brushed onto the distal part of one antenna or that had a crushing force applied *via* forceps. We also sought to determine if a local anesthetic (benzocaine), applied before the noxious stimuli were applied, would modify responses to these treatments. Finally, we asked if any activities directed toward the antennae were preferentially directed at the treated antenna rather than being directed to both. The experiment was conducted in two stages. First, a prawn was removed from a tank and placed on damp tissue paper. One antenna was randomly selected and was brushed with either benzocaine or seawater. The prawn was then placed in a small tank and the behavior observed for 5 min. Second, the prawn was then removed, and the same antenna was subject to one of the three noxious stimuli or seawater control. It was then placed in the observation tank and the behavior again observed for 5 min.

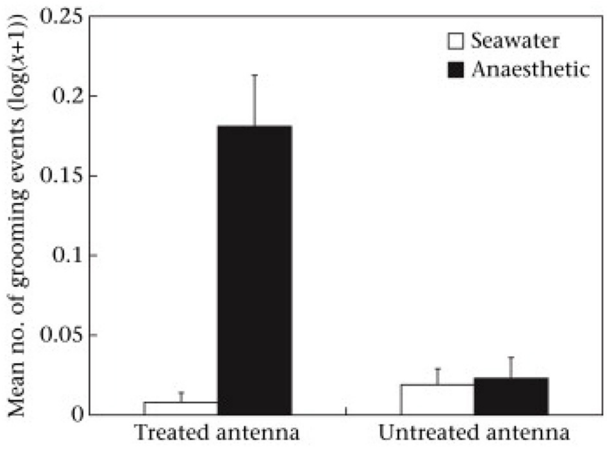

FIGURE 1 | Mean ± SE [log(x + 1)] of grooming of treated and untreated antennae following application of seawater or anesthetic in the first observation. Reproduced with permission from Barr et al. (10).

While the benzocaine was applied to the antenna about half the animals showed tail flicking, which is an escape response comprising rapid flexing of the abdomen that would normally propel the animal backwards. Because none of those having seawater applied performed flexing it showed that the benzocaine was initially aversive and presumably stimulated the nociceptors before silencing them in the way expected of a local anesthetic. When the animals were placed into water there was a high level of grooming of the antenna that had been treated with benzocaine, but very little directed to the alternative antenna and very little of either antenna if treated with seawater (**Figure 1**). Grooming involved repeated pulling of the antenna through the small pincers on the front legs of these animals. That is, the benzocaine did not have an immediate anesthetic effect and appeared to be aversive when first applied. Indeed, local anesthetics are reported to cause pain when first administered to humans, largely due to the acid medium (11), so it seems something similar happens in crustaceans. However, the benzocaine seemed to have the expected anesthetic effect by the time the second treatment was applied because no prawn with benzocaine then showed a tail flick response, but it was seen in those previously treated with seawater. Further, it was only seen in those animals receiving a noxious stimulus (chemical or pinching) and not those whose second treatment was again seawater.

When the prawns were placed again into the tank for the second observation period there were no differences between the groups in their swimming activity. However, there were differences in activities directed at the antennae that were attributable to both the first treatment (benzocaine vs. seawater) and the second treatments (chemicals, pinching, or seawater). In short, grooming was most frequently seen when the second treatment was chemical and applied to an unanesthetized antenna. Further, the grooming was directed specifically at the treated antenna rather than the alternative antenna (**Figure 2**). Prawns also showed rubbing of the antennae against the side of the tank and again this was of the treated antenna rather than the alternative antenna. It also occurred most with chemical treatment and if the first treatment had been seawater. Thus, we saw two responses involving the antennae that were reduced

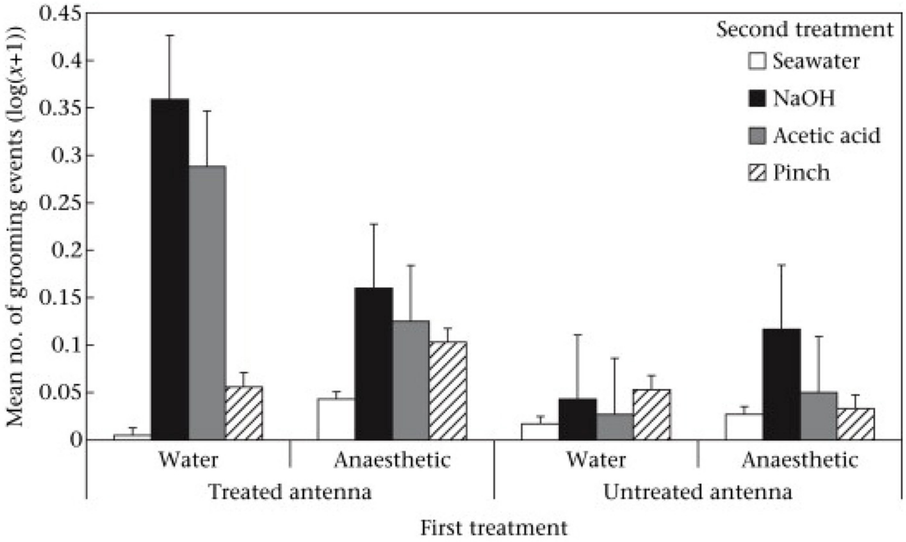

FIGURE 2 | Mean ± SE (log(x + 1)) of grooming of treated and untreated antennae in the second observation. Reproduced with permission from Barr et al. (10).

by pre-treatment with benzocaine and always directed more at the treated antenna compared to the untreated. Pinching with forceps elicited much less of a response than did the chemical treatment but there was more rubbing of the pinched antenna compared to that which was not pinched. However, rubbing was not significantly suppressed by benzocaine.

The results from our experiment on prawns showed remarkable similarities to those on trout (1, 8). Both trout and prawns responded to acetic acid by showing prolonged directed activity toward the site of application and trout had this response reduced by analgesic and prawns by local anesthetic. Further, shore crabs, *Carcinus maenas,* brushed with acetic acid on the mouth parts or an eye showed marked behavioral changes that, in the latter case, involved the specific treated eye (12). The responses of grooming and rubbing seen in these experiments appeared too prolonged and complex to be described as reflexive. Additionally, brown crabs, *Cancer pagurus*, that had a claw removed to simulate the fishery practice of collecting just the claws showed behavior directed at the wound and guarding of the wound when an intact competitor was encountered (13). Thus, there is a similarity with pain responses seen in humans and other mammals and these observations are consistent with the idea of pain. However, one should note that experiments using hydrochloric acid on three species of prawns found no such directed activities (14) and experiments on Atlantic cod, *Gadus morhua*, with acetic acid injection failed to show activities directed at the site of treatment (15).

SHIFTS IN ATTENTION

Another approach to examine potential pain in fish involved changes in the attention of trout to novel stimuli (16). Trout normally avoid novel objects but did not display this avoidance when they had been injected with acetic acid in the lips. Avoidance was seen, however, in fish injected with saline, and in fish treated with acid plus morphine. It was suggested that the pain caused by the acid dampened the attentiveness of the fish toward a novel item. Thus, it was suggested that a higher order process was involved in the attention decline while the fish was in pain (17). Studies in crayfish (*Procambarus clarki*) that examined responses after the cessation of the noxious stimulus (repeated electric shock) showed enhanced, relatively long-lived, fear responses (18). This finding was described as an enhancement of anxiety and shown to be mediated by elevated serotonin levels. Indeed, injection of serotonin without shock was sufficient to cause anxiety behavior. Remarkably, injection of an anxiolytic, originally produced to reduce anxiety in humans, also reduced anxiety in crayfish. These changes in anxiety and avoidance of risk are particularly interesting because they reduce further tissue damage over the longer term, which is presumed to be the key function of pain (19–21). This protection following noxious stimuli (wounding) has been demonstrated in squid during predation attempts by fish. Squid that were wounded attracted more predation attempts than unwounded squid, and survival was not as high as those without wounds. However, the lowest survival was seen in squid that were given an analgesic as well as the wound (22). It was suggested that the wound increased anxiety and enhanced attention to the predator, but this depended on nociceptive input to the central nervous system. It was consistent with a prediction of the function of pain. Similar findings on improved survival have since been noted in crustaceans (23).

LONG-TERM EFFECTS ON MOTIVATION AND LEARNING

Long-term changes in behavior after noxious stimuli have also been shown in hermit crabs (*Pagurus bernhardus*) (24, 25). Crabs were given shocks on their abdomen within their gastropod shells whereas control crabs were not shocked. The crabs were subsequently offered a new empty shell and the motivation to obtain a new shell was found to be markedly increased in those exposed to the noxious stimulus. Shocked crabs approached the new shell more quickly, moved to the new shell after a shorter latency, were more likely to change shells, and gave a less thorough investigation of the interior of new shell before moving in. Such shifts in behavior toward shells after shock clearly reflect enhanced motivation to switch shells and suggest that the shell is perceived as being low quality after shock (26, 27). The shift in motivation was found up to 24 h after the noxious stimulus. Thus, the resulting change in behavior could not be called a reflex.

Another way that pain might enhance fitness is that it increases the salience of the noxious stimulus and thus enables rapid avoidance learning. Such learning has been demonstrated in both fish (28) and in shore crabs (*Carcinus maenas*) (29). In the latter example crabs were repeatedly placed in an enclosure containing two dark shelters. In one shelter crabs were shocked if they entered and continued to be shocked if they remained, but the other shelter was safe. Crabs showed a significant shift in their entries to the shelters after just two trials and thus avoided the shock shelter. A further experiment used a different process of exposure to the shock and non-shock shelters (30). Whereas, in the first study the safe and shock shelter were simultaneously present in each trial, in the later study the animal was exposed to one shelter at a time, alternating with safe and shock experiences. Only after five exposures to each were the crabs given a choice of the two, but they showed no preference for the safe shelter. They did, however, reduce the number of shocks received during the trials because they simply left the shock shelter more quickly in later trials. Of course, one might ask why they simply did not refuse to enter either shelter as a means of avoiding shock. The problem with that is that crabs use dark shelters on the shore to avoid predation. Thus, entering a shelter is important for survival but, nevertheless they leave should they receive shock. This is likened to paying a cost to avoid shock. We see this repeatedly in studies in which animals leave a safe shelter such as a shell for hermit crabs (31) or giving up feeding opportunities to avoid the shock in fish (32). Paying a cost to avoid the stimulus is a clear demonstration that the animals find the stimulus aversive, and it is consistent with the idea of pain.

TRADE-OFFS WITH OTHER MOTIVATIONAL REQUIREMENTS

Observations of non-reflexive, long-term changes in behavior after noxious stimulation have been influential in our thinking about the occurrence of pain but persuasive experiments have also indicated that some swift responses are not purely reflexive. One way of showing this is to examine if escape from the noxious stimulus is traded-off against other motivational requirements. If they are traded-off, then they must be the result of a higher order decision rather than a reflex (33). There are examples of this with both fish and crustaceans. Trade-off between avoiding an area of electric shock and being near a companion fish has been demonstrated (28) as has that between shock avoidance and the requirement for food (32). Similarly, hermit crabs might leave their gastropod shells if shocked within, however, they evacuate from less preferred species of shell at a lower voltage than a preferred species (31) and, if the voltage is kept constant, they are more likely to get out of the less preferred species (25). Further, hermit crabs are less likely to leave their shell if the odor of a predator is in the surrounding water (34). Fish and crustaceans clearly trade-off various motivational requirements against shock avoidance. These studies demonstrate that even short-term or immediate responses may not be reflex escape responses but rather the result of centrally organized decisions that maximize fitness following tissue damage.

PHYSIOLOGICAL RESPONSES

Injury not only triggers behavioral responses that appear to indicate pain, but it also initiates a series of physiological changes that are termed stress responses (35). The main stress response in fish has similarities with those of other vertebrates (36). It is mediated by the hypothalamic-pituitary-interrenal axis and produces a cascade of hormonal changes. A key effect is the metabolism of glycogen to release glucose, thus providing an abundant energy supply to help deal with energetic activities required for fight or flight. An analogous system occurs in crustaceans with the release of crustacean hyperglycemic hormone (37). It too mobilizes intracellular glycogen and converts it to glucose. It also elevates lactate. After injury, such as the fishery practice of twisting and removing a claw, there is a rapid elevation of glucose and lactate in brown crabs (*Cancer pagurus*) (38). A sharp increase in lactate is also seen in shore crabs after a series of electric shocks (39). However, there is a potential problem with the interpretation of this finding because electric shock often induces escape responses, and the high lactate might be due to elevated activity rather than the shock *per se*. To get around this problem Elwood and Adams (39) recorded the behavior of crabs that were shocked and of non-shocked controls. Crabs that showed particularly high activity or particularly low activity were excluded from the data set, so that just those that walked around the enclosure were used in the lactate analysis. There was still a large difference in lactate between the shock and non-shock group, demonstrating that the stress response was specific to the noxious experience of electric shock rather than being mediated by a behavioral change. That is the shock was a stressful stimulus for the crabs.

SOME TAXONOMIC DIFFERENCES

The conclusion so far is that behavioral and physiological responses to noxious stimuli are similar in fish and crustaceans. However, they differ in some respects. This is the case with the analgesic effect of morphine in fish (8, 16). With crustaceans, early studies suggested morphine had similar effects. For example, mantis shrimp, *Squilla mantis*, and the crab, *Chasmagnathus granulatus*, showed a reduced response to electric shock if treated with morphine (40, 41). However, *C.*

granulatus given morphine also shows a reduced responsiveness to a moving shadow, a stimulus that normally elicits escape or defensive responses (42). Thus, the effect of morphine is not specific to analgesia and could be explained if morphine reduced responsiveness to all stimuli. This was tested by Barr and Elwood (43) using the responses of shore crabs being placed in a brightly lit arena with a single dark shelter. Crabs thus placed typically move into the shelter to escape the light. The experiment had two main factors, some crabs were injected with morphine while others received saline, and some received shock when in the shelter while others did not. Each crab had 20 trials with the shelter in the experiment. The rationale is that crabs might be expected to avoid the shelter if they receive shocks or at least hesitate before entry, however, those pre-treated with morphine should show less aversion of the shelter because the morphine should have an analgesic effect. The results did not uphold this prediction. During the early trials few of the crabs that had been given morphine entered the shelter irrespective of receiving shocks or not. They appeared to be limp, unable to move and unresponsive. However, over the course of the first 10 trials, the crabs with morphine recovered their responsiveness and started to move into the shelter but were not more likely to enter and did not enter more quickly than crabs without morphine. Thus, no analgesic effect was found, rather morphine causes the animals to become unresponsive to all stimuli and this lasts for a short period, a finding that would account for the apparent effect in *S. mantis* and *C. granulatus.*

A second difference in responses between crustaceans and fish occurs with capsaicin. Capsaicin causes a burning sensation in humans and appears to cause pain in most mammals, but not birds (44). When capsaicin or acetic acid were injected into the lips of cod there were similar behavioral changes, indicating that fish too have capsaicin receptors (15). By contrast, neither crayfish, *P. clarkii* (3) nor shore crabs, *C. maenas* (12) showed any responses to application of capsaicin. This variation in receptivity is dependent upon relatively minor differences in the molecular structure of vanilloid receptors that typically respond to high temperature, and some chemicals, but the ecological reasons for this variation is not clear (45). However, these minor differences have little or no effect on the evidence of behavioral and stress responses to noxious stimuli that are consistent with pain in fish and crustaceans.

OPPOSITION TO THE IDEA OF PAIN IN FISH AND DECAPODS

The experimental studies noted above have elicited fierce resistance from some authors (46–49). It is claimed that the experimental work has been "mission oriented" and has not used the "detached tradition expected of basic science" (46). Rose et al. (46) further claimed that it is often "faith-based research" and that "these biases have an insidious impact on the credibility of the "science" surrounding aquatic animal welfare." It should be noted, however, that the early work on fish pain was funded by the UK Biotechnology and Biological Sciences Research Council, whose teams of expert reviewers only support science of the highest rigor. The application process for that project was described in Braithwaite (17).

Diggles (49) states "scientific claims that fish or crustaceans "may feel pain" have been largely based on a few dubious and disputed studies done on a small number of animals and species." For my own part, I have published 13 experiments that relate to decapod welfare, using four species, in 10 papers. The mean sample size per experiment was 91.7 (range 40–244); the chosen numbers were dependent on the complexity of the experimental designs and the requirements of the proposed statistical analyses. These are not small numbers of animals. Other authors have used different decapod species so the additional claim of a few species by Diggles is unfounded.

A key aim of these highly critical opinion pieces is to prevent changes to fishery operations (46–48). However, these attempts to reject welfare improvements have, in turn, been heavily criticized [e.g., (33, 50–52)]. Rather than go through the detailed arguments made by each side I refer here to a few major objections.

It has been suggested that because we know the brain areas in humans that have been linked to the experience of pain then any animal lacking these areas cannot experience pain (46, 47). This contention is based on "the bioengineering principle that structure determines function" (47). Because fish and decapods do not have these human structures the idea of pain is dismissed. There is a complete denial that very different brains can have similar functions as has been noted for visual ability in humans, cephalopods, and decapods (21). We also see olfactory abilities in very different animals despite having very different brain morphology. Further, it has been stated that brain size is related to sentience and that those of fish and crustaceans are too small for the necessary neural computation. It should be noted, however, that the very surprising, complex cognitive abilities of bees is achieved with a very small brain (53). Such studies in comparative cognition have led to the idea that a "bottom-up perspective" might lead to a better understanding of basic building blocks of specific advanced functions (54). It is that approach reviewed here that should help our understanding of other mental capacities such as the ability to feel pain. Brains comprise many neurons, each with many dendrites, which enable a vast number of connections, distinct circuits, and functional compartmentalisations. It is those connections, rather than the embryological derivation of brain regions, that are important for computational complexity and function. Viewed in this way the similarity of function and abilities across taxa appears less surprising, and the possibility of pain in fish and decapods cannot be dismissed (21, 50, 51).

There is a repeated claim that the responses of fish and decapods to noxious stimuli are merely the result of nociceptive reflexes that do not require higher order processing (46). However, the experimental studies noted above unequivocally demonstrate that the responses to noxious stimuli cannot be explained by reflex alone. Rather, the animals respond by integrating information from different sources, and that requires central processing. They also show prolonged shifts in responsiveness that provide long-term protection from further damage, again that cannot be a reflex (33).

Key (47) states that human pain involves conscious neural processing and there are repeated calls for conscious feeling to be demonstrated when suggesting pain in animals (46–48). However, consciousness is known as the "hard problem" because it is impossible to demonstrate (55), and these demands for the impossible to be shown has caused confusion. For example, Stevens et al. (48) demanded that "conscious higher level neural processing" must be demonstrated to support the idea of pain. A few lines later, however, the authors retreat from that demand and state "We agree with Dawkins that one should address animal welfare pragmatically using stress-related indicators without reference to conscious experiences." This retreat is welcome because the numerous experimental studies noted in the present article have consistently used pragmatic indicators without reference to consciousness.

CONCLUSIONS

Responses to noxious stimuli that are consistent with the idea of pain are seen in both fish and crustaceans (7). It is important to note, however, that there is no conclusive proof of pain in any animal (33). Conversely, Key (47) acknowledges that he cannot prove that fish do not feel pain. Thus, pain in these taxa can neither be proven nor disproven but, in that case, we must at least accept the possibility of pain (21, 56). Given the large number of studies that are consistent with the idea of pain, that possibility is much higher than if those studies had not been consistent. If pain is possible, then the precautionary principle should be invoked (57). That is, although we accept that there is no absolute proof, we take measures to ensure that animals do not suffer by our actions, just in case. That approach is not questioned with respect to mammals and is increasingly accepted for fish and now for decapods. For example, there has been a recent legal change in Switzerland banning some slaughter methods for lobsters and crabs, and the British Veterinary Association now accept that decapods are sentient and calls for stunning before killing (58, 59). These moves suggest that the experimental work on both decapods and fish are not widely regarded as "dubious" or "faith-based," and that both taxa will soon gain further protection.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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When Victoria Braithwaite's 2003 paper in Proc Roy Soc B (with Lynne Sneddon and Mike Gentle) was published I was greatly impressed with their attempt to delve into the feelings of animals. Two years later I had a chance meeting with Rick Stein, the well-known television seafood chef, and he asked if crustaceans feel pain. We discussed the possibility, but I was not immediately convinced it was a question worth pursuing. However, after looking again at the 2003 fish pain paper, and after discussion with Victoria, I began to think there might be ways to grapple with the problem. Subsequent meetings with Victoria at workshops and conferences on pain and sentience provided excellent opportunities to discuss pain, animal welfare and sentience, and helped shaped my approach to the studies on decapods. Victoria was an enthusiastic scientist and original thinker. She was also a very lovely person. I acknowledge the excellent inputs over the years of Lynsey Patterson, Stuart Barr, Barry Magee, Claire McCambridge, Natasha Dalton, Laura Adams, and Mirjam Appel, who worked with me in this area. I also acknowledge the considerable technical support of Gillian Riddell, who did much of the field collection of animals and ensured the smooth running of the laboratory. I thank Felicity Huntingford and three referees for their help with this paper.

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Ontogenetic Change in Behavioral Responses to Structural Enrichment From Fry to Parr in Juvenile Atlantic Salmon (*Salmo salar* L.)

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Enrichment is widely used as a tool for studying how changes in environment affect animal behavior. Here, we report an experimental study investigating if behaviors shaped by stimuli from environmental enrichment depending on the stage animals are exposed to enrichment. We used juvenile Atlantic salmon (*Salmo salar*) in their first autumn. This is a species commonly reared for conservation purposes. Previous work has shown that environmental enrichment had no effect on long-term survival when the fry stage (smaller than 70 mm) was released, but that if late parr stages (larger than 70 mm) are released, enrichment is reported to have a positive effect on smolt migration survival. Here, we explored the effect of enrichment at two different stages of development. Both stages were reared and treated for 7 weeks (fry at 11–18 weeks and parr at 24–31 weeks after hatching) before tested for behavior. Responses known to be associated with exploratory behavior, activity, and stress coping were quantified by testing 18-week-old fry and 31-week-old parr in a six-chamber maze on 7 successive days after rearing in structurally enriched (plastic plants and tubes) or plain impoverished rearing environments. The data show that Atlantic salmon are sensitive to stimuli from structural enrichment when they are parr, but not when in the fry stage. Parr deprived of enrichment (control treatment) were reluctant to start exploring the maze, and when they did, they spent a longer time frozen than enriched parr, suggesting that deprivation of enrichment at this life can be stressful. Our data suggest that structural enrichment could have the potential to improve welfare for salmonids in captivity and for survival of released juvenile salmon if structural enrichment is provided at the parr stage and the fish reared for conservation are released at the parr stage.

Keywords: natal habitat enrichment, sensitive life stage, experience, exploratory behavior, stress coping behavior

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INTRODUCTION

Animals usually express behaviors that appear to be adapted to the environment in which they find themselves. In some cases, the development of adaptive behavior seems to be influenced and refined by early life experiences (1–3). For example, the developmental status of sensory systems such as vision depends on sensory experience in early life (4). This is particularly relevant for animals reared for release into the wild. Previous work with captive birds, mammals, and fish have already illustrated how increasing environmental complexity, often referred to as environmental enrichment, can increase behavioral and neuronal plasticity, improve cognitive performance, reduce stress responses, and increase survival in reintroduced species (3, 5–16). Some animals respond strongest to stimuli from environmental complexity at certain sensitive life stages and need specific stimuli for developing behaviors that are appropriate and effective in dealing with the changing conditions of life (2, 4, 17). Some species respond over a prolonged time period, while others are particularly sensitive for specific external stimuli over a short period at a specific life stage (4, 17). Others improve behavior after a few days of experience at any time during their first year of life (1).

Specific cues from external stimuli can shape certain behavioral phenotypes [e.g., (18–20)]. For example, rats showed enhanced cerebral plasticity after a few days of experience at any time during their first year of life, indicating that it is environmental stimuli *per se* that stimulated learning (1) and thus behavioral development rather than specific developmental phases. Others, such as chicks of ducks and hens, require auditory and visual stimuli within a few days after hatching to develop a social bond to their parents (21), indicating that for these species, auditory and visual stimuli are required at a specific life stage. The way external stimuli promote future decisions in animals seems thus to be context and taxa dependent.

A well-developed exploratory behavior may provide benefits during a life stage when animals shift into a new habitat or disperse over a larger area (22). If the mortality risk in the present habitat is increasing and/or prey abundance decreases, and an alternative habitat has better conditions, the theory of optimal habitat shift (23) predicts that it will be beneficial to shift habitat to minimize mortality risk per growth rate. For animals in which dispersal will reduce competition and provide access to novel resources, free-living dispersers should, according to the habitat selection theory, prefer new habitats that contain stimuli comparable to those experienced in their natal habitat (24–26). Some researchers suggest that stimuli of new habitats will have a stronger impact on preferences if encountered during a sensitive life stage (17, 24, 27–30). Others regard the phenomenon as more general and that experience with stimuli in an individual's natal habitat increases the probability that the individual later will select a habitat that contains similar stimuli (25, 31).

In Atlantic salmon (*Salmo salar* L.), eggs hatch in the nests in the spring and alevins remain there until they emerge as fry and settle in the vicinity of the nest site (32). In this vulnerable early life stage, a young fry increases its probability of escaping predation by aggregating and having a synchronous emergence pattern (29, 33). The young fry are drift feeders, but the propensity to actively search for prey increases with size (32) and involves expanding the spatial habitat to meet increased energy demands and changed feeding habits as the juveniles grow (34). In *S. salar*, a typical change is observed when juveniles develop from fry to parr: individuals longer than 70 mm (parr) have a larger spatial habitat than those shorter than 70 mm (fry). Foldvik et al. (22) showed that as the individuals grew, dispersal initially increases slowly until the fry reached 70–80 mm length (parr size), a size when dispersal rate almost doubled. It is known that juvenile Atlantic salmon shift feeding habit when they reach this size: Typically, as fry change from feeding primarily on benthic drift prey to become parr, they take up feeding stations and attack drifting invertebrates at the surface waters more often (30, 35, 36).

Here, we experimentally studied how the development of behaviors shaped by stimuli from structural enrichment depend on stage of exposure. We used juvenile Atlantic salmon (*S. salar*) in their first autumn. We reared for 7 weeks fry (11–18 weeks after hatching) and parr (24–31 weeks after hatching) in structurally enriched or plain impoverished (control) environments. After 7 weeks of treatment, we used six-chambered mazes and tested individual fish on 7 successive days for behaviors known to be associated with the propensity of exploratory behavior, with activity, and with stress coping. Exploratory behavior in fish is known to be stimulated by enrichment [e.g., (13, 14, 37, 38)] and to reduce stress response (15, 16). If stimuli from enrichment is important any time early in life for salmon, we predict that enrichment will stimulate exploratory behavior and stress coping in both fry and parr stages. We may however not rule out that Atlantic salmon could be a species with a sensitive life stage that need specific stimuli for proper behavioral developments to deal with the changing conditions of life as shown for animals from other taxa [e.g., (2, 4, 17)]. If so, an early life stage when dispersion is pronounced such as the parr stage (22) may need more sensitive experience than the fry stage, and the alternative hypothesis would then be that stimuli from structural enrichment are more relevant during a dispersal period (parr) than during a less mobile (fry) life stage.

MATERIALS AND METHODS

Experimental Fish and Treatments

We follow the ARRIVE guidelines by Percie du Sert et al. (39) in our description of our experiments.

Salmon (*S. salar*) fry of wild origin (hatching date, April 20, 2015) were transferred from Voss hatchery to the experimental facilities at the University of Bergen (UiB), Norway, on two occasions. The first group arrived as fry on July 1, 2015, 11 weeks after hatching ($n = 447$, mean weight 0.5 g). The second group arrived when at the parr stage on September 30, 2015, 24 weeks after hatching ($n = 501$, mean weight 8.0 g). In both cases, the fish were randomly distributed into six identical treatment tanks (100 × 100 cm, water level 60 cm) by transferring five fish at a time into each of the tanks (in the end, three fish at a time) to minimize potential unequal distribution of fish caught early and late among the rearing tanks. The tanks were next numbered using tank number on paper labels (1, 2, 3, 4, 5, and 6) and treatment (three labels enriched and three labels control), and then sampling without replacement to match three tanks to be structurally enriched and three tanks to be plain impoverished control tanks. The structural enrichment consisted of plastic plants and structures made by plastic pipes fitted together using aquaria silicone. When the tanks were cleaned once a week, the structures were moved around. The same amount of time of disturbance was given to the enriched and control tanks. Physical conditions were identical in control and enriched tanks; 12:12 light/dark cycle was used with natural day light. The water had a temperature of approximately 12°C, the tanks had flow-through, and the water renewal was 4 L min^{-1}. All tanks had the same flow direction, which was counterclockwise. Fish were fed continuously with commercial fish feed (EWOS microstart 40020 and microstart5), *ad libitum* in the light period, using automatic feeders (Hølland technology). All fish were kept under these conditions and reared for 7 weeks in enriched or control environments before we started the behavioral experiments. Fry were reared under these conditions 11–18 weeks after hatching and parr were reared 24–31 weeks after hatching.

We used a handheld dip-net and selected 18 enriched and 18 control fish from the rearing tanks: 6 from each of the three enriched rearing tanks and 6 from each of the three control rearing tanks and all of intermediate size. The mean length ± SE of fry was 5.0 ± 0.2 cm, and the mean body mass was 1.7 ± 0.1 g, whereas the mean length ± SE of parr was 10.3 ± 0.2 cm and the mean body mass was 16.0 ± 1.2 g. Four additional holding tanks were used to house these 36 fish: two were structurally enriched and two were plain (control). The 18 enriched fish were housed in one enriched tank before behavioral screening and in the other holding tank after screening. The 18 control fish were housed in one plain tank before screening and in the other plain tank after screening. In the holding tanks, the fish were hand-fed pellets two times per day, and bloodworms one time and in the evening.

Four days prior to the behavioral assays, the fish were individually marked to allow identification of test individuals. Each fish was first anesthetized using MS222 (80 mg 3L^{-1}) and then marked with a unique combination of yellow and red fluorescent Visible Implant Elastomers (NMT INC Northwest Technology) on two to three dorsal and ventral positions.

Experimental Arena and Experimental Test

Four mazes were designed; two were used to test 18-week-old fry, and the two larger ones were used to test the 31-week-old parr. The ratios between fish length and possibility for horizontal movements in the maze were kept approximately the same. Both maze sizes consisted of six rectangular boxes (**Figures 1A,B**). The height of both mazes was approximately 11 cm. Chamber 1 of each maze was used as the start box, and its exit to chamber 2 was initially closed by an opaque removable door that was lifted remotely when each trial started. The water of the other five chambers was connected *via* openings that differed in geometrical shape.

Each maze was placed in the center of a larger holding tank (100 × 100 × 60 cm) and surrounded by water to keep temperature stable and to minimize injuries if the fish managed to escape its maze. The maze was covered with transparent glass to prevent fish from jumping out and to allow video recording from

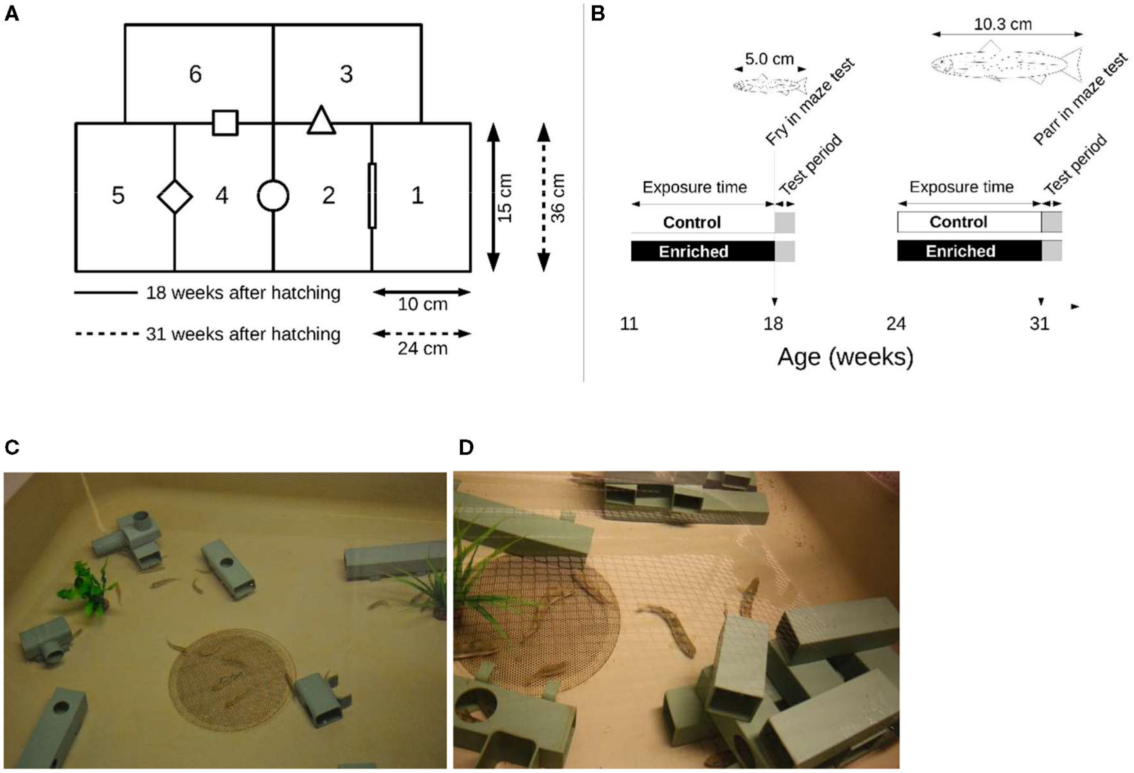

FIGURE 1 | (A) Schematic representation of the mazes used to test exploratory behavior and activity of juvenile Atlantic salmon reared 11–18 weeks after hatching in structurally enriched or control environments and those reared 24–31 weeks after hatching. The ratio between the fish length and the chamber size was the same for both groups. **(B)** The timeline of the experimental setup. The water of the chambers was connected via openings that differed in geometrical shape. **(C)** Structures used when rearing fry, and **(D)** Structures when rearing parr for 7 weeks in enriched environments. Control fish were reared in identical tanks but without structures.

above. The water level in the maze was kept at approximately 11 cm. A video camera (LegraHFR560 Canon) was mounted above each maze.

Fish were familiarized to be moved from their holding tanks by allowing them to swim freely in all chambers in the maze in groups of nine for 1–2 h over 4 days prior to start of the behavior experiments. The fish had then access to food (red bloodworms *Chironomidae* fitted into a green ring using Vaseline fitted with metal piece to keep it on the bottom) in all chambers during acclimation, and there were no doors blocking any entrance. An air stone in one chamber provided aeration of the water. Unfortunately, four enriched and three control parr fish jumped out of their holding tank at night between two acclimation days and were removed from the experiments. Two enriched and two control fry jumped out of their holding tank at night between two test days, as also did three enriched and one control parr. The number of individuals tested all 7 days were therefore reduced to 16 enriched and control fry and 11 from enriched and 14 from control treatments for large parr.

At the start of each trial, an individual fish was collected in a handheld dip-net, and its identity was recorded using UV-light (VI-light 405 nm, 82 mV) on the Visible Implant Elastomers, and then it was carefully released into the start box. To keep handling stress to a minimum, we tested the fish in the order in which they were netted. Thus, the order of testing was different for each trial. A Canon LegraHFR560 camera, mounted 1.5 m above the center of the maze, recorded the trials. Fish could not see the observer, but the observer could view the fish and maze from the display on the camera. After 5 min acclimation, the start box door was opened remotely using a pulley. The test lasted 10 min. The fish had access to food in chamber six during the experiment. We tested the fish once per day over seven consecutive days to test if enriched and control fry and parr differed in the way they responded to the behavioral tasks. Seven days were chosen as previous experiments using salmon in the smolt stage showed different learning over this length of time (14). The water was replaced between each test fish. We used J-watcher and analyzed the videos of individual behavior. The video coder was blind to the fish treatment. Each fish had an ID consisting of three letters. These three letters were visible on a label on the video, but the video coder was not informed which codes belonged to which treatment groups. The following data were collected: (i) the time the test fish took to leave the start box, which, for fish, is a common proxy for exploratory behavior [e.g., (11, 40–42)]; (ii) the number of chamber changes; and (iii) the time the fish stayed still (froze) after it had left the start chamber.

Ethical Note

All procedures have been completed according to the Norwegian Food safety Authority in compliance with "The regulation on the Use of Animals in Research with FOTS ID 7931." After the trial on the last day, the fish were euthanized by an overdose of buffered MS222 (0.5 g L^{-1}).

Data Analysis

All statistics were performed using R v4.0.3 [(43), http://www.r-project.org].

For individual fish, we first calculated the cumulative time to leave the start chamber, cumulative number of chamber changes, and cumulative time spent frozen for seven successive experimental days. Cumulative time to leave the start chamber was used as a proxy for exploratory behavior, cumulative number of chamber changes was used as a proxy for activity, and cumulative time spent frozen was used as a proxy for fear. Cumulative data were chosen for the analysis since both consistency in behavioral differences between the treatment groups and the link between behavior and survival is best considered if accumulated over several observation times. For the continuous response variables "body mass," "length," "time to leave the start box," and "time spent frozen," we fitted linear mixed-effects models using the lme function from the nlme library of R (44). For analyses concerning the discrete response variable "number of chamber changes," we fitted generalized linear mixed-effects models with Poisson error term using the glmmPQL function from the MASS library of R (45). Due to differences in both fish and maze size between the two age classes, we did separate analyses for the fry and parr life stages. In all models, "tank" was set as a random effect factor to account for the dependency structure caused by multiple fish in each of the six treatment tanks. "Treatment" (reared in structurally enriched or control environments) was specified as the predictor. We wanted to explore if motivation to explore and activity levels depended on stress coping in fry and parr. Therefore, we used the proxy for stress coping (time spent frozen), treatment, and interaction between these two as predictors in some additional analyses. All tests were done using cumulative data over 7 days.

RESULTS

Immediately after an individual had been transferred to the start chamber of the maze with a dip-net, all fish exhibited some level of fearfulness. Initially, the test fish froze before swimming a little around. Once the chamber was opened, the fish tended to move to the opening, standing still a little looking at the space outside the start chamber before entering the maze and swimming slowly around. Typical behavior in the maze was to change between swimming slowly around and standing still (freeze) while visiting different compartments of the maze.

There was no difference in size between structurally enriched and control fish when they were tested for behavioral differences. Fry did not differ in length [lme; $F_{(1,4)} = 0.71$; $p = 0.45$; mean length 5.0 cm] or in body mass [lme; $F_{(1,4)} = 0.005$; $p = 0.96$; mean body mass 1.7 g], and parr did not differ in length [$F_{(1,4)} = 0.11$; $p = 0.76$; mean length 10.3 cm] or body mass [$F_{(1,4)} = 0.29$; $p = 0.62$; mean body mass 16 g, respectively].

At 18 weeks of age, enriched and control fry did not differ in time to leave the start box [**Figure 2A** and **Supplementary Figure 1A**, lme; $F_{(1,4)} = 0.725$, $p = 0.443$], the number of chamber changes (**Figure 2C** and **Supplementary Figure 1C**, glmmPQL; $t = 1.685$, $df = 4$, $p = 0.167$), or the time spent frozen [**Figure 2E** and **Supplementary Figure 1E**, lme; $F_{(1,4)} = 0.207$, $p = 0.673$, respectively]. At 31 weeks of age, the enriched parr individuals

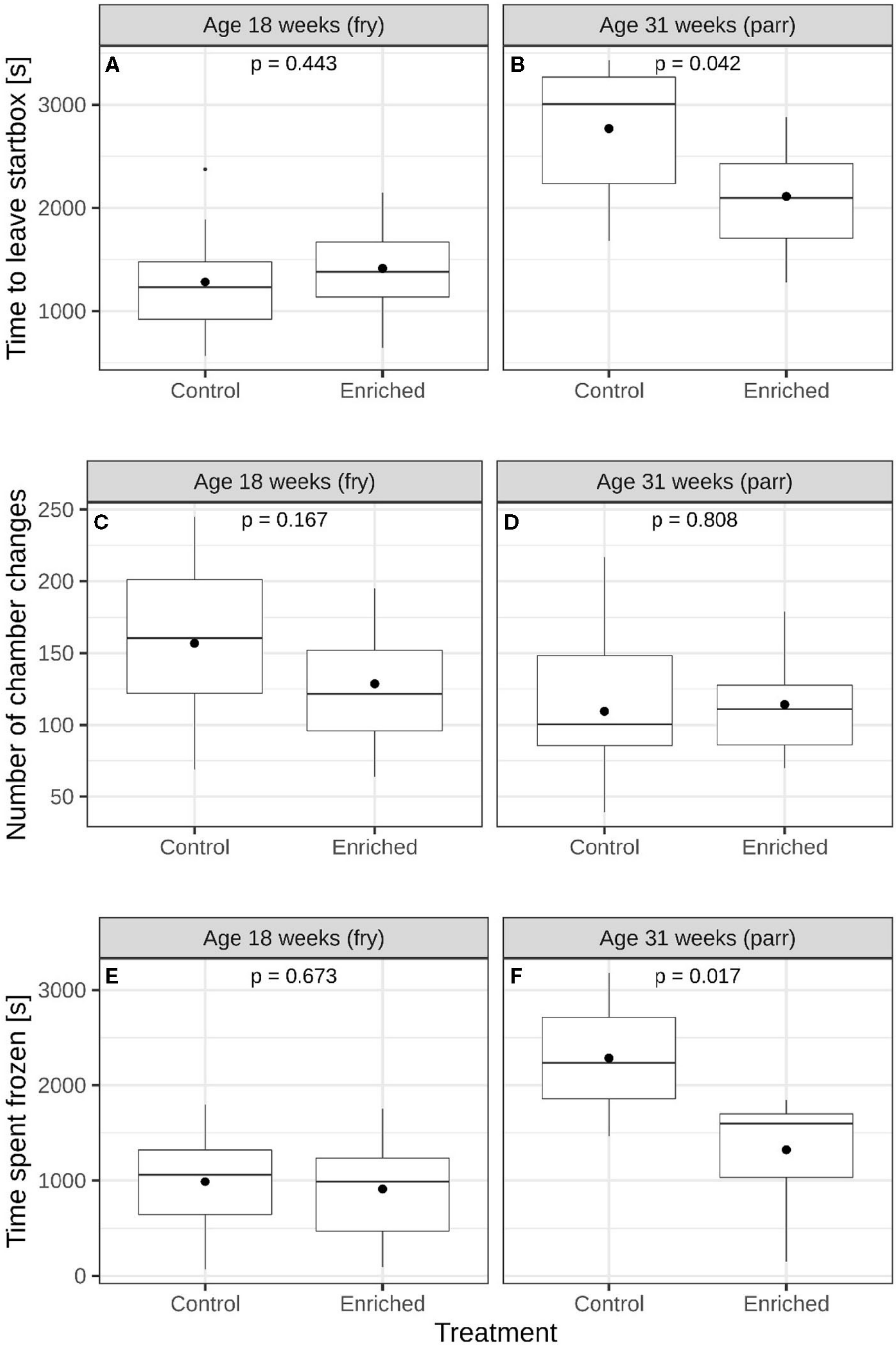

FIGURE 2 | Comparisons of enriched reared and control reared juvenile Atlantic salmon (*Salmo salar*). Time to leave start box of **(A)** 18-week-old and **(B)** 31-week-old juveniles. Number of chamber changes of **(C)** 18-week-old and **(D)** 31-week-old juveniles. Time spent freezing of **(E)** 18-week-old and **(F)** 31-week-old juvenile salmon. The boxes show the medians and quartiles while the whiskers show the extremes within 1.5 times the interquartile range. Black circles represent mean values. The *p*-values in each panel represent a comparison of mean values between the two groups.

left earlier [**Figure 2B** and **Supplementary Figure 1B**, lme; $F_{(1,4)} = 8.744$, $p = 0.042$] and spent less time frozen [**Figure 2F** and **Supplementary Figure 1F**, lme; $F_{(1,4)} = 15.721$, $p = 0.017$] in the maze test than control parr did. Enriched and control treatment groups did not differ with respect to the number of chamber changes (**Figure 2D** and **Supplementary Figure 1D**, glmmPQL; $t = 0.260$, $df = 4$, $p = 0.808$).

At 18 weeks of age, the effect of time spent frozen on the time to leave the start box [**Figure 3A**, interaction term from lme: $F_{(1,24)} = 0.016$, $p = 0.901$] and on the number of chamber

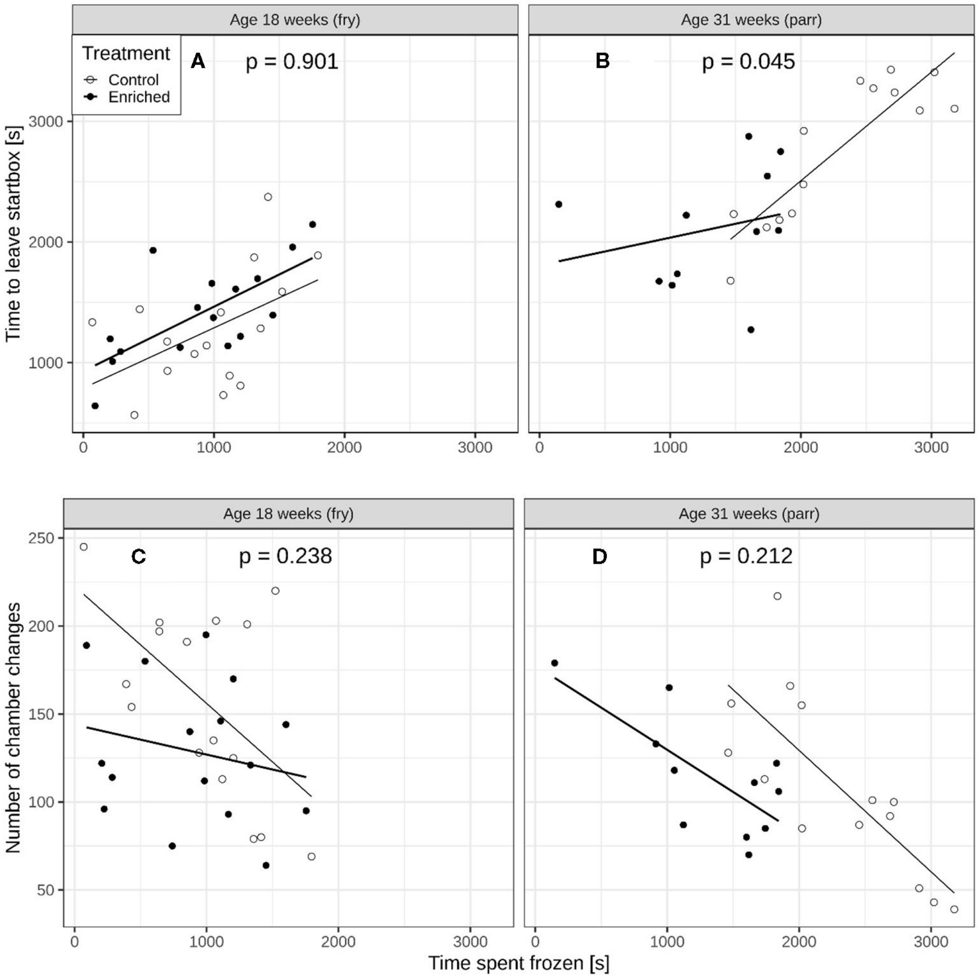

FIGURE 3 | Comparisons of enriched reared and control reared juvenile Atlantic salmon (*Salmo salar*). Time to leave start box vs. time spent immobile for **(A)** 18-week-old and **(B)** 31-week-old juveniles. The *p*-values on each panel refer to the interaction term and represent a comparison of slope between the two regression lines. Number of chamber changes versus time spent immobile for **(C)** 18-week old and **(D)** 31-week old juveniles.

changes was similar for enriched and control fry (**Figure 3C**, interaction term from glmmPQL; $t = 1.211$, $df = 24$, $p = 0.238$). At 31 weeks of age, however, the effect of time spent frozen after they left the start box on time to leave was significantly weaker for the enriched parr compared to the control parr [**Figure 3B**, interaction term from lme: $F_{(1,17)} = 4.699$, $p = 0.045$]. The effect of time spent frozen on number of chamber changes was, however, similar for the enriched and control parr (**Figure 3D**, interaction term from glmmPQL: $t = 1.30$, $df = 17$, $p = 0.212$).

