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Sleeping with the fishes: contradictory results on behavioural and neurophysiological assessments of chemical narcosis in rainbow trout

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Anaesthesia in fish is commonly assessed through visual inspection, yet behavioural indicators of consciousness are not always reliable. Complementary measurements of brain activity can enhance accuracy and generate evidence-based welfare guidelines. Here, we anaesthetized rainbow trout with five common anaesthetic agents and assessed presence or absence of visual evoked responses (VERs) on the electroencephalogram as a neurophysiological indicator of unconsciousness combined with the behavioural indicators of consciousness balance and mobility to determine the duration of induction-to and recovery-from narcosis. As expected, both induction and recovery times varied depending on anaesthetic compound and dose. Tricaine methanesulfonate stood out as the compound with the most rapid induction and recovery. Notably, we observed significant discrepancies between neurophysiological and behavioural indicators of consciousness. For example, metomidate induced immobility at relatively low concentrations, whereas much higher doses were required to achieve unconsciousness. Across compounds, trout typically regained motor function well before VERs. This may suggest biological prioritization of mobility during recovery from unconsciousness, or potential visual impairment caused by the anaesthetic agents. These mismatches have important implications for the interpretation of behavioural responses under anaesthesia and should be carefully considered in both research and applied settings.

1. Introduction

Anaesthetics are extensively used in research, environmental monitoring and aquaculture to protect workers and animals during handling and invasive procedures. Commonly used compounds for fish include tricaine methanesulfonate (e.g. Syncaine®, commonly known as MS-222 but hereafter referred to as tricaine), benzocaine (e.g. BENZOAK VET®), isoeugenol (e.g. AQUI-S®), eugenol (e.g. clove oil) and metomidate (e.g. Aquacalm™). The choice of anaesthetic depends on intended procedures, safety for both users and animals, cost and legislations [1–3]. The intended procedure determines the desired level of anaesthesia. Light sedation is often sufficient for transport and handling [4], whereas surgical procedures require deep anaesthesia or narcosis in accordance with prevailing legal and ethical standards to prevent unnecessary suffering (e.g. [5,6]). In the field and aquacultural settings, induction should ideally take no more than 3 min to minimize struggling and stress-induced hyperactivity, and recovery should not exceed 5 min to minimize risks associated with immobility such as predation and

oxygen depletion in aggregates of recovering fish [1,7]. These risks are less pronounced in laboratory settings where fish can be monitored individually, predators are absent, and recovery typically occurs in well-aerated water with ample space. Consequently, longer induction and recovery periods may be acceptable under laboratory conditions.

The depth of anaesthesia before, during and after surgical intervention in research is typically assessed through visual inspection (e.g. [8–10]). However, commonly used behavioural indicators of consciousness, such as balance and mobility [6], are not always reliable [11–16]. Several studies across species have demonstrated that fish may appear immobilized yet retain, or rapidly regain, brain function following interventions intended to induce unconsciousness, such as electrical stunning, percussive stunning and rapid cooling [17–22]. Even more conservative behavioural indicators of consciousness, such as eye rolling or ventilation can cease long before neurophysiological signs of consciousness are lost and therefore substantially underestimate the time required for a fish to reach narcosis [14,16]. Fortunately, methods for measuring neurophysiological indicators of consciousness in fish by recording electroencephalography (EEG) are available, which can be used to verify behavioural indicators. For example, exposure to a flashing light elicits a visual evoked response (VER) in the EEG of a conscious fish. The absence of VERs indicates a level of brain dysfunction incompatible with consciousness or awareness [23–25]. At present, VERs represent the most conservative and reliable indicator of consciousness in fish. Nevertheless, despite considerable global research interest in fish anaesthesia [26], behavioural and/or somatosensory evoked responses remain the most used techniques for assessing responses to anaesthetics (e.g. [27,28]).

Although largely impractical in pre- and post-surgical settings, neurophysiological assessment of consciousness prior to, during and after narcosis can be employed to validate protocols. Together with existing knowledge on the physiological effects of anaesthetic compounds (reviewed in [29]), such assessments can inform the development of evidence-based welfare guidelines. In this study, we applied a non-invasive EEG technique developed by Bowman *et al*. [30], in combination with the commonly used behavioural indicators balance and mobility to assess the duration of induction-to and recovery-from narcosis in rainbow trout (*Oncorhynchus mykiss*) exposed to five anaesthetics agents. The five compounds were selected based on their availability and our previous experience, although other compounds not included here, such as 2-phenoxy-ethanol, also warrant further investigation. Our primary objective was to identify ideal compounds and doses suitable for laboratory use, specifically those capable of inducing narcosis rapidly and non-aversively, while enabling smooth recovery. We hypothesized that induction and recovery times would vary between compounds and doses, and that discrepancies would arise between behavioural and neurophysiological indicators of consciousness.