DISCUSSION

These results reveal an interaction between experience from different types of environmental heterogeneity, age of experience, and subsequent behavioral responses. These results are in line with enrichment experiments on higher vertebrates [e.g., (2, 10, 19, 46)] and other fish species (11, 12, 47, 48) and also in line with experiments on later life stages of Atlantic salmon (14, 49, 50). In addition, these results follow the ideas of Immelmann (24) and Knudsen (17), suggesting that in animals having sensitive developmental periods, stimuli will have a stronger impact on the development of a behavioral response if it is experienced during a specific period/life stage. Our data show that 18-week-old enriched and control fry did not differ in exploratory behavior, activity, and stress coping, as indicated by the proxies used to study these behaviors (time to leave the start box, the number of chamber changes, and the time spent frozen). This suggests that stimuli from structural enrichment may not be relevant at the fry life stage. In parr, however, which were from the same cohort but both treated and tested when 13 weeks older (3 months later), the enriched individuals could be regarded as more exploratory as they left the start box earlier than control parr. Enriched parr did also show elevated stress coping compared to control fish as they spent less time frozen in the maze after they had left the start chamber than control parr did. Enriched and control parr had, however, similar activity as the number of chamber changes they did during the test did not differ.

Our data suggest that in Atlantic salmon, the parr seem to be more behaviorally plastic than the fry. Hence, stimuli for suitable exploratory behavior and stress coping would be needed at the life stage when Atlantic salmon juveniles typically expand their habitat and alter feeding habits. Although for all the parr, the longer they spent frozen once the door of the chamber was opened, the longer they took to leave the chamber, the enriched parr spent less time frozen than parr reared in impoverished control environments. As most fish exhibited some level of fearfulness just after transfer to the start box of the maze and where they initially froze before leaving, this could be interpreted as the start box was somewhat stressful for both treatment groups and similar for enriched and control fry. However, the control parr that were significantly more reluctant to leave the start box and also froze for a longer time after they had entered the maze than enriched reared parr (**Figure 3B**) appears to have been more fearful than enriched parr. Hence, a potential motivation of enriched reared parr for leaving the start box could be to leave a space associated with fear and seek for potential shelters. Presence of shelter has been shown to reduce stress in salmon and to reduce metabolic costs (15, 16, 51).

Previous researchers have shown that during the transition between fry and parr, dispersal rate increases and the feeding habits change from relying on benthic drift prey to more actively feeding on pelagic prey shallower in the water column (22, 30, 34–36, 52). Our data show that prior experience of stimuli from structural enrichment had a positive effect on our proxy for explorative behavior as enriched reared parr left the start box earlier than control parr that had been deprived from such stimuli. Previous studies have shown that enrichment causes decreased stress hormone levels in juvenile Atlantic salmon and Pacific salmon, although those studies did not investigate exploratory behavior *per se* (15, 16). It might be that the behavioral response in control parr could reflect higher stress level compared to those from structural enriched tanks, but further studies would be needed to investigate this in fuller detail. Our study was a pure behavior study and did not include physiological measures such as hormone levels in the test fish, though the behavioral responses we measured are known to be associated with exploratory behavior, activity, and stress coping.

Why did enriched reared salmon juveniles respond differently to stimuli from structural enrichment as fry and parr? Wild fry do experience complex habitats after emergence, but early in the fry life stage, wild fry of Atlantic salmon tend to stay fairly stationary in hiding places among the stones on the bottom, where they are supplied with small benthic crustacean prey transported *via* currents and eddies (32). The fry are small and have low energetic storage. For fry, prey capture and dispersing into a larger habitat would be too energetically costly and probably exceeding the potential benefit of encountering additional prey passing by. However, as fry grow, they build energy storage and become sufficiently large to have the capacity for successful capture of larger prey and also for dispersing further away from where they hatched [e.g., (53)]. As animals disperse, they will need to quickly and reliably be able to recognize a suitable habitat for settlement (25), using cues that resemble those experienced earlier (26).

Not all studies have reported positive effects of structural enrichments. Whether structural enrichment promote exploratory behavior seems to depend on the context and the model species used for the study. For example, structurally enriched reared cod (*Gadus morhua*) juveniles explored more than control juveniles reared in impoverished tanks (11), while enriched and control reared juvenile steelhead salmon (*Oncorhynchus mykiss*) did not differ in their exploratory behavior (54). *O. mykiss* was also studied by Bergendahl et al. (38) who found that both the timing and duration of experience from structural enrichment influenced the strength of the behavioral response, but enrichment had no effect on anxiety-related behaviors for this species.

Our overall findings are relevant for conservation biology and the welfare of fish. The use of enrichment is considered important in captivity for all stages of animal's life. Fishes are important laboratory animals and many species are housed in captivity as model organisms for research (55), and others are kept in captivity for aquaculture and ornamental pets. Housing conditions are important in the welfare of captive animals, of which environmental enrichment is an important component (48). Previous studies have demonstrated that environmental and social enrichment promote behavior and cognition in animals in general. Releases of hatchery-reared salmonids deprived from such stimuli into natural environments tend to be unsuccessful. Therefore, enriched rearing has, for the last decades, been widely used as a tool for managing stress in captive fish and with the ambition of producing fish with better survival in the wild. Studies that have released enriched reared early stages of salmonids (i.e., younger than 18 weeks; the fry life stage) did not find higher survival after release (56–58). Solås et al. (59) who released 12- to 17-week-old fry in three different years report higher survival from predation mortality 48 h after release only in the year when 17-week-old fry were released, but enriched fish did not have higher survival 12 weeks after release of fry. A few release experiments using older Atlantic salmon parr at release have, however, demonstrated higher smolt migration survival of enriched reared compared to 11-month-old (50) and 24-month-old (49) control fish. Previous findings and our data suggest that structural enrichment could have the potential to improve welfare for salmonids in captivity and for survival of released juvenile salmon at later stages than the fry stage. Further studies will be required to investigate the timing, amount, and type of environmental enrichment that produce the best welfare for captive fish and survival of released ones. At present, there is a lack of knowledge on what enrichment to provide for most fishes housed in captivity or the effectiveness of different kinds of enrichment that is used, and there is a need for the development of standardized enriched housing that can provide welfare benefits for captive fish at all life stages (48).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Norwegian Food safety Authority in compliance with the regulation on the Use of Animals in Research with FOTS ID 7931.

AUTHOR CONTRIBUTIONS

IA and ASa designed the study. IA reared the fish and did the experiments under supervision from ASa. IA did the initial analyses under supervision from KJ. KJ did the final statistical analyses and made all figures. IA wrote the first draft of the manuscript with input from ASa, KJ, and ASk. ASa revised the manuscript with input from IA, KJ, and ASk. All authors have approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.638888/full#supplementary-material

Supplementary Figure 1 | Comparisons of control and enriched reared juvenile Atlantic salmon (*Salmo salar*). The lines show cumulative values at the individual level over seven days of testing, where grey and black lines represent control and enriched reared fish, respectively. Time to leave start box of **(a)** 18-week old fry and **(b)** 31-week old parr. Number of chamber changes of **(c)** 18-week old fry and **(d)** 31-week old parr. Time spent freezing of **(e)** 18-week old fry and **(f)** 31-week old parr. Open and solid circles represent mean values for control and enriched reared fish, respectively.

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Qualitative Behavioral Assessment in Juvenile Farmed Atlantic Salmon (*Salmo salar*): Potential for On-Farm Welfare Assessment

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There is a growing scientific and legislative consensus that fish are sentient, and therefore have the capacity to experience pain and suffering. The assessment of the welfare of farmed fish is challenging due to the aquatic environment and the number of animals housed together. However, with increasing global production and intensification of aquaculture comes greater impetus for developing effective tools which are suitable for the aquatic environment to assess the emotional experience and welfare of farmed fish. This study therefore aimed to investigate the use of Qualitative Behavioral Assessment (QBA), originally developed for terrestrial farmed animals, in farmed salmon and evaluate its potential for use as a welfare monitoring tool. QBA is a "whole animal" approach based on the description and quantification of the expressive qualities of an animal's dynamic style of behaving, using descriptors such as relaxed, agitated, lethargic, or confident. A list of 20 qualitative descriptors was generated by fish farmers after viewing video-footage showing behavior expressions representative of the full repertoire of salmon in this context. A separate, non-experienced group of 10 observers subsequently watched 25 video clips of farmed salmon, and scored the 20 descriptors for each clip using a Visual Analog Scale (VAS). To assess intra-observer reliability each observer viewed the same 25 video clips twice, in two sessions 10 days apart, with the second clip set presented in a different order. The observers were unaware that the two sets of video clips were identical. Data were analyzed using Principal Component (PC) Analysis (correlation matrix, no rotation), revealing four dimensions that together explained 79% of the variation between video clips, with PC1 (Tense/anxious/skittish—Calm/mellow/relaxed) explaining the greatest percentage of variation (56%). PC1 was the only dimension to show acceptable inter- and intra-observer reliability, and mean PC1 scores correlated significantly to durations of slow and erratic physical movements measured for the same 25 video clips. Further refinements to the methodology may be necessary, but this study is the first to provide evidence for the potential of Qualitative Behavioral Assessment to serve as a time-efficient welfare assessment tool for juvenile salmon under farmed conditions.

Keywords: qualitative behavioral assessment, fish, salmon, aquaculture, welfare

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INTRODUCTION

While global fish supply from capture has remained relatively static since the mid 1980's, there has been a huge increase in both inland and marine aquaculture (1) with much of this increase in Asia, and China in particular. In 2018, it is estimated that 54.3 million tons of finfish were produced globally within aquaculture with salmon accounting for 4.5% of global production (1). In Scotland alone it is estimated that 47 million fish were transferred from freshwater rearing tanks to sea cages in 2018 (2). Farmed fish can be held in different types of rearing system, and are subject to varying husbandry routines and operations throughout the different stages of their life cycle. These systems will impact fish welfare in different ways, exposing them to different stress challenges and hazards, and presenting the risk that the animals' environmental and behavioral needs, both at individual and group level, are not met (3). Given the rapid increase in aquaculture production and the range of species now farmed, there is thus an urgent need to address the welfare of farmed fish, and, as with other farmed animal species, to develop methods that monitor the different species' needs (4).

Welfare appraisal in fish has frequently focused on disruption of biological function, illness, injury and mortality. However, a "feelings-based" consideration of animal welfare (5) has historically been neglected in fish welfare assessment, along with consideration of opportunities for positive affect and well-being (6, 7). For example, in Scotland, welfare inspection and enforcement, outside of assurance scheme requirements, is under the remit of local authorities, government and Animal Health and Veterinary Laboratories Agency. These inspections are often carried out in response to reports of mass disease or mortality (6) and in such cases, assessment is often primarily concerned with mortality, clinical indicators of disease and inappropriate usage of veterinary medicines (6).

Assurance scheme welfare guidance frameworks, including the Code of Good Practice for Finfish Aquaculture (8) and RSPCA Assured welfare standards for Atlantic salmon and rainbow trout (9), are based on the "Five Freedoms," which mostly focus on the avoidance of negative states such as pain and hunger. The importance of positive experiences for welfare are receiving growing recognition (10, 11), however suitable methodologies to robustly assess positive welfare in different species are still lacking and in need of development (12).

There is a growing body of evidence supporting that fish are intelligent, sentient beings that possess cognitive abilities of considerable complexity [e.g., (13, 14)], and are capable of emotion and experiencing pain (15, 16). These capacities do not in fact appear far removed from those observed among warm-blooded terrestrial vertebrates, yet the level of protection and moral concern afforded to fish remains far behind that given to terrestrial species (15, 17). For example, in the European Council Directive 98/58/EC[1] on the protection of animals for farming purposes, the detailed welfare provisions prescribed exclude fish (6), despite the explicit acknowledgment of fish sentience in EU law[2]. Fish species have been afforded greater protection when used for experimental purposes however, through national legislations, regulations and guidelines [e.g., (18, 19)]. In Europe[3] fish in scientific settings are protected from the time they are capable of independent feeding, on the assumption they are then capable of experiencing pain, suffering and distress. Following this directive, the UK Animals Scientific Procedures Act 1986[4] included fish for the first time as worthy of protection, but this does not apply to fish in commercial aquaculture.

Current monitoring methods within the aquaculture industry are limited to video surveillance measures of the physical environment such as water turbidity, however there is scope to expand the use of such technology to include a wider range of welfare indicators (20–22). Stien et al. (23) review existing welfare standards and assessment systems for farmed fish and suggest a system of Operational Welfare Indicators that can be monitored through video surveillance, as practiced for example in Norway. An additional problem in operating such systems, however, is that a progressive decrease of staff relative to fish numbers imposes time constraints on monitoring. Recent reviews of Scottish aquaculture have shown that tonnage of seawater fish produced in relation to number of employees has increased by 11-fold since 1985 (24). There is thus a distinct requirement for time-efficient fish welfare assessment tools which, as is increasingly the case with terrestrial animals, should not only focus on physical well-being, but also on emotional well-being, including both negative and positive experiences (11).

Qualitative Behavioral Assessment is an integrative technique which evaluates the "whole animal" in terms of the dynamic expressive quality of its behavior (25, 26). Different "styles" of behavior are summarized using qualitative descriptors such as relaxed, agitated, inquisitive and listless (27) that should cover the full range of both negative and positive emotional experience, and are quantified by scoring their prevalence on unstructured Visual Analog Scales. Numerous studies have validated the application of QBA to different livestock species (28). A perceived strong point of QBA is that it includes positive aspects of animal affect, which led to its inclusion in EU Welfare Quality® welfare assessment protocols for cattle, pigs and poultry as the only indicator for positive emotional state [e.g., for poultry: (29)]. In addition, integrative judgements of expressivity are time efficient (30), and so potentially provide a logistically feasible tool for practical on-farm welfare assessments. However, integrative judgments also bring vulnerabilities; people are known to vary in the way they calibrate unstructured Visual Analog Scales, potentially confounding outcomes with undesirable observer-based variation (26). It is therefore best to always use QBA

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[1] Council Directive 98/58/EC of 20 July 1998 Concerning the Protection of Animals Kept for Farming Purposes. Official Journal of the European Communities. L221/23-27. https://www.legislation.gov.uk/eudr/1998/58/pdfs/eudr_19980058_adopted_en.pdf

[2] Treaty of Lisbon Amending the Treaty on European Union and the Treaty Establishing the European Community 2007/C 306/01 of 13 December 2007.

[3] Council Directive 2010/63/EU of 20 of October 2010 on the protection of animals used for scientific purposes. Official Journal of the European Union. L 276/33-79. https://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2010:276:0033:0079:en:PDF

[4] Animals (Scientific Procedures) Act 1986 Legislation. The National Archives. https://www.legislation.gov.uk/ukpga/1986/14/contents

in combination with other validated animal- and resource-based measures, and adequate instruction and training are essential (31).

To date, QBA has not been applied to fish. Whilst in terrestrial species observers may integrate expressive qualities of elements such as posture (32), facial expression (33) and ear position (34) into assessments of overall body language, there may be fewer such elements available for assessment in fish. The scientific literature reports a range of health and welfare measures for fish, including behaviors such as food intake, swimming and stereotyped behavior (23, 35). However, there is a lack of measures for affective state such as facial expression (36), and also of efforts to integrate different measures of behavior into assessments of affective state. A challenge for assessing fish affect is the number of fish that are kept within sea cages and the aquatic environment, limiting their movement and expressivity. However, QBA has been applied successfully to large groups of terrestrial animals such as commercial broilers (37, 38), suggesting that potentially this is also possible for fish.

The aim of this study was to evaluate the inter- and intra- observer reliability of observer judgements of fish body language using a fixed list QBA methodology developed for juvenile Atlantic salmon. In addition, the association between QBA scores and measurements of ethogram-based categories of salmon behavior was investigated for the purpose of additional validation.

MATERIALS AND METHODS

Ethical Review

Approval for video recording was gained from the University of Stirling Animal Welfare & Ethical Review Body and SRUC's Animal Ethical Committee. Approval for observer participation in the QBA element was gained from the University of Edinburgh Vet School Human Ethical Research Committee (HERC: approval number HERC_79_17).

Animals, Housing, and Husbandry

The fish used in this study were juvenile Atlantic salmon (Salmo salar), kept at a hatchery and rearing unit in Scourie, Scotland between December and April 2017. The fish were 9–12 months of age and weighed around 30–45 g. The unit contained 23 freshwater circular rearing tanks (5 m diameter by 2 m deep) in a flow-through land-based system with no artificial current. There were ∼13,000 juvenile salmon in each freshwater tank and stocking density was on average 20 Kg/m^3. The 23 tanks were laid out in 5 rows of 4 with a row of 3 at one end, and the enrichment was spread throughout the layout.

One tank was not populated by salmon at any point in the study. Three of the tanks were excluded due to poor visibility in the water. Of the remaining 19 populated tanks in which salmon were assessed, 9 were randomly assigned to contain environmental enrichment in the form of artificial "kelp" which was suspended from above the water in the form of long plastic strips. The other 10 tanks did not contain artificial kelp but were otherwise identical in setup. The sex ratio of fish was unknown, as during the pre-smoltification stage, the salmon had not reached sexual maturity and displayed no external indicators of gender. All salmon in the rearing tanks were fed on standard salmon pelleted dry food (Skretting Nutra Advance/Supreme©), which was deployed by automatic feeders using a spinning arm every 10–20 min during daylight hours. Fish were routinely vaccinated at 7 months of age. Daily tank cleaning was carried out between 0900 and 1100 h (aside from on video recording days, where this was performed after each tank was recorded). During this process any salmon mortalities were removed.

Video Recording

Video recording was carried out in the 19 tanks populated with salmon with submerged GoPro Hero 3© cameras. That this included tanks both with and without enrichment was not considered a problem, as this difference could be expected to increase the range of salmon expressivity available for observers to assess. During the recording of each tank, the GoPro© cameras were submerged using a metal pole which was fixed in place for 17 min (the length of one undivided "block" of footage as recorded by the camera). The GoPro's field of view normally showed between 10 and 40 fish. All recordings were carried out between 10:00 and 13:00 h. During all recording periods, workers were instructed not to perform any husbandry procedures or use the walkways over the tanks during recording so as not to disturb the fish. In accordance with the standard feeding routine, the automatic feeders continued to deploy food every 20 min.

Qualitative Behavioral Assessment

Qualitative Behavioral Assessment was carried out in two phases. Phase 1 consisted of the generation of a list of terms for describing salmon expressivity, and Phase 2 consisted of applying these terms, by different observers, to the scoring of salmon expressivity as viewed in 25 video clips.

Phase 1 Term Generation

Participants

For the term generation stage, four employees of the salmon hatchery site where video recording took place, were recruited. All participants had at least 1 year of experience working directly with fish, with 3 of the 4 individuals having worked in the aquaculture industry for 7–15 years. Each observer was therefore considered experienced in monitoring fish behavior.

Clip Selection

For the term generation session, 12 video clips of 45 s duration were selected from footage of farmed salmon taken in December or March. Selection aimed to include a range of varied and contrasting behaviors, such as for example darting, drifting, startle responses, or interacting with artificial kelp or conspecifics. It was assumed that such footage would reflect a varied range of salmon expressions, ranging from high to low mood and arousal (39). To facilitate the generation of qualitative descriptors by observers, video clips were arranged in an order which demonstrated good expressive contrast between adjacent clips.

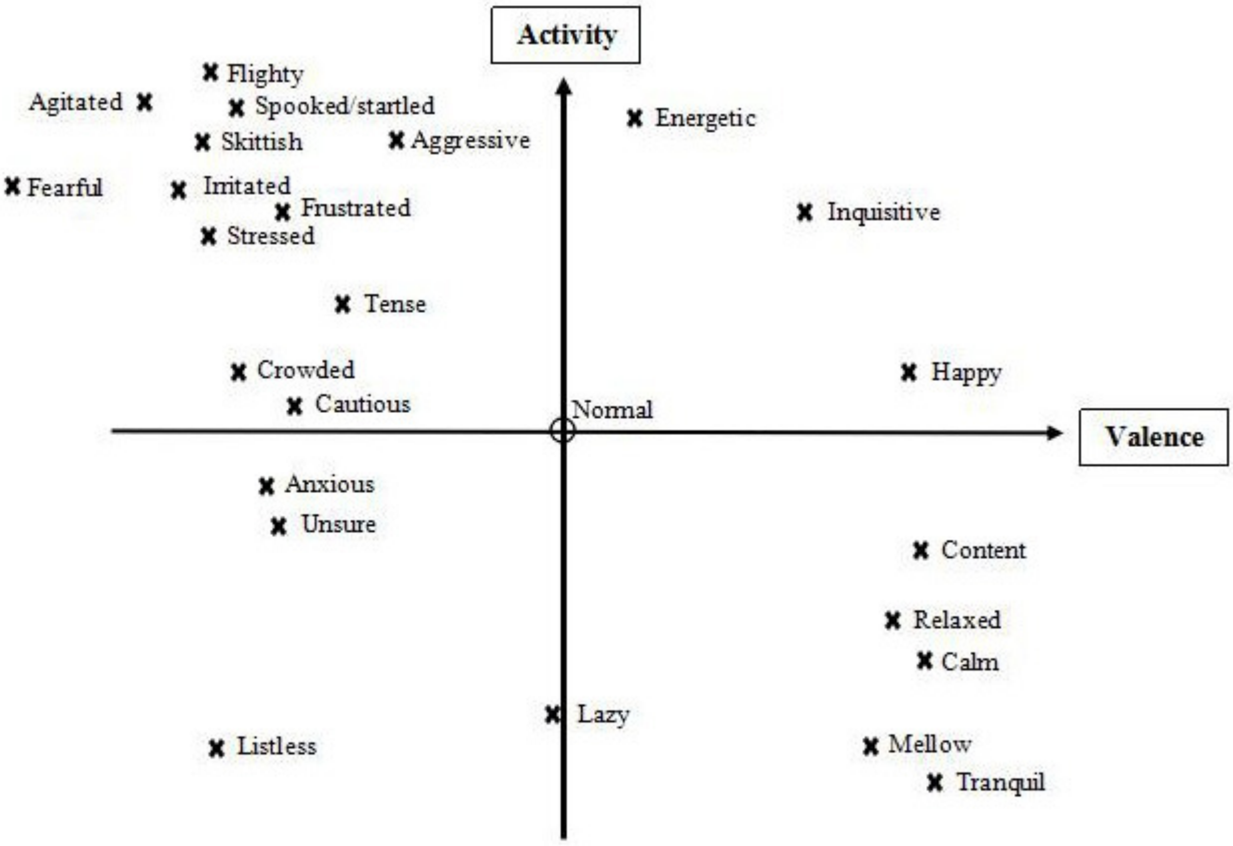

FIGURE 1 | Valence and activity (arousal) scales used to discuss terms, along with placement of all generated descriptors, subject to discussion with the farmers, before finalization of the Fixed List.

Video Viewing and Term Generation Session

The term generation session took place on-site at the salmon hatchery. Participating fish farmers were given instruction in Qualitative Behavioral Assessment and received guidance in how to generate QBA descriptors while watching the video footage. To minimize the influence of this briefing on descriptors generated by participants, any examples used referred to mammalian species and contained terms considered unlikely to be generated for fish. As the potential bank of terms was considered limited compared to other (terrestrial) species, no video practice or open discussion took place prior to the screening of the video clips. Participants were instructed to strictly refrain from any discussion during the term generation exercise.

Participants generated a total of 26 descriptive terms for salmon expression. Some of these were excluded because they described physical behavior rather than expressive demeanors (e.g., hunting, seeking), or referred to external conditions such as the fish "being controlled."

After term generation was completed, participants were invited to take part in a joint discussion with the aim of selecting a final list of terms. They were asked to place all individually generated terms in a diagram in which "valence" and "activity" dimensions framed four main quadrants (39) of salmon expressivity, and to consider whether any of these quadrants were underpopulated, or whether any key terms were missing because the video footage had not shown the relevant expressions. Based on this the group added four terms: fearful, listless, frustrated and aggressive (see **Figure 1**). They then discussed which terms, balanced across the four quadrants, were most suitable to characterize fish body language, and chose a final list of 20 descriptors.

TABLE 1 | Finalized fixed list of QBA descriptors for salmon behavioral expression.

Terms			
Inquisitive	Listless	Aggressive	Mellow
Unsure	Startled	Fearful	Anxious
Agitated	Tense	Tranquil	Energetic
Relaxed	Crowded	Irritated	Stressed
Flighty	Calm	Skittish	Content

It was felt that the term list developed by the fish farm participants was sufficiently in line with current knowledge of fish behavior and welfare (40), and no further terms were added. This Fixed List of Descriptive Terms (**Table 1**) was then used in Phase 2 of QBA scoring.

Phase 2: QBA Scoring Sessions

Participants

The 10 participants in this phase consisted of veterinary students ($n = 4$), animal welfare (MSc level) students ($n = 5$) and staff members ($n = 1$) recruited from the Dick Vet Behavior Society (University of Edinburgh). These participants had variable levels of experience in working with fish, ranging from no theoretical or practical experience ($n = 6$) to practical work in fish husbandry in a laboratory or aquarium setting ($n = 4$). No participants had experience in a commercial aquaculture setting or with salmonids.

Clip Selection

Twenty-five video clips of 1 min duration each were created from the on-farm footage taken in March. Clips were selected to cover as wide a range of behavioral expression in juvenile salmon as possible, and were arranged to display contrasting expressive qualities. No video clips were selected from the period 2 min before or after the feeders being deployed. Between video clips a period of 2–3 min enabled participants to record their scores for each clip on each of the 20 terms developed in phase 1.

Video Scoring Session

To align observers' understanding of the terms in the QBA descriptor list developed in phase 1 (**Table 1**), an open discussion was conducted with all participants for an hour before scoring commenced. The meaning of all 20 terms was discussed, with further time given if questions were raised against specific descriptors. Following this discussion, the term list was written out with agreed synonyms, identical in both video sessions, for each descriptor to clarify meaning (see **Table 2**).

TABLE 2 | List of agreed synonyms for fixed list terms, as generated and discussed with all participants.

Fixed list term	Agreed synonyms
Inquisitive	Interested, curious, engaged
Unsure	Cautious
Agitated	Disturbed, unsettled
Relaxed	At ease, no urgency (not necessarily motionless)
Flighty	Erratic, volatile, unpredictable
Listless	Lethargic, lifeless
Startled	Spooked, surprised
Tense	On edge, strained
Crowded	Claustrophobic, overwhelmed
Calm	Peaceful, undisturbed
Aggressive	Hostile, assertive (violent)
Fearful	Afraid, frightened
Tranquil	Still, quiet, serene
Irritated	Annoyed, frustrated
Skittish	Excitable, easily frightened
Mellow	Easy going, tolerant, unphased
Anxious	Worried, apprehensive
Energetic	Active, lively, dynamic
Stressed	Disturbed, upset, under pressure, mix of anxious and tense
Content	Satisfied, at peace, restful

The full set of 25 video clips was then shown to all participants in two separate face-to-face sessions, for logistical reasons. Verbal instructions were given on the fundamental principles of QBA, general information on how to assess body language (using posture, gaze, speed and character of movement) and how to score descriptive terms using Visual Analog Scales as described below. These instructions were identical on both days.

Scoring was carried out on paper-based forms. For each qualitative term, a 125 mm horizontal line was present as a Visual Analog Scale. Participants were instructed to make a single vertical mark on each line, corresponding with how intensely they felt a particular expressive quality was seen in the salmon's demeanor within each clip. The leftmost extreme represents complete absence of the expressive quality (e.g., not at all agitated), and the rightmost extreme represents a maximal judgement of an expressive quality (e.g., couldn't be more agitated). Vertical marks represent where the participant has judged the clip on this spectrum. Observers were told to mark every single term for each clip, and not to use the Visual Analog Scales as a yes/no or categorized response, but to consider the whole scale when judging expressive intensity. It was explained to participants that expressive qualities of demeanor can be assessed at group level by scoring how animals collectively move; if different expressions were seen in different individual fish or different parts of a group, observers were advised to score the different expressions according to the proportion of animals showing them.

A second viewing session was required in order to collect data for intra-observer reliability analysis, i.e., the repeatability of scores within individual observers. Because it proved not possible to find a date at which all participants were available, and no further collective instruction or discussion of methods was required, the second set of video clips was transferred electronically to all participants 10 days after the first scoring event, for observation in their own home environment. The clips ($n = 25$) were identical to those used in the first video session but arranged in a different order. The observers were unaware they were the same set of clips. As before, scoring was carried out on paper-based forms using Visual Analog Scales. Participants were advised at this stage to view the video clips once only, in the given numbered order, and to allow 2–3 min for scoring after each clip. A week after delivery of the clips, paper forms were collected for data input and analysis, as carried out for the first session.

Ethogram-Based Behavior Measurements

For the 25 video clips used for the Fixed List QBA scoring in Phase 2, an ethogram was developed consisting of categories of physical behavior that covered the different types of collective motion by fish observed in the video clips, and were sufficiently easy to visually identify to be quantified (see **Table 3**). These behaviors were recorded quantitatively as frequencies or durations (secs) by an independent observer who had not taken part in any of the QBA assessments.

TABLE 3 | Ethogram for juvenile salmon.

Behavior	Description
"Inquisitive" (frequency)	One or more salmon are orientated toward the camera or other visible environmental features/objects, swimming in place and observing object with no erratic movements or evidence of impedance of forward motion
"Aggressive" (frequency)	One salmon is observed to make a sharp (<2 s) movement toward a conspecific which brings the aggressor into close proximity.
"Startled" (frequency)	Any number, ranging from an individual, small group to all visible fish make sudden (<2 s), sharp movements
"Calm" [duration (s)]	Consistently slow movement (2 or fewer tail movements per second), swimming in place. Can involve some drifting in position but should be passive and not associated with significant propulsive effort.
"Active" [duration (s)]	Consistent movement, smooth and not erratic. Continuous propulsion. Can vary in speed - low speed (in camera view for >3 s) and high speed (<3 s)
"Chaotic" [duration (s)]	Erratic/sharp movements in different directions with no "consensus" on direction of travel. Should involve >50% of visible group with a fast rate of travel - traverse over 1/2 of camera view in <2 s

Behaviors were recorded as frequencies or durations as indicated in brackets.

Statistical Analysis of Qualitative Data

Measurements

On all paper forms collected from sessions 1 and 2, the distance between the vertical mark made on each completed Visual Analog Scale and the left "minimum" point was measured with a 300 mm ruler. The distance values (in millimeters) were entered into Microsoft Excel (2016), along with session and participant into a matrix formed by QBA descriptors listed horizontally in the first row, and video clip numbers listed vertically in the first column.

Principal Component Analysis

Principal Component Analysis (correlation matrix, no rotation) was carried out in R Studio® on the data for session 1 and session 2 separately, and also for the two sessions combined into one data set. Comparison of these 3 PCAs indicated their main dimensions of fish expressions were so similar that all further data analyses were executed with the combined data set only.

For the combined data set, Principal Components with Eigenvalues >1 were labeled by identifying the two or three highest positively and negatively loading descriptors on a Component. Where several high-loading terms were available for a Component, terms with complementary meanings were selected that together represented the larger pattern of expressivity reflected in the PCA.

Principal Component Analysis creates weighting factors allowing the scores attributed to each video clip in sessions 1 and 2 to be summarized with a numerical value on each Principal Component (the "PC score") for each observer. These scores were the basis for the inter- and intra-observer reliability analyses of the combined data set.

Inter-observer Reliability

Kendall's coefficient W was used to calculate the level of agreement between the 10 participants' PC scores in the combined data set, for each of the four Principal Components. Any W values under 0.4 were considered to reflect unacceptable inter-observer reliability. This analysis was carried out using Genstat 16.1.

Intra-observer Reliability

The degree to which observers showed agreement between their session 1 and session 2 scores within the combined data set was determined using partial correlation, by means of a one-way ANOVA on the PC scores with either session 1 or session 2 as treatment factor. This yielded two columns of residual scores for each PC, the normality of which was evaluated by generating histogram plots and Anderson-Darling test outputs, Residual data for all 4 PCs were evaluated as normal. Pearson's correlations were then performed on these residuals for all four Principal Components. This approach ensured that data was expressed relative to the individual participants' mean score value, eliminating the influence of individual participant scoring style, ranging from conservative (limited) to full use of the VAS scales, on the results of intra-observer reliability. This analysis was carried out using Genstat 16.1.

Analysis of Ethogram-Based Data

The ethogram-based scores for the 25 video clips were correlated with the mean PC scores for these clips (derived by averaging each clip's session 1 and session 2 PC scores in the combined data set) on each of the 4 Principal Components. A Spearman correlation test was used as the ethogram-based scores were not normally distributed and resistant to transformation. This analysis was carried out using R Studio®.

RESULTS

Qualitative Behavioral Analysis

Principal Component Analysis

On visual inspection of the loading plots for the separate sessions 1 and 2, and for the combined data set for both sessions, it was established that the distribution of the qualitative terms on all three plots was sufficiently similar to consider sessions 1 and 2 as representing the same "dimensions" of fish expressivity. Therefore, subsequent statistical analyses were carried out only using the combined data set for sessions 1 and 2 (**Figure 2**).

Four Principal Components were generated from the combined data set where the Eigen value exceeded or closely approached 1 (see **Table 4**) (41). Although PC4 had an Eigen value of slightly <1, it was considered to reflect an interesting dimension of fish behavioral expression, and was therefore included. The first dimension (PC1) explained the greatest

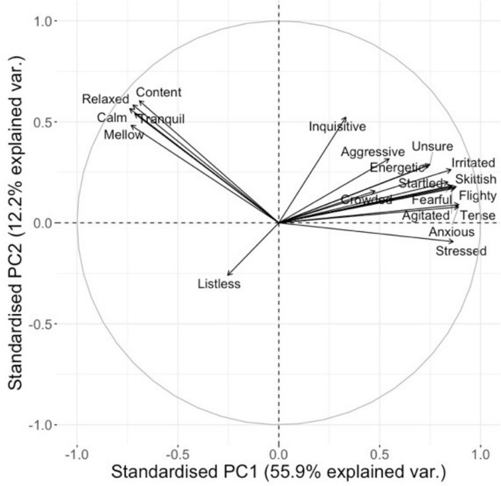

FIGURE 2 | Loading plot for qualitative descriptors for the combined data set. Axes represent the level of correlation at which the QBA descriptors for fish expression load onto the two main Principal Components of the analysis.

TABLE 4 | Eigen values and percentage of variance for each principal component.

Value	PC1	PC2	PC3	PC4
Eigen value	**11.17**	**2.441**	**1.275**	0.910
% of variance explained	56	12	6	5
Cumulative variance	56	68	74	79

Eigen values greater than 1 are in bold.

TABLE 5 | Descriptor loading values for each principal component.

Term	PC1	PC2	PC3	PC4
Inquisitive	0.099	0.335	**−0.164**	**−0.609**
Unsure	0.224	0.185	0.034	0.022
Agitated	0.257	0.116	−0.000	0.133
Relaxed	**−0.216**	**0.374**	0.039	0.090
Flighty	0.261	0.110	−0.004	**0.213**
Listless	−0.076	**−0.166**	**0.729**	0.092
Startled	0.251	0.129	−0.004	0.205
Tense	**0.267**	0.058	0.147	−0.024
Crowded	0.143	0.101	**0.431**	**−0.516**
Calm	**−0.221**	**0.362**	0.044	0.053
Aggressive	0.164	0.203	0.180	**−0.227**
Fearful	0.256	0.118	0.043	**0.223**
Tranquil	−0.214	0.346	0.191	0.184
Irritated	0.255	0.168	0.109	0.012
Skittish	**0.263**	0.114	−0.013	0.197
Mellow	**−0.219**	0.309	0.185	0.185
Anxious	**0.266**	0.050	0.065	0.137
Energetic	0.221	0.182	**−0.297**	−0.021
Stressed	0.259	−0.060	0.112	0.076
Content	−0.207	**0.387**	−0.094	0.068

The highest positively and negatively loaded terms for each PC are in bold.

percentage of variance at 56%, with the first four dimensions collectively accounting for 79% of the variation (see **Table 4**).

As can be seen in **Table 5**, PC1 ranges from Tense/anxious/skittish to Calm/mellow/relaxed, describing a shift from negative mood/high-energy to positive mood/low-energy. PC2 ranges from Content/relaxed to Listless, but as "Listless" is the only significantly negatively loading descriptor, this dimension seems to mainly reflect the salmons' degree of listlessness against all other possible expressions. PC3 ranges from Listless/crowded to Energetic/inquisitive, and appears to indicate an association between listlessness and crowded conditions. PC4 ranges from Inquisitive/crowded to Fearful/flighty and seems to indicate a contrast between inquisitiveness and fear.

Inter-observer Reliability

PC1 (Tense/anxious/skittish - Calm/mellow/relaxed) was the only Principal Component to demonstrate good inter-observer reliability for the combined data set ($W = 0.68$, $\chi^2 = 335.31$, $P = < 0.001$). The other 3 PCs had W values of below 0.4 for all data sets, which is considered unacceptable (42).

Intra-observer Reliability

Similarly Tense/anxious/skittish -Calm/mellow/relaxed (PC1) was the only dimension to show good intra-observer reliability between session one and session two PC scores in the combined data set (PC1: $r = 0.65$, $p<0.001$), with PC2-PC4 demonstrating significant but poor to moderate correlations.

The Association Between QBA Scores and Ethogram-Based Behavior Measurements

Given that PC1 was the only QBA dimension with significant inter- and intra-observer reliability, here we report only significant correlations between ethogram-based behaviors and PC1 that were of sufficient strength (i.e., $r > \pm 0.50$).

PC1 scores (Tense/anxious/skittish – Calm/mellow/relaxed) showed a very strong negative correlation ($r = -0.85$, $p =< 0.001$) with the duration of slow physical movement without active propulsion (**Figure 3A**). In addition PC1 scores showed a moderate positive relationship ($r = 0.65$, $p =< 0.01$) with the duration of erratic/sharp movement in different directions (**Figure 3B**). Correlations between PC1 scores and other ethogram-based measures were non-significant.

DISCUSSION

The aim of this study was to investigate the potential of QBA as a welfare assessment tool for Atlantic Salmon in the freshwater phase within aquaculture. In a first phase of the study, experienced fish famers watched a set of 12 videos created to cover a wide range of behavioral expression in juvenile salmon, and through discussion created a list of 20 descriptors for salmon expressivity. In a second phase, this fixed list of terms was used by 10 different observers, all inexperienced in fish farming, to score

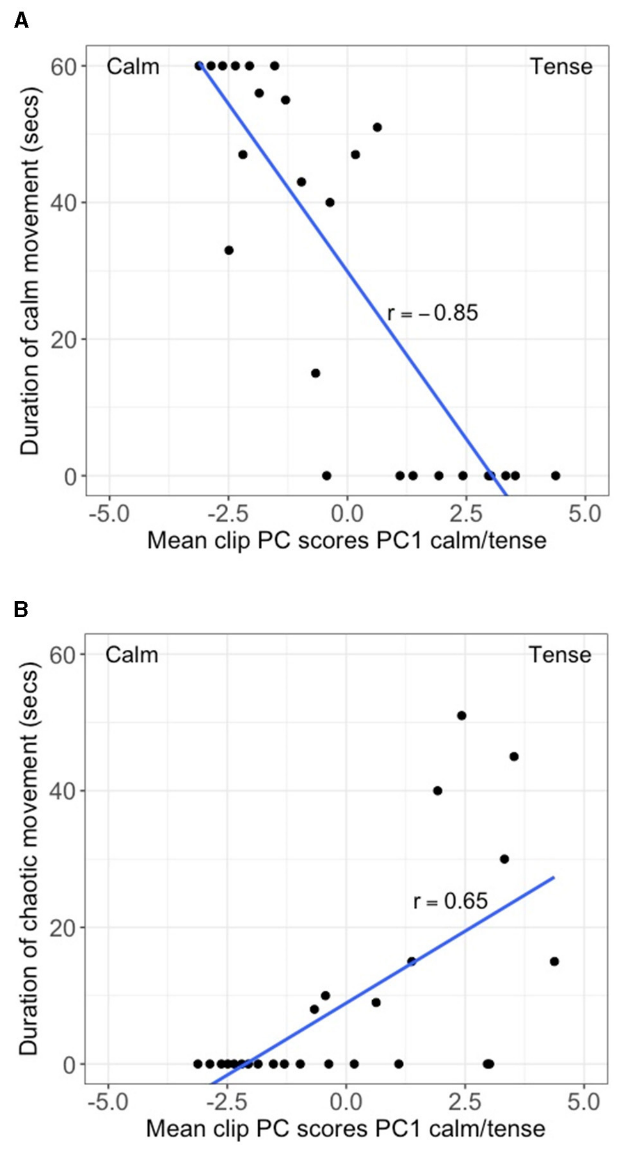

FIGURE 3 | Scatterplot of mean clip PC scores for PC1 (Tense/Calm) vs. **(A)** duration of calm movement in seconds, and **(B)** duration of chaotic movement in seconds. Line of best fit is included.

salmon expressivity from a new set of 25 video clips. In order to test the repeatability of their scores, these observers scored the same 25 clips a second time at least 10 days after the first session had been completed. The initial session was conducted face to face with all assessors together, but the repeat scoring of clips was performed online by participants individually at their own home. The ensuing data were all analyzed together using Principal Component Analysis, revealing four meaningful dimensions of salmon expression. However, of these dimensions only the first one (PC1) showed acceptable inter- and intra-observer reliability.

PC1 was characterized as ranging from "Tense/anxious/skittish" to "Calm/mellow/relaxed," explaining 56% of the variation, and showing good inter- and intra-observer reliability (the latter despite the different settings in which the two sessions were conducted). Significant correlations with measures of the salmons' physical movements found "Calm/mellow/relaxed" demeanor to be associated with slow, unpropelled movement, and "Tense/anxious/skittish" demeanor with erratic/sharp movement in different directions. Such meaningful mapping of qualitative assessments and ethogram-based measurements supports the validity of QBA (43), however we should not necessarily expect a full overlap of the two types of assessment. Qualitative assessments include and integrate subtle expressive aspects of an animal's demeanor in its environmental and social context, that may be difficult to quantify in ethogram-based categories. Thus, QBA is hypothesized to provide information on an animal's affective state that is complimentary to other measures and facilitates a more comprehensive evaluation of animal welfare, including positive welfare states (25). The current results indicate that observers with relatively little fish-based experience (but using descriptors developed by experienced fish farmers) were able to consistently judge a dimension of tense vs. calm expressivity in juvenile salmon, which is highly relevant to appraisals of fish welfare in aquaculture.

The remaining dimensions (PCs 2, 3, and 4) explained a lower percentage of variation, and did not show acceptable inter- and intra-observer reliability. A first reason for this may be that the expressions characterizing these dimensions (e.g., listless, content, inquisitive) were more difficult to perceive and assess for inexperienced observers than those characterizing PC1, particularly as the latter were associated with specific patterns of physical movement. "Listless" appeared as a key term in characterizing both PC2 and PC3, but for inexperienced observers it will not have been easy to distinguish "calm/relaxed" fish from "listless" fish. A contributing factor here may have been the large numbers of fish shown in the video clips, making it harder to clearly see expressive cues. In other QBA studies, video footage is often focused on individual animals [e.g., (44, 45)], or is focused on small groups of animals (<15) with low stocking density [e.g., (43)]. However, QBA methodology has also been successfully applied to larger groups of terrestrial animals, for example through the EU Welfare Quality® assessment protocols [e.g., (29)], or in studies of farmed broiler chickens (37). In order for QBA to be successful as an on-farm welfare assessment tool within aquaculture it has to be robust when observing very large groups of fish. This study provides the first evidence for the availability of a meaningful and reliable dimension of salmon expressivity, describing the difference between "Tense/anxious/skittish" and "Calm/mellow/relaxed" fish.

Similar dimensions have been reported for terrestrial animals, such as in cattle during transport (46), or at the abattoir (47). Many QBA studies of terrestrial animals find main dimensions of mood and energy that show acceptable inter-observer agreement and can be applied to practical farm-assessment, however as the single reliable dimension identified here characterizes a combined shift in both mood and energy, it could still be of use for practical application to welfare assessment in farmed salmon. In fact all four dimensions identified in the current study describe combined shifts in mood and energy, and so are potentially relevant to monitoring welfare in farmed fish. The question is whether further study could improve inter-observer agreement for these dimensions, through a stronger focus on the experience and training required for observers. Considering this study's observers' experience, a second reason for not finding more reliable dimensions could be that observers struggled to apply unfamiliar descriptors to an unfamiliar species. In this study we invited an experienced group of fish farmers to create the list of QBA descriptors, rather than asking the observers in phase 2 to create their own descriptors for scoring [e.g., (48)]. The reason for this was that QBA term lists should cover a comprehensive and varied range of a species' expressions, and that there is considerable risk that inexperienced observers will fail to include important aspects of this expressivity (49). The potential downside of this approach, however, is that prescribing a list of terms which observers have not developed themselves makes it harder for them to use it appropriately (31). Observers were asked to spend an hour discussing the meaning of the terms on the list. However, in the absence of much experience with farmed fish, this may not have been sufficient to reach agreement on dimensions beyond the first obvious one (50). On the other hand, some studies have reported that non-experienced observers show better agreement than experienced ones, arguing that factors such as observer personality and attitude are more powerful determinants of agreement than experience (51). Thus, regardless of observers' levels of experience, the use of pre-fixed QBA term lists in fish requires that adequate instruction and training in fish biology and behavior is provided (50, 52). The consequence of this for QBA's feasibility is that initially it may require considerable investment in observers' assessment skills. However, this is true for most assessment methods (31), and the investment should pay off over time in creating an efficient and informative assessment tool.

Further QBA research should extend to assessment of salmon at different lifecycle phases and in different production environments. It would be informative to combine use of QBA with other welfare assessment systems for salmon, such as for example reviewed by Stien et al. (23). In addition it could be fruitful to combine QBA with other monitoring modalities such as motion-detection of optical flow at group level, as studied in poultry by Dawkins et al. (53). This could be taken forward into remote sensing and machine learning, using QBA to evaluate the capacity of such new technologies to address valence aspects of animal welfare. QBA in its own right, when applied by trained farm staff assisted by mobile application technology, also has considerable potential as an on-farm welfare assessment tool within aquaculture, as it is observational, non-invasive, and, after initial investment in observer training, can be applied in a time efficient manner (26). Using all such development avenues, QBA descriptor lists and dimensions can be further validated and tested for efficacy. Given the growth of aquaculture globally and the large number of species and individual fish involved (4), there is a need to develop reliable tools for monitoring fish welfare (including positive welfare), that can inform guidance and legislation to meet the species-specific needs of fish.

CONCLUSION

This study provides the first successful application of QBA to the assessment of emotional expressivity in Atlantic salmon under farmed conditions. One dimension (Tense/anxious/skittish – Calm/mellow/relaxed) explained the majority of variation, showed good inter- and intra-observer reliability, and correlated significantly to durations of erratic vs. slow physical movement. Thus, QBA has the potential to provide a meaningful and, with further validation, time-efficient tool for welfare assessment in farmed salmon.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://hdl.handle.net/10283/3902.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by University of Edinburgh Vet School Human Ethical Research Committee. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by University of Stirling Animal Welfare & Ethical Review Body.

AUTHOR CONTRIBUTIONS

The collection of video footage was conducted by ME. The term generation and QBA sessions were conducted by ME and Laura Dunn under supervision of SJ and FW. SR and JT were also supervisors of ME's PhD project and were involved in experimental design and preparation of the manuscript. Statistical analysis was carried out by ME and Laura Dunn under supervision of SJ, FW, and SR. Preparation of the manuscript was conducted by SJ with input from all authors.

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Does Vaterite Otolith Deformation Affect Post-Release Survival and Predation Susceptibility of Hatchery-Reared Juvenile Atlantic Salmon?

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Sagittal otoliths are calcareous structures in the inner ear of fishes involved in hearing and balance. They are usually composed of aragonite; however, aragonite can be replaced by vaterite, a deformity which is more common in hatchery-reared than in wild fish. Vaterite growth may impair hearing and balance and affect important fitness-related behaviours such as predator avoidance. Captive rearing techniques that prevent hearing loss may have the potential to improve fish welfare and the success of restocking programmes. The aim of this study was to test the effect of structural tank enrichment on vaterite development in the otoliths of hatchery-reared juvenile Atlantic salmon *Salmo salar*, and to assess the effects of vaterite on immediate predation mortality and long-term survival after release into the wild. Fry were reared in a structurally enriched or in a conventional rearing environment and given otolith marks using alizarin during the egg stage to distinguish between the treatment groups. Otoliths were scrutinised for the presence and coverage of vaterite at 6, 13, and 16 weeks after start feeding, and the growth traits were measured for enriched and control fry when housed in tanks. In a subsequent field experiment, juveniles were released in the Rasdalen river (western Norway), and otoliths of enriched reared and control reared fry were scrutinised from samples collected immediately prior to release, from predator (trout *Salmo trutta*) stomachs 48 h after release and from recaptures from the river 2–3 months after release. Vaterite otoliths occurred as early as 6 weeks after start feeding in hatchery-reared *S. salar*. Vaterite occurrence and coverage increased with fish length. Enriched rearing had no direct effect on vaterite formation, but enriched reared fry grew slower than control fry. After release into the wild, fewer salmon fry with vaterite otoliths had been eaten by predators, and a higher proportion of fry with vaterite otoliths than those lacking vaterite were recaptured in the river 2–3 months after release. Contrary to expectations, this suggests that vaterite does not increase predation mortality nor reduce survival rates in the wild during the early life stages.