2. Methods

Rainbow trout (*Oncorhynchus mykiss*, mean ± s.e.m. weight 636.8 ± 16.1 g, n = 86) were obtained from a commercial fish farm (Vänneåns Fiskodling AB, Knäred, Halland, Sweden) and held in aerated, recirculating fresh water at approximately 11°C under a 12 L : 12 D photoperiod for at least 2 weeks before experiments. Fish were fed with 4 mm commercial pellets three times per week (Protec trout pellets, Skretting, Stavanger, Norway).

To measure EEG, a non-invasive silicone cup with three 1 cm diameter silver chloride plate electrodes (Electrode ARBO H98LG MOD, Tyco Healthcare, Ratingen, Germany) was positioned approximately above the left optic lobe (for details on cup fabrication and placement, see [30]). Prior to cup placement, the trout were lightly sedated in 5 mg l^{-1} isoeugenol (AQUI-S® Aquatic Anaesthetic; AQUI-S, Lower Hutt, New Zealand) and a thin layer of conductive paste (Ten20, Weaver and Company, Aurora, Colorado, USA) was applied to the surface of each electrode to ensure good contact between the skin of the trout and the electrodes. The cup was secured in place using suction generated by a peristaltic pump connected to a silicone tube attached to the cup. The trout were then introduced into a 12 l opaque monitoring chamber (length: 48 cm, width: 12 cm, water depth: 16 cm) covered with a glass lid and supplied with 1.5 l min^{-1} of clean fresh water. The EEG wires were then connected to a bio-amplifier (FE136, ADInstruments, Sydney, Australia) which continuously recorded EEG signals in response to light flashes from an LED strobe-light (10 ms light flashes at 2 Hz) in a dark room. The sensitivity range of the bio-amplifier was (± 2 mV) with a low-pass filter (50 Hz), high-pass filter (0.1 Hz) and 50 Hz notch filter activated to optimize EEG signals. Signals from the bio-amplifier and a light detector were relayed to a PowerLab (ML 870, 8/30, ADInstruments). Data were subsequently collected on a PC for analyses using LabChart Pro software (version 7.3.2, ADInstruments) at a sampling rate of 1 kHz.

When analysing the EEG recordings, a bandpass filter was used to separate the beta wave frequency (13–32 Hz). This is because activity in this frequency range relates to awareness and normal alert consciousness and is consequently also where VERs are found to be most distinct [30]. VERs were detected using the Scope View module in the software, which was set to display time windows starting 50 ms before and ending 450 ms after the strobe light flash. To reduce the effects of noise caused by strong muscular movements, 500 ms time windows where the amplitude of the beta wave exceeded 10 µV were automatically excluded from the analyses. To obtain specific determinations of when VERs were present or absent, the Scope View module was used to average 120 consecutive, non-overlapping time windows into a single 500 ms time window representative of the beta wave for 60 s of recordings. VERs were determined to be present or absent when the peak-to-peak amplitude of the respective VER was greater or less than double the peak-to-peak amplitude of the rest of the beta wave (see fig. 1 in [31]). If VERs were determined to be lost or recovered during a 60 s recording, that recording was divided into shorter segments to more precisely identify the exact time of loss or recovery. During the experiments, a custom-made IR-camera coupled with an IR-light facilitated visual observation of the fish.

Once calm behaviour and stable EEG measurements were attained, the water flow was turned off and one of the five anaesthetics was added to the tank at one of 2–3 different dosages (table 1). Water flow containing the corresponding anaesthetic dose was then immediately turned on into the monitoring chamber. The post-administration recordings lasted

Table 1. Experimental anaesthetic compounds and doses. Concentrations are reported in mg l^{-1}, with sample sizes in parentheses. Doses marked with an asterisk (*) were not tested because a higher dose failed to consistently abolish VERs in more than 75% of the fish.

anaesthetic	isoeugenol	eugenol	metomidate	benzocaine	tricaine
high dose	160 ($n = 8$)	80 ($n = 10$)	25 ($n = 8$)	80 ($n = 8$)	150 ($n = 10$)
medium dose	60 ($n = 9$)	30 ($n = 9$)	10 ($n = 3$)	30 ($n = 4$)	75 ($n = 9$)
low dose	20 ($n = 2$)	8 ($n = 4$)	*	*	40 ($n = 2$)

for a maximum of 10 min or until VERs were abolished. To determine effective doses, we started with the highest dose and proceeded to lower doses if the high dose consistently (i.e. in >75% of the tested fish) abolished VERs within 10 min. If any dose failed to abolish either VERs or behavioural indicators within 10 min in more than one individual, no more animals were tested.

While aware and awake, VERs appear as a distinct waveform in the trout's EEG, milliseconds after light stimulus. During the transition to narcosis, their amplitude declines until they are indistinguishable from the rest of the signal [31]. At this point, the fish is strongly indicative of unconsciousness, while fish reacting to visual stimuli may still maintain rudimentary consciousness [14,16,25]. In the comparison between neurophysiological and behavioural consciousness indicators, equilibrium and mobility were used as behavioural measures in the present study. These indicators are widely applied and can be readily observed remotely without interfering with ongoing non-invasive neurophysiological recordings. Behavioural indicators were graded in three levels: fully present (i.e. maintains upright position in the water column, displays rhythmic swimming pattern), partially lost (i.e. struggles to maintain upright position, displays reduced and irregular swimming and/or fin movement) and fully lost (i.e. flipped and completely immobile). Fish were considered behaviourally unconscious when both equilibrium and mobility were fully lost. Conversely, any sign of partially recovered mobility qualified the recovery of behavioural consciousness indicators. Significant differences ($p < 0.05$) between groups receiving different anaesthetics, in terms of the required range of times until loss or recovery of VERs and behavioural indicators, were assessed using the Kaplan–Meier method and a log-rank (Mantel–Cox) test in Prism (GraphPad Software, San Diego, California, USA), which was also used to visualize data.

3. Results

The highest dose of each anaesthetic compound abolished VERs and all behavioural signs of consciousness in all fish during the experimental protocol, except in one individual receiving 25 mg l^{-1} metomidate where VERs were still present after 10 min. The lowest doses required to abolish VERs in all tested fish were 150 mg l^{-1} tricaine, 80 mg l^{-1} benzocaine, 30 mg l^{-1} isoeugenol and 30 mg l^{-1} eugenol. At these doses, induction to narcosis varied significantly (figure 1A; $p < 0.001$) and was fastest with tricaine at 1.5–4 min, followed by isoeugenol at 3–6.5 min, while induction times using eugenol and benzocaine were at 5.5–8.75 min and 6–8.75 min, respectively. Importantly, metomidate did not consistently eliminate VERs in all tested fish at either dose within 10 min. Similarly, recovery times from these anaesthetic doses varied significantly (figure 2A; $p < 0.001$) and was fastest with tricaine at 4–15 min, followed by benzocaine at 6–12 min, and isoeugenol at 8–14 min. Using these doses, however, some of the fish did not recover VERs during the 15 min of recovery from narcosis. This proportion was lowest for tricaine at 1/10 trout, followed by isoeugenol at 2/9 trout and benzocaine at 3/8 trout. Notably, none of the fish recovered VERs after narcosis with eugenol and metomidate.

Dose significantly affected induction times, as illustrated by comparing different doses of isoeugenol and eugenol (figure 1B; $p < 0.001$). With these anaesthetic compounds, induction was generally quicker with isoeugenol relative to a comparable dose of eugenol. Recovery times also appeared to be affected by doses, although the effect was not significant when comparing doses of tricaine and isoeugenol (figure 2B; $p = 0.054$).

When comparing the time until loss of VERs with the time until full loss of behavioural consciousness indicators, there were marked discrepancies for both the medium dose of metomidate and the low dose of eugenol (figure 1C; $p = 0.003$). For 10 mg l^{-1} metomidate, this is illustrated by the rapid full loss of equilibrium and mobility, between 86 and 187 s, while VERs were abolished after 4.5 min in one fish but persisted throughout the whole 10 min induction period in the other two trout. By contrast, three out of four fish receiving 8 mg l^{-1} eugenol lost VERs within 10 min, whereas only one out of four was rendered observably unconscious. While being most pronounced when using these compounds, a general mismatch between VERs and behavioural indicators was observed across groups receiving different anaesthetics and doses. This is illustrated by the fact that across compounds and doses, behavioural consciousness indicators were typically fully lost before VERs (figure 1D).