Keywords: enrichment, fish stocking, otolith deformities, Salmo salar, vaterite

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INTRODUCTION

The inner ear of bony fishes contains calcareous structures (otoliths) that are part of the organs for hearing and balance. In teleost fishes, the largest pair of otoliths (the sagittae) are usually composed of a polymorph of calcium carbonate called aragonite. However, substitution of aragonite by vaterite, an alternative polymorph, has been documented in several species (1–7). Vaterite otoliths are larger, are deformed and have a lower density than aragonite otoliths (3, 6, 8). While relatively rare in wild fish, vaterite deposition is very common in hatchery fish and in aquaculture (2, 4, 6). Previous studies suggest that the presence of vaterite may impair hearing in salmonids (6, 8) and alter the escape kinematics (9) in salmonids as young as 6 months old.

The functional mechanisms underlying vaterite deposition and its consequences are largely unknown; hormonal (5), genetic, or biochemical (1) factors have been hypothesised as predictors, and there is growing evidence of the roles of different proteins in polymorph deposition at the molecular level (10). Vaterite prevalence during conventional hatchery rearing is reportedly associated with stress related to stocking density and handling practises (4), and with the typically faster growth rates mediated by diet, longer photoperiods that allow for continuous feeding, and temperature regimes (11). Consequently, vaterite is over 10 times more common in farmed than in wild fish, as demonstrated in a review spanning several species including a range of salmonids (6).

Hearing and balance are important sensory systems for fish's detection of auditory cues and for manoeuvring in a three-dimensional water body. Impaired hearing may generate biased soundscape-dependent swimming behaviour and challenge the welfare of captive fish. If the presence of vaterite is high in hatchery fish reared for release into natural habitats, impaired hearing may also bias the perception of predation risk and prey presence, sensory cues that are important for survival and growth (6, 9, 11).

Are there ways to reduce otolith deformities in hatcheries? Any captive rearing technique that prevents hearing loss in fish may have the potential to improve fish welfare. Growing research efforts in the last decades have shown that physical enrichment can improve fish welfare. Structural tank enrichment has been used as a method of improving behavioural phenotypes in juvenile fish reared for restocking programmes aiming to improve their chances of survival after being released into the wild (12–20). This means that captive fish are provided with more stimuli than in conventional tanks by increasing their structural complexity (e.g., provision of shelters). Field studies have shown mixed results regarding the effect of tank enrichment on the post-release survival of salmonids (21–25), suggesting that the relationship may be complex. Our research group has previously conducted field experiments to test whether structurally enriched rearing of Atlantic salmon *Salmo salar* reduced immediate (within 2 days after release) predation mortality by piscine predators (brown trout *Salmo trutta*), and improved long-term (2–3 months after release) post-release survival, and found that enrichment did not consistently reduce predation mortality or improve long-term fry survival (25). When scrutinising the otoliths during the study, which was required to distinguish between the treatment groups as determined by the number of alizarin marks given during the egg stage, we observed variations in the occurrence and extent of vaterite coverage among individual fish.

In the present paper, we have scrutinised otoliths from the same field experiment as of Solås et al. (25), and from enriched and control reared fish sampled in the rearing tanks on three occasions (July, August, September). We tested the effect of structural enrichment on vaterite presence and coverage using data from rearing tanks, and the effect of vaterite on survival using predation and release–recapture field experiments. In doing so, we could evaluate whether structural tank enrichment may represent a practical means to mitigate otolith deformities in juvenile hatchery fish, and whether the documented negative effects of vaterite on salmonid perception would translate to increased short-term predation mortality and reduced survival 2–3 months after release into the wild. Should the rearing of captive fish in structurally enriched environments reduce the incidence of otolith deformities and confer improved survival in the wild, the results could have implications for restocking programs and the aquaculture industry.

MATERIALS AND METHODS

Ethical Statement

All research was approved by the Norwegian Food Safety Authority in compliance with "The Regulation on the Use of Animals in Research" with FOTS id 7931.

Rearing and Experiments

We used juvenile Atlantic salmon *Salmo salar* reared at the Voss hatchery using eggs originating from the Vosso River population housed at the Haukvik live gene bank. Individuals were group-marked in the otoliths at the eyed egg stage using Alizarin Red-S, as described in Solås et al. (25), allowing us to differentiate between fry that would later be reared in an enriched rearing tank or in a conventional control rearing tank. Individuals of the enriched group were marked with two alizarin rings in their otoliths, while those in the control group were marked with one.

Hatching occurred around April 20th in each of the years 2014, 2016, and 2017. The fry were transferred to two large tanks (2 × 2 m) after yolk sac absorption, where they received natural river water from the Vosso river. The first feeding was 1–2 weeks after the fish had been transferred to the rearing tanks. The fish were fed under continuous light from above, with commercial pellets (Nutra XP, Skretting, www.skretting.com) dispensed five times per hour at the water surface by an automatic feeder and increasing pellet size with fish size. Structural enrichment was introduced to one of the tanks (hereafter referred to as enriched) at the onset of feeding (hereafter called start feeding), while the fish in the other tank continued being reared in conventional hatchery environments (no additional structures, hereafter referred to as control). The enrichment consisted of four plastic tube constructions and one green box to provide shelter, both with nylon ropes and plastic shreds attached, to simulate river flora [Figures 1A,B of (25)]. The structures were

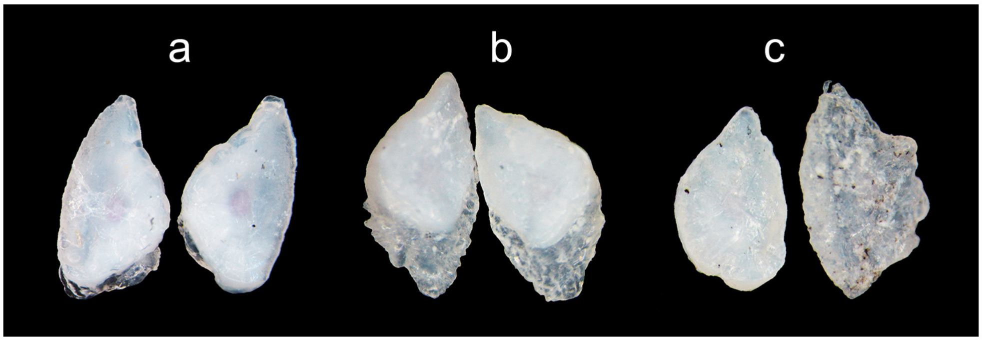

FIGURE 1 | Pictures of vaterite otoliths from fry consumed by trout predators 48 h after release into the Rasdalen river in 2016. **(a)** Vaterite deposition just started; **(b)** medium vaterite cover; and **(c)** nearly total vaterite cover for the right otolith.

cleaned approximately every other week during rearing in June and every week during rearing in July and August. Samples of fry from the tanks and at recapture were euthanised by an overdose of buffered MS222 (0.5 g l^{-1}) and frozen at −20°C until they were processed.

Pre-release Tank Fish

Pre-release juveniles from 2014, 2016, and 2017 were used to study vaterite presence and vaterite coverage in the early life stages of fish from tank rearing. In 2014, the rearing tanks were randomly sampled three times: 6, 13, and 16 weeks after start feeding (**Table 1**). This allowed us to study the effect of time after start feeding and tank treatment on the probability of vaterite present and the vaterite coverage in the sagittal otoliths. We also used the data on juveniles from 2014 to test if enrichment had an effect on growth traits.

In 2016 and 2017, the tanks were only sampled the day of release of fry into the river, which was 13 weeks after start feeding. We used these fry, together with the ones sampled 13 weeks after start feeding in 2014, to test the effect of fish size, tank treatment, and year on the probability of vaterite presence and the vaterite coverage in sagittal otoliths of fry 13 weeks after start feeding.

Predation Field Experiment

A predation field experiment was carried out in the Rasdalen river in western Norway in 2016 and 2017. In each of the years, a total of 3,600 individuals (1,800 from each treatment) were transported to Rasdalen and released into the river mid-August. The fish were aged 17 and 16 weeks when released in 2016 and 2017, respectively. At the day of release, we collected a random subsample of ca. 100 individuals from both the control and enriched rearing tanks to obtain pre-release fish size and otolith compositions (these were the same individuals as described under Pre-release tank fish, for 2016 and 2017).

The release area in Rasdalen was located upstream of a migration barrier and consequently did not have any other Atlantic salmon individuals apart from potential older year-classes from egg boxes set out in 2013 and 2014. No wild-born salmon occur in this area, but it does have a natural population of resident brown trout (*Salmo trutta*). The river stretch was ∼100 m long with an area of ∼1,230 m^2 and consisted of riffles, runs, and pools. The substrate was mainly composed of large stones and small boulders. Larger resident brown trout (standard length >10 cm) were considered as potential predators of the fry and were sampled 48 h after the release of fry. The trout were sampled by two people using point electrofishing with battery-powered backpack generators with a pulsed current of 1,400 V. It took 30–60 min to cover the entire release stretch and a few additional metres downstream. The stunned predators were collected with hand nets and transferred ashore in containers of river water for further examination. To collect the salmon fry that had been consumed by the brown trout predators, the sampled trout were either anaesthetised with MS-222 to enable evacuation of stomach contents in the field or euthanised with an overdose of MS-222 for later examination of stomach contents in the lab (see (25) for details).

Juvenile Atlantic salmon from the stomach contents of anaesthetised trout were collected using the gastric lavage technique (26). We inserted a 60-ml syringe fitted with a thin aquarium tube into the mouth of the trout, to the distal parts of the stomach, and flushed out stomach contents with water onto a sieve. Stomach contents were then put in a cooler to slow the decomposition process and later frozen. The brown trout predators recovered from anaesthesia in a 30-l tank containing river water before being released back into the river.

Release-Recapture Field Experiment

The fry released into the Rasdalen river in August of 2016 and 2017 were recaptured 2–3 months later using the same electrofishing technique as described above. Fish were also released in 2014, but recaptures from that year had to be excluded due to extreme weather conditions in the autumn. The Vosso river system had a 200-year-flood and the recaptures late in the autumn were not considered representative, and only data from before release in 2014 are included in the present paper. The whole release stretch and additional 50 m downstream were sampled (to sample fry that had dispersed downstream) with the aim of recapturing ∼100 fry released 2–3 months earlier.

Measurements and Otolith Examination

All fish were measured (standard length, to the nearest 0.1 cm) and weighed (to the nearest 0.01 g) prior to extracting their

TABLE 1 | The number of Atlantic salmon fry lacking vaterite (vaterite absent) in otoliths and the number of fry having vaterite (vaterite present) in at least one otolith for the tank data from 2014 at 6, 13, and 16 weeks after first feeding, pre-release (2016 and 2017), in fry eaten by trout predators 2 days after release into Rasdalen river (2016 and 2017) and at recapture from electrofishing 2–3 months after release in Rasdalen (2016 and 2017).

	Vaterite absent		Vaterite present	
	N	**Mean SL (cm)**	***N***	**Mean SL (cm)**
2014: Tank data				
Date: 6 weeks after first feeding				
Control	38	3.2 (0.4)	11 (0)	3.3 (0.3)
Enriched	41	3.4 (0.3)	9 (0)	3.2 (0.3)
Date: 13 weeks after first feeding				
Control	36	5.6 (1.2)	11 (4)	6.0 (1.2)
Enriched	40	5.5 (1.0)	15 (3)	5.9 (0.9)
Date: 16 weeks after first feeding				
Control	32	7.5 (1.7)	17 (1)	8.4 (1.4)
Enriched	41	7.4 (1.2)	9 (2)	7.0 (1.4)
2016: Pre-release				
Control	44	5.0 (0.5)	6 (1)	5.2 (0.6)
Enriched	41	4.7 (0.8)	4 (0)	6.0 (0.5)
2016: Predator stomachs				
Control	101	4.6 (0.5)	9 (0)	5.0 (0.4)
Enriched	69	4.6 (0.6)	6 (1)	4.9 (0.8)
2016: Recapture				
Control	9	5.3 (0.3)	27 (19)	5.5 (0.4)
Enriched	17	4.9 (0.6)	19 (13)	5.6 (0.5)
2017: Pre-release				
Control	25	5.7 (0.7)	21 (3)	5.9 (0.6)
Enriched	36	5.5 (0.8)	12 (3)	5.6 (0.9)
2017: Predator stomachs				
Control	11	5.3 (0.7)	5 (1)	5.6 (0.6)
Enriched	15	5.3 (0.5)	5 (0)	5.3 (0.7)
2017: Recapture				
Control	39	6.2 (0.5)	30 (4)	6.3 (0.5)
Enriched	27	6.0 (0.5)	12 (1)	6.0 (0.8)

The numbers in parentheses of mean SL are standard deviations of SL, and the number in parentheses of N refer to the number of the N salmon fry having two vaterite otoliths.

sagittal otoliths. Otoliths were cleaned of adhering tissue and air-dried, and their distal surface was photographed under a Leica M125 stereo microscope mounted with a Nikon Digital Sight DS-Fi2 camera at ×40 or ×50 magnification depending on otolith size such that the length of the otolith filled >30% of the field of view. Of the fry from the rearing tanks in 2014, 2016, and 2017, ∼50 individuals from each of the enriched and control treatments were examined for the presence and proportion cover of vaterite (**Table 1**). The presence of vaterite on the otoliths was noted before or after they were polished to categorise fry into treatment groups (enriched and control, see section about treatment groups below) and using a microscope.

Our first notice of vaterite was a serendipitous discovery, and it occurred after a large proportion of the otoliths from 2017 had been polished. The otoliths had to be polished for the alizarin marks to be visible, which was necessary to identify which fry had been reared in enriched and control tanks. Some otoliths do have a thin clear outer region, which may or may not indicate the onset of vaterite development. Two observers scored the otoliths for presence/absence of vaterite, and where they disagreed, a conservative approach was taken and vaterite was regarded as absent. For all otoliths, vaterite was not regarded as present unless crystallisation was clearly visible (**Figure 1**). Presence/absence scoring was done on all otoliths except for a handful that had to be excluded from the analysis as they were difficult to interpret (e.g., shattered while handling).

Coverage of the two-dimensional distal surface area of an otolith composed of vaterite (hereafter denoted vaterite coverage) was measured on the photos, except for fry from predator stomachs from 2017, to a precision of 1% (i.e., 0.01) using ImageJ (ver. 1.46r). For fry from predator stomachs from 2016, photos of otoliths were taken both before and after polishing the otoliths. These were used to evaluate whether we could measure the coverage of vaterite in polished otoliths from 2017. We decided to use a conservative approach for these otoliths by categorising them binomially by considering the otolith to either have vaterite coverage ≤5% or have vaterite coverage >5%. This approach was also applied to the pre-release otoliths for 2016 and 2017 so that we could compare the probability of vaterite coverage >5% of fry consumed by predators with that of fry pre-release for both 2016 and 2017.

Identifying Treatment Groups From Otoliths

Control and enriched rearing background of fry from predator stomachs and recaptures in the river was determined by examining otoliths for fluorescent alizarin rings. The otoliths were fixed on individual slides using temporary mounting wax (CrystalBond; www.aremco.com, or Quick-Stick; www.innovatekmed.com), and then otoliths were polished with grinding paper until the daily increments of otoliths became visible (27). Next, we identified the number of fluorescent rings using an epifluorescent microscope (Zeiss Axioskop 2 Plus, www.zeiss.com) and UV light. Control fish had one fluorescent ring whereas enriched fish had two.

Statistical Analysis

All statistical analyses were performed in R (ver. 3.6.2, www.r-project.org), and plots were produced using the additional package ggplot2 (28). The data were organised such that each fry was regarded as having vaterite present if at least one otolith contained vaterite. In cases where both otoliths had vaterite (**Table 1**), the otolith with the highest vaterite coverage was included in the statistical analysis. Since the majority of samples had only one affected otolith, we decided not to take the mean vaterite coverage of both otoliths so as not to generate a bias for the few samples with two affected otoliths. To ensure no effect of this on the results, we performed exploratory analyses taking the mean vaterite coverage from both otoliths, which had a minimal effect on the results (data not shown).

For the pre-release tank fish sampled at the same age among years (13 weeks after start feeding), we used generalised linear models (GLMs, logit link) to test for the effects of standard length (cm), treatment (control or enriched rearing), and year (2014, 2016, or 2017) on the probability of vaterite presence and vaterite coverage. Standard length was specified as continuous and treatment and year as categorical effects.

For the pre-release tank fish from 2014 sampled at different weeks after start feeding (6, 13, and 16 weeks), we used GLMs (logit link) to test for the effects of time (weeks) after start feeding and treatment on the probability of vaterite presence and vaterite coverage. Time after start feeding was specified as continuous. A linear model was applied to the data on standard length using log-transformed data to test if enrichment had effect on growth.

For the predation field experiment, we pooled the two treatments (enriched and control) and used GLMs (logit link) to test the effects of standard length and sample group (pre-release or predator stomach) on the probability of vaterite coverage >5% vs. vaterite ≤5%. Standard length was specified as a continuous variable and sample group as a categorical effect. We assumed quasibinomial distributions to account for over dispersion in the data for all the GLMs.

In addition, a Pearson's chi-test was used to evaluate whether the proportion of individuals with vaterite otoliths differed between fry sampled from the tanks the day of release into the river and those sampled from predator stomachs 48 h after release. The chi-test was also used to evaluate whether vaterite occurrence differed between fry sampled before release and those recaptured from the river 2–3 months after release. Since the proportion of fry with vaterite decreased from pre-release to predator stomachs and increased from pre-release to recapture 2–3 months later both in 2016 and in 2017 (**Table 1**; **Figure 4**), we pooled the data from these 2 years for both tests.

RESULTS

Vaterite was detected in the sagittal otoliths of fry after hatchery rearing in all 3 years. Of a total of 890 fish that were scrutinised, vaterite was present in at least one of the otoliths in 228 fish (25.6%), whereas 56 fish (6.3%) had vaterite in both otoliths. However, the extent of vaterite occurrence varied among years, sampling time, and treatment (**Table 1**), which could be a result of random sampling effects.

When comparing pre-release fish that were sampled at the same age among years (13 weeks after start feeding), the probability of vaterite being present in at least one otolith increased with fry length (GLM; deviance $= 15.86$; $p << 0.01$; **Figure 2A**), but did not differ between 2014, 2016, and 2017 (GLM; deviance $= 0.55$, $p = 0.47$). There was no main effect of treatment on the probability of vaterite being present (GLM; deviance $= 1.27$, $p = 0.27$), but a marginally non-significant interaction effect of year and treatment (GLM; Deviance $= 3.12$; $p = 0.08$). The vaterite coverage of the otoliths also increased with length (GLM; deviance $= 7.51$; $p << 0.01$; **Figure 2B**) and did not differ between years (GLM; deviance $= 0.43$, $p = 0.32$; **Figure 2B**). There was a marginally non-significant effect of treatment on vaterite coverage (GLM; deviance $= 1.61$, $p = 0.054$).

For the 2014 tank fish sampled at different times after start feeding, vaterite was detected in both control and enriched reared fry and as early as 6 weeks after start feeding. Overall, 72 out of 300 fish sampled from the tanks in 2014 had detectable vaterite in at least one sagittal otolith. The maximum proportion of an otolith covered by vaterite in control fry was 0.37 at 6 weeks after start feeding and increased to 0.84 and 0.83 at 13 and 16 weeks, respectively. For enriched fry, the maximum proportion cover was 0.39 at 6 weeks after start feeding and increased to 0.69 and 0.76 at 13 and 16 weeks, respectively. There was no significant effect of treatment on the probability of vaterite present in at least one of the otoliths from the 2014 tank data (GLM; deviance $= 1.29$, $p = 0.26$), and there was no change in the probability of vaterite presence from 6 to 16 weeks after start of feeding (GLM; deviance $= 1.18$, $p = 0.28$; **Figure 3A**). However, the vaterite coverage of affected otoliths increased with time after start feeding (GLM; deviance $= 10.64$, $p << 0.001$; **Figure 3B**). There was no main effect of treatment on otolith coverage (GLM; deviance $= 0.45$, $p = 0.22$).

The standard length of fry from both treatments increased significantly over time after start feeding [LM; $F_{(1,296)} = 33.58$,

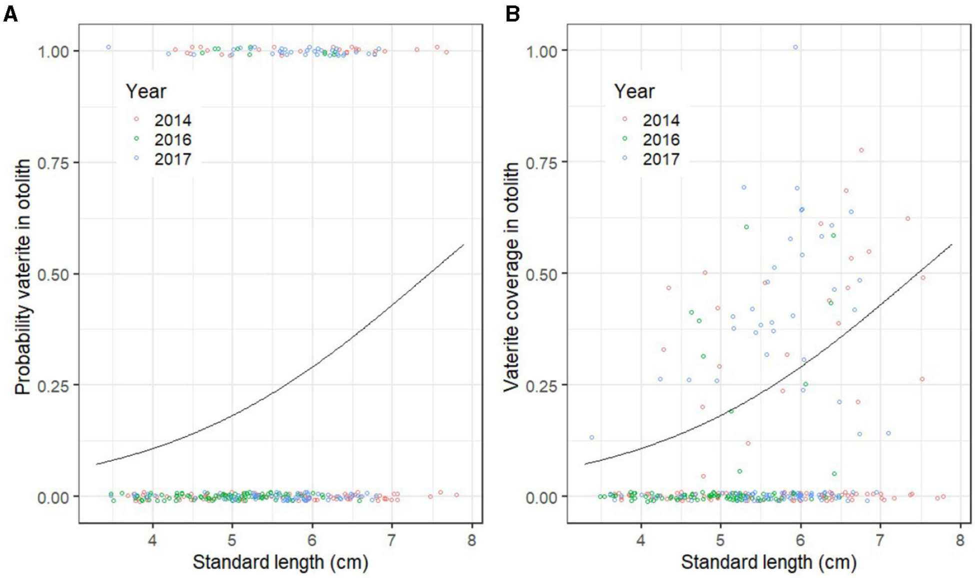

FIGURE 2 | (A) Probability of vaterite present in at least one otolith by standard length (cm) in Atlantic salmon fry ca. 13 weeks after first feeding in 2014, 2016, and 2017 (the three treatments have been pooled). **(B)** Vaterite coverage of the otolith with most vaterite as a function of standard length (cm). The points show the raw data, which are jittered to allow overlapping points to show. The lines represent model predictions generated by retransforming the parameters from logistic regression.

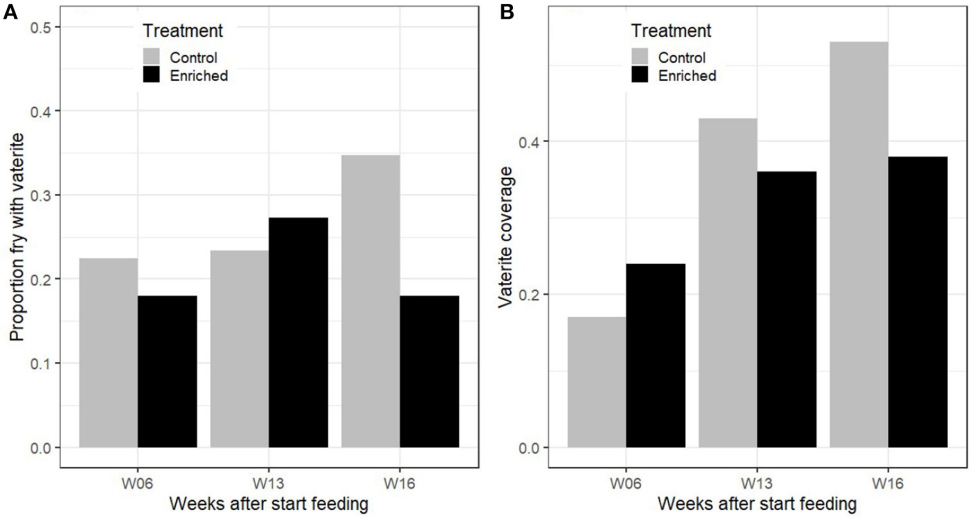

FIGURE 3 | 2014 Tank data. **(A)** Proportion of control and enriched reared salmon fry that had at least one vaterite otolith at 6, 13, and 16 weeks after first feeding. **(B)** Mean proportion vaterite coverage of otoliths, excluding otoliths without vaterite.

$p << 0.001$], but the enriched reared fry had a slower growth than control fry between 6 and 16 weeks after first feeding (LM; interaction of treatment*weeks since first feeding; $t = -2.20$, $p = 0.028$.

From the predation field experiment, the pooled data from 2016 and 2017 show that the proportion of fry with vaterite present was lower in predator stomachs (11% of fry) than pre-release 48 h earlier (23% of fry, chi-test: $\mathbf{X_1^2} = 9.29$; $p = 0.002$; **Figure 4**). The probability that at least one otolith of a fry had >5% vaterite coverage increased with fry size (GLM; deviance = 28.88; $p << 0.001$; **Figure 5**), and the probability was lower for fry in predator stomachs than pre-release 48 h earlier (GLM; deviance = 9.43, $p = 0.003$; **Figure 5**).

The pooled data from the release–recapture field experiment in 2016 and 2017 show that survival was higher in released fry with vaterite otoliths 2–3 months after release than in fry without vaterite (chi-test; $X_1^2 = 27.5$, $p \ll 0.001$; **Figure 4**).

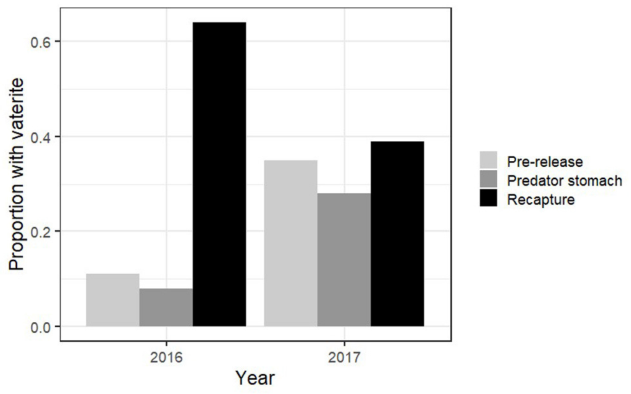

FIGURE 4 | Predation experiment and release–recapture experiment. Proportion of Atlantic salmon juveniles that had at least one vaterite otolith present pre-release, predator stomachs 48 h after release and 2–3 months after release in 2016 and 2017.

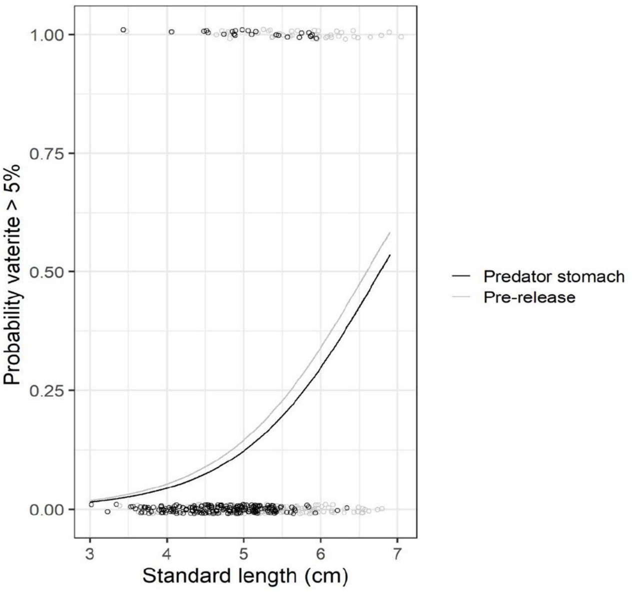

FIGURE 5 | Predation experiment. Prevalence of >5% vaterite in at least one otolith with increasing size of reared Atlantic salmon fry. Data from 2016 and 2017 are pooled. Black circles refer to observed data for fry from the predator stomachs, and grey circles to fry pre-release. The data points are jittered to allow overlapping points showing. The lines represent model predictions that were generated by retransforming the parameters from logistic regression.

DISCUSSION

The increased occurrence of otolith aberrations due to vaterite formation in captive-reared fish has recently received increased attention as its impact on hearing and balance may compromise fish welfare in aquaculture (6) and reduce the performance of hatchery fish released into the wild (29). In the present study, we demonstrate that vaterite formation may occur very early in the juvenile life stages of Atlantic salmon, and within the first 6 weeks after start feeding in hatchery tanks. We found no evidence that vaterite formation was directly affected by the structural enrichment of the rearing environment. However, vaterite was strongly associated with larger body sizes, suggesting that it may be linked to faster growth rates. After release into the wild, salmon fry with vaterite otoliths were not overrepresented in stomach samples of predatory brown trout but were rather underrepresented. Furthermore, salmon fry recaptured 2–3 months after release into the river had a higher occurrence of vaterite compared to samples at the time of release. Therefore, neither does vaterite necessarily result in increased susceptibility to predation nor does it necessarily affect mortality in the wild.

Vaterite replacement in sagittal otoliths occurs sporadically in the wild, but is typically 3–10 times more prevalent in captive-reared fish (4, 6). The strong relationship between captive rearing and increased vaterite occurrence has been attributed to stress caused by intense growth and rearing conditions in hatchery tanks (2, 4, 6). However, the underlying mechanism behind vaterite replacement is still unknown. Reimer et al. (11) found vaterite to be strongly related to rapid growth rates in Atlantic salmon juveniles and suggested that abnormally fast growth disrupts normal aragonite deposition and triggers replacement by vaterite in otoliths. This is in accordance with the results of the present study, where the extent of vaterite was strongly related to body size. However, Austad et al. (29) found no relationship between vaterite and body size in Atlantic salmon smolts but suggested instead that vaterite may have been influenced by crowding in tanks. Bowen et al. (2), on the other hand, found that the prevalence of fish with vaterite increased markedly after routine intervention including dip netting and weighing the fish, suggesting that handling stress may be the main cause. This suggests that vaterite may also be influenced by various physical and social conditions in the rearing tanks. We found no evidence of any direct effect of environmental enrichment, in the form of adding physical structures in the rearing tanks, on vaterite formation. However, enrichment appeared to have an indirect effect on vaterite; fish grew slower in the structurally enriched tanks than in the plain tanks (control treatment), corresponding to a somewhat lower proportion of vaterite in enriched tanks. The lack of growth effect on vaterite in the study of Austad et al. (29) may be due to the fact that size was measured at a later life stage (i.e., at the smolt stage vs. early juvenile stage as in the present study). Growth of juvenile Atlantic salmon may vary across the season, and individuals that experience slow growth initially may also show compensatory growth later in life (30). Any potential growth effect resulting in vaterite deposition during earlier life stages, when it would most likely have initiated, may therefore have been concealed by growth compensation during later juvenile stages. While the role of environmental factors in determining vaterite formation during tank rearing remains to be resolved, the present study strengthens the hypothesis of fast growth being a key factor in causing vaterite otolith deformation.

Most studies indicate that vaterite replacement predominantly begins during the first year of life (4). However, little is known about the precise age at which vaterite replacement is initiated and whether certain life stages are particularly sensitive to vaterite development. In the present study, vaterite was present in 20%

of the fish already 6 weeks after the onset of feeding in the tanks during the 2014 tank data. There was little change in the occurrence of vaterite after 13 and 16 weeks of feeding in the 2014 tank data, indicating that vaterite growth was mostly initiated during the early stages of tank rearing. Similarly, Bowen et al. (2) found that the majority of vaterite development appeared to occur within the first 5 months of life in lake trout *Salvelinus namaycush*, and indications of early vaterite development were identified during the first weeks after the onset of feeding in rainbow trout *Oncorhynchus mykiss* (5). The initial weeks of the juvenile stage are often critical in fish; during this time, individuals often prioritise rapid growth and development to outgrow the prey size range of gape-limited predators (31). Although more research is needed to resolve when vaterite forms in fish, our study suggests that the first weeks of juvenile rearing may be particularly sensitive for vaterite development.

Otoliths are a key component in the sensory system of fish, contributing to hearing, balance, the detection of gravity, and linear acceleration (32). Hence, otoliths are crucial for the ability of fish to manoeuvre in their surroundings and respond to immediate threats. Vaterite replacement in otoliths has been shown to result in a significant loss of hearing functionality (6, 8) and to affect escape response (9) and is therefore likely to reduce the ability of young fish to detect and escape from predators. Surprisingly, we did not find fry with vaterite otoliths to be overrepresented in the stomach contents of natural predators sampled 48 h after release. On the contrary, they were underrepresented when compared with fry sampled before release. Furthermore, the prevalence of fish with vaterite was significantly higher in samples of fry recaptured after 2–3 months in the wild, suggesting that the survival rate of fry with vaterite was higher and not lower than fry with normal otoliths. The latter result is likely to be due to size selective mortality; fry with vaterite otoliths were significantly larger, whereas predation was found to be selective toward smaller fry (see also (25)). Thus, despite high predation mortality and a high scope for selection, body size appeared to have a stronger effect on survival than the presence of vaterite in the sagittal otoliths.

The apparent absence of an effect of vaterite on predation susceptibility and mortality in the wild suggests that the loss of hearing may be of minor ecological significance early in life, or that the fish can compensate for reduced otolith function. For example, fish may partially compensate for reduced hearing by using the lateral line system for stimuli that are in close proximity (9). Furthermore, hearing may be more important under certain environmental conditions or during different life stages. Juvenile salmonids largely rely on vision to search for food and to detect predators (33), but auditory cues and hearing may become more important when visual conditions are suboptimal. Hearing and manoeuvrability may also be more important during pelagic phases, for example during smolt migration in fjords and post smolt feeding in open oceans. Austad et al. (29) found that the proportion of vaterite was lower in returning Atlantic salmon adults than in released smolts, suggesting that vaterite may have a negative effect on marine survival. On the other hand, Sweeting et al. (4) found the prevalence of vaterite to be higher in returning coho salmon *Oncorhynchus kisutch* than in released smolts, suggesting that the effect on marine survival may not be ubiquitous, at least across species. Nor was vaterite found to affect homing ability in chum salmon *Oncorhynchus keta* (34). The evidence is therefore mixed, and more data are needed to elucidate the effect of otolith deformities on the performance of fish across different environments, species, and life stages in the wild.

The underlying causes of vaterite formation in the sagittal otoliths of fishes, and the consequences in terms of fish welfare and fitness, remain unclear. It is evident that the prevalence of vaterite otoliths is higher in captive-reared fishes and is associated with conditions that facilitate rapid growth. We have also demonstrated that deposition is likely to begin within the first few weeks after start feeding or earlier. Therefore, experimental studies manipulating the rearing environment during these early life stages may help to identify methods of reducing otolith deformities in hatchery-reared salmon. Although the consequences of vaterite otoliths for fish hearing and behaviour have been documented, the results from the present study suggest that vaterite does not necessarily translate to lower fitness for juveniles released into the wild. Recapture experiments spanning the lifespan of salmonids from hatchery to maturity may help to elucidate the long-term effects of otolith deformities on long-term fitness after release.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Norwegian Food safety Authority in compliance with the regulation on the Use of Animals in Research with FOTS ID 7931.

AUTHOR CONTRIBUTIONS

AS, AD, and MS designed the study. AS raised the funding. AD and MS did the lab work and examined fish otoliths under the supervision of HS. AD and AS analysed the data. AS, AD, and HS wrote the first version of the manuscript. All authors have commented on and discussed the results, and all have contributed with comments and the writing of the final version.

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The Influence of an Enriched Environment in Enhancing Recognition Memory in Zebrafish (*Danio rerio*)

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Environmental enrichment is used to increase social and physical stimulation for animals in captivity which can lead to enhanced cognition. Fundamental to the positive effect enrichment has on the brain is that it provides opportunities for captive animals to recognize and discriminate between different stimuli in the environment. In the wild, being able to discriminate between novel or familiar stimuli has implications for survival, for example finding food, hiding from predators, or even choosing a mate. The novel object recognition (NOR) test is a cognitive task that is used extensively in the rodent literature to assess object recognition and memory, where the amount of time an animal spends exploring a novel vs. familiar object is quantified. Enrichment has been shown to enhance object recognition in rodents. More recently, the use of the NOR test has been applied to another animal model, zebrafish (*Danio rerio*), however, the effects of enrichment have not yet been explored. In the current study we looked at the effects of enrichment on object recognition in zebrafish using the NOR test. Adult zebrafish were housed in either enriched conditions (gravel substrate, plastic plants, shelter, heater and a filter) or plain conditions (heater and filter only) for 6 months before behavioral NOR tests were conducted. Enriched fish showed a preference for a novel object over a familiar one at a distance but did not show a preference during close inspection. Control fish did not show a preference at either distance. Our results suggest that enrichment can enhance zebrafish ability to discriminate between novel and familiar objects, but distance from the object may be an important factor. Future research is needed to determine whether any enhancements in object recognition are a result of an increase in sensory stimulation from being reared with enrichment, or whether it is due to a reduction in stress reactivity.

Keywords: cognition, environmental enrichment, learning and memory, zebrafish (*Danio rerio*), object recognition

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INTRODUCTION

Environmental enrichment is used extensively across many different animal taxa [e.g., fish: (1–3), birds: (4, 5), mammals: (6, 7)] to enhance physical and social stimulation in a captive setting. One aspect of enrichment that is fundamental to the benefits that it provides is the ability to recognize and discriminate between different stimuli in the environment. For example, animals react differently to a stimulus if it is novel vs. something familiar (8). In the wild, being able to discriminate between different novel or familiar stimuli has implications for survival, for example finding food, hiding from predators, or even choosing a mate (9).

Discriminating between different environmental stimuli requires that an individual assess if the stimulus is something they recognize from past encounters or not (10). The novel object recognition (NOR) test is a cognitive task that is used extensively in the rodent literature to assess object recognition and memory (11), where the amount of time an animal spends exploring a novel vs. familiar object is quantified and compared. Research on animal models of human disease, particularly rodents, has shown that they will spend more time exploring an object that is novel compared to an object that is familiar to them (11). More recently, the use of the NOR test has been applied to many non-mammalian animal models [for review see (12)].

The effects of environmental enrichment on performance in the NOR task has been explored in rodents, with most studies showing enhanced learning of novel objects by animals living with enrichment. For example, enrichment has been shown to increase the amount of time exploring a novel vs. familiar object in young (13, 14) and aged (15) rats compared to non-enriched conspecifics. Since there was no difference in total exploration rates between enriched and non-enriched groups, the results indicate that enhancement of memory could be attributed to learning alone and not just a difference in overall exploratory activity between the experimental groups. Rodents reared with more complexity in their environment (increased physical structures and objects to interact with) habituated faster to novel objects (8). Moreover, rats exposed to lifelong intermittent enrichment (3 1-h sessions a week for 18 months) showed enhanced learning in the NOR compared to non-enriched individuals (16). Different strains of rodent models used for various neurobehavioral disorders have also shown a similar effect of enrichment on increased exploration of a novel object, indicating enhanced learning (17–19). In contrast, Viola et al. (20) showed that young CF1 mice exposed to enrichment spent less time overall exploring objects than conspecifics not exposed to enrichment, however, enriched mice still spent more time exploring the novel vs. familiar object.

The NOR task has only recently been used to assess object recognition in zebrafish (21–24). Similar to rodent models, zebrafish show a propensity to explore novel objects over familiar objects (21, 23, 25) when the objects are simple geometric shapes such as spheres, however, this effect does not hold true with more complex or large objects (24). Moreover, it has been suggested that zebrafish are more sensitive to differences in color rather than shape or size of an object (22). Zebrafish color preference has been explored extensively with mixed results; a number of studies suggest that zebrafish are attracted to shorter wavelength colors such as blue and green and tend to avoid longer wavelength colors such as red and/or yellow (26–28), however, others have suggested they are attracted to red and green colors and avoid yellow and blue (22, 28–30). Although the effects of enrichment on novel object recognition have not yet been explored in zebrafish, several molecular pathways have been implicated in enhanced object recognition. For example, nicotine has been shown to enhance learning and memory in zebrafish subjected to the NOR test (21, 22) presumably through activation of acetylcholine receptors, which have been suggested to play a part in improved learning and memory in rodents (31). In addition, learning and memory in rodents has been shown to be inhibited by deacetylation of histones (32); zebrafish treated with phenylbutyrate (a drug that deactivates deacetylation of histones) exhibit improved learning in the NOR (22). Finally, the hormone 17β-estradiol which is known to be involved in modulating neural plasticity and neurogenesis, was shown to enhance novel object recognition in zebrafish (33).

In the current study we investigated the effects of enrichment on novel object recognition in zebrafish. We chose objects with simple, geometric shapes and colors that would not evoke strong responses either way (avoidance or attraction). We predicted that fish housed with enrichment would exhibit increased exploration of a novel object over a familiar one compared to control fish.

MATERIALS AND METHODS

Experimental Set Up

One-year old wild-type zebrafish ($n = 96$) were randomly distributed across two experimental groups, enriched and control, with equal numbers of each sex in each tank (8 tanks of each treatment, 6 fish in each tank). Enriched tanks had a small triple-flow corner biofilter (Lee's Aquarium and Pet Products, USA; Model number: 13405), heater (Penn-Plax Cascade Heat Aquarium Heater, 50 W), gravel substrate (rinsed and dried, grade <1 cm, 2 cm deep), two plastic plants (Pet Solutions, USA; one green and one red, 14 cm tall) and a small plastic shelter (black plastic plant pot, 9 cm). The shelter and both plastic plants in all enriched tanks were moved around once a week during cleaning, however, the final location of these enrichment items was consistent across all enriched tanks (**Figure 1**). Control tanks had a biofilter and a heater only. All home tanks were length = 35 cm × width = 19 cm × height = 28 cm, with a water depth of 25 cm. Tanks were placed on two shelving units standing side by side, each with four shelves, two tanks per shelf (one enriched and one control). The fish were maintained on a 12 L: 12 D cycle with a water temperature of 25 ± 1°C. The fish were fed daily with commercial flake food (TetraMin® Tropical Flakes) and live cultures of brine shrimp (*Artemia* sp.). All experimental and husbandry procedures were approved by the Pennsylvania State University's Animal Care Committee (protocol 201800369).

Novel Object Recognition Test

After 6 months of experimental conditions, all fish were tested in the NOR test. Six testing chambers were constructed using two large testing tanks (length = 76 cm, width = 76 cm, height = 30 cm) and dividing each into three equal sections (length = 25 cm, width = 25 cm) using gray non-transparent plexiglass dividers. A marker was used to place a black dot where each object would be placed (equidistant from all 3 walls of the chamber) to ensure the objects were being placed in the same location for each fish. Two different stimulus objects were used for NOR testing (**Figure 2**); a simple spherical pink bead (diameter = 2 cm) and a simple rod-shaped brown bead

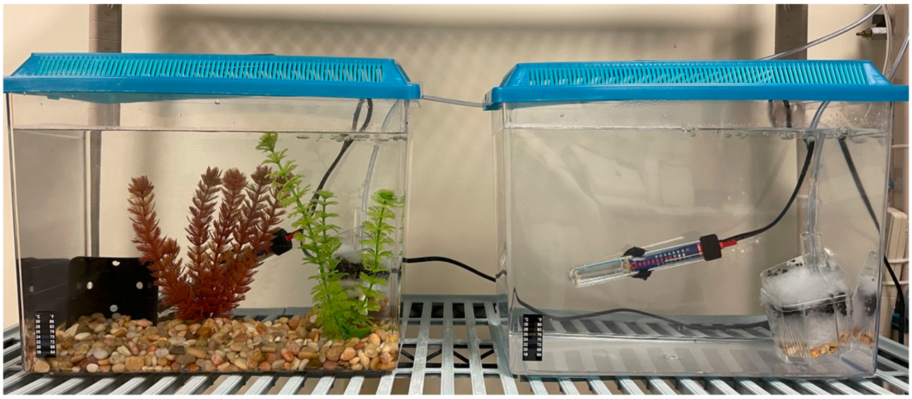

FIGURE 1 | Design of the home tanks. Enriched on left, Control on right. Enriched tanks contained a biofilter, heater, gravel substrate, plastic shelter, and two plastic plants. Control tanks contained a biofilter and heater only.

FIGURE 2 | The two objects used in the NOR test; a simple spherical pink bead and a simple rod-shaped brown bead.

(diameter = 1 cm, height = 2 cm). Objects were pre-tested for saliency using non-experimental fish to ensure that individuals investigated both objects equally, thus indicating that the objects were equally interesting to zebrafish. The objects were attached (with a small amount of blue sticky tack) to the bottom of each experimental chamber. The role (familiar or novel) of the two stimulus objects was counterbalanced and psuedorandomly exchanged for each fish so that equal numbers of both enriched and control fish received the brown bead vs. the pink bead as the novel object. The testing tank was filled with sump water to a depth of 18 cm that was kept at within the same temperature range as the experimental home tanks.

Over the course of 4 days, each fish was given 3 h to acclimatize to the testing chamber without any stimulus objects prior to testing. Each fish from all experimental home tanks was individually netted from their home tank and placed carefully in a testing chamber (1 fish per chamber). We chose 3 h because acclimatization of zebrafish to the testing apparatus in previous studies using the NOR have ranged from 5 min (24) to 24 h (23). In addition, 3 h is a sufficient period of time for any potential stress hormones from handling to decrease to baseline levels (34). The test tank was replaced with new sump water prior to testing starting to eliminate the effects of stress hormones that may have been released into the water by the fish during acclimatization. Once all fish had been acclimatized, testing started. The NOR test was split into two phases: an acquisition phase and a retention phase (**Figures 3A,B**). The acquisition phase involved exposing each individual fish to two identical objects within each chamber for 10 min. The objects were already present in the tank when the fish was placed into the chamber. Fish were quickly (<30 s) and carefully netted from their home tank (if enrichment was present it was removed to make it easier to net the fish) and was placed in the center of the experimental chamber (to reduce any bias related to the fish being introduced closer to one of the objects). Once the last fish was placed in the 3rd chamber, timing started. The experimenter was careful to minimize any disturbances to fish already in a testing chamber by approaching the tank quickly and quietly. After the 10 min was over the objects were removed and the fish stayed in their individual testing chambers for a further 4 h (retention interval). A retention interval of 4 h was chosen because it has been shown that zebrafish are able discriminate between familiar and novel objects after a retention interval of only 2 h and as long as 24 h (23). Immediately following the 4 h retention interval, the retention phase started with the experimenter carefully netting the fish out the tank and placing in a holding tank so that each chamber could be set up for the retention phase. The retention phase involved exposing each individual fish to one familiar object and one novel object within each chamber for 10 min. The relative position of the two stimulus objects (top of chamber or bottom of chamber) was counterbalanced and pseudorandomly exchanged for each fish to reduce any side biases. Again, the fish was carefully and quickly netted from their holding tank and placed in the center of the experimental chamber. Timing started once the last fish was placed in the 3rd chamber. During both phases the experimenter left the room to minimize any outside disturbances and the fish were free to explore the objects and the chamber. Once the retention phase was over, the fish were

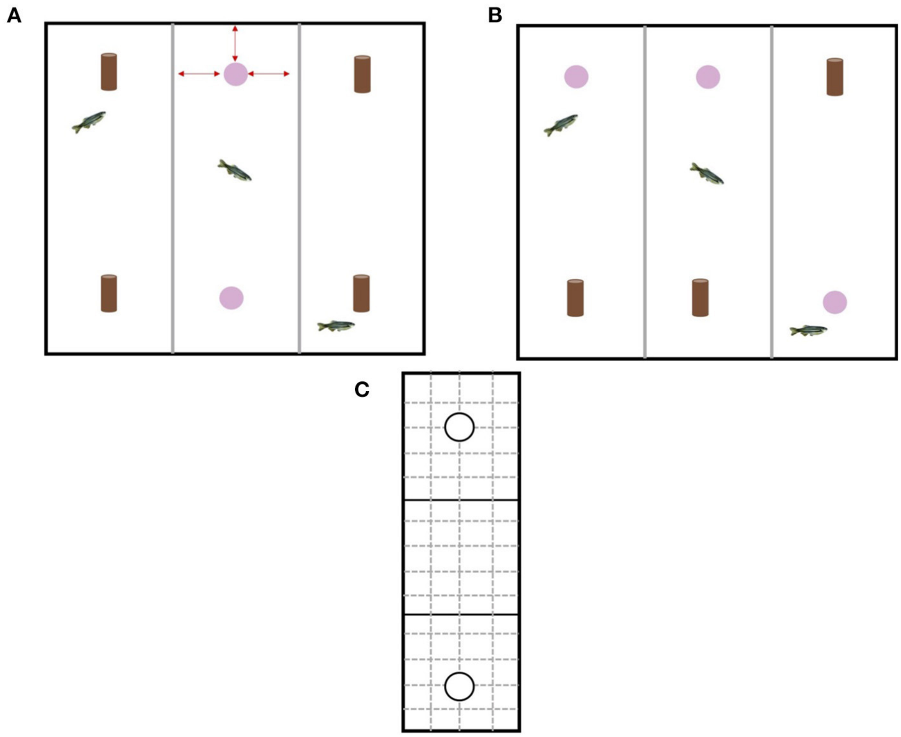

FIGURE 3 | (A) Design of the experimental tank during the acquisition phase. The experimental tank had three different chambers for individual testing of fish. Gray opaque plastic dividers separated each chamber. During the acquisition phase each fish received two copies of the same object, however, which type of object (pink bead or brown bead) was alternated to provide equal numbers of fish within each treatment that had been exposed to the different objects. Red arrows indicate the object was equidistant from the three walls surrounding it **(B)** Design of the experimental tank during the retention phase. During the retention phase each fish received one copy of the original (familiar) object and one copy of the new (novel) object. The location of the object was rotated to minimize any effects of side bias. **(C)** Schematic of the acetate used to analyze the behavioral videos. Solid black lines depict the three different zones (object 1 zone, neutral middle zone, object 2 zone) and solid black circles depict the encounter zone around each object. Solid black lines and dashed gray lines indicate lines used for quantifying movement rate.

removed from the test tank and placed in new experimental home tanks separate from fish still to be tested. The water in the test tanks was replaced with new sump water before stimulus objects and new fish were placed in each chamber. Video cameras secured to the ceiling were used to record fish behavior during the acquisition and retention phases. The videos were then analyzed using BORIS software (35). All video analysis was carried out by the same experimenter so as to reduce any experimenter bias. Acetate and a marker were used to divide each experimental chamber into a grid with three equal zones (object 1 zone, neutral middle zone, object 2 zone; 25 cm) and an encounter area (radius = 1 body length) around each object (**Figure 3C**). Variables collected included total time spent encountering object, total time in object zone, and movement rate (number of grid lines crossed/min).