Similar discrepancies were observed between the time until the recovery of VERs and behavioural consciousness indicators for the medium doses of eugenol and isoeugenol (figure 2C; $p < 0.001$). For 30 mg l^{-1} eugenol, this is illustrated by the rapid recovery of partial mobility between 106 and 286 s, while VERs were not recovered in any of the examined fish within 15 min. Similarly, all trout receiving 30 mg l^{-1} isoeugenol recovered at least partial mobility within 10 min but required at least 12 min to recover VERs, which failed to recover in 2/9 individuals. While being most pronounced when using these compounds, behavioural consciousness indicators were typically partially recovered before VERs across compounds and doses (figure 2D). For example, none of the individuals receiving metomidate regained VERs within 15 min, but 10/11 of them recovered mobility within 10 min (data not shown). Animals receiving doses that did not abolish VERs typically retained VERs during recovery and are represented as zeroes on the x-axis in figure 2D.

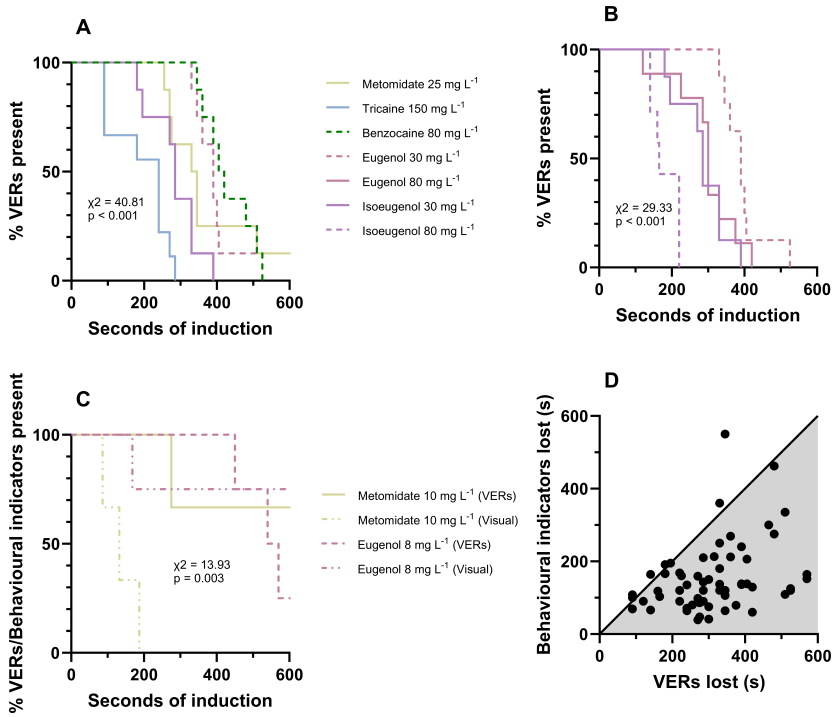

Figure 1. Induction times to narcosis and the relationship between visually evoked responses (VERs) and behavioural consciousness indicators. (A) Time to loss of VERs at the lowest dose required to abolish responses within 10 min for tricaine methanesulfonate, benzocaine, isoeugenol, eugenol and metomidate. (B) Time to loss of VERs at different doses of isoeugenol and eugenol. (C) Time to loss of VERs and full loss of behavioural indicators using eugenol and metomidate. (D) Time to loss of VERs versus full loss of behavioural indicators across groups. The black line illustrates a 1 : 1 relationship, and data points in the grey area represent a welfare hazard. Significant differences among groups are indicated by $p < 0.05$. Sample sizes are provided in table 1.