Statistical Analysis

Due to the position of the video camera, the sex of the zebrafish was not defined during statistical analysis because the identification of males and females was not reliable from the videos. Firstly, a linear model was used to determine if there were any differences between Enriched and Control fish in overall levels of exploration during the acquisition phase. The independent variables were treatment (Enriched or Control) and object type (pink ball or brown rod), and the dependent variable was movement rate. Linear models were then used to determine if there were any differences between Enriched and Control fish in the time spent encountering either object or entering either object zone during the acquisition phase. The independent variables that were included in the models were treatment (Enriched or Control), object type (pink or brown), and object location (top or bottom).

To determine if there was a difference between Enriched and Control fish in their preference to explore the novel object over the familiar one during the retention phase, a discrimination ratio was calculated. The discrimination ratio was used to analyze preference for one zone over the other as well as preference for approaching one object over the other and was expressed as:

Time spent exploring novel object/(Time spent exploring familiar object + time spent exploring novel object).

Where time spent exploring was either time in zone or time spent encountering. A discrimination ratio of 0.5 indicated no preference for one object over the comparison object.

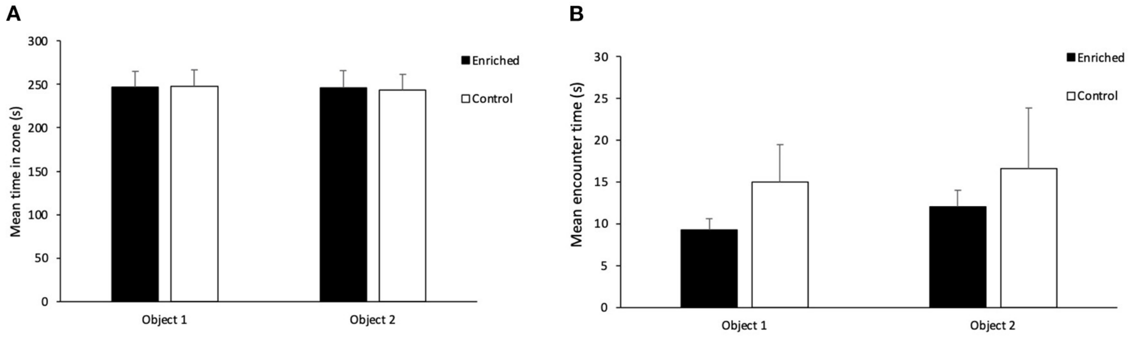

FIGURE 4 | Acquisition Phase on the NOR. **(A)** Mean time in each object zone for Enriched and Control fish and **(B)** Mean encounter time for each object for Enriched and Control fish during the acquisition phase of the NOR. Values are displayed as mean ± s.e.m.

Discrimination ratios higher than 0.5 indicated a relative preference for the novel object, and scores lower than 0.5 indicated a relative aversion to the novel object.

Again, a linear model was used to determine if there were any differences between Enriched and Control fish in overall exploration during the retention phase. The independent variables were treatment (Enriched or Control), object type (pink ball or brown rod), novel object location (top or bottom), and object novelty (novel or familiar). The dependent variables were time spent encountering the object, time spent inside the object zone, or movement rate. To determine if there was a difference between Enriched and Control fish in their preference to explore a novel vs. familiar object during the retention phase, generalized linear models were conducted using the quasibinomial model in R. The discrimination ratio for time to encounter the object and time inside the object zone were used as the dependent variables. The independent variables were treatment (Enriched or Control), object type (pink ball or brown rod), and novel object location (top or bottom).

Interaction effects were excluded from the models if they were not significant. Fish were excluded from analyses if they did not move and remained frozen during the entire acquisition phase or retention phase (Enriched, $n = 7$; Control, $n = 5$). During one session the cameras failed to record, so those fish were excluded from the analyses (Enriched, $n = 3$; Control, $n = 3$). Thus, the final number of fish from each treatment included in the analyses was Enriched = 38 and Control = 41. Four fish from the Enriched treatment and two fish from the Control treatment did not encounter either the novel or familiar object during the retention phase, therefore these fish were excluded from these analyses only. All data were checked for normality using Q-Q plots of residuals. All analyses were performed using R (36) and significance was tested at $\alpha = 0.05$. Values are quoted as mean ± s.e.m.

RESULTS

In terms of overall exploration during the acquisition phase, there was no effect of treatment ($t_1 = 0.08$, $p = 0.94$) or object type ($t_1 = 1.05$, $p = 0.30$) on movement rate. There was no effect of treatment ($t_1 = 0.02$, $p = 0.98$; **Figure 4A**), object type ($t_1 = 0.80$, $p = 0.43$) or object location ($t_1 = -0.11$, $p = 0.91$) on the amount of time fish spent inside each zone during the acquisition phase. There was also no effect of treatment (t_1 = -1.07, p = 0.28; **Figure 4B**), object type (t_1 = -1.81, p = 0.07) or object location ($t_1 = 0.48$, $p = 0.63$) on the time spent encountering the objects during the acquisition phase.

In terms of overall exploration during the retention phase, there was no effect of treatment in the time spent encountering the objects ($t_1 = -0.20$, $p = 0.84$), time spent inside each zone ($t_1 = -0.08$, $p = 0.93$), or movement rate ($t_1 = -0.66$, $p = 0.51$). There was no effect of object type ($t_1 = 1.44$, $p = 0.15$) or novel object location ($t_1 = 0.91$, $p = 0.36$) on time spent encountering the objects. However, there was an interaction effect; fish spent more time encountering the pink ball novel object if it was in the top chamber of the experimental tank, but the opposite was true if the novel object was the brown rod ($t_1 = 2.05$, $p = 0.04$; **Supplementary Figure 1**). There was no effect of object type or novel object location on time spent inside each zone (type: $t_1 = 0.62$, $p = 0.54$; location: $t_1 = -2.04$, $p = 0.06$), or movement rate (type: $t_1 = 1.12$, $p = 0.27$; location: $t_1 = -1.77$, $p = 0.08$). Finally, there was no effect of novelty on time spent encountering the objects ($t_1 = 1.07$, $p = 0.29$), but time spent inside each zone was marginally significant ($t_1 = 1.95$, $p = 0.05$); regardless of treatment, object type or location, fish spent more time in the novel object zone than the familiar zone (in seconds; novel: 293.57 ± 11.84; familiar: 257.82 ± 14.09).

During the retention phase, there was a significant difference in the discrimination ratio of enriched and control fish for time in novel zone vs. familiar zone ($t_1 = 3.04$, $p < 0.01$; **Figure 5A**). Fish reared in enriched conditions spent more time in the novel object zone during the retention phase than those reared in control conditions (Enriched: 0.61 ± 0.03; Control: 0.50 ± 0.03). There was no effect of object type ($t_1 = 0.81$, $p = 0.42$) or object location ($t_1 = 1.52$, $p = 0.13$) on the time spent inside each zone. There was no effect of treatment ($t_1 = -0.48$, $p = 0.63$; **Figure 5B**), object type ($t_1 = 0.50$, $p = 0.62$) or object location ($t_1 = 0.39$, $p = 0.70$) on the discrimination ratio for time spent encountering the different objects.

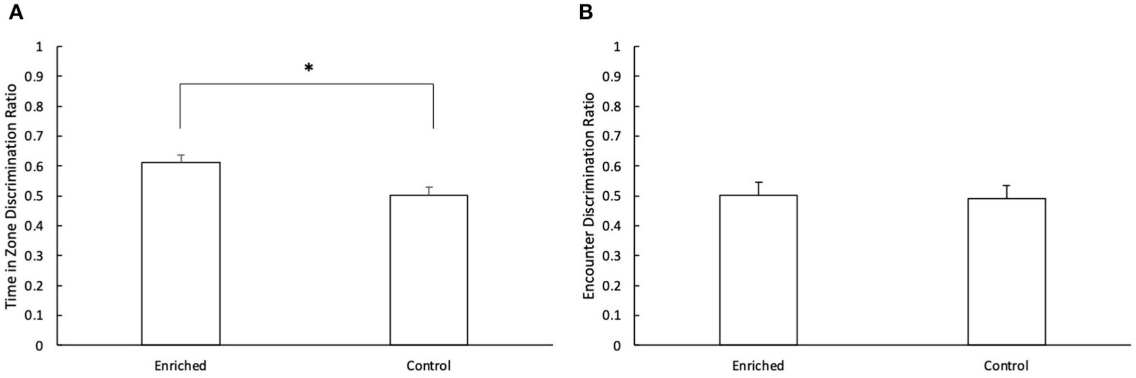

FIGURE 5 | Retention Phase of the NOR. **(A)** Comparison of the time in zone discrimination ratio for Enriched and Control fish and **(B)** Comparison of the encounter discrimination ratio for Enriched and Control fish during the retention phase of the NOR. *Denotes significant difference at $P < 0.05$. Values are displayed as mean ± s.e.m.

DISCUSSION

To our knowledge, this is the first study to look at the effects of enrichment on novel object recognition in zebrafish. The results show that zebrafish reared with enrichment were better able to discriminate between a novel vs. familiar object from a distance compared to zebrafish reared in control conditions. Interestingly, neither Enriched or Control fish were able to discriminate between the familiar or novel object on close inspection when encountering the objects. Therefore, the results from the current study are in partial agreement with our predictions and with previous studies conducted on rodents.

During the acquisition phase, there were no differences in overall exploration between Enriched and Control fish, and no differences in the amount of time fish spent exploring the objects, either close up or from a distance. Thus, both groups interacted with the two identical objects for a similar amount of time. When we look at the discrimination ratio for time spent inside each zone during the retention phase, Control fish did not exhibit a preference for the novel object over the familiar one. In comparison, Enriched fish had an average discrimination ratio of 0.61, suggesting increased motivation and/or interest to explore the novel object vs. the familiar object compared to Control fish. Interestingly, there was no effect of treatment during the retention phase when we looked at the absolute levels of exploration in each zone. The inconsistent results can be explained by the different statistical methods used (linear regression vs. a generalized linear model) and the number of independent variables used. A discrimination ratio is typically used as the standard measure of recognition memory across different NOR studies, including those on zebrafish (21, 22, 24, 25) because it is more resistant to individual differences in exploration (37). Thus, we consider the discrimination ratio a more reliable measure. Similar studies on rodents have also shown that enrichment has a positive effect on object recognition memory [(38–41) but see]. It was suggested that mice reared with enrichment have a higher propensity to explore novel objects because they have been exposed to a more challenging environment (39). In zebrafish, enrichment has been shown to enhance other forms of learning and memory, such as spatial learning (42–44). Moreover, enrichment has been shown to increase neurogenesis (growth of new neurons) and neural plasticity in a number of different animals, including fish (2, 45). Therefore, in the current study zebrafish reared with enrichment may have exhibited enhanced object recognition memory because they previously experienced more stimulating environmental conditions, and this had a positive effect on cognition. A number of studies have reported beneficial effects of enrichment on cognition in juvenile fish raised with enrichment during a critical period of development (2, 42, 46). In this study we investigated the effects of 6 months of enriched conditions on adult zebrafish, however, it would be interesting to know if Enriched fish would have shown a stronger response had the fish been raised in the different conditions from a younger age.

Stress has been shown to influence exploratory behavior in zebrafish, with many studies reporting a decrease in exploratory behavior in response to different stressors, including net chasing and social isolation (42, 47, 48). The NOR paradigm used in the current study potentially exposed subjects to isolation stress, not only during behavioral testing in the acquisition and retention phase, but also during the 4-h retention interval and the 3 h acclimatization period before testing started. Moreover, although we tried to minimize handling before and during testing, all fish had to be transferred from their home tanks to the testing chamber before the acquisition phase and were placed in a holding tank to allow new objects to be placed in the chamber before the retention phase, both of which could have exposed the fish to further stress. Past research on zebrafish has shown that enrichment can decrease the stress response (49) and decrease anxiety-like behavior (42), leading to a better ability to cope with stress. Enrichment has also been shown to reduce the fear response in fish, making them less likely to exhibit neophobic behaviors (50). Thus, even though fish from both treatments were exposed to the same handling procedures and social isolation during testing, Enriched fish may have been able to cope with these stressors and the testing environment more effectively than Control fish, allowing them to perform better.

Contrary to our predictions, we did not see any differences between Enriched and Control fish, nor did either treatment group show any preference toward encountering either object as exhibited by their discrimination ratios (Enriched: 0.50 ± 0.04; Control: 0.49 ± 0.04). The fact that we see a higher discrimination ratio for Enriched vs. Control fish in terms of the time spent in the novel zone compared to the familiar one, but we see no difference in the discrimination ratio for close encounters between the two treatment groups is evidence that close inspection was not needed for Enriched fish to gain enough information to identify whether the object was novel or not. It has been suggested that object recognition at a distance is possible in an aquatic setting, where sensory information can be received through the lateral line system or through detection of water movement (12). On the other hand, the lack of discrimination by both treatment groups when encountering the objects could be due to the relatively small amount of time spent encountering objects during the 10 min trial (in seconds; Enriched: 21.22 ± 3.16; Control: 22.29 ± 2.75). Moreover, we excluded six fish across both treatment groups that did not even enter either encounter zone during the retention phase. It has been reported that the amount of exploration during the NOR should be representative of normal exploratory behavior and allow for a meaningful statistical interpretation (37). We chose 1 body length (∼3 cm) as the size for the encounter zone which is similar to the 3.6 cm used in Luccon Xiccato and Dadda (Luccon Xiccato and Dadda, 2014), however, other NOR studies using zebrafish have used larger encounter zones of 8–10 cm (21, 22, 24, 25). Thus, the size of the encounter zone in the current study may have limited the amount of exploration we detected and thus not been a true representation of close inspection.

There was no influence of object type (brown rod or pink ball) on exploration during the acquisition phase, suggesting that both objects were equal with respect to motivation for exploration. Furthermore, there was no effect of object type on overall exploration in each zone, or on the discrimination ratios for either time spent encountering the objects or time in each zone. However, zebrafish did spend more time encountering the pink ball novel object if it was in the top chamber of the experimental tank, but the opposite was true if the novel object was the brown rod. Zebrafish have a propensity to exhibit color preference, however, the exact order of those preferences is still debated (28). Moreover, it has been documented that objects of a novel color and/or shape are known to increase exploration in zebrafish, but size does not induce such a response (22, 26). In the current study, the type of object and its location were counterbalanced among subjects, so our experimental design should have controlled for any bias effects. We also chose objects that were not known to have ethological significance for the zebrafish; we picked simple, geometric objects of the same size with neutral colors—brown and light pink and a ball and a rod of the same size. Similar objects (a small pink sphere made of glass and a yellow plastic hexagonal-shaped prism) have been used in a previous study on object recognition in zebrafish (23). However, in the current study there may have been subtle differences in the appearance of the objects from different angles depending on the lighting in the room that were only apparent on close inspection. For example, the shiny surface of the pink ball may have been more distinctive under a certain light compared to the matte surface of the brown rod. In addition, the limited size of the encounter area around each object, and thus the relatively short encounter times across all fish, could have made any small random difference in time spent during close inspection appear more significant.

In conclusion, the results of the current study show that enrichment can improve the ability of zebrafish to discriminate between a novel and a familiar object at a distance, however, any effect of enrichment on object recognition during close inspection was inconclusive. We investigated the effects of 6 months of enriched housing conditions on adult (1 year-old) zebrafish. It would be interesting to know if the results of the current study would have been different if the zebrafish had been raised in different housing conditions. Future research should take into account how the size of the area around the object is defined to quantify close inspection of the objects, as well as any differences in the appearance of the objects in the testing arena. Furthermore, more research is needed to determine whether any enhancements in object recognition are a result of an increase in sensory stimulation from being reared with enrichment, or whether it is due to a reduction in stress reactivity. Physiological and behavioral measures of stress, as well as neurological tools could help to answer these questions.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Penn State University IACUC (Protocol 201800369).

AUTHOR CONTRIBUTIONS

CD conceived the original idea and supervised the project. NK, NW, MY, and JW conducted behavioral experiments. JS analyzed the videos. XL conducted the statistical analyses. CD wrote the manuscript with support from NK, NW, MY, JW, and JS. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Fishnition: Developing Models From Cognition Toward Consciousness

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A challenge to developing a model for testing animal consciousness is the pull of opposite intuitions. On one extreme, the anthropocentric view holds that consciousness is a highly sophisticated capacity involving self-reflection and conceptual categorization that is almost certainly exclusive to humans. At the opposite extreme, an anthropomorphic view attributes consciousness broadly to any behavior that involves sensory responsiveness. Yet human experience and observation of diverse species suggest that the most plausible case is that consciousness functions between these poles. In exploring the middle ground, we discuss the pros and cons of "high level" approaches such as the dual systems approach. According to this model, System 1 can be thought of as unconscious; processing is fast, automatic, associative, heuristic, parallel, contextual, and likely to be conserved across species. Consciousness is associated with System 2 processing that is slow, effortful, rule-based, serial, abstract, and exclusively human. An advantage of this model is the clear contrast between heuristic and decision-based responses, but it fails to include contextual decision-making in novel conditions which falls in between these two categories. We also review a "low level" model involving trace conditioning, which is a trained response to the first of two paired stimuli separated by an interval. This model highlights the role of consciousness in maintaining a stimulus representation over a temporal span, though it overlooks the importance of attention in subserving and also disrupting trace conditioning in humans. Through a critical analysis of these two extremes, we will develop the case for flexible behavioral response to the stimulus environment as the best model for demonstrating animal consciousness. We discuss a methodology for gauging flexibility across a wide variety of species and offer a case study in spatial navigation to illustrate our proposal. Flexibility serves the evolutionary function of enabling the complex evaluation of changing conditions, where motivation is the basis for goal valuation, and attention selects task-relevant stimuli to aid decision-making processes. We situate this evolutionary function within the Temporal Representation Theory of consciousness, which proposes that consciousness represents the present moment in order to facilitate flexible action.

Keywords: animal consciousness, flexibility, Temporal Representation Theory, dual-systems theory, trace conditioning, unlimited associative learning (UAL)

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INTRODUCTION

A challenge to developing a model for testing animal consciousness is the pull of opposite intuitions. On one extreme, the anthropocentric view holds that consciousness is a highly sophisticated capacity involving self-reflection and conceptual categorization that is almost certainly exclusive to humans. At the opposite extreme, an anthropomorphic view attributes consciousness broadly to any behavior that involves sensory responsiveness. Yet human experience and observation of diverse species suggest that the most plausible case is that consciousness functions between these poles (1, 2). Subjectively, everyday conscious activity seems to occur without reflective thought, and a great deal of behavior (habits, conditioned response, reflex) seems to occur without consciousness. Objectively, single-celled animals respond to chemicals in their environment but display no other characteristics indicative of consciousness, whereas the behavior of mammals does seem to indicate consciousness despite the likely absence of self-reflection.

One source of opposing intuitions is the lack of agreement on how to define consciousness. Consensus is forming around the idea of *phenomenal consciousness* as the appropriate target for explanation (3–5). However, the definition of phenomenal consciousness is problematically vague: "what it's like" to have a sensation or thought, its feeling or qualitative character. Anthropocentric approaches emphasize the subjective awareness of conscious experiences while anthropomorphic approaches emphasize the quality of feeling. We propose that an evaluation of the pros and cons associated with a "high level" anthropocentric approach and a "low level" anthropomorphic account will help identify central features of consciousness.

The dual systems model is a "high level" approach. According to this model, *System 1* can be thought of as unconscious; processing is fast, automatic, associative, heuristic, parallel, contextual, and likely to be conserved across species. Consciousness is associated with *System 2* processing that is slow, effortful, rule-based, serial, abstract, and exclusively human (6, 7). An advantage of this model is the clear contrast between heuristic and deliberation-based responses, but rational deliberation is a very sophisticated cognitive ability that is difficult to demonstrate even in cognitively advanced species such as primates. We also review an example of a "low level" anthropomorphic model involving trace conditioning, which is a trained response to the first of two paired stimuli separated by an interval (8). This account highlights the role of consciousness in maintaining a stimulus representation over a temporal span, though it overlooks the importance of attention in subserving and also disrupting trace conditioning in humans (9).

Through a critical analysis of these two extremes, we develop the case for flexible behavioral response to the stimulus environment as the best model for demonstrating animal consciousness. Flexibility can be defined as the ability to adapt both goals and actions to situational demands. We discuss a methodology for gauging flexibility across a wide variety of species and offer a case study in spatial navigation to illustrate our proposal. Flexibility serves the evolutionary function of enabling the complex evaluation of changing conditions, where emotions establish the motivational basis for goal valuation, and attention selects task-relevant stimuli to aid decision-making processes (10–12).

We situate this evolutionary function within the *Temporal Representation Theory* of consciousness, which proposes a definition of consciousness as a representation of the present moment. "What it is like" to have conscious experience is to represent things (feelings, thoughts, events) as happening now. Critically, representation of the present moment is necessary for flexible action (13).

This article originated in a research group on emotion and consciousness led by Victoria Braithwaite, and she contributed significantly to the development of the approach we propose. Victoria was convinced that emotion contributed the differential valuation to environmental conditions that is essential to consciousness. This conclusion followed her ground-breaking work on fish pain, concisely laid out in her book (14) and subsequent work on emotion and consciousness (12, 15).

For Victoria, the question of whether fish can feel pain was a moral concern. Dismantling a laptop or a robotic vacuum cleaner is not an ethical problem, because these machines do not feel pain. In contrast, we have laws to protect dogs, cats, and livestock, because we have good reason to believe that mammals are conscious and therefore suffer when injured. If fish are also conscious, regulations should be developed to ensure suffering of these animals is limited as well (16).

MODEL 1: DELIBERATION BEYOND HEURISTICS

If "high level" approaches are correct, however, then fish are most definitely not conscious. On this sort of model, self-reflective deliberation or other sophisticated cognitive ability is necessary for consciousness. Several contemporary theories posit the sense of self as central, based on the way introspection reveals human experience [for a review see (17)]. When we attend to conscious thoughts and sensations, we always find a self as the subject of those experiences. Accordingly, philosophers and psychologists have long discussed two modes for reasoning. One is fast, automatic, associative, and implicit, while the other is slower, effortful, deliberate, and explicit [(6, 7); see (18) for review]. These modes have come to be known as System 1 and System 2 respectively (19) and have recently risen to prominence in their application toward decision-making, as many in the field of behavioral economics have embraced dual system constructs [e.g., (20)].

Given that decision-making is a fundamental survival process for all organisms that are candidates for consciousness, it is natural to wonder whether the dual systems model might lend insight into adjudicating conscious from non-conscious species. Correspondingly, System 1 has sometimes been assumed to share a lengthy evolutionary history with other species, while System 2 is typically cast as uniquely human [e.g., (18)]. However, there are real ambiguities between how System 1 and System 2 map on to the distinction between non-conscious and conscious processes (21).

In his book, *Thinking Fast and Slow*, Kahneman (20) describes a dichotomy between the Experiencing Self (System 2), and the Remembering Self (System 1). The contrast is based on a series of studies in which subjects endured unpleasant events or procedures and subsequently were asked to rate their experience or their willingness to reengage in a similar experience (22). The results were surprising; subject ratings of unpleasantness were not correlated with the overall amount of discomfort endured. Rather, evaluation of the experience was primarily influenced by the peak intensity of pain as well as the amount of pain at the end of the procedure (known as the peak-end effect), while neglecting the overall amount of time the event or procedure lasted (duration neglect). For example, using a procedure known as the cold pressor task, Kahneman et al. (22) immersed subjects' hands in very cold water. In the first condition, the temperature remained constant (14°C) for 60 s, while in the second condition, there was an identical 60 s immersion followed by an additional 30 s of immersion in which the water was one degree warmer (a perceptible difference). The majority of subjects reported a preference for repeating the second condition over the first, despite the fact that it entailed a prolonged period of discomfort. However, participants did prefer the shorter trial over the long one if the experimenters described the two conditions (22, 23). This suggests a dichotomy between the fast, automatic judgment (conforming to the peak-end heuristic) and the slow, deliberative judgment. As a model of consciousness, it is tempting to attribute fast, heuristic responses to unconscious processing, while slow, deliberative responses demand consciousness.

Applying this model to animals, the first question is whether the peak-end effect is evolutionarily ancient. This can be addressed using the comparative method and looking for homologous behavioral choices made by closely related species. While initial investigations suggested that peak-end effects were not shared with monkeys (24), later studies found that, like humans, rhesus monkeys do pay disproportionate attention to the peak and endpoint of an event (25, 26). For instance, rhesus monkeys preferentially choose sequences of rewards in which the highest values are located toward the end rather than middle of the sequence. These studies suggest that the peak-end rule likely shares a lengthy evolutionary history (although to the best of our knowledge, these studies have not extended beyond primates).

Given the strong possibility that peak-end effects are evolutionarily conserved, it is natural to wonder whether other species might exhibit a different set of preferences were they able to engage in deliberation, much like human subjects in the cold-pressor experiment described above. Here too, there is preliminary evidence that, like humans, non-humans express preferences that do not abide by peak-end effects when the problem is framed in a way that encourages deliberation. In a comparative study, Egan Brad et al. (26) found that humans and capuchin monkeys exhibited peak-end preferences when choosing reward sequences. However, in a follow-up experiment in which subjects were required to create their own reward sequences, neither humans (both adults and children were included) nor capuchin monkeys created sequences that accord with peak-end rules. Ultimately, there are likely too many differences between the choice task and the sequence-assembly task to infer that the latter involved rational deliberation [see (26, 27)].

According to the dual systems approach to consciousness, decisive evidence of rational deliberation would count as evidence of consciousness. The primate research falls just short of that bar in showing that capuchin monkeys are not subject to peak-end rules in a prospective reward task. These findings underscore the important difference between following a heuristic and making a deliberate choice. We would expect that animals capable of consciousness should not be "stuck" conforming to responses dictated by evolutionarily ancient heuristics [see (28)]. Rather, they should be able to overcome these rules in favor of choices that reflect anticipated preferences. In humans, deliberation of this kind always requires consciousness, so evidence for deliberate decision-making is reasonably taken as evidence for consciousness.

However, evidence of deliberative decision-making is a very high bar. Not only is it difficult to conduct appropriate experiments, as with the peak-end research, but there is a tendency to interpret all animal behavior as merely associative. Morgan's Canon advises that explanations of behavior involving simple mechanisms are preferable to explanations involving more complex cognitive abilities. While there is merit to this approach, it should not be used in every case (29). For example, evidence suggesting episodic-like memory in scrub-jays has been criticized as insufficient due to the possibility of accounting for the behavior in terms of associative learning (30, 31). Whether or not the ascription of episodic memory is appropriate, scrub-jays clearly demonstrate cognitive capacities beyond simple association (32).

The challenges of demonstrating homologs of System 2 processing in animals, coupled with the widely held view that mammals, birds, and possibly other animals are conscious (16, 33), provides a reason to look for a more tractable method. Later in the essay we will consider a case study for demonstrating flexible behavior that deviates from simple association yet bears none of the hallmarks of deliberation (e.g., slow, rule-based, abstract). Whereas it is unlikely that many non-human animals are capable of human-like System 2 processing, flexibility may be widespread in the animal kingdom.

MODEL 2: TRACE CONDITIONING BEYOND SIMPLE ASSOCIATION

In contrast to high-level approaches that adopt an anthropocentric self-reflective view of consciousness, low-level approaches take an anthropomorphic stance, where any form of sensory responsiveness is interpreted as conscious. For example, trace conditioning is a trained response to the first of two paired stimuli separated by a temporal interval. In their well-known study, Clark and Squire (8) presented a neutral conditioned stimulus (CS) such as a tone to participants, followed after a short interval by a motivationally significant unconditioned stimulus (UCS) such as an air puff. Because participants acquired a conditioned response to the CS only when they reported awareness of the tone-air puff contingency, Clark and Squire concluded that conscious knowledge of the CS-UCS relation is necessary for trace conditioning. A number of researchers have used this conclusion to argue that animals with neurologically simple systems such as honeybees and drosophila are conscious, because they too successfully learn by trace conditioning (34, 35).

While consciousness may be much more widespread in the animal kingdom than previously thought, it is counterintuitive to think that learning a very basic association is sufficient to demonstrate consciousness. Evidence that trace conditioning is successful even when the CS was masked and also when patients are in a vegetative state (36) further casts doubt on the link between consciousness and trace conditioning. Moreover, interpretations of trace conditioning experiments are various and subject to critique (37, 38), making it difficult to assess available evidence without a better sense of exactly what is involved in successful and unsuccessful trace conditioning.

Some insight into the source of the disagreements can be gained by examination of the important role played by attention in preventing *both* trace conditioning and awareness of the CS-UCS contingency. In the Clark and Squire (8) study, participants were instructed to watch a silent movie while the trace conditioning stimuli were presented, and there is good reason to think the distraction accounts for the failure of conditioning. What merits investigation is the way that such a basic association can be blocked by attentional inhibition.

Elsewhere (9) we hypothesize that only animals capable of task-directed attention will fail to trace condition under distraction when they are otherwise able to learn this sort of contingency. By filtering task-relevant stimuli, attention serves a critical role in maintaining focus. Consciousness has been linked with the selection and integration of stimuli in the performance of novel, context-dependent tasks, such as watching a movie (39, 40). Thus, both attention and consciousness are necessary to focus on a task, though the two processes are distinct (41, 42).

Trace conditioning without distraction forms a straightforward link between stimuli, albeit over a brief temporal interval. Simple coincidence detectors can account for the formation of these associations without the additional neural resources provided by consciousness or attention. Trace conditioning in insects likely involves strengthening synaptic connections either through prolonging the CS trace or anticipating US activation. Possible mechanisms include recurrent firing of CS-UCS pairing or neuromodulators to maintain the CS over the temporal interval (34). In contrast, trace conditioning in vertebrates requires more complex structures, such as the hippocampus and cerebellum (43).

We suggest that these more complex memory structures work in tandem with attentional selection. The ability to focus on one task over alternative possible tasks accounts for the difference in the Clark and Squire experiments between participants who successfully conditioned to the CS and those who failed to condition. Participants who successfully blocked the trace conditioning stimuli in order to focus on the movie failed to report those stimuli and failed to condition to the association. Participants who noticed the trace conditioning stimuli reported and conditioned to them. Attention to the stimuli was coupled with consciousness of the stimuli, as indicated by subjective report, and resulted in conditioning to the CS-UCS relation. The interesting result in the Clark and Squire experiments, on our analysis, is the *failure* to trace condition by participants who almost certainly would have successfully learned the association in the absence of distraction. Consequently, successful trace conditioning alone is insufficient as a test for consciousness [see (9) for further details]. Rather, we argue that having the flexibility to trace condition or not depending on attentional capacity might prove a better index, as we elaborate further in the final model under consideration.

MODEL 3: BEHAVIORAL FLEXIBILITY

The critique of anthropocentric high-level approaches on one extreme and anthropomorphic low-level approaches on the other extreme forms the basis of an emerging realization of the difficulties in establishing a methodology for investigating animal consciousness (1, 2). Theories of human consciousness cannot be extended to animals without running the risk of applying them too narrowly (high-level approaches) or too broadly (low-level approaches). Shevlin (2) calls this the specificity problem and argues that markers of consciousness (clusters of properties associated with conscious processes) can sort between likely and unlikely candidates for consciousness. Markers pick out candidate species for comparative analysis. According to Shevlin, behavioral and physiological similarities across candidate species help establish the correct level of specificity for application of a theory in order to minimize false-negatives and false-positives. For example, a neuroscientific theory based on human consciousness could use a set of markers to determine homologous neural structures in candidate species. Rather than say that fish do not feel pain, because they do not have a cortex (38), the presence in fish of a marker for consciousness such as multi-modal sensory integration (44) would support the suggestion that the telencephalon serves a similar integrative function as cortical structures (45, 46).

We are in favor of Shevlin's "dynamic equilibrium" between theory and a more open-ended cluster approach, with one crucial addition: the functional consequences of consciousness should be a guiding constraint. Evolutionary considerations favor the assumption that consciousness serves a selective advantage. Our suggestion is that flexibility best satisfies the functional, behavioral, and physiological considerations relevant for testing animal consciousness. Flexible behavior, that is, the ability to adapt both goals and actions to situational demands, is connected with the value of attentional selection and inhibition in accomplishing complex, novel tasks. In overriding peak-end effects, rational deliberation demonstrates the power of sustained attention to the individual elements of an experience rather than relying on a faulty memory of the events. Likewise, sustained attention to the movie disrupts trace conditioning. The challenge is to clarify exactly what flexibility involves, and how it charts a middle course between high-level reflective deliberation and low-level associative conditioning.

The first step in meeting this challenge is to identify the sort of cognitive and environmental factors involved in performing the proposed function (physiology is important as well but will not be included here for the sake of brevity). Droege and Braithwaite (12) offered four ways to demonstrate behavioral flexibility using fish as a model species. (1) Differential response to the environment involves the ability to alter behavior to a situation depending on a momentary means-ends assessment. As the most general description of flexibility, this category subsumes the three other forms of demonstration. We list them separately to highlight various ways flexible behavior appears in animals, not to suggest they are mutually exclusive. While all organisms capable of learning utilize past stimulus-response associations to determine action, flexible animals are capable of both goal-selection and action-selection. For example, the cleaner wrasse *Labroides dimidiatus* feeds on the parasites of client fish in a delicate relationship that requires evaluating multiple features of each interaction. Options include biting the flesh of the client fish instead of the parasites or providing a fin massage to soothe the client. Relevant factors in the assessment of options involve past interactions with the individual and the potential response of other clients that are observing the interaction (47–49). In other words, a cleaner wrasse demonstrates behavioral flexibility in its ability to tailor its goals and its actions to ongoing changes among multiple situational factors.

(2) Appropriate response in a novel situation requires the use of past information in a new way. This sort of behavior goes beyond simple conditioning, because it involves combining learned associations and applying them in a stimulus environment that has never been encountered. In a remarkable experiment, male cichlids observed conspecifics of similar size with variable fighting strength from A (strongest) to E (weakest). After watching A beat B, B beat C, C beat D, and D beat E, the observer fish was forced to choose whether to fight B or D. The cichlid appropriately combined the information about fighting strength to choose the marginally weaker opponent, D (50). Because the fish had not seen B fight D, its response to this novel situation, guided by inferential reasoning, demonstrates flexibility.

(3) Manipulation of the environment to accomplish goals exemplifies the ability to refrain from directly acting on a goal in order to better achieve it through indirect action. Tool use is one way to bring about a result by focusing first on something else. Facing the problem of a food pellet too large to eat, a six bar wrasse *Thalassoma hardwicke* carried the food to a pre-selected rock in order to smash the pellet into smaller pieces (51). Though this behavior fails strict criteria for tool use that rule out using substrate as a tool (52), the six-bar wrasse shows that it can refrain from acting on its goal of eating the pellet and pursue an alternate strategy as an intermediate step. Even if the discovery of the pellet-smashing rock was a matter of chance or trial and error, the use of the rock a second time required the ability to remember it and recognize its value as a tool in the new situation.

(4) A final way to demonstrate flexibility may seem too high level: the explicit representation of absent objects. However, this ability does not require that animals understand either the concept of an absent object or of an explicit representation. In fact, something as straightforward as the ability to represent an unrealized goal fulfills this condition (53). What makes the representation explicit is that it is not simply the end of a chain of behaviors; the goal is represented independently. For example, the small goby fish *Bathygobius soporator* needs to represent the terrain of pools surrounding its home pool in order to safely escape predators during low tide (54, 55). Though the adjacent pools cannot be seen, the goby can use a spatial representation learned at high tide to jump from one pool to the next. This sort of navigation demonstrates a more complex representation of spatial relationships than the ability to use a landmark or a series of spatial cues to achieve a goal (56).

An important justification for a behavioral flexibility approach to animal consciousness is the ability to situate functional, physiological, and ecological indicators within an evolutionary context. Our proposal is consonant with an extensive evolutionary argument by Ginsburg and Jablonka (57) that conceives of consciousness as an evolutionary stage rather than a property or process, a form of life rather than an acquired trait [see also (58)]. Conscious animals have "temporally persistent, dynamic, integrated, and embodied neurophysiological states that ascribe values to complex stimuli emanating from the external world, from the body, and from bodily actions" [(57), p. 7]. Their reason for this description of consciousness mirrors our own: consciousness evolved so that animals could respond flexibly to changing environmental and internal conditions. Emotions are critical to this evolutionary process, because they are means by which stimuli are evaluated. Anger, fear, and other negative emotions signal avoidance of stimuli, whereas joy, excitement, and other positive emotions signal approach to stimuli. Though emotional evaluation arguably can occur unconsciously (59), the capacity for assessing complex stimuli is necessary for flexible response.

The evolutionary frame provided by Ginsburg and Jablonka locates consciousness in the middle level of evolutionary development, where each of three levels is structured by a goal that determines its features. At the most basic level, the goal of life is survival and reproduction. The goal of the next level, consciousness, is value-based, action-guided learning. At the most cognitively advanced level, the goal of rationality is normative standards for cultural cooperation. Transitions from one level to the next involve the acquisition of necessary features that "accumulate, combine, and then become sufficient" to constitute the new level [(57), Ch 1]. Their evolutionary approach usefully articulates the mechanisms and dynamics that drive transitions from one level to the next. Gray areas between transitions can be better understood in terms of which mechanisms are operating (or not) and how they interact (or not) [(57), p. 10–17].

On this view, a transition marker is a key feature that indicates achievement of each level of development. Evidence for a transition marker demonstrates that the required coevolved set of mechanisms is in place. In contrast to a criterion, absence of a transition marker does not mean the absence of the system that enables a particular level of development. One or another of the mechanisms may be malfunctioning even though the system is in place. In locked-in syndrome, for example, complete loss of muscle control makes flexible sensorimotor behavior impossible with the exception of eye movements and blinking. Nonetheless, people with locked-in syndrome do not lose their capacity for consciousness (60). The malfunction of one component of the system, even a very important component such as muscle control, does not necessarily eliminate consciousness. This systems approach to analyzing evolutionary development is more coherent and specific than the tendency to produce lists of characteristics associated with messy concepts like life and consciousness. Yet it is less rigid than a set of necessary conditions.

For example, Ginsburg and Jablonka propose unlimited associative learning (UAL) as the transition marker that indicates consciousness. UAL is the "ability to attach motivational value to a compound, multifeatured stimulus and a new action pattern and to use it as the basis for future learning" [(57), p. 3]. Consciousness results from a system that enables UAL, so wherever UAL appears, consciousness appears as well. However, the absence of UAL in evolved animals does not imply lack of consciousness, since the enabling system may be malfunctioning in one way or another.

Generalizing from Ginsburg and Jablonka, we propose that an enabling system for flexibility includes the following: neuron structures to support learning; the development of neural patterns to integrate multi-modal stimuli in a novel situation and respond with complex action sequences; an emotional valence system to differentially weight the value of stimuli, actions, and goals according to a common currency; and an attentional system to select task-relevant stimuli for further processing and inhibit irrelevant stimuli [for more support of these features, see (12, 57, 58)]. Indicators for these elements of the enabling system could be tested in a variety of behavioral and physiological ways, and this evidence would add support to the tests for flexibility described earlier.

One essential piece missing from the evolutionary story offered by Ginsburg and Jablonka is a convincing reason to identify UAL with consciousness. They list hallmarks of consciousness, such as global availability, selective attention, and stimulus integration, and show how UAL depends on the structures that underwrite these hallmarks. Still, there remains the question of why global availability, selective attention and so forth must be conscious. That is, the explanatory gap remains between functions the brain performs and the subjective experience that correlates with it. In the final section, we will construct a bridge across the explanatory gap: a description of consciousness in terms of its function. Before we get to the abstract connection between consciousness and function, the next section will discuss a concrete study to test flexibility.

A CASE STUDY FOR TESTING FLEXIBILITY

In this section, we are proposing tests of navigation as a fruitful test of animal flexibility. One domain where flexibility may be particularly impactful is spatial navigation. Individuals must appropriately shift behavior if something in their landscape changes. Historically, spatial navigation research has been prioritized in mammals (61, 62), though fish have been shown to have remarkable spatial knowledge as well. Salmon are able to return to their birthplace to spawn using olfactory cues (63), and when faced with a novel maze, goldfish (*Carassius auratus*) are able to utilize allocentric cues outside of the tank to find a food compartment (46). These types of navigation, where an individual applies previously acquired information, demonstrate variability but not flexibility in the way we have defined it. The goldfish viewed the environment when completing the initial maze, encoded the allocentric cues, and then utilized these cues when completing a different maze. The fish were not required to transform the information in any way, nor decipher between multiple correct options. Flexibility can be seen in navigation by changing a past "correct" behavior in favor of a better solution.

While not everyone agrees on what a heuristic exactly is [see (64, 65)], heuristics are often studied in the context of reducing the cognitive burden of decision-making to arrive at an adequate solution. However, when choices are devoid of consequences, humans still conform to a consistent solution to a problem. Christenfeld (66) explored human choices from identical options by presenting participants with a maze containing three path options that yielded equivalent solutions with respect to distance traveled. Despite the apparent equivalence between options, participants preferred to take the final turn rather than the two turns that were available earlier (see **Figure 1** for abstraction of maze). This heuristic has been replicated and termed action continuation (67). Rather than changing routes between multiple iterations of the maze, participants tend to use the same strategy over multiple instances (67), perhaps as a means of reducing the cognitive burdens associated with generating a new plan by reusing a previously executed plan (68, 69). Humans undergo an automatic process of decision-making, despite no consequence of any decision (66, 67). However, heuristics can also lead to suboptimal strategies as well, for example, the peak-end effect discussed above (22).

Heuristics are widespread across different species of animals. Humans and capuchins (*Sapajus apella*) are suspectable to framing effects. Humans are more likely to take the same gamble when it is described as a likelihood of winning vs. a likelihood of losing (70). Similarly, capuchins have a preference receiving food framed as a gain (seeing one piece and receiving two 50% of the time) vs. a loss (seeing two pieces and receiving two 50% of the time) despite earning the same amount of food regardless of framing (71). Additionally, when presented with an undesirable third option, humans (72), honeybees (*Apis mellifera*) and gray jays (*Perisoreus canadensis*) violate the principle of irrelevant alternatives which states that a preference between two options should not change depending on the presence or absence of an additional option (73). Thus, there is evidence to suggest that some heuristics are evolutionarily conserved across multiple species and even taxa.

Our proposed study of flexibility utilizes the action continuation heuristic in a maze navigation task with fish (see **Figure 1**). This task would answer two questions: Do fish, like humans, use a heuristic to solve a navigation task in which all choices incur an equivalent cost? And if their choices conform to a heuristic, can fish change their behavior when presented

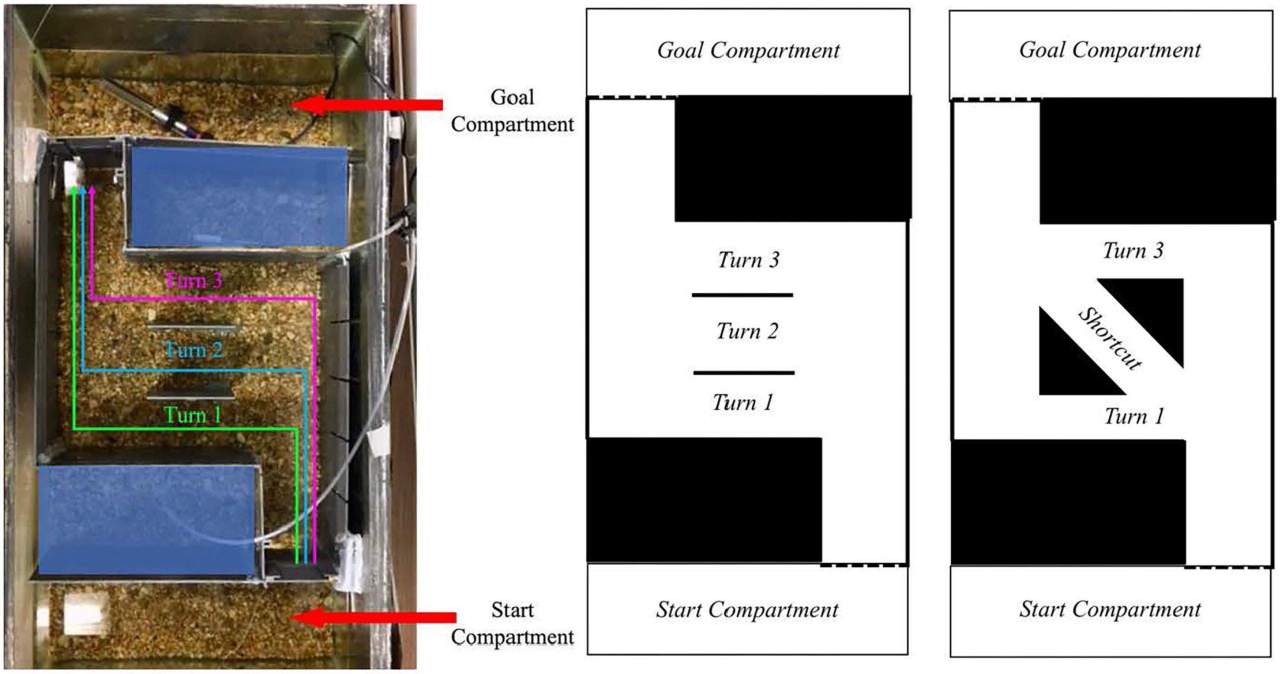

FIGURE 1 | (Left) Overhead view of the fish tank during a test trial. There were three possible turn options, all of equivalent length. During familiarization, the middle two walls, which create the turns, were not present. **(Right)** Schematic of the maze used and the proposed maze with a shortcut. Photo: Victoria Braithwaite; diagrams: Natalie Schwob.

with a more efficient shortcut? This type of flexibility would demonstrate an awareness that the context has changed with the availability of a more efficient option. To understand this process, we collected preliminary data on 12 zebra fish (*Danio rerio*) using a maze derived from experiments with humans (66, 67). The maze consisted of an entrance compartment that opened to a long straight arm, containing three possible turns into a shorter arm. From there, a longer arm led to the exit into a goal compartment where the fish received food (see **Figure 1**). After each fish ran the maze twice, we found that the fish preferred to take the first or last turn (50 and 41.66% respectively). Here, we were able to determine that the fish show a first turn and last turn bias, and mostly ignored the middle turn (8.33%). If a better, more efficient option were to become available, ignoring their original preference for this better solution would show flexibility. The original path would become suboptimal, though still a possible solution to the maze. To test whether fish would alter their behavior in this way, we planned to add a shortcut to the maze in place of the unpreferred middle turn. If the fish are not flexible in their decision making, they would continue to utilize their heuristic and take the same turn as the previous maze, not taking advantage of the shortcut option. If the fish were able to notice this change in the maze and take the shortcut, it would show they have a representation of where the end of the maze is in relation to their current location, and that they recognize the shortcut would be a faster route than the other two equivalent paths. However, if the fish fail to take the shortcut, this would not necessarily indicate that they lack flexibility.

Thus, we had a third condition planned to provide the fish with additional maze information. Rather than giving them experience in the tank with a shortcut, the fish would be placed in a tank with an overhead view that would allow visual access to the maze prior to entering it. We were interested in seeing if the fish would map their route prior to entering the maze for the first time by updating their representation of the maze where all options are no longer equivalent. Chimpanzees (*Pan troglodytes*), who most would agree are conscious and flexible, successfully transform spatial information. After viewing a hidden location of a juice bottle on a scaled-down model of their yard, chimpanzees directly went to the corresponding location outside (74). Rather than translating information from a model to the actual location, the fish in our study would simply need to update their representation of the maze. If the fish were to behave flexibly, they would prioritize taking the shortcut over taking the previously successful path. As noted above, goby fish demonstrate this sort of flexible behavior as part of their repertoire. If other fish species can also learn to map a maze by swimming above it, this is further evidence of flexibility over variability in behavior[1].

While we think a navigation task can add valuable information regarding flexibility and consciousness, we want to stress that not a single test is adequate for all species. Instead, we are suggesting

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[1] Sadly, we were not able to complete the shortcut experiment nor the overhead view experiment. Victoria conducted the initial navigation task but was unable to set up and run the other conditions. We encourage other investigators to take up this research and would happily share our plans for these experiments.

flexibility should be built into the search of indicators of potential consciousness in animals. For example, the ecology of the species needs to be considered. Research from Braithwaite and Girvan (75) found that three-spined sticklebacks (*Gasterosteus aculeatus*) can learn to use flow direction as a navigational tool to locate a hidden food patch. When the flow direction was reversed, fish reared in a river environment where water is often flowing were able to adapt to the reversed contingency faster than fish reared in a static, pond environment. Using water flow as a spatial cue could be appropriate in river fish, though it may not be for a species who does not live in a similar environment. To demonstrate flexibility and consciousness, multiple species-appropriate tests would be required: a mass of evidence is essential.

FLEXIBILITY AND CONSCIOUSNESS

This final section has the difficult task of explaining why flexibility indicates consciousness. The ability to evaluate complex situations and shift goals and actions accordingly is certainly adaptive. But why think it is conscious? As noted above, this is the explanatory gap. We need to explain why subjective experience is necessary for flexibility. While a full argument is beyond the scope of this article, the essential move is a description of consciousness in terms of function. On the Temporal Representation Theory, consciousness represents the present moment in order to facilitate flexible action (13). The function of consciousness is rooted in the adaptive benefit of flexibility.

In order to assess a situation and adapt goals and actions appropriately, an animal needs to represent how things are now. Task-relevant stimuli are selected and integrated into representations of the animal's external and internal environment to provide an ongoing update of current conditions. Because stimulus processing and coordination take time, conscious representation generally lags somewhat from the timing of the original signal. In time-sensitive contexts such as motion-detection, however, predictive processes anticipate shifts in order to better represent the stimulus location (11, 76–78). Consciousness is the integration of top-down and bottom-up stimulus selection into the best representation of the world at the present moment.

There are two main reasons that a representation of the world at the present moment is necessary for flexible action. The conceptual reason is that a division between past and future is needed to open the possibility of alternative actions. Simple associative response follows algorithmically from stimulus input. Past training determines behavior in a 1–1 input-output relation. In contrast, flexible behavior involves a more complex and dynamic mapping relation from input to output. Past training figures prominently, of course, in weighting various goals and their motivational valence. The critical difference is how the past is utilized: in simple conditioning, the past determines a single response, whereas in flexible response, past learning is one factor in assessing the situation. The distinction between past and future is probably not explicit for non-linguistic creatures (79); only an ability to represent now relative to not now is involved in consciousness, as described earlier in the representation of absent objects.

The second, pragmatic reason for regularly updating a representation of the world is that decision-making processes need information about what is happening now in order to ensure that progress is continuing on task or to initiate a change in course. Accumulating evidence suggests that decisions are made unconsciously, and action is initiated prior to the conscious feeling of decision (80–82). Nonetheless, conscious monitoring of the current situation coordinates information about obstacles and opportunities relative to ongoing goal pursuit. Consciousness grounds decision-making in the present by means of a unified representation of relevant information [see (83) for a similar view].

Of course, representations of the past and the future can also be conscious, so it may seem that consciousness cannot be adequately defined as a representation of the present moment. However, memory and imagination are forms of self-consciousness that arguably depend on the prior development of a representation of presence (84). Moreover, there is reason to think that these explicit representations of past and future are embedded in a representation of the present moment. That is, a conscious memory of last summer's vacation is a matter of representing this past event as in some way present (85). As in the case of dual systems, evidence for a sophisticated mental ability such as self-consciousness is sufficient but not necessary for the attribution of consciousness.

Throughout this article we have argued for a characterization of consciousness that falls between an anthropocentric approach like self-reflection and an anthropomorphic approach like basic association. In the previous two sections we highlighted the functional connections between flexible behavior and consciousness. It may be tempting to stop there and not attempt to situate flexibility within a particular theory of consciousness. Although great advances have been made in the science of consciousness (86), controversy and confusion continue to plague the field, particularly regarding animal consciousness.

At this point it is appropriate to credit Victoria for inspiring us with the courage to take this difficult and important additional step. Victoria's commitment to both science and animal welfare convinced her that the question of whether fish consciously feel pain should be answered, and she set about to collect the people and data to help her find the answer. The final answer will require further research to develop tests applicable to animals of widely varying groups. No single test is likely to be decisive. Instead, we should expect a gradual convergence of evidence—behavioral, physiological, and evolutionary—to develop in favor of or against ascription of consciousness in any particular case. We have suggested four general ways to test for flexibility. Physiological evidence for systems that enable flexibility—motivation and attention systems, for example—would also add strength to a case for ascribing consciousness.

Evolutionary considerations may provide the most compelling means of determining where consciousness is found in the animal kingdom. According to the Temporal Representation Theory, consciousness is a representation of the present moment, and the capacity for this form of representation evolved to facilitate flexible action. If this view is correct, demonstration of flexible behavioral responses by fish or other animals is evidence of consciousness.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

PD wrote most of the text. NS and DW each wrote one section. DW provided comments and edits. NS coined the term fishnition. The ideas were a collaboration of all authors.

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Although it is unusual to recognize the work of editors in the production of a journal article, this case is an exception. The wide range of Victoria's work can only be grasped by a volume with a wide range of contributors. We are honored to represent a part of that range and grateful to the editors for motivating us to complete the project Victoria initiated.

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The Complex Influences on How We Care for Farmed Fish

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As a veterinarian and academic in aquaculture, in my personal experience, most farmers are concerned for their animals and want to take good care of them. There has been substantial improvement in the welfare of farmed fish in recent decades, but improvements have been inconsistent across culture systems and species. Where there has been a lack of progress, it is not simply due to the more obvious barriers, for example, lack of clear messages, lack of effective dissemination, or cost of implementation. Why have the good intentions of farmers and research by academics failed to improve the care of many farmed fish? The reasons would appear to be complex; however, human behavioral theory (this term is used to differentiate from animal ethology) offers both a conceptual framework and practical guidelines for improving the care of fish by influencing the behavior of farmers. Here, I present some background context and apply human behavioral theory to examples of on-farm care of fish.

Keywords: fish, welfare, salmon, behavioral theory, aquaculture, behavioral science

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PERSONAL PERSPECTIVE ON FISH WELFARE AND HOW TO PROTECT IT

I have been fortunate to work as a veterinarian, researcher, and teacher in aquaculture in many parts of the world for over three and a half decades. In all that time, meeting and talking with many fish (and shrimp) farmers, I have never encountered one who intentionally mistreated their animals. Most expressed considerable concern for their animals [e.g., (1)]. Here, I refer to farmers as the people actively involved in the day-to-day care of the animals. While many owners or investors also share this concern for the animals, in some cases, these individuals or organizations have a more abstract relationship with the animals and have priorities driven by business concerns.

While there has been some significant progress in the practical care of farmed fish over the last three decades, progress has been inconsistent across culture systems and species. I have observed many cases of extremely poor fish welfare, especially around the time of harvest and slaughter. In my experience, such treatment of fish did not appear to be due to malice; in some cases, it was due to lack of understanding or resources, but in many cases, it was failure to implement existing viable strategies. Even in the best farming systems, there is still the potential for improvements in fish welfare and the question remains how should we achieve such gains?

Personally, working in both the applied and academic contexts, the relationship between improved academic understanding of welfare and practical care has been far from clear to me. Where translation from academic to applied context has been ineffective, it was not always due to the more obvious barriers, for example, lack of clear messages, lack of effective dissemination, or cost of implementation. While complex and unresolved scientific or ethical arguments are difficult to translate into husbandry practices, not all aspects of animal welfare are complex or still widely debated. Even where scientific issues are clear and effectively disseminated, and there is a demonstrable benefit for the business, progress remains limited.

Therefore, there must be other barriers to the translation from academic endeavors to practical animal welfare. Reflecting on how theoretical understanding has influenced my own practical actions regarding fish welfare, I find that some information has remained academic theory while other examples have affected my behavior. For example, work by Victoria Braithwaite on simple manipulations in the environment and feeding of juvenile cod (*Gadus morhua*) (2) opened my eyes to the possibility that fish might be capable of more complex and rewarding lives than I had previously thought possible, but this did not significantly affect my actions. In contrast, discussions on pain in fish based on studies conceived by Victoria Braithwaite and Mike Gentles (3, 4) convinced me that fish should be given the benefit of the doubt with regard to pain. As a result, I have changed my behavior, with the prevention, or alleviation of (potential) pain in fish becoming a personal priority.

Seeking to understand why some evidence affects behavior and some does not (personally and at an industry or national level), I looked outside the field of animal health, welfare, and husbandry for a conceptual framework. The relationship between information and subsequent action by people is central to public policy and many other areas. Further reading on influencing behavior introduced me to what is referred to as "behavioural theory" in the literature. Here, I use the term "human behavioural theory" to differentiate it from animal ethology. There is a large body of literature and many examples of successful application of the theory to influence peoples' behavior.

Human behavioral theory is used to understand and influence human behavior by governments and others. It demonstrates that evidence is only one aspect of the suite of influences and contexts that affect our decisions. The concept that our decision making can be influenced was developed by Richard Thaler, a Nobel Prize-winning behavioral economist. The concept is based on influencing our behavior by utilizing our cognitive shortcuts and biases. Influencing people's behavior is a complex issue and has the potential to be used for unsavory purposes; however, given its success and positive benefits in many areas, it also has the potential to improve the way people care for fish and other animals.

Below, I present evidence for some of the statements above (e.g., farmers want to take care of their fish, barriers to applying better care, and example of a successful welfare initiative) and then apply human behavioral theory to examples of applied fish welfare. The aim is to explore the potential of this approach with a view to stimulating further work in this area and facilitate additional gains in the welfare of farmed fish.

BARRIERS TO THE IMPLEMENTATION OF IMPROVED CARE OF FARMED FISH

Do Farmers Want to Take Care of Their Fish?

Our caring relationship with animals appears to extend back tens of thousands of years, as discussed by Bradshaw (5) and providing good animal welfare is important for people who have an affinity with animals. Attitudes to care for animals, whether pets or livestock, is not ubiquitous across cultures or individuals, and even in individuals, attitudes may change over time. While farmers are often more concerned with health and productivity rather than less easily measured aspects of welfare, participation in welfare schemes can increase awareness and concern for ethical and moral aspects of welfare (6). In terrestrial animal farming, it has been demonstrated that believing the animals under one's care are intelligent and can benefit from positive experiences will lead to a more pleasant experience for the farmer and more positive behavior toward the animals (7). Conversely, working in an environment where the animals are treated as economic units or purely mechanical devices can lead to a deterioration in the relationship between the farmer and the animals (7). This emphasizes that information or even understanding are not the only determinants of our behavior toward animals.

Is the Lack of Academic Consensus a Barrier to Improved Practice on Farms?

Several key concepts that are important when conceptualizing and protecting fish welfare are still debated in the academic literature. There is a lack of agreement on definitions for, or the existence of sentience, consciousness, cognition, pain, positive, and negative emotions in fish. Therefore, the debate is likely to continue for some time (8). In the absence of consensus, there may be no clear path to follow, and disagreements may throw doubt on, or disguise related but more widely agreed aspects of welfare. However, the public's attitudes to animal welfare are not always rational or based on scientific evidence or theories (9) and there is broad agreement on some key welfare issues.

Humane slaughter is widely accepted as essentially for good animal welfare (10) and potentially achievable but is still not universally adopted. It is still limited to a very small proportion of the fish killed for human consumption every year. This is an issue that affects a very large number of animals; estimates are highly uncertain, but may be in the tens or hundreds of billions (11). The complex relationship between the evidence base and our behavior toward animals would suggest progress is not entirely dependent on academic consensus.

Is Lack of Effective Dissemination a Barrier to Improved Practice on Farms?

There are examples of effective information dissemination between academics and industry. These range from original research papers, through summaries of the information (12), and assurance schemes with detailed sets of standards for farming practices (13), to practical applied training (14), although such training is still limited to a small number of options. However, these are limited in terms of husbandry systems and geography. There is, for example, widespread adoption of higher welfare standards by the Atlantic farming industry in the northern hemisphere and evidence of growing awareness in some tilapia faming sectors but a lack of any significant progress in much of Asian aquaculture. It would therefore seem that lack of dissemination may be a barrier to progress in some contexts.

Is the Cost of Implementation a Barrier to Improved Practice on Farms?

Improving fish welfare can increase productivity and allow access to markets and therefore does not always have a net cost. Even when there is a net cost, there may be good reasons for bearing that cost, including compliance with legislation and worker satisfaction.

There is a common intuitive notion that animals that are well-cared for will be more productive and there is also quantifiable evidence for the relationship (15, 16). However, there are still few examples where bioeconomic models have been used to clearly identify the costs and benefits of improving farmed fish welfare (17). There are additional business benefits, including fulfilling the demands from the value chain. Demonstrating that the fish are being provided with adequate care has a value in terms of both protecting and developing markets (15, 18). As a result, businesses have seen advantages in participation in accreditation or certification schemes that allow them to market fish with a high standard of welfare, for example, RSPCA Assured [e.g., (13)] and the Code of Good practice for Scottish Finfish Aquaculture (19).

Legislative instruments are another incentive for protecting the fish welfare even if there is a net cost. Regulation has been introduced to protect the welfare of farmed animals, for example, in Europe [e.g., (20–22)] backed up to some extent by national legislation [e.g., UK (23, 24).

As discussed above, farmers may wish to take care of animals based on personal beliefs, and therefore, the benefits of better fish welfare may go beyond improved productivity or profits. For example, "*happy fish equals happy farmer, and a happy farmer equals job satisfaction/employee retention*" (J Wiper. Cooke Aquaculture. Personal communications).

What Has Worked?

Despite the uncertain links between information and impact, some initiatives would appear to have been successful at disseminating information and promoting better welfare practices. The UK RSPCA Assured program (formerly Freedom Foods) has been adopted by more than 70% of the Scottish salmon farming industry (25) and the standards have also been used as the basis for other international initiatives including Fishwell (12) and MERCK animal health's Aqua Care 365 program (14). Objectively measuring the impact of the RSPCA scheme is very challenging, given the complex interacting influences involved, but some research is currently examining this problem (26). The scheme is based on comprehensive welfare-based standards that were developed in consultation with a wide range of stakeholders. According to news reports, the level of adoption was, at least in part, driven by the demands of the retail sector. It also provides incentives; following regular inspections and investigations of any complaint, there is the capacity to impose sanctions or remove accreditation. It has a very large formative component where the RSPCA Assured staff work with farmers to help them understand fish welfare and improve the welfare of the fish on their farms. However, it is difficult to determine the value of these various aspects of the scheme without a conceptual framework to understand how they might affect people's behavior.

HUMAN BEHAVIORAL THEORY AND FARMED FISH WELFARE

Human behavior theory offers such a theoretical framework and practical guidelines to better understand the influences on how people care for farmed fish and help us to achieve more effective change in the future. A document on the relationship between public policy and changes in human behavior based in behavioral theory (27) provides a framework (checklist) to examine what affects decisions. The document presents the most robust (non-coercive) influences on our behavior as a mnemonic (MINDSPACE):

Messenger—we are heavily influenced by who communicates information.

Incentives—our responses to incentives are shaped by predictable mental shortcuts such as avoiding losses.

Norms—we are strongly influenced by what others do.

Defaults—we "go with the flow" of pre-set options.

Salience—our attention is drawn to what is novel and seems relevant to us.

Priming—our acts are often influenced by sub-conscious cues.

Affect—our emotional associations can powerfully shape our actions.

Commitments—we seek to be consistent with our public promises and reciprocate acts.

Ego—we act in ways that make us feel better about ourselves.

This is not an exhaustive list, reflective (conscious) and automatic (unconscious) thought processes are affected by different sub-sets of these influences (27).

Human behavioral theory has been used in a wide variety of contexts, for example, reducing the spread of HIV in sub-Saharan Africa. The UK's Department for International Development recognized and utilized the complex drivers of human behavior in a successful scheme to reverse the spread of HIV (28). Another example is the reduction of gang violence in Western Central Scotland. Traditional approaches, such as increased foot patrols and stricter enforcement of knife crime legislation, had a positive effect but it was of limited duration. The Violence Reduction Unit successfully adopted an approach based on a model from the USA using norms and messengers to influence behavior (29).

In the context of MINDSPACE, hypotheses regarding potential influences can be developed and tested to improve strategies for change. Below are some conjectures on why some farmers take better care of their fish or in some cases fail to do so.

Why Might Some Farmers Take Better Care of Their Fish?

Messenger—peers or other respected figures promote good fish welfare.

Incentives—good behavior is rewarded, and bad behavior is discouraged.

Norms—positive attitudes are emphasized, negative attitudes are played down, promoting a culture of care, for fish and people.

Defaults—training in good practices and making it harder to do the wrong thing through appropriate infrastructure.

Salience—welfare information is presented in a relevant and interesting way.

Priming—a supportive stimulating work environment.

Affect—innate affinity with the fish and positive emotional associations among staff.

Commitments—making explicit commitments to good fish welfare.

Ego—caring for the fish makes them feel good (see Affect).

Why Might Some Farmers Fail to Take Care of Their Fish?

Messenger—lack of peer support and either no promotion of good welfare or promotion by people who are not respected.

Incentives—no rewards or punishments for good or bad behavior.

Norms—a culture where no one appears to care for the fish or people.

Defaults—in the absence of good training and poor infrastructure, the go-to option will probably be the easiest option.

Salience—any fish welfare information presented in a dry or irrelevant format.

Priming—poor social and physical working environment.

Affect—employing people without an innate affinity with fish and either neutral or negative emotional associations.

Commitments—lack of any agreement on the need for good fish welfare and lack of appreciation of the consequences for others resulting from bad behavior.

Ego—lack of awareness of what is the "right thing" to do.

Specific Example

We can also look at specific initiatives in this context, for example, the UK RSPCA Assured scheme (formerly Freedom Foods).

Messenger—the individuals involved in management and implementation of the scheme have had an extremely good reputation and relationship with farmers.

Incentives—the level of adoption (>70% of the industry) was at least in part driven by the demands of the retail sector. The scheme conducts regular inspections and investigations of any complaint and has the capacity to impose sanctions or remove accreditation.

Norms—most of the industry is in the scheme and staff training and behavior are components of the assessment.

Defaults—good practices have become embedded in standard practices.

Salience—these comprehensive but welfare-based standards were developed in consultation with a wide range of stakeholders and are regularly reviewed to keep them relevant.

Priming and **Affect**—the scheme has a very large formative component where the RSPCA Assured staff work with farmers to help them understand fish welfare and improve the welfare of the fish on their farms.

Commitments—membership of the scheme involves explicit commitment to the standards.

Ego—the scheme helps workers to understand what the "right thing" means in terms of fish welfare.

CONCLUSION

Working both in the academic and applied arenas of fish welfare gave me a personal perspective on the barriers or challenges to effective communication and implementation. Considering the implications of academic studies through the lens of human behavioral theory has the potential to develop more realistic pathways to impact. In the future, bringing expertise in human behavioral theory together with those interested in applied animal welfare has the potential to improve understanding and develop more effective strategies for change. Whether change is industry wide or more localized, the application of human behavioral theory offers a more effective approach than simply provision of information, training, and incentives.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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Research Before Policy: Identifying Gaps in Salmonid Welfare Research That Require Further Study to Inform Evidence-Based Aquaculture Guidelines in Canada

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Aquaculture is a growing industry worldwide and Canadian finfish culture is dominated by marine salmonid farming. In part due to increasing public and stakeholder concerns around fish welfare protection, the first-ever Canadian Code of Practice for the Care and Handling of Farmed Salmonids was recently completed, following the National Farm Animal Care Council's (NFACC) rigorous Code development process. During this process, both the Scientific (responsible for reviewing existing literature and producing a peer-reviewed report that informs the Code) and Code Development (a diverse group of stakeholders including aquaculture producers, fish transporters, aquaculture veterinarians, animal welfare advocates, food retailers, government, and researchers) Committees identified research gaps in tandem, as they worked through the literature on salmonid physiology, health, husbandry, and welfare. When those lists are combined with the results of a public "top-of-mind" survey conducted by NFACC, they reveal several overlapping areas of scientific, stakeholder, and public concern where scientific evidence is currently lacking: (1) biodensity; (2) health monitoring and management, with a focus on sea lice infection prevention and management; (3) feed quality and management, particularly whether feed restriction or deprivation has consequences for welfare; (4) enclosure design, especially focused on environmental enrichment provision and lighting design; and (5) slaughter and euthanasia. For each of these five research areas, we provide a brief overview of current research on the topic and outline the specific research gaps present. The final section of this review identifies future research avenues that will help address these research gaps, including using existing paradigms developed by terrestrial animal welfare researchers, developing novel methods for assessing fish welfare, and the validation of new salmonid welfare indices. We conclude that there is no dearth of relevant research to be done in the realm of farmed salmonid welfare that can support crucial evidence-based fish welfare policy development.

Keywords: salmonid, aquaculture, fish welfare, policy, future directions, Canada

INTRODUCTION

The number of fish bred, raised, and slaughtered each year for food is on the rise as the human population continues to rapidly increase (1). Due to a decline in capture fisheries worldwide (2, 3), there has been a subsequent expansion of the aquaculture industry to match fish production with increasing consumer demand (4). This trend has led to public interest and concern around aquaculture practices worldwide (5–9) and particularly their impact on fish welfare, which is now a high priority concern for consumers (10, 11) and a policy agenda item (12, 13). However, compared with farmed terrestrial species, fish have not been a priority for welfare researchers for nearly as long (9, 14–16), and thus there exists an urgent need to further our understanding to protect and improve their welfare in aquaculture.

Though elsewhere much of the recent industry expansion has centered around freshwater species, in Canada, the aquaculture industry is dominated by marine salmonid farming, which is valued at ∼$1.1 billion per year (17). Accordingly, the first-ever Canadian Code of Practice for the Care and Handling of Farmed Salmonids was recently completed [available at (18)], following the National Farm Animal Care Council's (NFACC) rigorous Code development process [see (19) for details on the development process]. Briefly, the process began with an online survey (reply window: February 26th-March 18th, 2019) asking stakeholders (including those in the farmed finfish industry), key partners, and concerned citizens for their "top of mind" welfare concerns for farmed fish in Canada [see (20) for survey results]. Two committees were then formed: (1) the Scientific Committee, comprised of experts in fish physiology, behavior, health, and welfare, who were tasked with reviewing scientific evidence on priority welfare issues and writing a peer-reviewed report [see (21) for Scientific Committee report]; and (2) the Code Development Committee, who used the Scientific Committee's report to develop the Code's specific requirements and recommendations. Members of the Code Development Committee were a diverse group of stakeholders including aquaculture producers, fish transporters, aquaculture veterinarians, animal welfare advocates, food retailers, government officials, and researchers. Dr. Victoria Braithwaite served as the National Animal Welfare Representative on the Code Development Committee and was an integral contributor to preliminary drafts of the Farmed Salmonids Code of Practice.

OBJECTIVE AND IDENTIFICATION OF RESEARCH GAPS

During the NFACC Code development process, both the Scientific and Code Development Committees identified research gaps in tandem, as they worked through the literature on salmonid physiology, health, husbandry, and welfare. When those lists are combined with the results of the public "top-of-mind" survey, they reveal several overlapping areas of concern where scientific evidence is currently lacking (**Table 1**), to the point where making specific and measurable Code requirements and recommendations was difficult for the Code Development Committee. Thus, herein, our objective is to highlight five of these overlapping welfare-relevant research areas that contain significant knowledge gaps (**Table 1**): (1) biodensity; (2) health monitoring and management, with a focus on sea lice infection prevention and management; (3) feed quality and management, particularly whether feed restriction or deprivation has consequences for welfare; (4) enclosure design, especially focused on environmental enrichment provision and lighting design; and (5) slaughter and euthanasia.

For each of these five research areas, we provide a brief overview of current research on the topic and outline the specific gaps present in the current literature, with the final section of this paper identifying future research avenues that will help address these gaps, ideally in advance of future Code revisions. Specific research gaps we report on within each research area were identified by the Scientific and Code Development Committees during numerous meetings over the course of the 3-year Code development process, using both their own extensive reviews of the literature and their collective expertise spanning long research careers in fish physiology and aquaculture [see the Scientific Committee's membership, detailed in (21)] and as aquatic veterinarians and aquaculture professionals (see the Code Development Committee's membership in the Code available at: https://www.nfacc.ca/codes-of-practice/farmed-salmonids). Similar approaches that incorporate multiple perspectives from a variety of stakeholders have been encouraged in the field of animal welfare [e.g., (18, 23, 24)]. Importantly, we do not attempt to provide a comprehensive review of current salmonid welfare research nor a value judgment on what the most pressing future welfare research priorities are. Rather, we are reporting on and extending the work of a unique grouping of aquaculture experts, to highlight future research that is necessary for the continued development of evidence-based salmonid welfare policy in Canada, and thus likely elsewhere as well.

DEFINITION OF WELFARE AND HOW IT IS ASSESSED

There exist numerous definitions of "animal welfare" [c.f. (25–27)]. NFACC's current definition includes consideration of affective states, as well as health and biological functioning, and exhibition of both normal and important behaviors. This definition mirrors the "three circles of welfare" approach outlined by Fraser (26), which posits that welfare is comprised of three overlapping concepts (in no particular order): (1) health and biological functioning, (2) affective states, and (3) natural living. Similarly, the Five Freedoms concept, as employed by the OIE (28), includes reference to affective states with words like "comfortable," "suffering," "fear and distress," and "pain." Though these different concepts have each received criticism [c.f. e.g., (29–32)], a unifying characteristic among them is that the ability to experience pain, suffering, or any other objectionable, negative affective state (i.e., to be capable of sentience) is relevant to welfare. So, following Duncan (25), we take an affective states

TABLE 1 | Illustration of Research Gaps arising from the "top-of-mind survey" conducted by NFACC, the list of "outstanding issues not addressed in current literature" created by the Scientific Committee and circulated internally, and the list of "research needs" published online by the Code Development Committee (22).

"Top of mind" survey	Scientific Committee	Code Development Committee	Research gaps
Top five concerns raised: - **Stocking density[1]** - **Health monitoring and management[2]** - **Humane euthanasia and slaughter[5]** - Water quality - Humane handling Additional concerns raised: - **Feed quality[3]** - **Enclosure design and maintenance[4]** - Behavioral monitoring and management - Emergency preparedness - Transportation	Report chapters with the most "outstanding issues not addressed in current literature" identified by chapter authors: - **Biodensity[1]** (6 issues) - **Sea Lice: Infestation and Treatment[2]** (8 issues) - **Feed Deprivation[3]** (5 issues) - **Lighting[4]** (4 issues) - Stress Indicators (4 issues) - Water Quality Issues in Recirculating Aquaculture Systems (4 issues) - **Ice Slurry Slaughter[5]** (2 issues)	Preliminary "research needs" list identified by the entire Code committee: - **Rearing Units** (5 issues; including topics on biodensity, environmental enrichment, and lighting)**[1,4]** - **Feeding Management** (2 issues)**[3]** - **Sea Lice** (5 issues)**[2,4]** - **Other** (5 issues; including topics on euthanasia and stress)**[3,5]**	Top five overlapping research areas containing significant knowledge-gaps: 1. **Biodensity** 2. **Health monitoring and management (with focus on sea lice)** 3. **Feed quality and management** 4. **Enclosure design (with focus on environmental enrichment and lighting)** 5. **Slaughter and euthanasia**

Superscript numbers indicate which issues identified by each group were combined to become the research gaps discussed herein.

approach to welfare herein. There still exists some debate around whether fish are capable of sentience [cf. e.g., (33–35)]; however, similar to the Code Development Committee, in this paper we will be taking a precautionary approach that assumes fish are sentient and capable of suffering and experiencing other negative affective states.

The scientific assessment of animal welfare is dependent on validated and standardized measurable parameters known as "welfare indicators." Welfare indicators can be used to gain insight into an animal's welfare state and can either be direct, animal-based indicators (e.g., weight loss, fin damage, increased gasping at the surface) or indirect, environment-based indicators, centered on the resources and environment the animals are subjected to (e.g., water temperature, oxygen levels) (36–38). Most animal welfare assessment protocols use a combination of both animal and environmental indicators [e.g., (22, 39, 40)] and "operational" welfare indicators are those which are relevant, easy to use, reliable, comparable, suitable for aquaculture and appropriate for specific systems or routines (38). Although a number of validated operational welfare indicators have been developed for salmonids [e.g., (38, 41)], currently there is an ongoing debate and no consensus on the best set of indicators to use [e.g., Salmon Welfare Index Model (SWIM 1.0); the FISHWELL handbook] for assessment of salmonid welfare in aquaculture. The literature reviewed herein uses a variety of operational welfare indicators that we have reported where possible.

RESEARCH GAPS

Biodensity

Salmonids have a wide range of social behaviors, depending on life-stage [c.f. e.g., for Atlantic salmon: (42, 43)] and species [c.f. e.g., juvenile Arctic charr vs. Atlantic salmon: (42, 44)], so inappropriate biodensities can impact their welfare in captivity. "Biodensity" (often used interchangeably with "stocking density") is defined as the fish biomass per unit volume of water (usually in units of kg/m^3). Though biodensity can facilitate useful comparisons, it is important to recognize that fish are rarely distributed consistently throughout a tank or net pen (21) and can instead cluster together or break into smaller groups depending on the species and enclosure conditions. As well, stocking density is constantly changing over time and will increase as fish grow or may decrease following grading or other farming procedures. Considering that fish density can influence water quality depending on flow of water per unit time through the system and that living in water enables fish to move freely in three dimensions (45), the concept of minimum space for fish is thus more complex than for terrestrial animals.

In the context of welfare, biodensity has important implications for managing water quality in net pens, tanks, and recirculating aquaculture systems. But changing the spatial relationship between conspecifics (i.e., altering biodensity independent of water quality considerations) in and of itself can have important implications that change depending on the species and life-stage in question, which makes it challenging to provide blanket guidelines, much less legislation on maximum densities (46). For example, stress response activation increases with increasing biodensity for Atlantic salmon [e.g., highest at 70 kg/m^3; (47), 125 kg/m^3; (48)], but increases with decreasing biodensity for Arctic charr [highest at 30 kg/m^3; (49)]. Aggression follows a similar pattern, with young rainbow trout being most aggressive at high biodensities [e.g., 316 fingerlings/m^3: (50); > 1000 fingerlings/m^3: (51)] and young-of-the-year Arctic charr showing the most aggression at low biodensities [44 kg/m^3; (44)], with adult Atlantic salmon exhibiting increased aggression during feeding (43). Moccia et al. (21) review further examples of how biodensity can impact the health and social behavior of several different salmonid species.

Concerningly, most of the data on optimal biodensities come from experiments conducted in small rearing tanks with relatively small fish (typically parr), due to financial and spatial constraints on research. Thus, findings from experimental manipulations may not be scalable to large production systems, which may use different tank materials or have different tank wall surface to water volume ratios. For example, a typical rearing tank with 1 m diameter and 1 m depth has a ratio of tank wall surface area to water volume of 5:1, while a tank with 5 m diameter and 2 m depth has a ratio of 0.9:1 (21). This might be pertinent when assessing welfare indicators such as fin erosion, a condition in which fins are injured that is hypothesized to be due to abrasion against tank walls and/or conspecific aggression that persists likely due to secondary infection (52). Furthermore, net pens are flexible structures that can change shape in response to tidal and other hydraulic conditions and/or biofouling, which may affect how much living space is available at any given time (53). Finally, a number of biodensity studies are confounded with water quality, such that the results cannot be strictly attributed to the changing number of conspecifics but might be instead a response to deteriorating water quality with increasing density. It is logistically challenging to control water quality in these types of studies, but this can limit how well we can draw clear conclusions on the impact of biodensity independent of other factors.

Beyond the applicability limits of the current research, there exist several crucial gaps in our understanding of how biodensity might impact salmonid welfare. First, we were unable to find studies where salmonid behavioral preferences for different biodensities were tested. Determining what densities different species and life-stages might choose for themselves would be challenging but may provide additional information about which biodensities could optimize salmonid welfare. Second, comparative studies, where species-specific responses to identical experimental parameters are compared, would be of considerable value, especially when trying to extend existing results from one species to many. Third, furthering our understanding of natural salmonid social behavior and how social interactions change with life-stage is important for making biodensity adjustments throughout rearing. As mentioned previously, species-level differences in responses to biodensity can be pronounced, but the salmonid life cycle is also complex, with variation in responses even between life-stages. For example, we know that Atlantic salmon conspecific interactions change a great deal from the parr to adult stages [e.g., (54–58)]. But how much variation is there between life-stages for other salmonids? And what is the relationship between fish size and optimal biodensity? For details of the salmonid life cycle and the dynamic ecology of different life-stages, see Aas et al. (59). Fourth, biodensities are often higher during situations involving acute stress, such as handling and transport. We do not have a strong grasp of what species-specific biodensities could protect welfare in those situations while remaining logistically feasible, nor do we know what biodensities optimize recovery from those acute stressors. Finally, there is evidence that non-optimal biodensities may impact immune parameters and subsequent vulnerability to pathogens [(60–62): reviewed in (53)], but we need further research to elucidate how different biodensities might contribute to pathogen transmission within a given enclosure or system; something that is likely pathogen- and host species-specific as well as multi-factorial.

Health Monitoring and Management

Disease is a major cause of diminished health and increased mortality in salmon aquaculture (63, 64). Regular monitoring of fish appearance and behavior can help to facilitate early identification of health problems that affect welfare and may be associated with bacterial and viral pathogens, parasites, and/or pollutants [e.g., skin lesions, loss of equilibrium, decreased activity, change in feed intake; (7)]. However, even with regular health monitoring in place, sea lice infestations remain one of the most persistent and highly publicized challenges in salmonid aquaculture.

Sea lice are parasitic copepods (within the family Caligidae) of both wild and farmed marine fish, but the rearing densities and conditions present in salmon aquaculture can exacerbate infection intensities when compared with natural conditions (65, 66). Multiple species of sea lice have been found to infect farmed salmon and sea lice biology, infection, and development are highly dependent on water temperature and salinity [reviewed in (21)]. For example, *Lepeophtheirus salmonis salmonis*, *Caligus elongatus*, and *C. curtis* represents the greatest concern in the North Atlantic (67, 68), and *L. salmonis oncorhynchii*, *C. clemensii*, and *L. cuneifer* represent the greatest concern in the Northern Pacific (69, 70). Sea lice feed on the skin, mucus, and blood of their hosts and cause tissue damage (71, 72). In cases of severe infestation, sea lice may also cause significant lesions that lead to increased stress, reduced swimming performance, anemia, reduced growth, and they may even act as a vector for other diseases and pathogens [reviewed in (71); reviewed in (72, 73)]. It is thus essential for aquaculture managers to implement appropriate management and intervention strategies that maintain the welfare of farmed fish and attempt to reduce the impacts of severe sea lice infestations on wild salmonid populations in the area (74, 75).

The primary management approach in all major salmon-producing countries is to regularly monitor and report sea lice densities on salmon in sea pens, with mandatory delousing or other sanctions implemented before levels reach pre-determined limits (21). Sea lice thresholds at which intervention is required (i.e., numbers of sea lice of a particular sex or life stage per fish) are different between and even within countries (21). Currently, however, sea lice thresholds are set for conservation purposes rather than out of concern for captive fish welfare, due to the alleged role of sea lice in the decline of wild salmonid populations as a result of louse spillover infections [e.g., (66, 76–78)]. Although prior work has suggested that sea lice infestations become lethal around 0.12–0.15 lice per cm^2 of fish (37, 79), the impacts of sea lice are largely dependent on host species and size [e.g., salmon lice are rejected more rapidly by Coho, *Oncorhynchus kisutch*, and pink, *O. gorbuscha*, salmon than by Chinook, *O. tshawytscha*, and chum, *O. keta*, salmon; (69, 71, 72)]. Although, lethal limits are a late-stage indicator of welfare impacts; fish may be negatively affected long before their infection burden induces mortality [e.g., (80)]. There is currently very little research on the sub-lethal effects of sea lice infestations on fish welfare across different salmonid species and life-stages. Research that addresses this gap would help policy makers establish firmer, welfare-based sea lice thresholds for when intervention is required [e.g., (81)].

There also exist a couple of important gaps in our understanding of how to control sea lice infestations. First, lice-infected fish are typically treated by applying chemical treatments in tarpaulin-enclosed net pens (to contain the chemicals), as a bath in well-boats, or by including them in feed (82). Currently, in feed treatments are considered advantageous due to their passive implementation (82), in comparison to bathing treatments which may cause stress and mechanical harm to fish through withholding feed and transfer prior to, and crowding and oxygen deprivation during, bathing (45, 83, 84). However, incorrect dosages of chemical sea lice treatments have been shown to cause mortality post-treatment in salmonids, which typically increases with increasing water temperature [e.g., hydrogen peroxide; (84, 85)]. As well, because some treatments are not completely effective and sea lice are becoming resistant to them (86–89), fish are often treated repeatedly over a 2–3 week period. We do not have a complete understanding of how repeated exposure to chemical therapeutants may impact fish welfare. Second, treatment-resistance has prompted a rapid and recent shift to non-chemical approaches to control infections including the use of altered temperature, salinity, and lighting, physical removal, mechanical barriers, and cleaner fish (21). However, preliminary studies suggest that some of these methods may compromise host salmonid welfare. For example, recent work suggests that temperatures used during thermal delousing treatments (28–34°C) may be noxious to fish (90), can initiate panic reactions [exposure <5 min; (90)], and may cause thermal injury [exposure to 34–38°C for 72–140 s; (91)] and even death [exposure to 34–38°C for >2 min; (91)]. Physical de-lousing systems such as the "Hydrolicer" also require fish to be crowded prior to treatment (85), which may induce an acute stress response. In severe cases, some of these methods can lead to elevated post-treatment mortality in comparison to the use of chemical methods (85). Thus, extensive research is needed to determine the potential impacts of these non-chemical sea lice treatments on fish welfare.

Furthermore, the use of a number of cleaner fish species (e.g., lumpfish and wrasse) that eat sea lice directly off host salmonids are gaining popularity as a biological alternative for infestation control. In the context of salmonid welfare, one of the most important considerations is the role that cleaner fish may play in pathogen transfer to salmonids [e.g., *Tenacibaculum maritimum*; (92); and others reviewed in (93)]. The close mixing of cleaner fish with salmon in net pens creates favorable conditions for the emergence and transfer of diseases, especially considering cleaner fish broodstock are often wild-caught and may pose a biosecurity risk (94, 95). However, the welfare of the cleaner fish themselves is of considerable concern because the biology, ecology, and population dynamics of these species are poorly understood. For example, individuals of some species are territorial (96) and territorial behavior may expose cleaner fish to attacks from the larger captive salmon and thus, exposure to injury and unavoidable chronic stress [e.g., (97–99)]. Reports of poor cleaner fish survival in commercial salmon sea nets [e.g., (99, 100)], with some individual farms observing up to 100% mortality or loss [e.g., (101)], add to this concern. There are also important ethical questions to consider when using cleaner fish. For example, cleaner fish are commonly euthanised after each production cycle when salmonids are slaughtered for harvest (95, 102). This leads to demand for additional, replacement cleaner fish at the beginning of the next salmon production cycle [e.g., (94, 95)] and raises the question: do the ethical implications of this practice outweigh the efficiency of cleaner fish as a sea lice control method? So before introducing these species as a legitimate alternative for sea lice control, we need considerable research at both the basic (e.g., describing cleaner fish ecology, behavior, etc.), applied (e.g., investigating welfare of cleaner fish in sea pens, comparing efficacy of cleaner fish to thermal de-lousing), and philosophical (e.g., is this practice ethical?) levels.

Feed Quality and Management

The quality of the diet, including feed formulation, affect salmonid health and welfare. For example, feeds with insufficient phosphorus cause potentially painful skeletal deformities [reviewed in (103)]. Popular salmonid feeds usually use fish meal and oil as their primary protein sources, which are limited resources whose harvest can have considerable environmental impact (104). In an effort to improve the sustainability of feeding farmed salmonids, alternative protein sources such as insect meals, poultry by-products, plant-based meals (e.g., soybean, canola, etc.) are being investigated and used (105, 106). However, emerging research suggests that some of these products may have welfare-relevant health impacts. For example, feeding unfermented soybean meal to Atlantic and chinook salmon may cause an intestinal inflammatory response (enteritis) that renders fish more susceptible to diseases like furunculosis (107, 108) but supplementing soybean meal with bacterial meal containing *Methylococcus capsulatus* appears to mitigate enteritis in Atlantic salmon (109). This enteritis also appears to differ in severity between species; unfermented soybean meal does not induce enteritis in pink salmon, and is less severe in Atlantic than in chinook salmon (108). Further research is needed to address species- and life-stage-level differences in how these alternative feed formulations may impact salmonid health and welfare.

Similarly, feed restriction (i.e., feeding a reduced ration) or withdrawal (i.e., not providing any feed) can have welfare consequences that are not well-understood. Feed is withheld in a variety of situations during salmon farming; before acutely stressful procedures like grading, vaccination, etc., it is often considered prudent to empty the gut through short-term feed restriction to maintain water quality during holding, lower hypoxia risk through lowered metabolic rates, and reduce the risk of needle damage during peritoneal injections (6). Prior to slaughter, feed may also be withheld for human food safety and product quality reasons [e.g., (110, 111)]. Further, during rarer events like superchill (112, 113), harmful algae blooms (114), and high temperature events (115), feed withdrawal is often required as it prevents death due to freezing, exposure to algal toxins at the water's surface, or elevated activity in temperatures outside a species' optimal range [reviewed in (21)].

Under the assumption that fish have conscious affective states [as (34, 116), and others claim], the most obvious potential welfare consequence of feed restriction or withdrawal would be hunger, an aversive interoceptive state that can include aspects of pain and frustration and may involve considerable individual variation [e.g., (117)]. However, it is still unknown whether fish experience hunger, both because of doubts surrounding whether they are sentient [cf. e.g., (33, 118, 119)] and because most species (and all salmonids) are ectotherms. Warm-blooded farm animals have consistently high energy demands and therefore require regular meals to avoid hunger and maintain metabolism; however, the feed requirements of fish are dependent on temperature, the principal controlling factor of their metabolic rate (120). Recent research indicates that, when held at optimal temperatures, Atlantic salmon post-smolts can tolerate up to 4 weeks without food with negligible impacts on welfare (121). Some species of fish (including salmonids) also exhibit a natural decrease in appetite to the point of fasting during certain periods of their life cycle [e.g., (122)], so it is possible that hunger is either not as strong a motivator for these fish as it is for mammals, or fish have a physiological mechanism that decreases the aversiveness of hunger during these periods. Work done on transgenic salmon has contributed to our understanding of fish appetite [e.g., (123–125)], but much remains unknown about the endocrinological and neurological mechanisms controlling it and the affective component remains elusive.

Furthermore, welfare consequences may vary depending on the severity and duration of feed restriction or withdrawal, with very long-term situations potentially forcing fish into a stage of starvation requiring protein catabolism to mobilize stored nutrients, wherein vital organ function can be compromised (126). But even less severe feed restriction can have behavioral consequences; the sudden onset of restriction can increase aggression rates and subsequent fin damage severity (127), with these behavioral changes potentially becoming permanent, possibly depending on the life-stage at which feed is restricted [e.g., (128)]. There have been multiple calls for further research on the effect of feed withdrawal of varying lengths on stress physiology, behavior, and welfare (6, 110, 129). Currently, feeding regimes are often based on water temperature and calculations made using known relationships between body size and metabolic rate [for fish: on a log-log scale, body mass and standard metabolic rate are linearly related, with a slope of 0.8: (130), explained in (21)], with the aim of maintaining or increasing body mass. However, this method does not incorporate the numerous other factors that may play a role in how severe the welfare consequences of varying periods of feed restriction or withdrawal are such as water quality, species, life-stage, biodensity, and disease status, among likely many others. In contrast, over-feeding (as a possible result of strong dominance hierarchies, incomplete training of personnel, etc.), though less studied, may have welfare consequences such as fouling of the holding tank or net-pen and/or obesity resulting in possible immunological disorders (131).

Enclosure Design

A variety of rearing unit types and conditions are used in the farmed salmonid industry, ranging from ponds, sea and lake net pens, and land-based flow-through and recirculating systems. Despite this diversity, aquaculture rearing conditions typically lack complexity, most often being plain, impoverished enclosures containing only water. Deliberately adding resources to the environment with the aim of improving fish welfare by meeting their needs and preferences is often termed "environmental enrichment" (132, 133). Environmental enrichment can take many forms, from physical objects added to the rearing unit that increase structural complexity to sensory, social, nutritional, or even occupational enrichment (133). Providing fish with environmental enrichment that increases the complexity of their rearing units while mimicking their natural environments may be an effective way to offer choice (134) and decrease stress responses. Although enrichment strategies are highly dependent on the natural history of the fish species and their preferences, there are some principles that have been found to hold true for several salmonid species used in research and aquaculture. For example, the use of dark tank backgrounds, tank floor substrate, and shelters, has the potential to reduce aggression and consequent fin damage [Rainbow trout: (135–137); Arctic charr: (138); Coho salmon: (139)] and increase survival [Atlantic salmon: (140, 141)].

For a comprehensive overview of environmental enrichment research for cultured salmonid fishes, see Näslund and Johnsson (133); however to date, environmental enrichment research has been conducted mainly under laboratory conditions in small rearing tanks at relatively low biodensities. While several types of environmental enrichment have been adapted to aquaculture out of necessity (mainly in terms of reproduction success), almost nothing is known about the effects of environmental enrichment on fish welfare at the scale of intensive aquaculture. Furthermore, we do not know what, if any, forms of environmental enrichment are preferred by salmonids at different life-stages, nor what types of enrichment might be important for positive salmonid welfare. There are also some concerns about the application of environmental enrichment that require empirical study: some suggest that enrichment may exacerbate accumulation of food particles and feces [e.g., (142)] or act as a vector for pathogens [e.g., (143)] such that the drawbacks may outweigh the benefits. Accordingly, aquaculture managers are often concerned about effective and safe application of environmental enrichment, especially in a large-scale production context. Much more research is needed to investigate what types of environmental enrichment might be effective and feasible to deploy on-farm.

Lighting is another important aspect of housing design in the farmed salmonid industry. Light has three components: color, intensity, and duration (daylength or photoperiod); all of which can potentially influence animal welfare and can be manipulated by increasing or decreasing the number of lights on the farm, or by changing their strength or type (21). Currently, the manipulation of both photoperiod and light intensity represents key management tools used in salmonid aquaculture. For example, various artificial lighting regimes (e.g., extended or reduced day length or continuous, 24-h lighting) are used to induce smoltification, advance or delay the timing of spawning, manipulate sexual maturation, promote fish growth, and prevent suffocation in the early swim-up stages of the salmonid life cycle (21). Concerningly, there are a number of welfare-relevant health and production issues associated with continuous lighting, including disrupted neurological development, reduced bone strength, poor smolt quality, failed smolting, and failed spawning (144–147). Similarly, sudden changes in light intensity or regime cause fear responses, increased oxygen consumption, injuries, or even suffocation in fish (148–150). So although artificial lighting is readily used and manipulated across the salmonid aquaculture industry, research is needed to investigate these welfare concerns. Furthermore, considering that light intensity influences the spatial distribution of fish within a tank, light intensity may be too low at depth in larger, deeper tanks, which could potentially inhibit feeding, growth, and smoltification (151). We need more information about how light distribution differs with depth in a variety of salmonid housing enclosures and how this impacts fish welfare.

Slaughter and Euthanasia

Generally, when farmed salmonids reach a certain size, they are slaughtered for human consumption, but it is sometimes also necessary to euthanize fish to prevent them from experiencing excessive pain or suffering (e.g., ill, injured, or diseased fish that do not have a reasonable prospect of improvement or do not respond to treatment). A "humane death" is one that is quick, causes minimal stress and pain, and results in a rapid loss of consciousness followed by death without the ability to regain consciousness (152–154). Under the assumption that fish have conscious affective states, humane approaches to the slaughter and euthanasia of farmed salmonids are expected by both society and the aquaculture industry. Importantly, humane slaughter and euthanasia of fish can only be fully achieved by minimizing stress and injury during, as well as, before the killing procedure itself. Considering procedures such as crowding, loading, and transporting fish from their pens to the place where they will be slaughtered or euthanised (e.g., by use of braille nets, pipes, and/or well boats) has the potential to induce stress and injury in fish [e.g., (155–158)], they must be minimized as much as possible in terms of intensity and duration [e.g., (22, 39, 40, 153)].