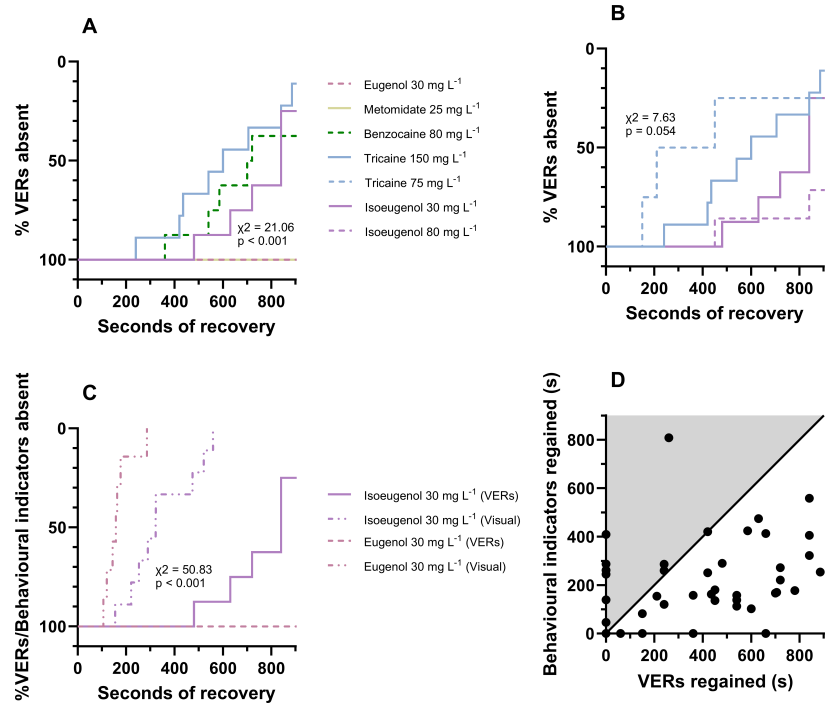

Figure 2. Recovery times after narcosis and the relationship between visually evoked responses (VERs) and behavioural consciousness indicators. (A) Time to regained VERs after narcosis with the lowest dose that abolished responses within 10 min using tricaine methanesulfonate, benzocaine, isoeugenol, eugenol and metomidate. (B) Time to regain VERs after narcosis with tricaine and isoeugenol. (C) Time until VER recovery and partial recovery of behavioural consciousness indicators after narcosis using eugenol and isoeugenol. (D) Time until VERs were regained versus time until behavioural indicators recovered across all groups. The black line illustrates a 1 : 1 relationship, and data points in the grey area represent a welfare hazard. Significant differences among groups are indicated by $p < 0.05$. Sample sizes are provided in table 1.

Few clear behavioural signs of distress (e.g. thrashing, jumping) were observed apart from in 1/9 individuals receiving 30 mg l^{-1} eugenol, 1/9 individuals receiving 75 mg l^{-1} tricaine, 1/9 individuals receiving 30 mg l^{-1} isoeugenol and 3/8 individuals receiving 80 mg l^{-1} isoeugenol. However, all compounds caused cardio-ventilatory depression (data not shown). In addition, faeces were repeatedly found in the experimental tank after administering benzocaine.

4. Discussion

As the efficacy of anaesthetic compound varies and may depend on biological and physical factors [2,11,32–34], species-specific validation of anaesthesia induction protocols is required to ensure ethically acceptable standards. In this study, non-invasively acquired EEG data were used to evaluate various common anaesthetic compounds for their ability to rapidly and non-aversively induce narcosis in rainbow trout. We demonstrate that induction and recovery times are highly dependent on compounds and their dosage. Tricaine had the shortest induction and recovery times, achieving narcosis in all trout within 5 min and recovery starting at 4 min at a concentration of 150 mg l^{-1}. These findings agree with those of a previous study, which reported 2.5 to 5 min for loss of VERs in similar sized trout under similar thermal conditions using the same dose of tricaine [30]. However, even at this relatively high dose, the induction time of up to 5 min with tricaine remains significantly longer than the <3 min recommended in earlier studies [1,7]. This highlights the importance of careful monitoring when fish are anaesthetized in batches, such as in aquaculture settings. Monitoring may be especially critical when using benzocaine without a preparatory fasting period (e.g. [6]), as this compound induced vomiting and/or defaecation in trout in the present study. Additional care should be taken to ensure that health and welfare are maintained in fish that are repeatedly exposed to anaesthetic compounds. For example, proteomic analysis indicates that apoptotic activity increases in the brain of rainbow trout anaesthetized with tricaine, which may have long-term implications [35]. On a brighter note, the lowest required doses to induce narcosis with each compound did not elicit any clear aversive responses. Even so, as we did not measure primary or secondary stress responses (e.g. heart/ventilation rate, cortisol), the possibility remains that the experience was stressful for the fish. As anaesthesia commonly induces cardio-ventilatory depression in fish [34], appropriate monitoring and supportive care is essential even in laboratory settings to maintain adequate welfare standards. One straightforward method to mitigate the adverse effects of reduced ventilation during surgery is to provide a continuous flow of water containing a maintenance dose of the anaesthetic compound over the gills.