Aquaculture slaughter and euthanasia techniques are diverse, and fish species vary in their response to different methods [e.g., sensitivity to oxygen deprivation; (159)]. Unfortunately, some of the current methods are unacceptable under the definition of a "humane death" and have instead been developed with a focus on product quality and ensuring personnel safety (45). For example, immersion in CO_2 saturated water is sometimes used to kill farmed salmonids; however, it is losing popularity because it has been shown to cause narcosis and loss of brain function [e.g., (160)] over several minutes, during which time the fish exhibit pronounced distress and escape behaviors (161, 162). Thus, considering the negative welfare consequences of these methods, they are being phased out and are only permitted for emergency situations [e.g., CO_2 may still be used for emergency depopulation events; (22)].

Of the methods presently available, when applied correctly, percussive and electrical stunning appear to be among the more humane methods for salmonid slaughter (163, 164), with electric stunning becoming the preferred method in Canada (21). Considering fish can only be stunned by the use of electricity [i.e., not killed; (162, 165)], electrical stunning must be followed by a kill method that prevents recovery of consciousness in order for it to meet requirements for humane slaughter [e.g., (22, 39, 153, 154)]. However, selection of the most appropriate (i.e., humane) method of slaughter in any situation will depend on the fish species, size, life-stage, number of individuals involved, available means of restraint, and personnel skill level [e.g., (22, 39, 153, 154)]. To date, electrical and percussive stunning methods have been tested on a limited number of fish species at harvestable size, mainly in laboratory conditions [e.g., Atlantic salmon, Common carp, Rainbow trout, Gilthead sea bream, European sea bass; reviewed in (166)], leaving gaps in our understanding of the potential of welfare impacts of these methods in additional fish species, at different life-stages, and in commercial settings. This is concerning because, for example, when the electrical current or voltage is too low, or the application duration too short, electrical stunning can be ineffective at stunning fish and thus, has the potential to cause pain [(154, 166); for a review in fish pain see (167)]. As well, additional considerations need to be taken into account for in-water vs. dry/semi-dry electrical stunning procedures such as the conductivity of the water [e.g., stunning a fish in sea water requires more power than fresh water; (154, 168)] and the orientation of the fish [e.g., incorrect orientation of the fish increases the risk of ineffective stunning; (154, 166)], respectively.

Despite existing research on humane salmonid slaughter and euthanasia, a number of research gaps remain that are hindering our understanding of how these different methods might impact salmonid welfare. First, comparisons between fish and mammalian brains are difficult [due to eversion during embryonic development; see (169)]. Thus, what we know about relationships between mammalian brain regions and their functions cannot be directly applied to fish. It is therefore imperative that we continue to research teleost brain region function in commercially relevant lineages. Second, electroencephalography (EEG) has been used to assess brain electrical activity in fish in a number of laboratory experiments and has been shown to be one of the most reliable methods of assessing consciousness [e.g., (162, 165, 170–173)]. However, in a commercial fish farm setting, registration of EEGs is impossible to perform, instead forcing farmers to rely exclusively on behavioral indicators to evaluate the degree of consciousness in fish [e.g., coordinated swimming and escape behaviors, ability to maintain equilibrium, "eye roll" reflex, and ventilatory reflexes; (170)]. The use of behavioral indicators alone are problematic: for example, some commercially used slaughter methods may only induce sedation and/or paralysis in fish without loss of consciousness [e.g., ineffective electrical stunning, ice slurry slaughter; (163, 166, 170, 174, 175)]. Thus, in order to fully validate the use of behavioral indicators of unconsciousness in the absence of EEGs on farms, more research is needed to investigate additional commercially-relevant fish species and a variety of types of slaughter. Third, we do not fully understand what the actual cause of death is during some of the currently used slaughter and euthanasia techniques. For example, the cause of death during ice slurry slaughter, a method of trout (*O. mykiss*) slaughter used in Canada, is unknown but likely to be asphyxiation from either a lack of gill irrigation or hypoxia [(176); reviewed in (21)]. The chilled water reduces the fishes' activity level but may not render the fish insensible to pain and may thus cause a prolonged period of distress before death (163, 176). Understanding the cause of death can thus be important for assessing welfare impacts, since it can play a role in how long a slaughter method takes to cause death and how potentially painful it may be, especially if another method that causes insensibility (e.g., anesthesia) is not used immediately prior. Thus, methodological studies of how to measure fish brain activity and investigation into the improvement or possible further development of humane slaughter and euthanasia methods would be of use for both fundamental and applied work. Finally and importantly, though electrical and percussive stunning methods are the recommended methods of slaughter at present, this does not preclude the discovery of more humane methods in the future.

FUTURE DIRECTIONS

Herein, several gaps in the field of salmonid welfare have been identified, with pertinent questions to guide future research summarized in **Table 2**. However, further, more in-depth work is required to review the full extent of relevant salmonid welfare research and a complete suite of research gaps, beyond those most relevant to policy development in Canada that we have presented in this review. We strongly suggest that researchers consider performing a scoping review (177) of the literature to provide a complete picture of the state of research and identify a full suite of research deficits. Some valuable reports like this already exist, such as the gap analysis study conducted by the Standing Committee on Agricultural Research [SCAR: (178)], and narrative reviews on various relevant topics by Ashley (6), Overton et al. (85), Macaulay et al. (179), Hvas et al. (180), among others. However, since the aquaculture industry includes a variety of expert stakeholders hailing from different backgrounds, we also recommend borrowing methods from the social sciences [e.g., a systematic review of text and opinion (181), survey-based research (182): Chapter 9], and/or participatory methods [discussed in (24)] to help reveal important anecdotal or experiential understanding from working aquaculture professionals that could inform novel research questions or policy developments [as suggested in relation to the issue of surplus dairy calves, by (24)].

With regards to the research questions summarized in **Table 2**, there are many promising methods that may assist in addressing them, particularly non-lethal physiological indicators of salmonid health such as the quantification of water-borne cortisol [e.g., (183)], fin erosion scoring schemes [e.g., (184)], bioelectrical impedance analysis (185), and hematological indicators of health [reviewed in (186)] and stress [reviewed in (187)]. However, many of these research questions remain unanswered, possibly for several reasons: some of the aforementioned indicators and methods have yet to be fully validated [e.g., (188, 189)], some research questions are yet unanswerable because we lack the necessary tools, and/or we, as fish biologists, have not yet pursued interdisciplinary research to its fullest extent.

The field of animal welfare has been largely focused on terrestrial species but offers many experimental paradigms that can be used to investigate the welfare of aquatic species as well. For example, preference tests commonly used by poultry and cattle welfare researchers [e.g., (190)] have been used to investigate what types of environmental enrichment are most preferred by laboratory zebrafish [e.g., (191)]. These simple preference tests can be extended into investigations of motivation, in which a cost is titrated against access to a resource to determine how valuable it is to an animal [e.g., (192)]. For example, using motivation tests, welfare researchers discovered that farmed mink will pay a high "price" for access to pools for swimming and experience a stress response indistinguishable from that elicited by food deprivation when they are prevented from accessing their favorite resource (193). Further, validated tests of judgment bias, a concept borrowed from human psychology in which one's underlying mood state affects whether neutral stimuli are perceived as potentially rewarding (optimistic) or threatening [pessimistic; e.g., (194)], are gaining popularity for assessing non-human animal mood states [e.g., (195)]. For example, a judgment bias task was recently validated for laboratory mice, wherein mice housed with preferred and welfare-improving environmental enrichment and tumor-bearing nude mice showed optimistic and pessimistic responses, respectively (196). Judgment bias tasks have been attempted for zebrafish [e.g., (197)], but a validated method for salmonids has yet to emerge.

Going forward, a focus on methods development (both building on existing tools and experimental paradigms and creating new ones) would help facilitate the necessary research on salmonid welfare. Of particular interest might be the development of validated judgment bias tasks, ways to assess fish motivation for resources, and other behavioral measures of fish distress, fearfulness, etc. for use on-farm, as well as other non-invasive techniques for investigating fish physiological responses. Considerable work describing salmonid natural ecology exists [e.g., (59, 198, 199), among many others]; however, deepening our understanding of their natural behavior across life-stages (especially during enigmatic at-sea life-stages), would help us further develop and validate behavioral indices of welfare. We may also need to explore how other sensory modalities are affected in production, both as potential welfare implications but also to discover new indices—for example, what sounds can salmon in net pens and land-based enclosures detect and/or produce, and are they relevant to welfare state? How do different enclosure designs affect how salmon use their lateral line, and are there properties of the lateral line that are affected by overall

TABLE 2 | Examples of outstanding research questions that exist in each Research Gap identified herein, as informed by the Scientific Committee, Code Development Committee, and the authors' perspective as fish welfare researchers.

Biodensity	▪ What biodensities are preferred by different salmonid species and life-stages? ▪ How does social behavior change throughout the entire salmonid life cycle? Are these patterns species-specific? ▪ What is the relationship between fish body size and optimal biodensity? ▪ Do patterns and relationships identified in the current biodensity literature scale up to large production systems? ▪ How does biodensity affect salmonid recovery from acute stress? ▪ For different welfare-relevant pathogens, how does biodensity contribute to pathogen transmission?
Health monitoring and management	▪ What are the sub-lethal effects of sea lice infestations on salmonid welfare, and at what threshold number of lice per fish do they occur at welfare-compromising levels? ▪ What are the welfare impacts of repeated exposure to chemical therapeutants for managing sea lice infections? ▪ How do alternative sea lice treatment methods (e.g., thermal and physical de-lousing, etc.) impact salmonid welfare? ▪ How does the introduction of cleaner fish species to a given enclosure impact the welfare of captive salmonids? ▪ What are the potential areas of concern for cleaner fish welfare?
Feed quality and management	▪ Do fish experience hunger as an aversive affective state? ▪ If hunger is aversive to fish, how motivating is it? ▪ How might the aversiveness of hunger interact with different social dynamics (e.g., dominance hierarchies) to impact welfare? ▪ What protein alternative is best for the welfare of different salmonid species? ▪ What period of feed restriction or withdrawal is appropriate (i.e., does not compromise welfare), and how does it change with different environmental conditions?
Enclosure design	▪ What types of if environmental enrichments do farmed salmonids prefer at different life-stages? ▪ What types of environmental enrichment positively impact fish welfare at different life-stages? ▪ What types of environmental enrichment are feasible to deploy on-farm? ▪ What effect does the spectral composition of light have on fish welfare at different life-stages? ▪ What effect do differing photoperiods have on fish welfare? ▪ How is light intensity distributed in differing tank depths and how might this affect fish welfare?
Slaughter and euthanasia	▪ What brain region(s) is/are responsible for consciousness in fish? ▪ How do we measure brain function in fish? ▪ At what point does unconsciousness occur during differing slaughter and euthanasia methods? ▪ What is the cause of death in slaughter and euthanasia methods (e.g., ice slurry slaughter and electrical or percussive stunning)? ▪ Are there more humane methods of slaughter and euthanasia than presently available?

welfare? Longer-term, a non-invasive way to measure brain activity in tanks, and further work on fish brain neuroanatomy and function [e.g., (200, 201)], would help us understand and potentially validate new welfare indicators. Overall, developing a suite of validated, non-lethal welfare indicators that facilitate rapid and reliable assessment of welfare on-farm would be of considerable value. Such a panel of indicators could help us understand welfare at the fish level on-farm, since there is likely to be high individual variation in welfare and stress coping ability within a given group of farmed salmonids [e.g., (202)].

There is obviously no dearth of relevant research to be done in the realm of farmed salmonid welfare. In particular, it is essential to address these and other research gaps to ensure that policy guidelines do not rest solely on assumptions about whether these gaps represent welfare issues or not. Evidence-based policies safeguard welfare in meaningful ways while preventing pointless and potentially damaging impacts on valuable industries. Together with the work done by both the NFACC Scientific and Code Development Committees, we hope that this review serves to guide future studies toward the most pressing and policy-relevant research questions, ideally in advance of future NFACC Code of Practice revisions. But regardless of timelines, it is important that we support and conduct basic and applied research that can address some of the gaps in our understanding of how to safeguard farmed fish welfare, especially considering increasing expressions of concern for fish well-being from farmers and the general public and the continuing expansion of the salmonid aquaculture industry.

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REVIEW
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It Began in Ponds and Rivers: Charting the Beginnings of the Ecology of Fish Cognition

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But fish cognitive ecology did not begin in rivers and streams. Rather, one of the starting points for work on fish cognitive ecology was work done on the use of visual cues by homing pigeons. Prior to working with fish, Victoria Braithwaite helped to establish that homing pigeons rely not just on magnetic and olfactory cues but also on visual cues for successful return to their home loft. Simple, elegant experiments on homing established Victoria's ability to develop experimental manipulations to examine the role of visual cues in navigation by fish in familiar areas. This work formed the basis of a rich seam of work whereby a fish's ecology was used to propose hypotheses and predictions as to preferred cue use, and then cognitive abilities in a variety of fish species, from model systems (Atlantic salmon and sticklebacks) to the Panamanian *Brachyraphis episcopi*. Cognitive ecology in fish led to substantial work on fish pain and welfare, but was never left behind, with some of Victoria's last work addressed to determining the neural instantiation of cognitive variation.

Keywords: cognitive ecology, fish, homing pigeon, navigation, predation, spatial cognition

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INTRODUCTION

In the past three decades, our understanding of what fish can perceive, attend to, learn, and remember has gone from little and assuming less, to the inclusion of fish in any course on animal cognition. Gone is the mention of the three-second memory of the goldfish, in is the awareness of pain, the ability to count, navigational abilities rivaling those of a homing pigeon, and much more.

Indeed, it was work with homing pigeons that first brought about some of the major changes in our current understanding of fish cognition. This is because much of the responsibility for our deepening understanding of the cognitive capacities of fish lies with Victoria Braithwaite, and her story starts with a flock of pigeons. Braithwaite's contributions come from asking questions about fish cognition in the context of their ecology and evolution, and how natural selection might have shaped their cognitive abilities. In this review, we therefore have two aims: first and foremost, to examine the impact of Victoria Braithwaite's work on current understanding of orientation and navigation in fish and other vertebrates, and second, to reflect on how bringing this adaptationist view of fish cognition brought fish into the mainstream of a field previously dominated by mammals and birds. Our particular focus on Braithwaite's work is unabashedly firstly as a memorial to our friend and colleague whose untimely death in 2019 we mourn but also because we contend that her work was pivotal in the establishment of fish as mainstream, even conventional, in work on animal cognition.

NAVIGATION AND VISUAL LANDMARKS

That work began, as alluded to above, with the avian model for navigation: homing in pigeons. By the late 1980's it was firmly established that birds, among other animals, used all manner of cues to guide their journeys, long or short. Homing pigeons formed the basis for a large part of the "real-world" experimental investigation into vertebrate navigation. One reason for their popularity as a model was that multiple features could be readily manipulated including their rearing, housing, transport, sensory input, and experience. However, there had been relatively little investigation into their use of landmarks and other visual cues. This was perhaps because of the famous 1970's experiment in which pigeons returned to their home loft even when wearing frosted lenses (1), leading to the belief that visual cues were not important to homing pigeon navigation. And so it took until the early 1990's before Braithwaite and Guilford (2) showed that even 5 min of viewing a familiar landscape prior to release was sufficient to reduce the time it took homing pigeons to return to their loft in comparison to control birds confined in a box with opaque walls. A subsequent experiment confirmed that the recognition of familiar landmarks visible at the release site was the key to the difference in homing time and not other factors such as a reduction in confidence of the pigeons in homing. In this latter experiment, birds homed from familiar and unfamiliar release sites and were allowed to view or not view the landscape for 5 min prior to release. Only the birds allowed visual access to the familiar site prior to release homed faster (3).

These data, followed by confirmatory experiments in the next few years (3–5), showed that visual information (landmarks) could be important in enabling pigeons to home, in addition to the other cues (especially magnetic and olfactory) that had long been the focus of the pigeon homing community. More than two decades on, the degree to which visual landmarks influence the route a pigeon takes toward its home loft and the speed at which it does so continues to be debated and elucidated [e.g., (6–9)]. Some of the questions that arose from those early pigeon release data, such as the importance of landmarks at points later in a journey rather than just at the starting point, had to wait until the development of appropriate technology such as GPS tags for tracking animals [e.g., (10, 11)]. But the early data also initiated an interest in the role of other visual information that homing pigeons might use, such as the identity (12) and experience [e.g., (13)] of their flock partners. These studies also laid the foundation for the examination in other species of the role played by visual information in navigation in other species. Alongside birds and mammals, attention began to be paid to fish too.

It is a common observation in navigation texts that fish can perform remarkable navigational feats. The most famous examples come from data showing that migratory fish like salmon successfully return from the open ocean to their home stream by using olfactory cues (14–16). Even non-migratory fish are known to be expert navigators: ironically, given the popularity of the myth of their three-second memories, there are century-old data showing successful learning and navigation of a maze by goldfish (17). However, much like with homing pigeons, the body of research on memory and cue use in fish [e.g., (18–20)] contained surprisingly few attempts to investigate their use of *visual* cues. This omission is striking, considering that visual cues have always been the focal cue type for spatial cognition work in rats and mice. This is perhaps because visual cues are so much more readily manipulable by human researchers, dependent as we are on our visual capacities. Braithwaite's early fish experiments were among the first to ask what visual cues fish might use to navigate around a familiar area. In her first experimental manipulation on visual cue use by fish in 1996, using a flume tank and colored plastic Lego bricks as landmarks, Braithwaite and her co-authors showed that Atlantic salmon *Salmo salar* could use conspicuous visual cues to track a moving resource (21).

A second experiment contained within that 1996 paper showed that when conspicuous visual cues were no longer available, the fish would switch to another preferred cue type for navigation. This secondary cue was probably chemosensory. But it was clear that fish differed in their preferred cue type, as only some fish switched to the chemosensory option when the conspicuous visual cues were no longer available. This evidence that the salmon might use more than one cue type, or have a hierarchy of cues, also echoed work from homing pigeons. Furthermore, the variation among the fish in cue preference and in performance on the task prefigured the current enthusiasm for understanding differences among individuals [e.g., (22)] and coping strategies [e.g., (23)]. The simplicity of the experimental method and the salmon data themselves formed the basis for work that continues today. Some of that work involves identifying the kinds of information used by fish when moving within and between locations, familiar or novel, just as Braithwaite et al. (21) did 25 years ago. There is plenty of scope for such work, as shown by the large and growing number of species examined. An incomplete list of this species includes French grunts *Haemulon flavolineatum* (24), freshwater stingrays *Potamotrygon motoro* (25), Amarillo fish *Girardinichthys multiradiatus* (26), and rainbowfish *Melanotaenia* spp. (27). Perhaps not surprisingly (but one still has to collect the data), it is now typical to find that these fish, just like homing pigeons and salmon, have a hierarchy of cues when navigating [e.g., (24, 28, 29)].

Rather amazingly, however, the spatial movements of which fish are capable continue to surprise. For example, the three-spined stickleback *Gasterosteus aculeatus* is familiar to behavioral ecologists as a model for sexual selection [e.g., (30)] and speciation (31) among others, but it was not until 2013 that its ability to home after displacements of up to 180 m was demonstrated (32). Likewise, the ability of female cardinal fish *Apogon notatus* to return to the exact location of the territory they had held up to 6 months earlier could still be described as remarkable in 2010 (33). The apparent surprise in the demonstrations of the navigation abilities of these fish put us in mind the time it took to refute the belief that goldfish have memories older than 3 s: the idea that fish have any degree of capaciousness to their learning and memory abilities seems to have taken a long time to really take hold (34).

COGNITIVE ECOLOGY AND CUE USE

Braithwaite's work on cue use in fish was, however, even more influential beyond the narrow focus of cue use and memory capacity in different fish species. Indeed, this work was one of the forerunners to the field we now refer to as 'cognitive ecology' (35). Around the middle 1980's and into the early 1990's behavioral ecologists began to ask questions about animal cognition that differed from the questions typically asked by experimental psychologists. Where the previous questions had included the nature of associative learning, whether timing is scalar, and the difference between working and reference memory, researchers in this new field asked whether and how cognitive abilities might have been shaped by natural selection. One of the first of these questions was whether spatial memory abilities are better when a species' ecology appears to depend heavily on spatial memory. One, now textbook, example centered around asking whether food-storing species had better spatial memory than did species that do not store food [e.g., (36, 37)]. Although a convincing demonstration of a difference between storers and nonstorers in spatial cognition took some years and multiple experiments [e.g., (38, 39)], both correlational and experimental data showed that (a) food storers had a larger hippocampus (the region of the vertebrate brain heavily involved in spatial information processing) than did nonstorers [e.g., (40, 41)], and (b) damage to the hippocampus in food storers reduced their ability to retrieve their stores and to solve spatial memory problems (42, 43).

These data set the scene for Braithwaite to bring together two worlds: cognitive ecology and fish. For the first set of experiments, Girvan and Braithwaite (44) chose to ask how the ecology experienced by three-spined sticklebacks *Gasterosteus aculeatus* was related to their performance on a spatial learning task. To do this, they used populations from a highly variable environment (a river) and contrasted them with populations from a stable environment (a pond). The task came in two versions, both using a linear maze in which the fish were trained to swim from the release compartment to the end of the maze for a food reward, through a series of choices (e.g., a set of open or closed doors). In one maze the route was marked by a visual landmark (a plant) at each of the correct decision points, while in the other maze there were no visual landmarks. The hypothesis was that fish from a stable environment might be more likely to use visual landmarks, whereas fish from a less visually stable environment might be more likely to rely on movement cues when orienting themselves in their habitats. Although the data did not entirely neatly dovetail with these predictions, the sticklebacks from two different ponds did take longer to learn how to navigate the maze when there were no landmarks than did the fish from the river populations. In addition, fish from one of the river populations took longer than fish from the other three populations to relearn the maze when the sequence of choices was reversed, which was consistent with these fish having learned a pattern of turns for successfully navigating the maze.

Much like the earlier food-storing work on birds [e.g., (45)], this experiment showed immediate support for a relationship between the ecological demands of the habitat and cue preferences, with the added flourish of the difference being within, rather than between, species. Although this work was soon cast in an adaptive framework (46), and indeed was consistent with that framework, it did not actually yet demonstrate differences in memory among the populations. Furthermore, one should always be aware that a wide variety of factors can and do affect the motivation of an animal to pay attention to, to learn or to remember an object, location, event or other. This difference in motivation or attention can result in an animal performing in such a way that looks poorer, or better than another. If an animal does not pay attention or does not value the reward, similarly to another individual, testing on a cognitive task is not occurring on a level playing field (47).

Since these early experiments, a multitude of experiments using ecology to predict cue use and cue preferences have ensued in birds, fish, and other taxa. In some, ecology does seem to explain those preferences, while from other experiments we have learned more about yet other ways in which context can affect test outcomes. For example, there are multiple experiments showing that when both color and spatial cues are available, nonstoring birds do not have a cue preference whereas storing birds prefer to use the spatial information. In a typical instance, a one-trial associative memory task, nonstoring blue tits *Cyanistes caeruleus* and jackdaws *Corvus monedulus* had no cue preference when the locations were specified by visual or spatial cues (48), while food-storing marsh tits *Parus palustris* and jays *Garrulus glandarius* preferred to visit the location specified by spatial cues. And yet, animals can learn to shift from using one cue to another even across the course of an experiment. For example, in another associative memory task, nonstoring great tits were trained to find food in the same location on 10 consecutive visits, always covered by a cloth flap of the same color. When given the choice between the familiar location covered with a cloth flap of a different color or a new location with a cloth flap of the familiar color, the nonstorers overwhelmingly chose the familiar location (i.e., using spatial cues) rather than the cloth flap of a familiar color (49). As seen in a variety of tests of spatial learning including rats in a Morris water maze, sticklebacks in a T-maze, and wild hummingbirds in the field, stability of cues seems to be important, and spatial cues are very often more stable than are visual cues (50–52).

In other cases, the relationship between ecological context and cue preferences is less obvious. One example is an experiment demonstrating the preference of two ecotypes of a facultative Caribbean cleaning goby *Elacatinus prochilos* for spatial over pattern cues (53). In that experiment, there were two ecologically-based predictions: (1) that cleaning gobies would perform better in a task relying on pattern cues, because the task (identifying a pattern on a plate) was analogous to deciding which clients to clean, while (2) sponge-dwelling gobies would perform better on a spatial task. In fact, both species did well on the spatial task and poorly on the pattern-cued task. The authors could only speculate as to the meaning of these results, but these data give notice that predictions about cue use may well test a researcher's understanding of the key attributes of the ecological environment in which their animals live.

In yet other situations, discrimination ability or salience may underlie apparent preference. For example, in a visual discrimination task zebrafish *Danio rerio* learned color cues very readily but not shape cues, until the shape cues were much enlarged (54). Here, a cue-use test led to the uncovering of a species' sensory abilities that had not been previously obvious. Yet more issues may be raised whereby the structure of the task itself has an impact on the animals' performance. For example, in an early examination of the neural bases of spatial learning in frogs, the animals were tested in a Morris water maze, a task in which the frogs, like rats, were thigmotaxic (keeping close to the edges of the pool). Frogs in that test did learn to use a visual cue to locate the platform hidden below the water's surface but would not swim across the center of the pool to reach it, even if that was the shortest distance to the platform (55). Rather, they swam around the edge and then used the pool wall to push off when they got close to the platform. If the authors had used the directedness of swim paths or speed to reach the platform exclusively to measure whether the frogs had learned the platform's location, as is typical in Morris water maze studies, these frogs would not have provided very convincing evidence that they could learn a spatial location. It is increasingly evident that frogs, like fish before them, are capable of learning spatial locations (56–59), a rather unsurprising confirmation if one considers the ecology of these species. Indeed, any animal that needs to find its way home, unless utterly dependent on volatile cues is likely to have some need for spatial memory. But then, examining cognition in frogs lags well behind even the work on fish.

SOURCES OF CUE PREFERENCES

For anyone attending navigation conferences through the 1980's and 1990's (as was Braithwaite), the often-heated debate as to which was the primary cue used by pigeons to home between the Italian and German groups was a regular feature. The Italians argued that olfaction was key while the Germans argued that magnetic information was by far the more important. There was more than one accusation of poor science during such debates. What was needed was an experimental test. And when homing pigeons were experimentally raised in Frankfurt in the 'Italian' manner i.e., in a wind-exposed roof loft rather than in the typical-Frankfurt mode of an enclosed garden loft, these "Italian-Frankfurt" birds subsequently relied more heavily on olfactory than on magnetic information when homing (60), which was not the cue hierarchy of birds raised in the more standard Frankfurt manner. Importantly, this experiment provided rather good evidence that cue dependence seen in adults could depend very heavily on early experience, removing at least this point of contention from homing pigeon debates.

Given this background, when (61) investigated the sources of cue preference in sticklebacks, the obvious place to look was early environmental conditions in sticklebacks. Stickleback fry from pond and river populations, each raised with and without stable landmarks were tested in two ways: in a maze in which the fish needed to use visual landmarks to locate rewards and in an apparatus in which water flow was the relevant cue. The key result is that there was no difference in performance on either task between fish derived from river and pond populations. For all those working in the field of cognitive ecology, there were two important associated take-home messages from this result: (1) ecology can shape cue preference and use, but (2) a preference cannot be interpreted as evidence of an adaptation. Preferences are very likely to be at least somewhat flexible, and the nature of this flexibility may differ between species, or even between populations within a species.

One major source of flexibility, as shown by Girvan and Braithwaite, is the early environment, and particularly, the physical environment. Not only do fish pay attention to cues from their physical environment, but this early experience can also have a major effect on their capacity to cope with later-life complexities such as the release from hatchery conditions into the wild. Braithwaite, together with long-term collaborator Salvanes, showed that the provision of visual cues into tanks of juvenile cod can increase their reaction to novel prey and their speed to switch to natural, wild prey (62). They also showed that spatial structure in the early environment led to better anti-predator skills (63). These data on cod reared in hatcheries have not only led to a plethora of work conducted on cue use in an ecological context, but have also had a substantial and broad impact in both welfare and economic terms. A small sample of those contributions are more fully described and appreciated in other papers in this Special Issue.

More recent studies from Braithwaite's group have shown that the developmental stage of enrichment provision impactful [e.g., (64)], its duration [e.g., (65)], and its nature are all impactful. Braithwaite's focus was on the role that the physical, rather than the social, environment played on subsequent information use and learning [e.g., (66–68)]. She and colleagues also showed that at least zebrafish preferred an environment in which they could combine physical enrichment with swimming opportunities (66). Although many others had previously demonstrated impacts of physical and social enrichment on performance in learning and memory tasks of a wide range of species [e.g., (69, 70)], a recent meta-analysis (71) provides strong support for Braithwaite's own emphasis: asocial factors (physical enrichment, enclosure space, sensory enrichment, exercise) lead to larger impacts on learning than do social factors (isolation, parental deprivation, group size). Furthermore, duration of that enrichment also plays a major role, and apparently greater than the specific timing of the enrichment. Although the majority of the data on which this meta-analysis was based came from rodents [also now a meta-analysis on aquatic animals: Zhang et al. (72)], Braithwaite's work on fish is consistent with the broader taxonomic patterns.

NON-MODEL SYSTEMS

No consideration of Braithwaite's work and its impact on the way we now regard fish cognition is complete without mention of her work using the tropical poecilid *Brachyraphis episcopi.* Like her stickleback work, working with Brachyraphis found Braithwaite out in the field collecting fish, but in this case, from streams along Pipeline Road, near Gamboa, Panama. The question to be addressed here was no longer the role that cues in the environment played in cognitive performance, but the role other species played, specifically that of predators. In the streams along Pipeline Road, Brachyraphis found above waterfalls typically share their stream with few fish species other than killifish *Rivulus brunneus*, while Brachyraphis living downstream below the waterfalls face a barrage of predators, the waterfalls being a considerable barrier to movement upstream for the downstream fish. Although boldness (speed of emergence from a shelter) does not always differ between fish from the two environments (73, 74), a variety of performance measures in a spatial task did: upstream (low predation) fish were more active within the maze, were faster to find the rewarded patch, and learned the rewarded location cue with fewer errors than did the fish from the high predation (downstream) sites (75). Recent tests of Trinidadian guppies *Poecilia reticulata* in the Lower and Upper Aripo (i.e., tested in the wild) showed that guppies from the high predation site were also less active and slower to complete a maze (76) and it would appear that at least some of these responses are learned in early life (77) and from parental behavior (78). Although begun with an interest in cognitive ecology, the Brachyraphis work then followed the growing enthusiasm for examination of individual differences and personality, leading to data on associations between environmental conditions and variation in aggression and boldness (79) and exploration (80). However, it never left cognitive ecology entirely behind, with the demonstration that Brachyraphis that explored more were also faster to learn to associate a cue with reward (81).

NEURAL WORK

No serious work on the role that natural selection plays on cognitive abilities (cognitive ecology) can avoid the part played by the brain. One example of this is the role of the hippocampus in the research on spatial in food storing mentioned above [e.g., in food-storing songbirds: Sherry and Vaccarino (43), Clayton and Krebs (82)]. In Braithwaite's own work, she and collaborators examined the role of neural plasticity in visual navigation in Atlantic salmon (67). This work showed that enrichment with physical landmarks that changed locations weekly led to enriched fish (Atlantic salmon) learning the correct exit from a simple maze with fewer mistakes than did the control fish. Examination of neural plasticity in the telencephalon (the part of the fish brain pertinent to spatial cognition) showed that enriched, but not control, salmon had upregulated expression of Neuro 1D mRNA expression. Just a few years on, it is becoming increasingly clear that environmental enrichment leads to neural cell proliferation (83–87) and it will now be interesting to determine which components of enrichment have this effect and why [e.g., (64)].

Evidence is also appearing for variation in brain regions involved in early stages of processing sensory information in recently diverged stickleback species. Limnetic species that are heavily reliant on visual information have larger optic tecta and smaller olfactory bulbs than do benthic species, which are much more dependent on olfaction (88). A similar effect is seen in killifish from sites with and without predation: killifish from sites with predators have large eyes and large optic tecta compared to killifish from sites without predators, but the whole brains of the two groups do not differ (89). Cognitive ecology in the round now has fish examples of the neurobiology of cognition to add to those from birds and mammals, a point reached in no small part thanks to Braithwaite and her collaborators.

NOT JUST FISH

Finally, but very much not least, much as Braithwaite loved to work with and on fish, she was never just a "fish person". She was always alert to systems that best addressed the question in which she was currently most interested. Braithwaite's continuing interest in spatial cognition led her first foray into examining spatial learning in rodents when she and collaborators examined the impact of parasitic infection (90). Her next and more substantial venture was inspired by the rich literature concerning the role of sex in spatial memory abilities in mammals, and especially rodents [e.g., (91–94)]. One feature of especial interest was the variation across this literature: some researchers found sex differences and some did not. When Braithwaite, together with a student and one of the current authors (Healy), collected some empirical data we also found no differences between the sexes in a spatial cognition task [Morris water maze (95)]. However, we did find that the number of swims the females needed to learn the location of the hidden platform performance differed across the 4 days of their oestrous cycles: they needed an extra swim on oestrous days. These data seemed to present a possible explanation for at least some of the cross-study variation as if females performance depends on the day on which they are tested, on some days they may perform as well, and on other days more poorly, than males.

Hormonal variation is a possible mechanistic explanation for sex differences in spatial cognition, but the question is why such hormonal differences would exist in the first place. There are also a multitude of evolutionary scenarios proposed to explain why the sexes might differ particularly in spatial cognition. Our subsequent consideration of the rationale and empirical data for the evolutionary explanations for differences between the sexes in spatial ability was, and still is, rather well-received by a greater diversity of fields than just evolutionary biology or animal cognition (96). Nearly 20 years later this review is being cited in such diverse work as bumblebee cognition (97), effects of binge drinking (98), stereotyped threat (99), gender differences in seminomadic pastoralist children (100), and behavior during COVID-19 lockdown in Russia (101). Although it has not led to policy changes as did her work on fish welfare (again see other papers in this Special Issue), the impact of this paper has been sustained and broad. Not bad for a paper that was addressed to a sideline interest.

LEGACY

When Les Real labeled cognitive ecology as an emerging field in 1993, he believed there was a sufficiently novel approach to deserve the name (35). Seven years later, present author Healy and Braithwaite wrote a cheekily early assessment asking if it was a field of substance (102). We wrote at the time that "there are those who will dispute the value of yet another label for yet another sub-discipline, and if little has happened in seven years, such critics will be right." Now, over 20 years later, we can say with confidence that not only is cognitive ecology a field of substance, but that substance is in large part thanks to the work of Victoria Braithwaite. The field itself has truly begun to come of age with an increasing diversity of species under examination, in an increasing variety of contexts. Importantly, fish are now a mainstream taxon, along with mammals and birds, for addressing questions regarding cognition in general, and cognitive ecology specifically. This major change in the field is just one of Victoria's scientific legacies, and we know that she would look forward with keen interest to where the field will go next.

AUTHOR CONTRIBUTIONS

SH wrote much of the first draft. Both authors contributed to revisions.

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What Is It Like to Be a Bass? Red Herrings, Fish Pain and the Study of Animal Sentience

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Debates around fishes' ability to feel pain concern *sentience*: do reactions to tissue damage indicate evaluative consciousness (conscious affect), or mere nociception? Thanks to Braithwaite's research leadership, and concerns that current practices could compromise welfare in countless fish, this issue's importance is beyond dispute. However, nociceptors are merely necessary, not sufficient, for true pain, and many measures held to indicate sentience have the same problem. The question of whether fish feel pain – or indeed anything at all – therefore stimulates sometimes polarized debate. Here, we try to bridge the divide. After reviewing key consciousness concepts, we identify "red herring" measures that should not be used to infer sentience because also present in non-sentient organisms, notably those lacking nervous systems, like plants and protozoa (P); spines disconnected from brains (S); decerebrate mammals and birds (D); and humans in unaware states (U). These "S.P.U.D. subjects" can show approach/withdrawal; react with apparent emotion; change their reactivity with food deprivation or analgesia; discriminate between stimuli; display Pavlovian learning, including some forms of trace conditioning; and even learn simple instrumental responses. Consequently, none of these responses are good indicators of sentience. Potentially more valid are aspects of working memory, operant conditioning, the self-report of state, and forms of higher order cognition. We suggest new experiments on humans to test these hypotheses, as well as modifications to tests for "mental time travel" and self-awareness (e.g., mirror self-recognition) that could allow these to now probe sentience (since currently they reflect perceptual rather than evaluative, affective aspects of consciousness). Because "bullet-proof" neurological and behavioral indicators of sentience are thus still lacking, agnosticism about fish sentience remains widespread. To end, we address how to balance such doubts with welfare protection, discussing concerns raised by key skeptics in this debate. Overall, we celebrate the rigorous evidential standards required by those unconvinced that fish are sentient; laud the compassion and ethical rigor shown by those advocating for welfare protections; and seek to show how precautionary principles still support protecting fish from physical harm.

Keywords: fish, sentience, consciousness, pain, welfare

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INTRODUCTION

Debates around fishes' ability to feel pain are essentially debates about consciousness. In other words, the central issue is: when fish react to actual or threatened tissue damage, does this indicate true pain, with its "phenomenal" character ["an unpleasant sensory and emotional experience", e.g., (1)]? Or just mere nociception: an unconscious process by which noxious stimuli are responded to? [c.f. e.g., (2); see also (3–7)]. Today, thanks in part to the trailblazing work of Dr. Victoria Braithwaite celebrated in this Special Topic collection, there is no disputing what an important issue this is, and also no argument as to whether bony fishes possess functioning nociceptors [e.g., rainbow trout (*Oncorhynchus mykiss)*: (8, 9), goldfish (*Carassius auratus)*: (10), common carp (*Cyprinius carpio*): (11)]. But the question of whether fish are *aware* of noxious stimuli, and feel true pain, remains contested and controversial, stimulating considerable debate. Views can be polarized: at one extreme some argue that fish have no awareness of anything at all, including pain (Key in prep., pers. comm), while at the other extreme some argue that they feel not only pain but also fear (12) and even maybe joy (13). Yet *most* seem uncertain: 83% of the 43 responses to Key (14) in *Animal Sentience*, for example, do not take a firm stance on whether or not fish can feel pain. And this reflects a much broader, harder problem: that the functions of consciousness are still not understood. Trying to identify what would make for stronger evidence of sentience was therefore one of Victoria's last pieces of scholarly work (15).

So, as authors of yet another "fish sentience" review, what can we add that will constructively promote her legacy? Our aim is to celebrate the rigorous evidential standards required by those remaining unconvinced that fish are sentient [e.g., (6, 16)], and to leverage the high levels of evidence they require into a research agenda for the future. But we also laud the compassion and ethical rigor shown by those advocating welfare protections for fish [e.g., (17–19)], and seek to show how the high current levels of agnosticism about fish sentience are consistent with adopting practical guidelines that aim to protect fish [e.g., see discussions from (16, 20–23)]. To put this in the context of our own views, personally and professionally we treat fish as though sentient (e.g., we are working on a zebrafish enrichment and welfare project in which Victoria was involved). But we do this because we are taking a precautionary approach; like her we feel that fish should be treated as if sentient [e.g., (24)]– despite not yet being convinced there is strong evidence that fish *definitely are sentient*. This paper therefore aims to illustrate why being uncertain, while simultaneously treating fish as sentient, is a reasonable stance; and to outline what types of data could decrease this uncertainty in the future. In this way we hope to honor our friend and colleague.

To do this, first we review some key consciousness concepts, including "sentience" (a term with two meanings); and the deductive "theory heavy" vs. inductive "theory neutral" approaches typically used to infer capacities for pain in non-human animals. We follow this by identifying types of measure that should not be used to infer sentience, because they can be performed by organisms reasonably assumed to be non-sentient (such as spines disconnected from brains, decerebrate mammals and birds, and humans in states of unawareness). We argue that these are "red herrings": measures that add little to debates about fish pain or animal sentience. We then suggest some experimental approaches more likely to rely on sentience, and thus to be valid indicators: certain operant tasks, tasks reliant on working memory, methods that ask animals to self-report their feelings, and higher order abilities like episodic memory. We also suggest how future research could test the validity of these potential indicators. Finally, returning to the fish pain debate, we attempt to alleviate concerns raised by key skeptics that sentience is irrelevant for welfare consideration, and that classifying fish as sentient will be harmful to people. We also summarize what the indicators most likely to be valid reveal about fish.

What Is It Like to Be a Bass? Some Consciousness Basics

Primary or "phenomenal" consciousness (often abbreviated to P-consciousness) is raw experience or sensation: the ability to feel or be aware, sometimes described – to parallel Nagel's famous essay "What is it like to be a bat?" (1974) – as the "what it is like" aspect of a state [e.g., (25), p. 32–37]. Many equate this with "sentience", using this term to mean all forms of P-consciousness [e.g., (26–30)].

When consciousness can influence actions (including, for humans, speech), it is often termed "access consciousness" [e.g., (26)]. It is therefore *through* access consciousness (e.g., self-report in humans) that researchers make inferences about P-consciousness. Combined with its subjective, private nature, this means that P-consciousness is most readily (indeed perhaps only) empirically detectable in humans, via experiments that rely on self-report [e.g., (25, 31), p. 187–210; (32), p. 224, and many others]. An important sub-type of access consciousness comprises higher order forms [cf. e.g., (29)], involving self-reflection or introspection: self-consciousness (an awareness of self), for example, or the "mental time travel" implicated in the episodic recall of past experiences [e.g., (33, 34)]. Such higher order forms of consciousness are generally seen as reliant on P-consciousness [e.g., the taste of past food, the visual recall of past foraging sites, the sensory inputs from one's own body, etc.; e.g., (35)]. Passing experimental tests for self-reflection or self-awareness is therefore usually taken as evidence of P-consciousness – although what *types* of P-consciousness are necessary to pass such tests depend on what exactly is being tested for (something we discuss further below).

Putting these concepts together, in this paper we therefore assume hierarchical levels of consciousness as illustrated in **Figure 1**'s Venn diagram. Here, higher order forms of self-reflection ("I know that I feel pain"), as well as non-introspective self-reported states ("Pain is present"), reveal and rely on P-consciousness. However, P-consciousness may occur without higher order self-reflection, or indeed perhaps without any form of access consciousness. This conception follows e.g., Blackmore and Troscianko (25), Birch (33), Birch et al. (36), and Ginsburg and Jablonka (37). But it is at odds with some other authors: as for so many other aspects of consciousness,

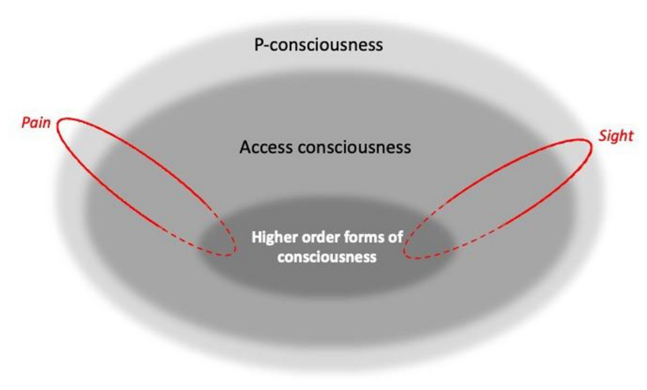

FIGURE 1 | A Venn diagram illustrating three broad, arguably hierarchical levels of consciousness [Inspired by Blackmore and Troscianko (26), Block (25), Birch (33), Birch et al. (36), Ginsburg and Jablonka (37)]. Boundaries between levels are blurred to capture how they likely vary by degrees rather than being "all or none". Spanning the hierarchical levels are components that represent independent, dissociable sensory modalities and affective states, such as sight and pain, shown here in red (along with many others: see text). For these, solid outlines indicate parallel processing, while dashed outlines indicate the potential for diverse dimensions to be bound together (as for example during multi-modal recognition).

there is no consensus here, and so some argue there can be no P-consciousness without higher order forms [e.g., (38–40)]; no P-consciousness without access consciousness [e.g., (41)]; and even that there can be access consciousness without P-consciousness [e.g., (42, 43)]. Nevertheless, this figure provides a model of our practical assumptions (and those often used by researchers interested in animal consciousness), including that self-report provides a window into P-consciousness, and that failing tests for higher-order consciousness (or even other forms of access consciousness) does not prove a lack of sentience.

Figure 1 also illustrates another feature of consciousness: we drew the boundaries between hierarchical levels as blurred because they seem to exist in degrees rather than being "all or none" [cf. e.g., (29)]. For example, we know from human research that some types of stimuli may be on the threshold of conscious detectability, with subjects being barely aware of them [e.g., (44)]. Furthermore, P-consciousness can be a reversible state (as well as a trait) that we lose during anesthesia or sleep; and researchers studying its waxing and waning in humans find that this state is graded: not just present or absent, but instead occurring by degrees [e.g., (25); p. 115, 119, 189; (45), p. 33; (37), p. 187]. By analogy, this could suggest that the *trait* of consciousness can also vary by degrees.

In addition, as well as having hierarchical levels, consciousness also comprises parallel, non-hierarchical components such as the phenomenal pain and phenomenal vision (sight) shown in **Figure 1**. Although often bound or unified together to give a multi-modal representation of the world [e.g., (46)], these are independent and dissociable. Thus, in their heuristically valuable framework, Birch et al. (36) proposes that "perceptual richness" (the perception of aspects of the environment), and "evaluative" or affective richness (emotional experiences with valence, such as pain), are categories or dimensions of P-consciousness. In turn, each of these broad dimensions can be broken down into components, with sight being dissociable from conscious olfactory or proprioceptive experience, pain being distinct from hunger or pleasure, and so on. Other authors make similar distinctions [see e.g., (45), p. 54; (37), p. 7, 203], and data from humans clearly illustrate the separability of these components. For example, people who lack sight because of retinal or visual cortex damage can still consciously experience sound, smell, touch, pleasure, pain and so on [e.g., (32)]; people who lack phenomenal olfaction (perhaps because of olfactory cortex damage) can still taste, detect olfactory irritants that cause pain, and experience all other forms of P-consciousness [e.g., (47)]; people with certain cortical or thalamic lesions may become unable to perceive tactile, thermal, or high pressure somatosensory stimuli to the body [e.g., (48)], yet despite this numbness and lack of proprioception, still have all other forms of P-consciousness; and finally, people who lack the capacity to feel pain (due to a lack of receptors or central change in how pain signals are processed) likewise are still able to feel pleasure, and to consciously see, hear, taste, and so on [e.g., (48–50)].

Appreciating these different components is important in several ways for the fish pain debate. First, it illustrates how the word "sentience" is used to refer to two different concepts: a potential source of confusion. As mentioned above, some use this term to mean all forms of P-consciousness. But others (including ourselves) use sentience to refer to *just* the sub-type that is most ethically relevant: the dimension that Birch et al. term "evaluative", or the capacity for felt emotions [e.g., (51–54)]. Second, because P-consciousness comes in these diverse dissociable forms, this means that evidence for one component tells us little (and perhaps nothing at all) about the presence of other components. Thus, evidence for visual awareness tells us nothing about the presence of olfactory awareness, for instance, or the ability to feel pain. This is not just an abstract issue. Appreciating such components is useful because it highlights how the cognitive data typically treated as evidence for P-consciousness in animals (sentience in its broadest sense), reveal little or nothing about *affective,* evaluative consciousness (sentience in its narrower, more ethically relevant sense). For example, mirror self-recognition tests probably indicate some forms of P-consciousness: those reliant on sight and phenomenal proprioception. But this does not reveal anything at all about an animal's capacity for pain or the other forms of conscious affect at the heart of sentience. In the section What *Could* Be Evidence of P-Consciousness and, More Narrowly, Sentience? we develop this argument further, and use it to propose some new types of experiment.

INFERRING SENTIENCE IN NON-HUMANS

For non-human animals, for whom we cannot use verbal self-report, researchers interested in inferring sentience typically take one of two approaches. One is to look for what are argued to be neurological prerequisites for sentience (i.e., particular structures or types of organization within the brain). This is a deductive approach that assumes that we know what these prerequisites are (thanks to research on adult humans), and is what Birch (33) would term "theory heavy". A second approach– one especially useful for species lacking clear homology with humans – is to look for behavioral and cognitive responses that are at least consistent with sentience, and use these to make inferences: an inductive approach that would be called "theory neutral" by Birch (33). In the fish pain debate, the former approach is most often used by those arguing that fish cannot feel pain (or indeed anything at all), while the latter is most often used by those arguing that they do. In both cases, however, the protagonists are limited by their underlying assumptions.

Thus some authors have argued that fish do not have the types of brain necessary to be capable of pain, and these follow the first approach. Early versions based their argument on fishes' lack of our mammalian six-layered cortex and the assumption that this is crucial for any type of P-consciousness (3, 4, 6, 7, 14, 55). This stance thus equally rules out the possibility of any kind of P-consciousness in, for example, birds [which do not have a layered cortex at all, despite forebrain cyto-architecture of arguably similar complexity (56)]; in reptiles (which do have a layered cortex, but one with "only" three layers [e.g., (57)]); and of course all invertebrates (no matter how neurologically or behaviorally complex). This in turn raises an obvious question: do we know *for sure* that having six cortical layers represents the crucial, unique requirement for consciousness? And this in turn flags a general problem with such approaches: even if we can say that a neurological system with property X is sufficient for consciousness, this tells us nothing about the capacity of a system with 95% of that property, or 90%, or 80%, and so on [after (5, 33)].