The comparison between loss and recovery of VERs and behavioural indicators revealed a clear discrepancy, with behavioural indicators generally being lost and recovered before VERs. Hence, our data support the notion that visual monitoring of anaesthesia induction may present a welfare hazard [30]. This approach can lead observers to mistakenly believe that the fish have reached narcosis, thereby increasing the risk of performing invasive procedures on a conscious animal. The most pronounced mismatch between VERs and behavioural consciousness indicators was observed when using metomidate. Despite the high dose of 25 mg l^{-1} being well above the typical range, this compound failed to abolish VERs within 10 min in one out of eight trout despite loss of observable consciousness. In addition, using a lower dose of 10 mg l^{-1}, all tested fish were rendered behaviourally unconscious within approximately 3 min, and yet two of three fish retained VERs throughout the 10 min of induction. These observations suggest that while metomidate immobilizes rainbow trout at relatively low doses, extremely high doses are required to induce narcosis. Importantly, anaesthesia with 10 mg l^{-1} of the closely related compound etomidate abolished responsiveness to a painful tail pinch within 10 min in rainbow trout [28]. As the loss of this somatosensory response is generally regarded as indicative of deep anaesthesia, the present findings highlight the need to validate somatosensory evoked responses against VERs. Further investigations are necessary to determine whether fish exposed to metomidate are truly sedated and unconscious or are only merely immobilized while retaining intact afferent sensory pathways. Until such clarity is achieved, its use during invasive procedures should be carefully weighed against the potential suffering and may be best reserved for non-invasive applications where sedation is sufficient. For example, the immobilizing quality of metomidate makes it very useful for grading and general non-invasive handling. The choice of anaesthetic compound in research must also be considered in the context of its pharmacological mechanisms (reviewed in [5]). Although a detailed mechanistic discussion is outside the scope of the present study, we note that metomidate is known to inhibit activation of the hypothalamic–pituitary–interrenal (HPI) axis [36,37], which may be desirable in research as to prevent cortisol release from obscuring results.

Another significant but contrasting mismatch was observed during recovery from anaesthesia, where a large proportion of fish recovered at least partial mobility before VERs. Neural processes supporting locomotion appear to recover earlier than those supporting visually evoked processing during emergence from general anaesthesia. In mammals, early reactivation of pathways involving the cerebellum and motor cortex has been demonstrated to facilitate conscious recovery, preceding the full restoration of integrative sensory and cognitive functions [38]. This observation aligns with our findings and supports the notion of a conserved neurophysiological hierarchy governing recovery dynamics across vertebrate species. The observed mismatch may thus suggest biological prioritization of mobility over the ability to visually assess the environment during recovery from unconsciousness. Alternatively, this could reflect impaired vision. Interestingly, the low dose of eugenol abolished VERs before immobilizing during induction, and the absence of VER recovery within the 15 min recovery period further suggests that visual function was affected. Future investigations are needed to verify and further explain this unexpected finding. Identifying the duration of this potential impairment could influence the future use of anaesthetics in e.g. field studies, as visually impaired fish generally have reduced foraging and escape abilities (e.g. [39,40]). These findings further highlight that those neurophysiological indicators of consciousness dependent on visual stimuli, such as VERs, can be obstructed in visually impaired animals. Therefore, the potential of complementary evoked responses on the EEG, such as auditory or somatosensory stimuli, should be explored for such investigations. Additionally, the commonly used behaviourally observable indicator of consciousness, VORs (vestibulo-ocular reflex or eye-roll reflex; see [15]), may also have limited applicability for validating protocols of anaesthesia induction with eugenol. Its usefulness remains if this reflex is gyroscopic, but we are unaware of any study that confirms or refutes this.

In conclusion, our results highlight several welfare hazards during anaesthetic induction to narcosis in rainbow trout. Visual monitoring of the depth of anaesthesia can be misleading, especially when using metomidate. In addition, anaesthesia may

be associated with impaired vision, notably when using eugenol. Overall, the most viable anaesthetic compound for invasive procedures, of those tested here, appears to be tricaine.

Ethics. The study was covered by ethical permit no. 1873–2018, approved by the animal ethical board at the regional court in Gothenburg.
Data accessibility. The dataset analysed during the current study are available in the figshare repository [41].
Declaration of AI use. We have not used AI-assisted technologies in creating this article.
Authors' contributions. N.W.: data curation, formal analysis, investigation, methodology, visualization, writing—original draft, writing—review and editing; E.P.: conceptualization, funding acquisition, project administration, visualization, writing—review and editing; A.G.: conceptualization, funding acquisition, methodology, project administration, resources, software, supervision, writing—original draft, writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest declaration. We declare we have no competing interests.
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