Key and Brown (58) propose instead an agenda of "identifying the algorithm (sequence of neural functions) necessary for subjective experience and then seeking to define the specific neural structures (e.g., neural architectures and neural circuitry) that could possibly execute that algorithm among different species". This seems sensible – if, and only if, it can escape from the problem outlined above. But even then, it is an extremely challenging task. For one, because humans are currently the best (only?) model in which P-consciousness can be studied, this approach is limited to principles derived from understanding human brains. Any neural correlates of P-consciousness must also disentangle it from the self-report behaviors used to access it experimentally [e.g., (25), p. 87; (45), p. 45]. Additionally, human consciousness researchers need to agree on what does or does not constitute good evidence for different hypotheses, and then systematically test them: a process that is still very much ongoing [e.g., (37), p. 142–147; (59, 60)]. Until then, there is no consensus on what neurological substrates are required [e.g., (45), p. 21; (37), p. 142–146], and so to quote Blackmore and Troscianko [(25), p. 260], "we should not just guess which features are needed for consciousness".

Perhaps more fruitful is to look for behavioral or cognitive responses that seem consistent with sentience. This is the approach typically taken by those arguing that fish do feel pain. For example, Sneddon et al. (61) developed a list of behavioral, and also physiological, responses (e.g., rubbing, limping, or guarding; self-administration of analgesia; paying a cost to avoid a stimulus; etc.) that they argue would demonstrate pain perception in mammals and so should be assumed to do so in fish. However, this "theory neutral", inductive approach is also problematic. As Key and Brown (62) cogently summed up: "the difficulty here of course is distinguishing whether the behavior truly demonstrates an underlying experience of pain". Similar concerns are raised by Birch (33). And so as LeDoux and Brown (39) summarize, "deciding whether a non-verbal behavior reflects conscious vs. unconscious cognitive processes requires not only that the behavior be explainable in terms of conscious processes, *but also that non-conscious explanations are inadequate*" (our emphasis). This issue is important because, as we review in the section *Red Herrings:* Responses That Do Not Require Sentience, a large corpus of research shows that many superficially persuasive behavioral phenomena can actually occur without P-consciousness. Identifying responses that indicate sentience thus involves looking for types of affective response that humans can *only* make when they are aware [cf. e.g., (36, 39, 63, 64) and others]. The search for these is still on-going (see sections *Red Herrings:* Responses That Do Not Require Sentience and Discussion and Conclusions: Applying Our Approach to the Fish Pain Debate). But a second, complementary strategy is important too: identifying responses that do *not* require P-consciousness, so that these can be ruled out as likely "red herrings". This is the focus of our next section.

RED HERRINGS: RESPONSES THAT DO NOT REQUIRE SENTIENCE

For a response to be used as evidence of pain, we need to know that it could not just reflect mere nociception. This understanding has already led most of those interested in this topic away from measuring physiological responses to harm [long recognized as automatic reactions that do not require awareness; e.g., (65)], and instead toward behavioral or cognitive measures whose nature and functionality is more likely to rely on sentience. But even here, are researchers being stringent enough? To assess this, we must identify responses that do not require sentience. And to do this, we need data from subjects assumed not to have sentience, or indeed any kind of P-consciousness.

For these, here we look to four types of subject: plants and protozoa (P), spines (S), decerebrate mammals (D) and unaware humans (U), a group we came to term "S.P.U.D. subjects". To explain these choices, let us lay out the underlying assumptions. First we are assuming that P-consciousness requires a nervous system, such that responses by organisms without one (e.g., plants) are occurring without consciousness. This is not a consensus view [e.g., (66)], but it is the conventional one [cf. e.g., (37), p. 192; (67)]. Second, in animals with brains, we assume that responses that can be performed by the peripheral nervous system alone (e.g., the spine) do not require P-consciousness. This is not an assumption that organisms must evolve brains to be sentient [though many hold this to be reasonable, e.g., (45),

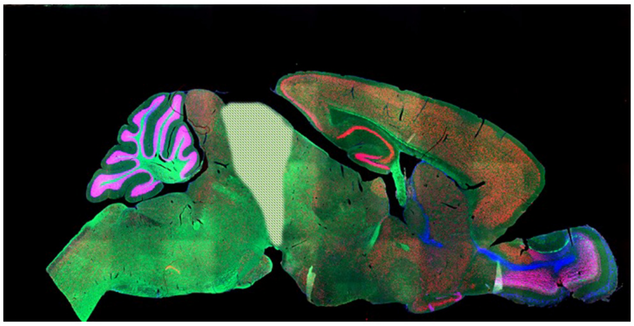

FIGURE 2 | After Woolf (71), showing what is removed in a decerebrate rodent: everything to the right of the pale shaded area (thus cerebral cortices, subcortical gray matter, hippocampus, olfactory bulbs and diencephalon). The pale shaded area (superior colliculi) may be removed too. Note that despite its superficial complexity, the mammalian cerebellum is not involved in P-consciousness [(45), p. 54–55, 83]. Such subjects are generally argued to be pain-free and thus not to need anesthesia for further surgeries [e.g., (5, 72)]. Note that the same is not true for decorticate mammals subject to less severe surgery (e.g., losing only the cortical layers of the cerebra but retaining the thalamus): anesthesia is required for these. (Note that while we find data from these subjects very revealing, we also acknowledge that this work–along with that on spinally transected rats– is highly invasive and disturbing). Photo: Mouse brain, NIH National Institute of Child Health and Human Development.

p. 153–161; (68)], but an assumption that if organisms do have brains, *then* these brains are required for any sentience or broader P-consciousness [cf. e.g., (33, 65, 69)]. It is also based on evidence that damage to the peripheral nervous system does not impair P-consciousness in humans. Third, in mammals and birds, we assume that P-consciousness requires their cerebra [e.g., (59, 70)], such that any responses made by decerebrate subjects (**Figure 2**) cannot require sentience. Again, this is not an assumption that organisms must evolve cerebra to be sentient, but instead an assumption that for mammals and birds which have evolved to have these, they are essential for any sentience or broader P-consciousness. Though not all agree [e.g., (73)], this view is sufficiently widely-held, and with enough certainty, that it already informs research guidelines [e.g., (5, 72); see **Figure 2**]. Fourth and finally, we take responses performed by human subjects who are unaware of stimuli, for instance because they are anesthetized, asleep, or being exposed to subliminal cues they report as undetectable, as occurring without P-consciousness [cf. e.g., (69) and many others]. Again, this is an assumption, and its reliance on self-report is not perfect. The presence of residual, low levels of awareness is sometimes raised as a concern in such studies [e.g., (32), p. 21, 201–204; (37), p. 220; (74, 75)], as is distinguishing the "not experienced" from the "experienced but forgotten" [e.g., (37), p. 134]. Furthermore, our use of such data assumes that the absence of the *state* of P-consciousness (in us, a conscious, sentient species) reveals responses that do not require the *trait* of P-consciousness – and perhaps this is incorrect [c.f. (76)]. Nevertheless, such experiments are often used to study the nature of P-consciousness in humans; they use diverse manipulations to modify awareness (suggesting findings are not artifacts of a single methodology); humans' verbal reports provide unique insights into their subjective experiences; and furthermore, using human consciousness research to yield measures for use in animals is an orthodox approach [e.g., (63)]. In addition, the evidence below does not rely on these human data alone.

The types of behavioral response performed by such S.P.U.D. subjects are compelling (and sometimes unsettling). That they are able to show unlearned avoidance or approach responses is perhaps the least surprising [and consequently, that reflex withdrawal can occur independent of the experience of pain has long been appreciated; e.g., (5, 54)]. Thus, plant stems and leaves will move away from adverse cues like shade, and toward light (77). Similar responses occur in single-celled organisms [e.g., (78)], such as "backwards jerks" if *Paramecia* encounter AC shock (79). The reflex retraction of limbs from noxious stimuli has long been known to occur in spinally-transected cats, rats and humans, despite no involvement of these subjects' brains [e.g., (80), reviewed in (5)]; and they occur in decerebrate rats too [e.g., (71)]. When fed, decerebrate chicks "followed the grain with striking pecking precision when it was moved in front of them by a tweezer" (81). Likewise, "blindsighted" humans, unable to see because of damage to the visual cortex, are still able to avoid walking or reaching into obstacles, as well as to visually track or grasp stimuli that they report that they cannot see [(32), p. 33, 90; although as outlined in the next section, the position of such obstacles cannot be remembered]. Further unconditioned reactions to harmful stimuli in decerebrate mammals are notable because of their seemingly affective nature. Decerebrate animals can react to noxious stimuli "by flight or attack" (82). Decerebrate rats, "respond to noxious stimuli with a flexion withdrawal response, vocalization, turning to the site of the injury, licking or biting the site of the injury, complex escape response and attack responses", although removing the noxious stimulus causes immediate return to passivity or grooming as if nothing had occurred [(71); see also (83) for similar reports]. They show startle responses to sudden sounds [e.g., (84)]. And decerebrate chicks "emitted contentment calls [sic] when warm and distress calls [sic] when cold" (81).

Such unlearned responses to stimuli can also be *modulated* in S.P.U.D. subjects, including by emotionally-relevant cues: they are not fixed and stereotyped. For example, faced with a startling stimulus, "jump" reflexes, and increases in heart rate and skin conductance (reflecting the sympathetic activation of sweat glands) are typically greater in fearful than relaxed humans, including subjects exposed to distressing images. Yet such images can still have this modulatory impact on the startle reflex even when presented in a way that precludes their conscious perception [e.g., (85–87)]. Thirsty humans also drink more (and rate the drink as more positive) if exposed to happy faces than angry faces, even when these are subliminal (88). Likewise, decerebrate rats show a greater ingestive response to sucrose if food deprived rather than sated (89). Furthermore, tail withdrawal reflexes in decerebrate and spinally transected rats are reduced by morphine (83, 90). And even in plants like the sensitive mimosa, whose leaves close when touched, responses to aversive stimuli like lit matches are dampened when the leaves are sprayed with lidocaine (91). These responses by S.P.U.D subjects thus show that modulation of avoidance or ingestive behaviors by affectively-relevant manipulations does not require sentience.

Being able to discriminate between presented stimuli does not seem to require P-consciousness either. For example, the same mimosa plants mentioned above habituate to repeated stimulation, but this appears to be stimulus-specific: thus after a plant habituates to repeated water drops, ceasing to react to them, their leaves will still close in response to a new stimulus-finger touch (92, 93). Tendrils of the perennial vine *Cayratia japonica* are also more likely to coil around neighboring non-self plants than neighboring self plants, again revealing discrimination (94). Decerebrate chicks show the same preferences for moving over still objects, and for particular colors, as intact birds (81). Humans exposed to cues that they have no awareness of can also still discriminate between them, if asked to choose: they report feeling as if they are completely guessing, yet they respond correctly significantly above chance. Thus, subjects can do this with masked, subliminal and other visual cues presented to prevent them being consciously perceived [(25), p. 188–192]. Humans with blindsight can also correctly identify, at levels above chance, whether two items are the same or different, which of two items is the larger, direction of movement, whether something is protruding or receding in a presented figure, the nature of an item portrayed in an image, and even the emotional expressions of faces that they cannot consciously perceive (but which they subconsciously imitate) [(32), p. 6–21]. Subjects with no conscious sense of smell due to olfactory bulb damage ("blindsmell") can also discriminate and identify odors, despite no phenomenal olfaction (47); and similarly subjects with "numbsense" (no somatosensory awareness) can make relevant discriminations: e.g., correctly identifying the bodily location of an applied stimulus they cannot consciously feel [e.g., (95) citing (48, 96)]. Thus discriminating between available stimuli does not require P-consciousness. (If stimuli are not *presently* available though, but instead were presented a short interval before the task, such abilities seem lost: something expanded on in the section What *Could* Be Evidence of P-Consciousness and, More Narrowly, Sentience?).

Discrimination during some *learned* tasks can also occur without awareness, as we outline next. Thus, simple Pavlovian conditioning, in which subjects associate a predictive cue (a "CS") with a reinforcer (a "UCS"), generally does not require P-consciousness. Thus, this type of learning may occur in plants, for instance: pea seedlings can learn to grow toward a breeze that predicts light, or away from one that predicts no light [(97); although this has not been replicated: (98)]. Likewise, *Paramecia* exposed to vibration-shock pairings will learn to jerk backwards to just the vibration alone (79), while a related protozoan can learn to avoid bright locations paired with a shock (99). And *Paramecia* can show conditioned approach to a needle previously baited with food [(100); see also (101)]. The pairing of a cue with a shock can also be learned by mammalian spinal columns [e.g., (102)]; by cats lacking mid- and forebrains (103, 104); by other decerebrate mammals [e.g., rabbits: (105), guinea pigs: (106)]; and by humans who are asleep [e.g., (107)]. Decerebrate cats are even able to show discrimination: they could learn (albeit slowly) that one tone predicted a shock while another did not, and when this contingency was reversed, they could learn the reversal (103). Human subjects exposed to "backward masked" visual cues and similar manipulations that prevent awareness, along with people with blindsight, can all similarly learn to associate a shock with visual cues that they report they cannot see [e.g., (108–111)]. And finally, in a reward-motivated form of Pavlovian learning, subjects asked to rapidly categorize words (e.g., male vs. female names) came to respond more accurately if the words of each category were reliably preceded by a consistent visual CS (e.g., one CS before all female names, another CS before all male) – even when these CSs were subliminal (112). Perhaps unsurprisingly then, diverse forms of Pavlovian conditioning have also been shown in mammals anesthetized with drugs that induce unconsciousness in humans [e.g., (113–115), and reviewed (116)].

One sub-type of Pavlovian learning has often been suggested to differ: trace conditioning. Here, there is no temporal overlap between CS and UCS: the CS (including its offset) fully precedes the UCS. It had been long believed that human subjects need to explicitly understand the relationship between CS and UCS to show this type of learning [see (74, 117)]: an understanding requiring them to be aware of both stimuli. However, some of the studies in the preceding paragraph involved no overlap between CS and UCS, despite authors not always using the term "trace conditioning" here [e.g., see (108–110) as examples; possibly also 101]. Learning to avoid certain flavors presented as CSs can also occur even if the UCS of sickness is induced after an interval, in animals rendered unconscious with anesthesia [e.g., (118)]. Furthermore, trace conditioning can occur in decerebrate guinea pigs [e.g., (119)], and humans who are asleep (120). Human data also indicate that trace conditioning can occur with subliminal CSs (112, 121). As a final example modeled on human blindsight, in macaques whose visual cortices were experimentally damaged so that parts of their visual fields were blind, trace conditioning of a visual cue to a juice reward occurred even if that cue was presented to the animals' blind fields (122). Nevertheless, specific sub-types of trace conditioning involving learning the length of the delay, perhaps thanks to working memory, may still require awareness: see Birch (33) and next section.

What about learning that an *action* leads to a reinforcer? Following Grau and colleagues, here we distinguish what they term "instrumental" learning, where innate responses to UCSs become modified in form and timing (123), from true "operant" learning in which the subject acquires arbitrary responses not in their unlearned, evolved behavioral repertoires (such that a diverse range of responses can potentially be reinforced *de novo* by valenced stimuli, and correspondingly, a variety of difference reinforcers can be used to train a particular response). Neither form of learning occurs in plants (98), but instrumental learning of this type does occur in spines. Spinally transected rodents can learn to retract their hindlegs for particular periods of time, to avoid shocks to the foot (123, 124). These subjects also show "positive transfer": if they have already learned to do this with one leg, they are faster to do it with the other (123). Furthermore, if the hindlimb of a spinally transected cat encounters an obstacle as it is swung forward, then the spinal cord rapidly learns to flex the leg to a greater extent to reduce contact with the obstacle (125). Similar instrumental learning occurs in decerebrate animals. Thus, decerebrate ferrets can learn to alter how they flex a limb to avoid colliding with an obstacle [reviewed (126)]. Another case involves modifying compensatory "up-hill responses". If placed on a slope, with their heads lower than their rumps, rats typically alter their position via a compensatory "up-hill response". Normal rats readily learn to suppress this response if it is punished with an electrical shock to the tail, but so too do decerebrate rats (82). The spontaneous, stereotyped pecking of pigeons can also become directed to a stimulus key by reinforcement, even in decerebrated birds (127). Furthermore, such birds can learn to modify that response according to a light signaling whether or not this pecking will yield food (a discriminative stimulus, DS) (128). Finally, in another study of monkeys whose visual cortices were experimentally damaged to induce blindness in parts of their visual field, Kato et al. (129) tested whether instrumental learning could occur. They successfully used a conditioned (secondary) visual stimulus (paired with juice) presented in the lesion-affected blind field, to reinforce looking in a particular direction.

As for true operant learning involving more arbitrary responses, this seems far more equivocal. It seems uninvestigated in plants or spines [with (123) stating: "there is no evidence that spinal mechanisms can meet the criteria of operant learning. .... In vertebrates, such learning may require a brain"]. Likewise, operant learning is rarely studied in decerebrate animals – in all three cases, perhaps because of the challenges of shaping responses in organisms that do not spontaneously emit variable behavior. There are just two rare potential exceptions here. Research on decerebrate duck embryos found they were able to learn to flex their feet in order to avoid shocks to their wings (130), although whether this is a true operant vs. an instrumentally modified reflex is not clear. In contrast decerebrate rats could not learn to climb onto a platform to escape from a tank of water [while decorticate rats, who retained the thalamus and other structures (**Figure 2**) could; (131)]. The remainder of our evidence here comes from unaware humans. In one complex study, human subjects could manually alter the temperature part of their hand was exposed to, and were told to make this temperature as stable as possible. However, in reality they were being reinforced for something different (e.g., increasingly greater tolerance of a hotter and hotter temperature), the reinforcement being a potentially painful increase. Subjects learned this task – despite not even realizing it was a task – but independently of their ability to report the reinforcer (31). The authors argued that awareness of reinforcing stimuli may therefore not be necessary for operant learning. In another experiment, subjects were set an operant button-pushing task, with backward masking used to render visual discriminative stimuli (DS+ and DS- image cues) non-visible. Subjects could still learn to push the button during only the DS+ condition (121, 132), thus learning to respond when shown subliminal cues associated with monetary rewards, and to withhold responding when shown subliminal cues associated with monetary penalties. However, whether these subjects could have learned to push this button *de novo*, without any instruction and [in (121)] without explicit feedback on their financial losses and gains, is unknown [and indeed seems unlikely: (132)]. Furthermore, not everyone has been able to replicate such results [c.f. (133, 134)].

Other related studies have assessed the effects of unconscious rewards on the *performance* rather than acquisition of operant tasks; thus tasks that were instructed rather than acquired by reinforcement. Some were simple motor responses, others more cognitively demanding and reliant on working memory. In one, former opioid addicts were found to lever-press to receive very small doses of morphine they reported they could not feel (135). Another used a manual gripping task, in which subjects were told that forceful grasps would win them bigger rewards, but that the sums at stake would vary. Presented with subliminal images of the monetary rewards on offer, subjects responded more forcefully when these were large rather than small [(136); see also (137)]. Ziauddeen et al. (138) obtained similar findings using subliminal cues of high/low value food items. In these four studies, more intense responding thus occurred for high than low rewards, despite subjects being unaware of subliminal incentive signals. However, it is unknown whether such differential operant responding would occur if subjects had not been instructed how and why to perform that task but instead had to learn it *de novo* via associative conditioning. Again using subliminal images of high/low financial rewards, Zedelius et al. (139) also found that high rewards promoted better performance of a working memory (word memorization) task, Likewise, subjects were better at an executive task reliant on working memory (140), and at switching between different tasks (141), if the potential rewards were high, even when they were unaware of the signaling images. But once more, subjects were given instructions on what the task involved, as well as explicit feedback on how well they were doing financially. Finally, Correa et al. (142) found that subjects could learn which of two buttons to press, based on images of money rewards that were designed to be subliminal (thanks to backward masking). However, again whether they could have learned to press button at all without instructions (i.e., with only subliminal rewards) is less clear; and importantly, the backward masking seemed unsuccessful in this study, as there was evidence of residual awareness. Overall, this leaves the ability of purely subliminal rewards to modify actions (with no explicit feedback) unclear. The ability of subliminal incentives to *condition* tasks this complex, from scratch, via associative learning also seems unknown.

Overall, a range of learned and unlearned behavioral responses thus do not seem to require P-consciousness, and even responses that "appear" emotional do not require sentience. Of course, *absolute certainty* about the absence of sentience in the plants and protozoa, spines, unaware humans and decerebrate mammals reviewed here, can always be challenged. Sentience is a subjective, private state that currently is not directly measurable: to be as definitive about its absence is nearly as hard as to be definitive about its presence. (And this makes the responses of decerebrate mammals especially disturbing, because so often exposed to harms that would cause pain in intact subjects). Nevertheless, these caveats acknowledged, it is highly defensible to propose that any responses performed by S.P.U.D. subjects do not require sentience, especially for responses that occur across the whole diverse group. And as a consequence, no response shown by S.P.U.D. subjects would convince a skeptic that fish can feel pain: they could argue, with good evidence, that a fish could show one, or even all, of the responses reviewed here, and yet still be no more sentient than a potato. We discuss the evidence previously used in debates about fish sentience in the section Discussion and Conclusions: Applying Our Approach to the Fish Pain Debate.

But what are S.P.U.D subjects *unable* to do? Can we use this information – as well as current ideas about the correlates and even functions of P-consciousness – to suggest more convincing measures of sentience?

WHAT *COULD* BE EVIDENCE OF P-CONSCIOUSNESS AND, MORE NARROWLY, SENTIENCE?

Biologists and psychologists typically assume that P-consciousness is functional, being somehow crucial for flexible, strategic behavior of a greater complexity than the responses of S.P.U.D. subjects [e.g., (25), p. 196, 287–292; (37), p. 186, 189; (33, 65, 116, 134, 143)]. Baars, for instance, proposes nine functions of P-consciousness that include integrating perception, thought and action, adapting to novel circumstances, and providing information to a "self system" [summarized by Blackmore and Troscianko (25), p. 196]. Similarly Ginsburg and Jablonka [e.g., (37), p. 233–237] argue that amongst its hallmarks are: the binding of information about multiple features of the world, and the global accessibility of this information (thanks to long-term memory) for evaluation and use in selective, flexible goal directed behavior. Perhaps these attributes thus capture what's missing in Woolf (71)'s striking contrast between decerebrate and intact rats: the former "react to noxious stimuli with an 'indifference' such that immediately after application of a noxious stimulus the animals will carry on grooming etc. as if nothing had happened. … no immobility or sustained licking of the injury, and no avoidance of the experimenter".

Is it possible to be more precise than this, and identify specific, well-operationalized behavioral or cognitive attributes that *require* P-consciousness? The answer is "not quite yet", which leaves a "theory heavy" or deductive approach currently impossible. But Birch (33) suggests a pragmatic alternative: a "theory light" approach of inference to the best explanation, that "commits to a broad hypothesis about the relation between phenomenal consciousness and cognition… the motivating idea being that phenomenal consciousness does something for cognition". To build on this constructive proposal, here we suggest four types of candidate measure that seem particularly promising as indicators of sentience. One concerns working memory, and another, forms of operant conditioning: two types of ability that S.P.U.D. subjects seem not to convincingly have. A third type of candidate measure concerns self report. As we saw in the section *Red Herrings:* Responses That Do Not Require Sentience, unaware humans, and also monkeys with blindsight, report sensing nothing (thence having no P-consciousness), even when presented with stimuli that elicit other kinds of response – with the human subjects consistently describing themselves as merely guessing. Finally, higher order cognitive processes such as episodic memory and self-recognition, can yield insights into animal awareness. These have not been studied in S.P.U.D. subjects, and this would likely be impossible, but are arguably reliant on perceptual consciousness. We review each of these below, and also outline how they could be modified to now address questions about sentience: the ethically relevant dimension of P-consciousness at the heart of the fish pain debate. We also suggest how their validity as indicators of sentience could be assessed.

Tasks Involving Working Memory and Aspects of Operant Conditioning

Working memory tasks require subjects to retain and use information after a delay. The potential role of P-consciousness in these tasks is revealed by some responses that humans with blindsight and similar deficits are unable to make. Above, we reviewed how subjects with blindsight can make visual discriminations, despite their lack of phenomenal sight, including reaching out with the appropriate space between fingers and thumb when asked to grasp objects of different sizes. However, they *cannot* make these size-appropriate adjustments if there is a 2 second delay between the stimulus presentation and the reaching task (95, 144). Likewise, blindsighted subjects successfully reach around obstacles currently presented to their blindfield, but they cannot do this if a 2 second delay is interposed between being presented with the set-up and then performing the task (145). Similar effects of delay have also been reported for the somatosensory equivalent of blindsight, "numbsense", for both tactile and proprioceptive stimuli [(95) cited by (96)]: subjects can react appropriately only to current inputs, not recent ones whose properties require recall. Furthermore, specific sub-types of trace conditioning, in which the length of the delay is is learned, may also require awareness [see (33)], perhaps because similarly reliant on working memory (146). The role of awareness in human working memory is therefore under intense current investigation, as well as some debate [e.g., (147)]. Specifying particular tasks for animals is thus probably premature at this stage, but this does seem like an exciting research area for those interested in animal consciousness to follow.

One caveat, however: as normally run, working memory tasks typically involve affectively neutral sensory stimuli (e.g., visual cues). These are only relevant for fish and other animals if one is just interested in the general conscious perception of stimuli [cf. (32)]: P-consciousness in its broadest sense. But because of the componential nature of consciousness (see Introduction), such tasks are not useful if one is specifically interested in sentience [i.e., conscious affect, cf. (116)]. Thus, if in the future, certain working memory tasks were robustly validated as requiring the conscious awareness of, say, visual cues, they could then be used to probe whether fish have phenomenal sight: something disputed by those who believe fish have no P-consciousness at all (e.g., Key in prep., pers. comm.). That would be very useful. However, such tasks would *not* address whether fish can experience pain or other conscious affective states. Instead, operant learning might be more suitable for this type of question because of the central roles that valenced stimuli (punishers and rewards) play in the learning of these responses.

Broadly speaking, aspects of operant learning do seem to hold promise as indicators of P-consciousness: for some human operant tasks, both experiment [e.g., (132)] and self-reflection (e.g., recalling or imagining learning such skills as riding a bicycle [(25), p. 94; (37), p. 75]) suggest a role for awareness in their acquisition. As yet, there is also little evidence that S.P.U.D subjects can do this form of learning. This is therefore another highly relevant research topic to keep abreast of. But again, those interested in sentience specifically (not just perceptual aspects of P-consciousness) need to pay careful attention to whether awareness of the operant task, of any discriminative stimuli, and/or of the reinforcer and its affective significance, are being manipulated and assessed in these human studies [cf. e.g., (31, 75)]. Only tasks that manipulate the awareness of reinforcers (or punishers), and find effects, are candidates for identifying sentience, because only these are investigating the role of conscious affect.

Given this, we suggest three possible ways in which operant tasks might fruitfully be investigated for their reliance on sentience. We agree with Paul et al. (116) that "evidence that reinforcement learning in humans requires rewards and punishers to be experienced as conscious feeling states (i.e., of positively or negatively valenced affect) would help shed further light on the potential utility of inferring conscious animal affect from reinforcement". So, first, one obvious research question is: can humans learn novel operant tasks, from scratch, to gain rewards or avoid punishers of which they are not aware? To investigate this, we thus need to design an experiment to assess whether arbitrary responses can be conditioned *de novo* by subliminal reinforcers. Subjects could perhaps be presented with a variety of manipulanda (e.g., a lever, a push button and a switch). Interacting with one of these yields subliminal reward cues (e.g., imperceptible levels of a rewarding drug, masked smiling faces or piles of money, or incentive cues presented to the blindfields of blindsight subjects); interacting with another yields subliminal punishment cues (e.g., masked angry faces or images of trauma); and interacting with the third yields nothing. Given minimal instruction, and no explicit information on success, would subjects eventually acquire preferences for the positive operant and aversions to the negative operant? A related approach could be to see if a particular novel motor response could be "shaped" (by successive approximation) in naive human subjects by such subliminal reward cues, or perhaps even to see if the other types of S.P.U.D subjects could, despite the challenges involved, likewise be shaped to display novel, arbitrary operants. Failures would be consistent with sentience being necessary for operant learning, as hypothesized, while successes would show it to be unnecessary.

In the latter instance, drilling into *sub-types* of operant learning might then be more useful. Focussing on goal-directed forms of operant learning is one potential strategy. "Conscious awareness of a reward enables individuals to change the strategies they employ to attain that reward", claim Capa et al. (141). "The possibility remains that some forms of reinforcement-learning, such as goal-directed learning, do indeed require consciously experienced affect to occur“, suggest Paul et al. (116) more modestly. Technically, goal-directed learning is often defined as being sensitive to reinforcer devaluation [e.g., prior satiation of a subject with the particular food they are responding to: reviewed (35); see also (36)]. Now, work by Ziauddeen et al. (138) has already shown that subliminal incentive cues illustrating food winnings of pizza or pie *will* selectively modify the responding of pre-sated subjects. But would such effects of subliminal incentive hold for operants that were conditioned *de novo*, rather than performed to follow instructions? That seems as yet unknown. Other research approaches could be to ask whether subjects will respond flexibly to attain a subliminal reward, for example performing an operant with their mouth if they cannot use a limb, or overcoming obstacles to do so (e.g., moving away items blocking or obstructing the operant apparatus). These are the types of study that would reveal whether goal-directed operant responses do indeed require conscious affect.

Implicit here, especially in those last scenarios, is the idea that an operant response can become motivating and potentially even reinforcing in its own right. And indeed Ginsburg and Jablonka [(37), p. 231–233] propose that operant conditioning involves sentience if it involves second-order conditioning (e.g., the learning of compound sequences by successive chaining, in which each reinforced action becomes a secondary reinforcer that then conditions others). Whether this is correct seems unknown as yet: their idea needs testing. But it is empirically testable. For example, we have seen that decerebrate rats, and spines disconnected from brains, can display Pavlovian conditioning, and also learn responses to avoid shock. But if a neutral tactile cue was repeatedly paired with shock, could they then learn instrumental responses to avoid these CSs? In other words, can a CS become a secondary reinforcer without awareness? If Ginsburg and Jablonka are correct, then the answer would be no. Ginsburg and Jablonka's hypothesis could also readily be tested in humans, in paradigms manipulating awareness of reinforcers and/or awareness of potential CSs: if correct, humans would not be able to chain responses together for subliminal rewards, for example, nor learn operants for subliminal Pavlovian CSs. Such findings, were they to emerge, would be of huge importance, validating new tools for investigating sentience in animals.

The Self-Report of Sensation

A third deficit in unaware humans is that because of the self-reported lack of relevant sensation, subjects have little to no confidence in the validity of their responses to stimuli that they feel they cannot detect. This can manifest as an unwillingness to wager on choices dependent on those stimuli [even when they make those choices correctly at above-chance levels: e.g., (31, 148), p. 25–26]. So, can we develop tasks that ask animals to self report in ways that are as reliant on P-consciousness as subjective self-report in humans? First, we should clarify what is meant by "self-report". In human experiments, subjective self reports are elicited responses to requests to introspect, often manifest as freeform verbal descriptions, ticking off subjective states on a written checklist, or marking a Likert Scale. They thus involve arbitrary, learned responses, performed flexibly according to the specific context or task at hand, and their function is conveying information on internal state to an outside observer. Yelping or pulling away a hand from a hot item would thus not be deemed self report, because these are innate, stereotyped responses that would occur even without an audience.

One potential approach to ask animals to self-report how (or even if) they feel, is to see if they can use internal states as discriminative stimuli [(149), Mason et al. in prep]. This is a method widely used in psychoactive drug research, on both humans and animals. Here, subjects are trained to use the presence or absence of a drug-induced state as a cue that guides which of two operants will be reinforced (with food or money, depending on species). Such research reveals some compelling findings, especially for rats. For example, rats' abilities to use a drug as a discriminative stimulus (DS) in lever-pressing tasks are altered by states that in humans would make that drug easier or harder to detect. Thus their use of aspirin as a DS is enhanced if they have potentially painful arthritis (150); while their use of the anxiogenic drug "PTZ" as a DS is abolished by anti-anxiety drugs [e.g., (151)]. Rats also "generalize" between drugs that feel similar to humans, even when the chemical modes of action differ; for instance if trained to use having an alcohol hangover as a DS, rats then choose the hangover lever if subjected to morphine withdrawal (152). Similarly, rats generalize between drug and non-drug treatments that seem likely to have similar subjective effects. For instance, the rats trained to self-report alcohol hangovers also chose the hangover lever if exposed to "jetlag" [an 8 hr time shift: (152)], while rats trained to use PTZ as a DS would pick the PTZ lever if exposed a cat, as if self-reporting similar states of anxiety (153). Such powerful experimental data led Emmet-Oglesby et al. (154) to conclude that this paradigm "provides the most sensitive and accurate behavioral analog in animals to what humans verbally report about subjective drug experiences" [with similar conclusions from other authors in this field; Wood and Lal (155), for instance, argued that this type of animal test is "a bioassay for detecting subjective effects"]. Similar data for fish might therefore be highly persuasive.

But could such results merely reflect a "blindsight-like" guessing: a mere discrimination response that need not reflect underlying awareness? After all, as we have seen for S.P.U.D. subjects, decerebrated pigeons can use colored lights as DSs (128), and humans can use subliminal visual stimuli as DSs [e.g., (121)]. We think several refinements could reduce this risk. One is requiring a performance criterion that exceeds the rather poor discriminative responding shown by these unaware humans and birds [e.g., to require that $> 90\%$ responses are cued by the DS+, rather than the 60 or 70% that unaware subjects seem to achieve at best: (121, 128, 132–134), Skora pers.comm.; also Mason et al. in prep.]. To further increase the task's sensitivity to subjects' confidence levels, a second refinement could be to make incorrect guesses costly, and to include an option to "opt-out" [cf. the use of "commentary keys" when studying blindsighted humans [(32), p. 47–48, 230–231] and monkeys (156); see also the opt-out key used in metacognition research by Hampton (157)]. This would essentially ask animals "are you *sure* you sense something?". Finally, a third option is to mimic some creative recent work on corvid visual perception by Nieder et al. (158), in both imposing a post-stimulus delay before the task and also requiring subjects to select a response from a *choice* of possible actions. This respectively adds an element of working memory (as discussed above), and also requires subjects to use information about their states in a flexible manner [cf. eg., (62)]. We think that Nieder et al. (158)'s (unstated) assumption that their task specifically detected conscious perception is extremely plausible. But importantly, we also think the validity of this assumption is *testable*, for instance by running humans through an identical experiment (with people replacing crows in this visual task) while actually asking them what they can see. Were the resulting data to support Nieder et al.'s assumptions, modified versions of such a task could be powerful tools for accessing animals' subjective states. Thus, overall, refined experiments using internal states as discriminative stimuli in operant tasks could prove very useful for investigating animal sentience.

Tasks Reliant on Higher Order Consciousness

Turning to cognitive processes seemingly reliant on insight, higher order capacities like self-awareness are generally seen as reflecting P-consciousness (as reviewed in the Introduction). Passing experimental tests for self-reflection or self-awareness is therefore usually taken as evidence of P-consciousness. Below, we discuss two forms of such evidence – mirror self-recognition and episodic memory – and, following the same logic as the previous section, suggest how they might be made more relevant to sentience and thence the fish pain debate.

Mirror self-recognition, as assessed in the famous "mirror mark test", is one ability of great significance to those interested in P-consciousness. Gallup, who devised this test, sums up what passing it means: "the observer needs to come to the realization that it is their behavior that is the source of the behavior that is being depicted in the mirror" [(159), in a paper that also emphasizes the needs for careful controls in such work]. McFarland [(160), p. 132] also offers a nice analysis of this "kinaesthetic visual matching": "if an organism has this ability, it looks in a mirror and recognizes that the visual display in the mirror matches its kinaesthetic experience". In other words, parsing this out into components, to pass this test a subject must have both visual awareness and proprioceptive awareness, as well as the cognitive capacity to spot the contingency between their own movements and their reflection's. Thus, it need not indicate something as grand as a "concept of self" [(25), p. 265], but it still relies on P-consciousness.

So, since cleaner wrasse (*Labroides dimidiatus*) have this ability (161, 162), this is strong evidence of both visual and proprioceptive awareness that, as discussed in the section Tasks Involving Working Memory and Aspects of Operant Conditioning, refutes those who argue that fish have no P-consciousness at all. But this does not yet indicate that the wrasse have a capacity to feel pain or be sentient. To achieve *this*, we therefore need a new type of mirror experiment that is explicitly designed to probe affective states. That is a challenging task, but one approach might be what we call the "mirror test with biting parasite", in which the mark is not affectively neutral. For example, the subject is exposed to a mirror, to provide opportunities for kinaesthetic visual matching. The subject is then repeatedly exposed to two marks, differing in color or shape, placed on their body in a location they can only see via their reflection (e.g., the head). To add the affective component, one of these (let's say a black triangle; obviously this would be counterbalanced across subjects) is rendered uncomfortable or even painful; the other (let's say a white circle) is not. After several such trials, a mark (now without any associated discomfort) is then placed on the animal's body in a location it *can* see (e.g., its tail, for many fish) (with controls involving presenting the same mark elsewhere, e.g., on an object or the side of the tank). The question would then be, does the subject react differently to the previously nasty black triangle, vs. to the previously benign white circle, when placed on its own body?

Likewise, evidence for episodic memory, the "what, where and when" of past events, is seen as reliant on the flexible use of information about sensations, locations and the passage of time, in a "conscious experience of recollecting" [e.g., (37), p. 440–441], or what Birch et al. (36) call "conscious mental time travel" [see also (33)]. In an elegant experiment on rats, for instance, Ergorul and Eichenbaum (163), taught animals to recall and effectively report on single training episodes, each composed of a series of four odors presented in different places on an open field. The rats were then probed for their abilities to flexibly use different aspects of their experience to solve a new task. Thus, after each training episode, in a probe test differing in format from this episode, rats were offered a choice of two of the four stimuli just presented, chosen at random (either two odor-location pairings, or just two locations, or just two odors), and rewarded for picking the stimulus that had occurred the earliest in the preceding sequence of four. Rats could pass this test. They thus remembered the order of events in unique experiences, and from this flexibly extracted combinations of odor and place information. This, the authors argue, is "consistent with current characterizations of human episodic memory as the capacity to 'replay' memories as a sequence of events and where they occurred in a previous experience" (although they carefully note this does not prove that rats have the subjective experiences that characterize episodic memory in humans). Again, running humans through identical experiments would be a good way to *test* the intuition that such tasks can only be passed with conscious recall, for instance by seeing if the order of subliminal cues cannot be "replayed" to win a reward (while only that of supraliminal cues can be). But for now, if we assume that this *is* good evidence of P-consciousness of odor, location and the passage of time, then if fish convincingly passed such a test [c.f. (164, 165)], this would reveal that they too have similar capacities. Again, this would defy those who argue that fish do not have the brains for any forms of awareness, but not help those specifically interested in affective, evaluative aspects like pain.

To use episodic memory experiments relevant to sentience specifically, once more we therefore need specifically designed tests that deliberately incorporate an affective component. For example, a "what, where, and *in which state*?" version of Ergorul and Eichenbaum's (163) task might expose animals to locations or sensory cues (e.g., odors) while induced to be in different affective states: perhaps different states of food deprivation (thence potential hunger), different threats of injury (thence potential pain), or differential exposure to rewarding brain stimulation via electrodes or drugs (thence potential pleasure). The probe test could then ask such questions as, can the subjects successfully identify which of two stimuli (locations or odors) had been presented while they were in more negative affective states, versus while they were in more positive states?

WHY IS SENTIENCE ETHICALLY RELEVANT AND WHAT ARE THE CONSEQUENCES OF RECOGNIZING IT?

As Birch et al. (54) sum up, "if a being is sentient, there are limits on what a human can ethically do to that being". And currently, fish experience practices that are likely to cause suffering, if they are aware. These include shark-finning, live sushi ("ikezukuri"), and globally, the wild-capture fishing that causes significant bodily injury [e.g., (166, 167)] and affects billions of individual fish (168, 169). So, given uncertainty about fish sentience, what should be done? Some argue that the potential feelings of fish should play no role in their protection, concerned that this could incur costs to industry, research and consumers which – if fish are actually *non*-sentient – would be needless. Thus, Browman et al. (16) worry that "the impact of increasing welfare-related constraints on aquaculture… will leave society less able to produce high-quality protein to feed a still-growing global population". Indeed this "appeal to consequences" seems to be why, instead of being agnostic, such authors argue that fish are definitely non-sentient [e.g., (6, 16, 170)]. Yet these authors *also* argue that this need not mean that fish are unprotected: that an animal's welfare can be considered separately from whether it is sentient, such that even non-sentient beings should well be cared for. So in this section we ask, is sentience important, or not? And does declaring an organism sentient impose enormous costs on humans?

For many ethicists, sentience *is* crucial for determining whether an animal is entitled to moral consideration of its welfare [e.g., (51–53, 171)]. Varner (172), for example, summarizes a utilitarian ideal of maximizing "aggregate happiness", from which it follows that moral concern should extend to anything capable of happiness or its opposite, suffering. But even outside utilitarianism, sentience can be held as the key prerequisite for moral concern. For example, DeGrazia [who defines sentience as the ability to have pleasant or unpleasant experiences: e.g., (173)] states: "only beings with interests have moral status, and only sentient beings—who, by definition, have certain mental states—have interests" (53). Consistent with this, much legislation and policy confers special status to sentient organisms. Thus, in Canada, research animals are protected by the CCAC (Canadian Council for Animal Care), but there is no "CCPC" for plants used in research; and for agricultural species there is a National Farm Animal Care Council (NFACC), but no "NFPCC" for crops. Sometimes policy-makers lay out the crucial role of sentience explicitly. The EU, for example, requires its member states to "pay full regard to the welfare requirements of animals" [using a definition of welfare that prioritizes affective states: (174)]

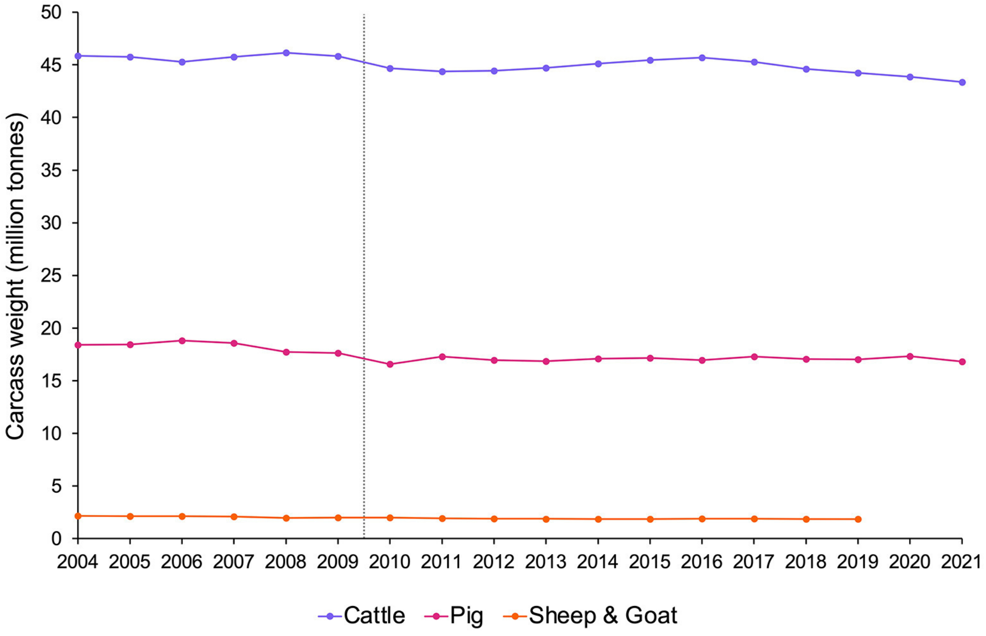

FIGURE 3 | Meat production (million tonnes of carcass weight) in EU countries from 2004–2021 by species, estimated from Eurostat data [sources: (apro_mt_lscatl), (apro_mt_lspig), (apro_mt_lssheep), and (apro_mt_lsgoat)]. The grey dotted line indicates the point at which the Treaty of Lisbon recognized that all animals are sentient (2009). Note that after the Treaty of Lisbon was signed, although animal protections subsequently changed, meat production levels remained the same.

since "animals are sentient beings" (175); and Australia's Animal Welfare Strategy states that "sentience is the reason that welfare matters" [also noting that animal welfare reflects the ethical imperative to minimize suffering and consider quality of life: (176)]. In other cases, the role of sentience is instead implied via the use of terms reliant on conscious affective states, such as "distress", "suffering", and "humane treatment". For example in Canada, the CCAC "utilizes affective states as the primary determinant of animal welfare" (177), while internationally, the World Organization for Animal Health (OIE) says that "an animal experiences good welfare if the animal is… not suffering from unpleasant states such as pain, fear and distress" (178). Thus if fish are deemed sentient, this *does matter*: it means they *should* be treated differently from plants, and given extra protection as are mammals and birds.

But what if we do not know? "It is sometimes necessary to act on the basis of evidence that does not deliver complete certainty" (54), and when there is scientific doubt about whether the animals covered by these policies are sentient, legislators often implement versions of the "precautionary principle". This is an approach used to reduce risk when relevant scientific evidence is uncertain or incomplete, especially in cases that may be near a tipping point [e.g., (33, 179)]. For fish, this means balancing the potentially needless cost to industry, research, consumers, etc. that could result from assuming fish *are* sentient when they *are not*, against the risks of significant and potentially unnecessary animal suffering that could result from assuming fish *are not* sentient when they *are*. For many ethicists, policymakers, and welfare scientists (including Victoria Braithwaite, and ourselves) [e.g., (23, 180–182)], the latter's moral weight tips the balance. Will this compromise human interests, as some worry? In an attempt to reassure those concerned about this, **Figure 3** presents evidence that recognizing sentience need not impact the numbers of animals used by humans: the Treaty of Lisbon (175) was *not* followed by declining meat production (183), although animal *well-being* is increasingly well-protected by law in EU countries.

DISCUSSION AND CONCLUSIONS: APPLYING OUR APPROACH TO THE FISH PAIN DEBATE

What *is* it like to be a bass? Some claim that fish have rich experiential lives, able to feel pain, fear, and possibly joy [e.g., (12, 13, 24, 184, 185)]. Others claim that fish are essentially unconscious zombies: that being a bass is like nothing, because fish have no phenomenal experience, not even sight [(6); Key in prep., pers. comm.]. The "fish pain" debate is thus rather polarized. Furthermore, discussion and comment still outweigh new data: only 43% of the citers of Sneddon et al. (9) are experimental papers. This research inertia may stem, at least in part, from the untested assumptions of both "sides". And of course, it also reflects that the whole broad field of P-consciousness is extremely challenging, rife with complexity, debate, and struggles to tackle what is infamously known as "the hard problem".

As we have reviewed, P-consciousness is currently impossible to measure, and impossible to assess in non-humans. Indeed even in humans, assessment is imperfect, relying on the veracity and accuracy of self-report. This makes the claims at both these extremes too strong – and the high levels of agnosticism about fish abilities to feel pain, appropriate. Perfect, "bullet-proof" diagnostic markers of sentience simply do not exist, at least as yet. Elwood (186) therefore lamented, "the idea of feelings or consciousness … is impossible to access, and [leads to] arguments that cannot be resolved". We are sympathetic to this, but somewhat more optimistic. We believe one argument that *can* be resolved is whether sentience matters: it does. Another is whether categorizing fish as sentient will destroy human livelihoods: European data suggest the resulting protections would not necessarily radically hinder animal use (something some may find reassuring, but others, sad). A third argument that we think can be resolved is whether or not to withhold protecting fish until we know they are sentient. The precautionary principle, and the great potential harms done to fish, both indicate that inaction would be ethically worrying; agnosticism about fish sentience is therefore consistent with supporting practical guidelines that choose to protect fish. Indeed, where practices risk extreme pain, we suggest that the guiding question should perhaps not be "is there evidence that this species is sentient?" but instead "are we sure it is not?" Finally, we suggest that empirical research on animal sentience can advance, doing so faster and more constructively, if it is deemed reasonable to treat plants and protozoa, spines disconnected from brains, decerebrate animals and unaware humans (i.e., S.P.U.D. subjects) as not conscious; to treat human self-report as a "gold standard" (as many consciousness researchers do, despite even this being imperfect); and to treat perceptual and evaluative dimensions of P-consciousness as separate and dissociable. We recommend that the questions *"Can S.P.U.D subjects do this?"* and *"In humans, does this always correlate with self-reported feelings, and if so, what type?"* are used to screen all potential indicators of animal P-consciousness. Answering these questions will weed out "red herring" measures that fail to distinguish between the sentient and non-sentient, identify types of indicator that best permit strong inference [e.g., *sensu* (33)], and so assist with both data interpretation and designing new studies.

Applying this approach to current "fish pain" data is revealing: many measures do not survive this screen. For one, S.P.U.D. subjects show diverse unconditioned behavioral responses to noxious stimuli, including avoidance or wound attendance, making it hard to argue that similar responses in fish require awareness or demonstrate true pain rather than mere nociception [cf. e.g., (61, 187), citing (188) on rocking in trout and (189) on tail-beating in zebrafish]. Furthermore, in S.P.U.D. subjects such responses can be modulated, including by analgesics. These means that contrary to several authors [c.f. e.g., (61, 187), citing (188, 190)], the modulation of fish responses to noxious stimuli by analgesics *cannot* be said to indicate pain over nociception; and the same applies to their modulation by food deprivation [c.f. e.g., (61, 187), citing (191)] or conspecific presence [c.f. e.g., (61, 187), citing (192)]. Furthermore, nor does the conditioning of escape responses to locations where a shock was delivered [c.f. e.g., (61), citing (192)], or of approach responses toward locations where nociceptive input is reduced by analgesics [c.f. e.g., (61, 193), reporting unpublished work on zebrafish], demonstrate pain rather than nociception: S.P.U.D subjects are similarly capable of Pavlovian conditioning. This includes trace conditioning in its broadest sense, often erroneously held up as a marker of P-consciousness [c.f. e.g., (194), citing (195)]: this too occurs in S.P.U.D subjects. Braithwaite and colleagues were therefore right to conclude, in one of Victoria's last papers, "trace conditioning is widespread and by itself does not indicate consciousness" (15), a conclusion echoed in this Special Topic collection by Droege et al. (143). And the same also holds for instrumental learning, where pre-existing innate responses change in timing or form to become more effective (e.g., at avoiding punishment). This means that, for instance, shuttlebox learning by fish, where escape responses become directed to particular locations to avoid shock [e.g., (196)], is also not proof of awareness or pain.

Now, authors using such responses to infer pain typically present lists of multiple different responses, to be treated as more convincing if demonstrated *en masse* [e.g., (197) p. 52; (61, 187, 193); see also (33) "theory neutral" approach]. At first this seems reasonable. However, attributes or responses that are as consistent with a lack of awareness as they are with P-consciousness can have little or no value for inferring true pain, regardless of how numerous they are. Of course, some measures might still be useful if their *absence* is revealing; in other words if they are deemed *necessary* for inferring P-consciousness. If a species of fish fails to have nociceptors, for instance [as seems true for at least some elasmobranchs: reviewed by Rose et al. (6), Sneddon (198), Smith and Lewin (199)], then perhaps logically, this is evidence that they cannot feel pain? One challenge would be knowing which these necessary indicators are; and another, distinguishing between true negatives and Type II errors. Nevertheless, thinking more formally about *necessary conditions* for various forms of animal consciousness (as well as the still elusive *sufficient* conditions), and parsing these out clearly, would be useful, not least for making assumptions more explicit than they often are.

In contrast, identifying responses that S.P.U.D subjects seem *unable* to make and ones that in contrast, at least in humans seem to require awareness, highlights other indicators as more useful: better able to permit strong inference (even deduction, if validated using the experiments we suggest). These are measures based on higher order abilities, the self-report of state, working memory, and aspects of operant conditioning. So, what might such indicators reveal about fish?

The apparent intelligence of some fish species [e.g., tool use by tuskfish (200), cooperative hunting by moray eels and groupers (201), numerical competency in angelfish (202), and the mirror self-recognition and episodic memory tasks mentioned above] has sometimes been taken as already sufficient evidence for sentience [e.g., (18)]. For two reasons, we believe this is premature. First, as Massimini and Tononi argue [(45), p. 153], "the mere fact that a behavioral repertoire is complex and "cognitively sophisticated" is not sufficient to clinch the case". Second, as we have reviewed here, the perceptual and evaluative dimensions of P-consciousness should be considered distinct [following (36)], and these tasks typically provide better evidence of the former than the latter. Nevertheless, the apparent intelligence of such species does bode well for tasks specifically designed to probe sentience, making this an exciting area for future work. Thus, in the section What *Could* Be Evidence of P-Consciousness and, More Narrowly, Sentience?, we suggested modifying existing tests for self-awareness and episodic memory, in order to make them sensitive to sentience (not just the visual, proprioceptive and temporal P-consciousness that they rely on in their current forms). We hope our outlines for a "mirror test with biting parasite", and for a "what, where, and in which state?" episodic memory task, indicate how such paradigms could be tweaked – both in new assumption-testing experiments on humans, and in new experiments with animals. In that same section, we also suggested ways in which self-report paradigms could be developed for animals, including fish (since when animals are trained to use their internal states as discriminative stimuli, highly revealing insights can emerge, as the drug discrimination literature reveals). We believe there are ways to avoid such tasks' risks of just capturing blindsight-like guessing with no awareness, and again, that the intelligence of some fish bodes well for applying these. Were such affect-sensitive higher-order tasks applied to fish (especially once better validated), we would therefore recommend starting with these species with impressive cognitive abilities.

What about other, seemingly less impressive cognitive abilities? We and others [e.g., (116)] have suggested operant learning as a relevant topic to explore. Our own suggestions about operating learning arise partly from looking for tasks that S.P.U.D. subjects seem unable to do. Of course, absence of evidence is not evidence of absence: the lack of data from S.P.U.D. subjects on the learning of arbitrary operants could reflect a lack of research effort, not a failure of such attempts to work. This therefore identifies another topic for future research, using both S.P.U.D. subjects and humans whose awareness of reinforcers is manipulated: the assessment of whether operant behavior relies on reinforcers producing conscious affective states. (Though note that for such work, we suggest using plants and temporarily unaware humans over highly invasive decerebrate or spinally-transected animals). If this hypothesis was supported, then at least some fish have already shown that they are sentient. These include Siamese fighting fish trained to swim through hoops to display to rivals [e.g., (203)], barramundi (*Lates calcarifer*) trained to touch arbitrary targets (204) and goldfish trained to bump a lever for food (205), as well as all farmed species that use demand feeders to deliver pellets.

More stringently, it could be that not all operant learning requires sentience, but that only some sub-types do. Thus, it could be that Ginsburg and Jablonka's (37) hypothesis about second-order conditioning is correct: again something not yet known, but amenable to empirical test. If their hypothesis is supported, then again there are already some cases demonstrating conditioned responses to secondary reinforcers by fish. In one, for example, Foerder (204) successfully used shaping to train a barramundi to swim 10 feet to touch a secondary reinforcer (a target previously paired with food). In another, an extraordinary experiment demonstrated the abilities of goldfish to learn to jump over a hurdle to modify a CS (lights that must either match or not), in order for this CS to predict no shock rather than shock (206). Thus, if future research does show that second-order conditioning relies on the conscious awareness of reinforcers, then these fish are displaying good evidence for positive affect and pain, respectively. What about the other sub-type of operant learning that we highlighted: goal-directed? It is not yet known whether goal-directed operant responding for food, say, require this reward to induce consciously-experienced positive states, but again this idea is testable. And were the hypothesis supported, then species with flexible forms of foraging, like those using the cooperation and tool use mentioned above, seem ideal for formally investigating whether they can show goal-directed operant behavior, in novel experiments specifically designed to assess this ability.

Operant learning is just one of the many topics tackled in considerable ongoing research by human consciousness researchers (with the use of discriminative stimuli, and working memory being others). We recommend following this fascinating work, for example by attending meetings such those hosted by the *Association for the Scientific Study of Consciousness*: learning about this complex, fast-moving human research is both useful and humbling. Furthermore, increased contact with and interest from those working on animal welfare might encourage such researchers to focus more on understanding sentience (since currently, most of their research efforts focus on perceptual forms of P-consciousness). We hope that our suggestions represent a constructive contribution to the deeply interesting, important, but sometimes frustrating field of animal consciousness. Inspired by Victoria, one of the first to wade into this fray, we look forward to the refinement of these ideas and improved methodologies for probing the puzzle of animal sentience.

AUTHOR CONTRIBUTIONS

GM and JL participated in the development and writing of this manuscript. GM developed novel experimental paradigms for probing affective sentience and reviewed "red herring" S.P.U.D. subject responses that do not require sentience. JL reviewed the ethical context of sentience and the fish pain literature. All authors contributed to the article and approved the submitted version.

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Curiosity in zebrafish (*Danio rerio*)? Behavioral responses to 30 novel objects

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Curiosity—the motivation to seek out information—has been studied widely across the animal kingdom. To investigate curiosity in zebrafish we presented 30 novel objects to groups of zebrafish housed in semi-naturalistic tanks (6 tanks; 10 fish/tank; 10-min presentations). During the first 100 s and final 100 s of each object's 10-min presentation period, we recorded each group's: (i) latency to approach the object, (ii) attraction to the object, (iii) social dynamics: agonistic behavior and group cohesion and coordination, and (iv) diving behavior, a stress response in zebrafish. Comparing these behaviors to a 100 s baseline period when no object was present, we tested for neophobia (avoidance of novelty), neophilia (overall attraction to novelty), sustained interest (prolonged attraction to at least some presentations), discriminant interest (certain objects eliciting more attention than others), habituation (loss of interest over time), and alterations to social and stress behaviors. Zebrafish groups readily approached all objects (1 s median latency), were neophilic throughout all object presentations, and showed systematic sustained interest only for some object presentations at the beginning of the study (object presentations 1–10). Over the course of the study, zebrafish also showed signs of habituation such that by the final ten object presentations (21-30), there were no signs of overall sustained interest. During the beginning of the study (object presentations 1–10), we also found evidence for specific object-driven interest, with object ID accounting for 11% of the variability in interest scores ($p < 0.01$), and object-driven interest corresponding to alterations in social behavior: decreased aggression ($p < 0.02$), increased group cohesion ($p < 0.02$), and increased group coordination ($p < 0.05$). By explicitly investigating curiosity in fish, this work reveals that under certain conditions, zebrafish voluntarily engage in cognitive stimulation opportunities. More work is needed to clarify what types of information zebrafish find most rewarding and how long-term exposure to such opportunities may affect fish welfare.

KEYWORDS

exploratory motivation, cognitive enrichment, naturalistic housing, fish welfare, positive welfare, animal agency

Introduction

"Curiosity has its own reason for existing." -Albert Einstein Old Man's Advice to Youth: "Never Lose a Holy Curiosity". Life Magazine (2 May 1955) p. 64

In its purest form, curiosity refers to the drive to gain information in the absence of clear instrumental goals such as food or shelter (1). From a behavioral biology perspective, curiosity is understood to have evolutionary value in that gaining information may ultimately enable an individual to better exploit resources and manage threats (2). In the immediate-term, however, information-seeking itself can become its own reward and is sometimes prioritized over clear, material gains (1). As such, curiosity has fascinated scholars throughout history, especially in the middle of the 20th century when psychologists and ethologists intensively studied curiosity in species across the animal kingdom (3–5). The past decade has seen a resurgence of interest in the study of curiosity across multiple disciplines, with investigations of curiosity in a range of species including raptors (6), tortoises (7), orangutans (8), honeybees (9), and in one multispecies study, sheep, penguins, lemurs, cockatoos, tortoises, and kangaroos (10).

To understand what features may drive curiosity, Loewenstein (11) proposed that information seeking behavior is elicited when there is an information gap between what is known and what is unknown, without going beyond the cognitive capacity of the individual. Two ways for perceptual objects to have enhanced information potential are by (i) being complex or (ii) being unusual. From a visual perspective, complex objects are those with many parts, shapes, patterns, and/or colors. Previous work has confirmed that visual complexity attracts greater attention from, for example, raptors (6), rats (12), and humans (13). Unusual objects are either ones that are very different from those that the individual has experienced in its own lifetime or, from an evolutionary perspective, those which would not normally be encountered in the ecological niche of that species—in other words, objects that are ecologically implausible. Recent work in orangutans, for example, has shown that they are particularly interested in unusual, ecologically implausible objects (8). Interestingly, some prey animals are also known to be highly motivated to gather information about predators, readily inspecting potential predators despite the evident risks (14, 15). Thus, previous theoretical and empirical work suggests that complexity, unusualness, and predatory potential are all relevant dimensions in eliciting perceptual curiosity.

As these proposed curiosity-eliciting dimensions indicate, experiencing curiosity and curiosity-producing situations are ambivalently charged—they have the possibility of taking on a positive or negative valence, thus complicating the relationship between curiosity and welfare. Human studies have found that heightened curiosity can be associated with positive affect and wellbeing [e.g., (16)], but also frustration (17, 18), sensation-seeking, boredom-avoidance, and substance abuse (19). When measured as exploratory behavior, curiosity's bivalent nature is evident in nonhuman animals as well. On the one hand, opportunities for exploration and exploratory behavior are associated with improved welfare and cognition (20–23). For example, rats maintained in complex social housing were found to have enhanced exploratory tendencies (giving up known rewards and risking aversive conditions in order to explore new spaces) compared to isolated rats in barren cages (24). Conversely, other forms of exploration have been associated with poor animal welfare. For example, undifferentiated exploratory behavior of novel stimuli (i.e., neophilia) in mink was more apparent in individuals housed in non-enriched cages compared to those housed in enriched cages (25, 26).

These and other studies suggests that exploratory behavior in the form of indiscriminate neophilia (quick, fleeting exploratory behavior) may be reflective of an avoidance motivation (escaping aversive or fear-inducing conditions), especially in situations likely to induce boredom. Boredom, a negative state caused by a lack of behavioral opportunities, is identified as a serious welfare concern (27) and an important area of study in animal behavior (28). In contrast, exploratory behavior in the form of free-choice, targeted, and sustained information seeking has been associated with positive wellbeing in both humans and other animals (16, 22, 23). Distinguishing between these two types of exploratory behavior—indiscriminate neophilia vs. targeted and sustained information-seeking—is necessary when attempting to understand the relationship between curiosity and current welfare state.

By investigating curiosity in zebrafish (*Danio rerio*), the present study aimed to provide crucial data for interdisciplinary, comparative work looking at curiosity and welfare across the animal kingdom. Knowledge about curiosity in zebrafish represents a particularly useful starting place for studying curiosity in fish because it can indicate whether similar issues may be at stake for other species of fish, including the welfare of fish in aquaculture (29). As one of the most studied species of fish (30, 31), information about zebrafish capacities and interests has the potential to set expectations for fishes in general and can influence fish welfare standards. Moreover, as a member of the *Cyprinidae* family, zebrafish can provide insights into patterns of behavior and motivation for *Cyprinidae*, one of the most farmed families of fishes (32).

By characterizing the exploratory behavior of groups of zebrafish toward novel objects, we hypothesized that initial interest could range from neophobic (manifesting as avoidance and fear responses), to indifference (little to no behavioral changes in response to the objects), to curiosity (exploration of and attraction to the objects).

We further distinguished between indiscriminate neophilia and targeted information-seeking. We operationalized indiscriminate neophilia as fleeting and uniform or haphazard interest across objects, i.e., short, irregular, nondifferentiated interest durations. In contrast, we operationalized targeted information-seeking behavior as sustained, differential attention, i.e., the presence of distinctive and consistently high interest for protracted durations of time for only some object presentations.

Pending evidence of targeted information-seeking behavior, we also sought to determine whether there was consistency across tanks as to which objects attracted the most sustained attention. General consistency in object-level attraction across tanks would indicate that some perceptual property of the objects themselves were driving the differential interest. Accordingly, we aimed to select objects that varied in complexity, ecological plausibility, and predator resemblance, allowing us to assess (i) which of these dimensions might produce the most sustained information seeing, (ii) how the introduction of objects may affect welfare, and (iii) what object properties might provide appropriate cognitive enrichment for this species (31).

To achieve these aims, we observed the behavior of groups of zebrafish toward a range of 30 different novel objects presented one at a time in their home tanks. The zebrafish were housed in relatively large (110 L), semi-naturalistic aquaria that could accommodate multiple behaviors, thus providing the fish with a low-stress, free-choice (explore or not) set up that allowed them to engage with a particular novel object or not. In addition to object-directed behavior, we also recorded aggression, shoal cohesion, swimming coordination, and diving behavior during a baseline period without a novel object and while the object was in the tank. Zebrafish social dynamics are sensitive to environmental manipulations (33), with recent empirical work suggesting that free-choice exploration can increase prosocial behavior and decrease diving (34), whereas potential stressors have the reverse effects (35). Together, this suite of behaviors can provide indications of information-seeking motivation in zebrafish as well as preliminary indications as to the valence (positivity/negativity) of the zebrafishes' response to the novel object exploration opportunities.

Materials and methods

Ethics statement

All husbandry and experimental procedures were approved by the University of British Columbia's Animal Care Committee, protocol number A14-0119.

Animals and housing

Sixty adult (> 4 months of age) wild-type, short-fin zebrafish (*Danio rerio*) were purchased from a local pet store (Vancouver, BC). Three months prior to the start of the experiment and throughout the experiment itself, fish were housed *via* random assignment into mixed-sex groups of 10 fish per group in stand-alone tanks furnished with sloping gravel substrate, rocks, artificial plants, and wrapped black plastic on three sides to minimize visual disturbance (fish/tank = 10; tank: $n = 6$, 110 L, 30 cm x 91 cm X 40 cm; see Figure 1). Tanks were the experimental unit in this study and were placed on a rack with two tanks per shelf, such that the top two and bottom two tanks had the slope on the right, the middle two tanks had the slope on the left. Zebrafish were fed to satiation with standard flake food (Nutrafin Max Tropical Fish Flakes, Hagen, Canada) twice per day. Room lights were on a 12L:12D schedule with tank lights turning on an hour after room lights and turning off an hour before room lights to graduate light-intensity changes. Water was kept at 26–28 degrees Celsius. Twenty percent water changes were performed once a week, followed by water quality checks 2 days later to confirm minimal ammonia (<0.1 ppm), nitrate (<20 ppm), nitrite (<0.25 ppm), and a pH of 7. After the conclusion of this research, the fish were adopted into private homes.

Experimental procedures

All procedures were conducted in the home tanks. Over the period of a month on 16 test days, thirty different novel objects (see Appendix) were inserted into the deep end of the tank (see Figure 1), one-at-a-time for a period of 10 min each with at least 30 min between presentations. Objects were fixed to the lid of the tank *via* a clear plastic rod so that they were suspended ~2 cm below the water surface. After positioning the object, the experimenter retreated to a corner of the room out of sight of the fish. Fish behavior was video recorded *via* video cameras (Swann NVR8-7200; resolution: 1000 TVL/1080p) fastened 1 meter in front of each tank.

Video recordings for each tank-object combination were edited to create 100-s clips for each of 3 observation periods: baseline (an hour or more before object presentation), first-100 s (from when the object was placed in the tank for the next 100 s), and final-100 s (the last 100 s before the object was removed from the tank). All videos were overlaid with two outlines indicating (i) the area within two-body lengths of the object and (ii) the area within four-body lengths of the object (see Figure 1). Only the first area (within two body-lengths) was used in the analyses. Thirty objects, six tanks, and three observation periods resulted in 540 video clips; however, 18 clips could not be used due to technical issues with the video, leaving 522 clips for analysis.

FIGURE 1
Example zebrafish tank provided from stills of video data collected during **(A)** baseline and **(B)** corresponding object presentation. Two tanks were set-up with this orientation and four tanks were set-up in the reverse direction (slope on the right and deep-end/object presentation area on the left). The baseline period **(A)** was defined as at least 1 h before a novel object was introduced into the tank. The concentric outlines around the object in panel **(B)** are overlain on both videos to indicate the areas within approximately two zebrafish body-lengths from the object and four zebrafish body-lengths from the object. Fish presence in the inner area (two body-lengths) was used for the analyses. Each object-tank combination had its own outline to control for differences in object size and shape so that interest scores could be calculated as the number of fish in the inner area beyond the number observed during baseline: interest score = fish in inner area$_{\text{object presentation}}$ − fish in inner area$_{\text{baseline}}$.

Objects (all made of inert and insoluble materials) were selected by human coders in an attempt to capture a range of characteristics along non-orthogonal dimensions of complexity, ecological plausibility, and predator resemblance. Power analysis indicated that a sample of 30 novel objects would allow us to detect correlations of at least 0.50 between object dimensions and zebrafish interest with ∼80% power. Eight coders with varying degrees of zebrafish expertise (from none to 10+ years of experience) showed a high degree of inter-rater reliability in their characterization of the objects along these dimensions (Cronbach alpha > 0.85), which allowed us to average their ratings together to create one composite score for each object along each human-generated dimension.

All tanks were exposed to all objects in a pseudo-randomized order across the entire study. The order could not be fully randomized as object presentation periods occurred simultaneously for the six tanks and we only had one version of each object. However, the pseudo-randomization schedule ensured that all objects were presented equally during the beginning, middle, and end of the study.

Behavioral coding and scores

All behavior was coded from the video clips in a randomized order by observers who were blind to condition and study predictions and trained to a minimum of 0.70 inter-rater reliability. Approach latency was scored as the time in seconds until the first fish entered the inner object area, i.e., approached the object within two body-lengths. Scan samples of the number of fish in the inner area, all social behaviors (agonism, cohesion, and coordination), and diving behavior were recorded every 10 s (ten scores per video clip) and then averaged to create a single score for each tank-object observation period. Agonistic behavior—chasing, charging, fleeing, biting, and lateral displays—was coded as present/absent. Cohesion—the degree to which fish occupied the same space—was coded on a scale from 0 (uniformly spread out) to 4 (tightly clumped). Coordination—the degree to which fish were swimming in the same direction—was coded on a scale from 0 (swimming in all different directions) to 4 (all fish swimming in the same direction). Diving behavior was scored as the proportion of visible fish near the gravel (in the bottom third of the tank) vs. higher in the water column. Previous studies used this methodology and describe it in more detail with video examples [see (34)].

We coded zebrafish behaviors during the first 100 seconds (first-100 s) and the final 100 s (final-100 s) of the 10-min object presentation. We also coded 100 s of behavior during a baseline period, ∼1 h before when there was no object present. It is also important to note that these behaviors are not mutually exclusive, do not constitute an exhaustive ethogram, and can, in principle, vary independently of each other—e.g., agonism can increase or decrease along with increased fish presence in the inner area, coordination can increase or decrease along with an increased proportion of fish diving to the lower parts of the water column.

All behaviors scores were calculated for statistical modeling as a difference between the average level of behavior across scan samples for a tank during an object presentation period (either during the first-100 s or the final-100 s) and the average level of behavior across scan samples for that tank during that object's baseline period. For example, if we consider tank 4, object presentation number 4 and hypothetically pose that we found (i) an average of 2.2 fish in the inner area during baseline (across the 10 scan samples), (ii) an average of 7.7 fish in the inner area during the first-100 s (across the 10 scan samples), and (iii) an average of 3.3 fish in the inner area during the final-100 s (across the 10 scan samples). These data would yield an interest score of 5.5 for the first 100-s (interest score = 7.7–2.2) and 1.1 for the final-100 s (interest score = 3.3–2.2). In general, interest scores could range continuously from −10 to +10 because the average number of fish in the inner area could range from 0 to 10. Agonistic behavior scores and diving behavior scores could range continuously from −1 to +1 because they were calculated as proportions (i.e., 0 to 1; proportion of scan samples with agonistic behavior and proportion of fish in the bottom of the water column). Cohesion scores and coordination scores could range continuously from −4 to +4 because they were coded on scales from 0 to 4.

Data analyses

We treated tank as the unit of analysis ($n = 6$). With repeated measures of tanks and objects, our general approach to account for pseudoreplication was to apply multilevel models (identity link, Gaussian error structure) to control for crossed-random effects of both tank and object (36, 37). Each model contained the continuous outcome variable of interest (e.g., interest score, agonistic behavior score), at least one fixed effect to test study predictions (e.g., object presentation period), and had crossed-random effects of tank ID and object ID. This modeling approach allowed us to treat the tank as the unit of analysis while also controlling for repeated sampling of tanks and objects across the study duration. We used the Satterthwaite method to calculate approximate degrees of freedom for the t-statistics of the fixed-effects null-hypothesis testing.

To assess changes to behavior during object presentation periods vs. the baseline period, outcome variables included: interest scores, social behaviors, and diving behavior. To determine the consistency of differential sustained interest, we modeled interest scores during the final-100s as the outcome and the corresponding interest scores during the first-100 s as a fixed effect predictor (with tank and object as crossed-random effects). Variability in interest was assessed with Likelihood Ratio Tests (36, 37) comparing a base model with tank but not object (or object but not tank) as random effects to an augmented model that included both object and tank as crossed-random effects. These model comparisons test whether object (or tank) ID can account for a significant proportion of the variability in interest-scores.

All data manipulation, plotting, and statistical analyses were conducted with R (38) in RStudio (39), using the following packages: tidyverse (40), psych (41), gridExtra (42), lme4 (43), and lmerTest (44).

Results

Zebrafish began exploring objects almost immediately with a median approach latency of <1 s, a maximum approach latency of 48 s, and 95% of all first approaches occurring within 6 s.

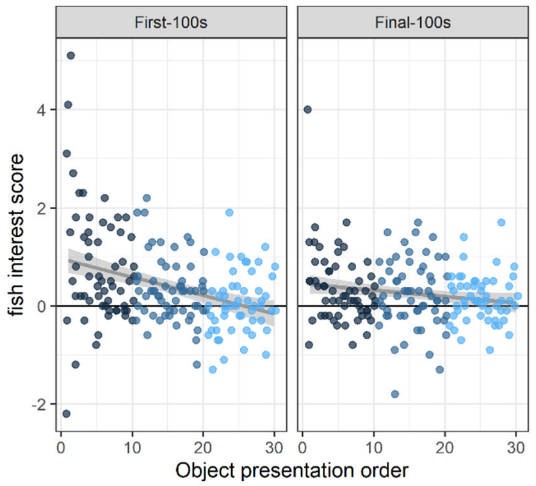

FIGURE 2
Interest across novel object presentations. Fish interest scores were calculated as the difference in the average number of fish in the inner area (within two body-lengths of the object) during the object presentation period vs. a baseline period when there was no object present. These data show that fish were significantly attracted to the novel object at the beginning of the study (i.e., the first 10 objects they saw) during the first-100 s of an object presentation ($p < 0.0001$) *and* during the final-100 s of object presentation ($p < 0.01$). By the end of the study, (i.e., the last 10 objects they saw), there was no strong evidence of overall attraction to (or avoidance of) the novel object area (*p's* > 0.05). Across presentations, therefore, interest in the novel objects decreased during both periods (first-100s: $p < 0.0001$; and final-100s: $p < 0.02$). Dots indicate the number of fish in the object area above the average number of fish in the object area during baseline. Dots are colored according to object presentation order (dark blue: 1–10; medium blue: 11–20; light blue: 21–30) and are jittered slightly (random noise added to avoid overlapping). Gray lines and shaded areas represent the best linear fit and its 95% Confidence Interval.

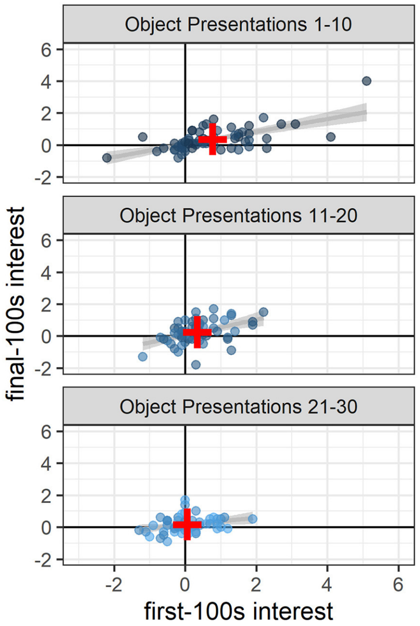

FIGURE 3
Sustained interest across the 10 min of novel object presentations. Throughout the study, high interest in the first 100 s generally corresponded to high interest in the final 100 s of the 10-min novel object presentations (presentations 1–10: $p < 0.0001$; presentations 11–20: $p < 0.001$; presentations 21–30 $p < 0.02$). Dots indicate the number of fish in the object area above the average number of fish in the object area during baseline and are colored according to object presentation order (dark blue: 1–10; medium blue: 11–20; light blue: 21–30). Gray lines and shaded areas represent the best linear fit and its 95% Confidence Interval. Red plus symbols indicate the center of each bivariate distribution (see online version for color).

At the beginning of the study (i.e., object presentations 1–10), fish were in the object area (within two body-lengths of the object) more often than expected above baseline, during both the first 100 s and the final 100 s of the 10-min novel object presentation (first-100 s: 0.77 above baseline, 95% Confidence Interval [0.51, 1.03] (henceforth indicated by square brackets only), t(154.66) = 5.77, $p < 0.0001$; final-100 s: 0.36 [0.10, 0.62] above baseline, t(154.66) = 2.67, $p < 0.01$; Figure 2). By the end of the study (i.e., object presentations 21–30), overall fish presence in the object area was indistinguishable from baseline (first-100s: 0.06 [−0.10, 0.22] above baseline, t(128.97) = 0.69, $p < 0.4$; final-100s: 0.16 [0.00, 0.32] above baseline, t(128.97) = 1.93, $p < 0.06$; Figure 2).

High interest during the first 100 s of a novel object presentation generally corresponded to higher interest during the final 100 s of that presentation, a pattern that remained significant throughout the study (object presentations 1–10: 0.40 [0.28, 0.52], t(58.00) = 6.36, $p < 0.0001$; object presentations 11–20: 0.51 [0.27, 0.75], t(55.62) = 4.19, $p < 0.001$; object presentations 21–30: 0.26 [0.06, 0.46], t(49.31) = 2.62, $p < 0.02$; Figure 3).

In addition to the greater overall interest at the beginning of the study compared to the end of the study, there was also greater *variability* in interest at the beginning compared to the end: The range in interest for presentations 1–10 was −2.2 to 5.1 vs. the range in interest for presentations 21–30 was −1.3 to 1.9 (Figure 3). At the beginning of the study (object presentations 1–10), object ID accounted for 11% of the variability in interest, with some objects consistently attracting more attention and some objects consistently attracting less attention than other objects [Likelihood Ratio Test: $\chi 2(1) = 7.08$, $p < 0.01$; Figure 4]. Tank ID accounted for 21% of the variability in interest [Likelihood Ratio Test: $\chi 2(1) = 15.30$, $p < 0.0001$], leaving 68% of the variability in object interest unexplained. None of the human-scored dimensions of object characteristics—complexity, ecological plausibility, predator-resemblance—explained variability in zebrafish

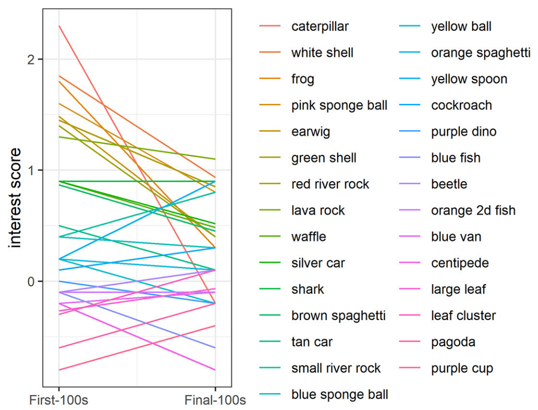

FIGURE 4
Variability in object interest at the beginning of the study. During the first 10 object presentations, object ID explained a significant proportion of the variance in zebrafish behavior ($p < 0.01$). Interest was quantified as fish presence in the object area (within two body lengths of the novel object) above a baseline period when there was no object present. Colored lines represent object interest scores at the beginning of the study averaged across tanks. The line colors and order of the novel objects in the legend correspond to the plot such that the object that appears first in the legend ("caterpillar", dark-red line), corresponds to the object with the highest interest score in the First-100 s, followed by objects that attracted less interest in the First-100 s and colored lines progressing through the rainbow, ending with the last object in the legend ("purple cup", magenta line), which corresponds to the object with the lowest interest score in the First-100 s.

interest during this period (p's > 0.4). At the end of the study (object presentations 21–30), neither object ID nor tank ID accounted for the (relatively small) variability in interest (p's > 0.2).

The experimental procedures of introducing of novel objects altered zebrafish social behavior and diving behavior, but unlike interest, these effects remained consistent throughout the study. Compared to baseline: agonistic behavior decreased during the first 100 s (-0.26 [-0.32,−0.20], t(13.73) = 7.86, $p < 0.0001$) and returned to baseline levels by the final 100 s (−0.01 [−0.07, 0.05], t(13.73) = 0.21, $p > 0.8$); cohesion increased during the first 100 s (0.47 [0.33, 0.61], t(5.91) = 7.04, $p < 0.001$) and returned to baseline by the final 100 s (0.05 [−0.09, 0.19], t(5.91) = 0.80, $p > 0.4$); and coordination also increased during the first 100 s (0.68 [0.54, 0.82], t(6.31) = 10.31, $p < 0.0001$) and returned to baseline by the final 100 s (0.13 [−0.01, 0.27], t(6.31) = 2.05, $p < 0.09$).

Throughout the study, diving behavior decreased during the first 100 s of object presentations (−0.26 [−0.30, −0.22], t(15.22) = 10.46, $p < 0.0001$) and returned to baseline by the final 100 s (-0.03 [−0.07, 0.01], t(11.90) = 1.37, $p > 0.1$).

Importantly, at the beginning of the study (object presentations 1–10), changes in social behavior and diving behavior corresponded to zebrafish interest: novel object presentations that elicited greater interest (vs. less interest) corresponded to decreased aggression (−0.06 [−0.10, −0.02], t(107.40) = 2.42, $p < 0.02$), increased cohesion (0.10, [0.02, 0.18], t(92.22) = 2.64, $p < 0.01$), increased coordination (0.08 [0.00, 0.16], t(67.24) = 2.02, $p < 0.05$), and decreased diving (−0.07 [−0.13, −0.01], t(30.99), = 2.10, $p < 0.05$). At the end of the study (object presentations 21–30), the linkage between interest scores and these other behaviors was no longer significant for any behavior (all p's > 0.1).

Discussion

Across the study, zebrafish readily approached novel objects introduced into their home-tanks with a median approach latency of 1 s. For comparison, a zoo study investigating curiosity across several taxa (sheep, tortoises, penguins, kangaroos, cockatoos, and lemurs) reported that latency to orient to novel objects ranged from 25 s to over 15 min, with many taxa never coming within two body-lengths of some objects (10).

At the beginning of the present study, *i.e.*, object presentations 1–10, we found evidence for sustained information-seeking: differential, prolonged (up to 10 min), interest consistently displayed toward some objects and not toward others. During this period, we found that interest varied systematically within tank such that objects that attracted high attention during the first 100 s, typically continued to attract above-average attention nearly 10 min later. Further, object ID accounted for 11% of the variability in interest across tanks, indicating that there was some object property driving the zebrafish interest behavior. This type of differential, object-driven sustained attention is more consistent with information seeking than it is with sensation-seeking or indiscriminate neophilia.

None of the dimensions coded by human observers—complexity, ecological plausibility, predator-resemblance—predicted differential zebrafish behavioral responses to the objects. One possible interpretation of this null result is that while we classified objects based on their visual characteristics from a human perspective, the zebrafishes' interest may have been determined by multiple sensory features as perceived by them, including, for example, chemosensory properties and fluid dynamics [zebrafish are sensitive to water flow; (45)]. Thus, it is possible that the theories about what predicts greater curiosity may have been borne out from the fishes' perspective: the objects with the most complex and most unusual features in multi-sensory space in zebrafish perception could have attracted the most attention from the zebrafish. For instance, across tanks, a small white shell and a large neon pink artificial ball both attracted a great deal of attention at the beginning of the study, despite being markedly different in human-coded complexity, ecological-plausibility, and predator-resemblance, and different in terms of shape, size, color, and material. In a multisensory space from a zebrafish's perspective, however, perhaps the white shell and pink sponge ball were equivalently unusual or complex.

Determining what parameters drive intrinsically motivated exploratory behavior in zebrafish is an area for future research, but as indicated in the present results, serial presentations of multiple static objects (the design employed for this study) is unlikely to yield the best data. At the end of the study, approaches remained fast, but interest was relatively uniform (variability in interest between object presentations was low), fleeting (interest did not extend throughout even the first 100 s of object presentation), and any variability that did exist was not driven by object characteristics. In other words, by the end of a serial presentation of 30 novel static objects (by object presentations 21–30), there was little evidence for information-seeking, instead the pattern of behavior was more consistent with sensation-seeking.

Importantly, objects were presented in a pseudo-randomized order, ensuring that across tanks, the objects during the early period were the same as the objects at the end of the study. As such, the drop-off in information seeking cannot be due to some object-level feature. Instead, the loss of interest could be due to some form of habituation to the task. At the beginning of the study, the task itself contained new information as the zebrafish learned what to expect from the procedures. By the end of the study, there was less information to be gained about the situation itself, with the only unknown aspects being those associated with the stationary, nonmanipulable, static objects themselves. It is possible, therefore, that at the beginning of the study, one source of the zebrafishes' curiosity was figuring out what to expect with the task, including what the object itself might do or not do and what could or could not be done with the object. If so, introducing more dimensions of variability into the study procedures—e.g., using objects or images that move, change, or are interactive—might facilitate continued information-seeking behavior throughout repeated presentations.

In sum, these results show that in addition to neophilia, zebrafish engage in behaviors that are consistent with the motivation for information gain. That such a motivation exists in zebrafish falls into a long tradition of animal behavior research pointing toward the deep evolutionary roots of an intrinsic motivation to explore, learn, and make sense of the world and suggests the possibility that zebrafish, like other species, may find information acquisition rewarding (12, 21–23, 46).

Welfare and cognitive enrichment

Neophilia in the absence of sustained attention, as observed at the end of the study, could be interpreted as a sign of boredom with the task and/or boredom in general. Despite being housed in semi-natural tanks, daily life in these tanks was likely less varied and less cognitively stimulating than it would be for a zebrafish living in the wild (33, 47). As such, it is possible that the zebrafish in this study were under-stimulated, i.e., experiencing a restriction of behavioral opportunities. Future research could look to establish the evidence of boredom in zebrafish by, for example, assaying for distorted time perception and tolerance of or even attraction to mildly aversive experiences, both of which are considered evidence of boredom in other species (27, 28).

Nonetheless, evidence that zebrafish have the capacity to engage with information-seeking for its own sake suggests that certain forms of cognitive stimulation could be beneficial zebrafish enrichment. Providing free-choice cognitive stimulation opportunities is known to increase welfare in other species (48) and may contribute to positive welfare (49). Crucially, the present work shows that not all novel objects were equally captivating to zebrafish, so not all novel objects are likely to be equally suitable candidates for enrichment. Moreover, the objects themselves, even those that appeared to stimulate information-seeking for the tanks that saw them at the beginning of the study, were not able to produce an information-seeking response for the tanks that saw them at the end of the study. As such, while these results point toward the possibility that zebrafish could benefit from long-term cognitive enrichment, the form that enrichment should take was not identified in the present work. Dynamic, changeable, and interactive objects or images (e.g., providing videos or vistas) may be more promising targets.

Along these lines, it is worth noting that the zebrafish in the present study were provided with environmental complexity that is more typical of home aquaria than laboratories, where small, barren tanks are currently the norm (30). Given that barren housing is aversive to zebrafish (45, 50) and detrimental to their welfare and cognition (31, 51), some of the work identifying sensation-seeking behavior in zebrafish, including impulsivity (52) and drug-seeking behavior (53), may be a product of these barren abnormal environmental conditions rather than a feature of normal zebrafish behavior. Similarly, if the present research had been conducted with zebrafish housed in the barren tanks typical of most laboratory research (30), we might not have been able to detect intrinsic information-seeking behavior, which is understood to depend on non-aversive housing conditions and low-stress conditions (21, 23, 54). As such, the implication of this work for barren-housed zebrafish is the "in principle" observation that some form of cognitive enrichment may be worth considering given zebrafish capacities and interests; the form that the cognitive enrichment takes is likely to be conditional on background housing conditions.

Positive welfare and emotional valence

The added value of a positive welfare approach is that it brings a "full life" perspective to animal welfare research and animal protection efforts (55). In the past, much of animal welfare science focused on alleviating suffering, rather than on considering and promoting positive states. Recent work across the animal kingdom, including promising indications in fishes as well (56), has established that while the alleviation of suffering is an urgent priority requiring immediate attention, on its own, a sole focus on suffering alleviation does not produce long-term welfare. For instance, as mentioned above, in the absence of opportunities for positive engagement, animals can slide into inescapable boredom and poor welfare (27, 28). Thus, in addition to an absence of negative experiences, ensuring even neutral animal welfare requires some positive experiences, including perhaps, opportunities for cognitive engagement and exploration (49).

Information-seeking behavior is consistent with a positive welfare framework, but it is unclear from the exploratory behavior alone whether the zebrafish in this study experienced positive affect as a result of the novel object exploration opportunities. The consideration of affective behavioral responses (i.e., positively/negatively valenced emotion) in zebrafish is now recognized as an important area of inquiry (57) and can be informed by examining their social and diving behavioral responses.

In the current study, the suite of social and diving behavioral changes observed in response to the presentation of novel objects is consistent with positive affect, or at a minimum, the absence of negative affect. Compared to a baseline period an hour before the introduction of an object, the first 100 s of object exposure caused agonistic and diving behavior to drop and shoal cohesion and group coordination to increase. In zebrafish, diving behavior is recognized as an important indicator of stress and negative affect (58) and has been observed in these fish in response to potentially stressful husbandry procedures (35). Despite the possibility that the fish could have responded similarly to the present procedures (i.e., responded by diving to the bottom of the water column), we found no evidence of increased diving. Indeed, we observed *less* diving in the presence of novel objects compared to a baseline period 1 h earlier. The suppression of diving behavior compared to baseline shows, at a minimum, that the free-choice exploration opportunity of novel objects did not produce an overriding stress response in the zebrafish. Decreased aggression and increased affiliative behaviors, especially group synchrony, are often taken as indicators of positive emotions in other species (59, 60), with similar patterns found in zebrafish as well (61). Accordingly, while some of these behaviors are not good indicators of valence on their own (e.g., increased cohesion can also be a stress response in zebrafish), as a group, the overall pattern of behavioral changes during the first 100 s of object presentations is not consistent with a stress response and could indicate the presence of positive affect.

Importantly, at the beginning of the study, when there was the strongest evidence for information-seeking, greater exploration of the objects corresponded to greater signs of positive affect (or low negative affect) in these additional behavioral measures. In other words, when fish were potentially engaged in high levels of object-driven information-seeking, they showed the least diving behavior and the most prosocial behavior. Later in the study, when there was less evidence for object-driven information seeking and only evidence for neophilia or perhaps even arousal or stimulation from the experimental procedures themselves, the correspondence between exploration and the other behaviors was no longer evident.

Finally, alterations in diving and social behaviors only occurred during the first 100 s of object presentation and returned to baseline by the final 100 s of the 10-min object presentation period. As a whole, this pattern of results suggests that information-seeking opportunities may have temporarily increased positive affect in zebrafish, but that this effect was short-lived and may not have improved welfare overall. Future work could investigate how long-term cognitive stimulation opportunities may affect overall welfare outside the immediate presentation periods, potentially with a between-tank experimental design in which only some tanks receive cognitive engagement opportunities while others are maintained in baseline conditions or are exposed to the same object repeatedly.

Conclusion

We found evidence that, in addition to being neophilic, zebrafish engage in sustained information-seeking behavior. As such, this study provides justification for future work exploring the extent to which fish might find cognitive stimulation rewarding and the role cognitive engagement cognitive engagement plays in their overall welfare. Further investigation into cognitive enrichment for fish will contribute to the growing literature on positive wellbeing, which has become an important topic of research across disciplines. Within the human literature, positive psychology theories have proved to be generative (62) and in the animal literature, a similar pattern is beginning to emerge (63–66). Extending the existing research exploring whether and how fish experience pain (67–69), the present research contributes evidence that fish also have the capacity for positively valenced experiences and are thus good candidates for future research on positive welfare.

Fish welfare is threatened by multiple forms of human activity—from industrial fishing to farming to scientific research to entertainment. There is an urgent need to understand and protect their wellbeing, yet comparatively little species-specific research investigates fish welfare (32) and fish are generally perceived as less deserving of enrichment than other vertebrates (70). One source of this discrepancy may be the general perception that fish are "primitive" or "lower" vertebrates, a notion that is demonstrably false—as a group, fish are just as evolved as any other extant animal taxa and are cognitively sophisticated (68, 71), with some species of fish even outperforming primates on cognitive tests (72) and passing the mirror-self-recognition test (73, 74). The misclassification of fish as somehow inferior to other vertebrates suggests that research on positive welfare in fishes may also have a special role to play in changing the narrative around what fishes can experience and, therefore, what sort of care and protections they require.

Fishes are also some of the most studied organisms in modern science, but they are rarely studied for their own sake. Much of the research involving fish instead focuses on modeling human biological processes [e.g., gene expression in the brain (75)], testing general theories in behavioral biology [e.g., Optimal Foraging Theory (76)], or determining efficient ways to increase farming and fisheries production. In compliment and in contrast to these anthropocentric forms of fish research, adopting a fish-centric research agenda can facilitate unique basic science contributions (e.g., evolutionary patterns of curiosity across the animal kingdom), ethical insights (fish are capable of positive experiences and may suffer from boredom and other forms of poor welfare in their absence), and practical solutions [e.g., cognitive stimulation may be a valuable source of enrichment for fish; (56)]. Moreover, studying fish in natural or semi-natural conditions can help elucidate the degree to which barren laboratory housing induces abnormal biological states that may reduce the generalizability of data collected.

In conclusion, while we did not find that zebrafish follow the same patterns of curiosity as those found in previously studied (terrestrial) animals, we did find evidence that they find some objects to be more interesting than others. As these exploration opportunities decreased agonistic and diving behavior while also increasing affiliative behavior, it is possible that zebrafish, like many other species, benefit from activities that engage their cognitive abilities and preferences. This research builds on our understanding of the determinants and consequences of curiosity across species and opens new avenues of investigation regarding the role that exploration and learning play in the lives and welfare of fishes.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

All husbandry and experimental procedures were approved by the University of British Columbia's Animal Care Committee, protocol number A14-0119.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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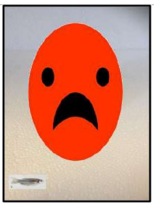
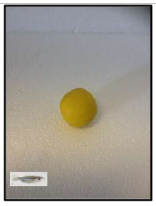
Appendix

TABLE A1 Novel object characteristics and photos.

Object and photo		Object and photo		Object and photo	
Beetle C: 3.88 PR: 0.5 EP: 0.5		Frog C: 4.62 PR: 1 EP: 0.74		Purple dinosaur C: 4.12 PR: 0.62 EP: 0	
Blue fish C: 2.12 PR: 0.5 EP: 0.5		Green shell C: 3.38 PR: 0 EP: 1		Red river rock C: 1.25 PR: 0 EP: 1	
Blue sponge C: 3.75 PR: 0.12 EP: 0		Large leaf C: 2.62 PR: 0 EP: 1		Shark C: 3.88 PR: 1 EP: 0.75	
Blue van C: 4 PR: 0.38 EP: 0		Lava rock C: 2.75 PR: 0 EP: 1		Silver car C: 3.25 PR: 0.25 EP: 0	
Brown spaghetti C: 3.12 PR: 0 EP: 0.5		Leaf cluster C: 3.38 PR: 0 EP: 0.88		Small river rock C: 1.5 PR: 0 EP: 1	
Caterpillar C: 2.62 PR: 0 EP: 0.5		Orange 2d fish C: 3.5 PR: 0.75 EP: 0.25		Tan car C: 4 PR: 0.12 EP: 0	
Centipede C: 3.88 PR: 0.12 EP: 0.38		Orange spaghetti C: 3.25 PR: 0 EP: 0.38		Waffle C: 2.88 PR: 0 EP: 0	

(Continued)

TABLE A1 (Continued)

Object and photo		Object and photo		Object and photo	
Cockroach C: 4.25 PR: 0.25 EP: 0.75		Pagoda C: 3.5 PR: 0.38 EP: 0.12		White shell C: 3.25 PR: 0.12 EP: 0.75	
Dark shell C: 3.88 PR: 0.12 EP: 1		Pink sponge ball C: 4 PR: 0.12 EP: 0.12		Yellow ball C: 1.5 PR: 0 EP: 0.12	
Earwig C: 3.5 PR: 0.12 EP: 0.5		Purple cup C: 2 PR: 0.14 EP: 0.14		Yellow spoon C: 2.38 PR: 0.12 EP: 0	

A list of the 30 novel objects used for this study (presented in a pseudo-randomized order across tanks) and the human-coded dimensions of interest: C refers to complexity (visual complexity of the object average score on a scale of 1 to 5), PR refers to predator-resemblance (the degree to which the object resembled a predator of zebrafish average score on a scale of 0 to 1), and EP refers to ecological-plausibility (the degree to which the object represented something a zebrafish might plausibly encounter in its natural environment average score on a scale of 0 to 1). These dimensions were generated by eight human coders that together had a Cronbach alpha of greater than 0.85 agreeability for each of these dimensions. NB: The object identified as “Orange 2d fish” is a rendering of the image that was printed on a sheet of paper and then laminated before being presented to the fish. Small white rectangles within each picture represent an estimate of a zebrafish’s size in relation to the object.

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