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Validation of the HemoCue Hb 801 portable haemoglobin analyser for fish blood

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Abstract

Haemoglobin concentration ([Hb]) assessment in fish blood has become a routine parameter to measure the health and welfare status of the animals. The original method (haemoglobincyanide method, best known as the Drabkin method) for measuring Hb in human and animals is not well suited for work outside of a laboratory setting. It is relatively time consuming, contains hazardous cyanide elements, and requires specific laboratory material. As an alternative to the Drabkin method, portable analysers have been developed for human blood, but they need to be first validated for fish blood before being used in experiments. In this study, the performance of the new HemoCue Hb 801 portable haemoglobin analyser was compared to the validated Drabkin method to determine [Hb] in three fish species. Hb readings between the two methods were not different for any of the species tested (rainbow trout, *Onchorynchus mykiss*, Atlantic wolffish, *Anarhichas lupus*, and Nile tilapia, *Oreochromis niloticus*). Therefore, this new portable device can be readily used to measure Hb in fish blood. Unlike the previous model from HemoCue, the Hb 201+, this device does not need an incubation time or a correction factor, representing a major gain of time and precision.

KEYWORDS
Drabkin's reagent, fish haemoglobin, HemoCue Hb 801, haemoglobin analyser, haematology, haemoglobincyanide method

1 | INTRODUCTION

Blood haemoglobin concentration ([Hb]) has been widely used to provide an indication of general physiological health and welfare status of vertebrates, including fish (Andrewartha et al., 2016; Artacho et al., 2007; Bańbura et al., 2007; Clark et al., 2008; Frisch & Anderson, 2005; Imsland et al., 2007; Schwieterman et al., 2019).

The original and still one of the most commonly used and accurate methods to measure [Hb] in vertebrates is the haemoglobincyanide method (HiCN, hereafter referred to as the Drabkin's method) (Balasubramaniam & Malathi, 1992; Drabkin & Austin, 1935). This method estimates [Hb] spectrophotometrically through a reaction between blood and a cyanide-based reagent called the Drabkin's reagent. Although still being widely used in laboratory settings, this method is not optimal for fieldwork measurements, as it is relatively time consuming, contains hazardous elements, and requires specific laboratory material. The use of a portable and practical device to assess [Hb] is, therefore, clearly beneficial for work with animals carried outside of

the laboratory setting, such as blood samplings on wild animals or in farms. Portable analysers for human [Hb] developed by the medical industry could house potential for these purposes, provided that they are validated for the species of interest. Because there is a variation in blood properties among animal species, it is essential to validate the methodology before applying it to a new species. For example, red blood cells in fish are nucleated, whereas these cells in humans lack a nucleus (De Souza & Bonilla-Rodriguez, 2007). Furthermore, Hb properties can also differ between fish species, and fish blood often possesses several types of Hb (Binotti et al., 1971; Clark et al., 2008; De Souza & Bonilla-Rodriguez, 2007; Falk et al., 1998). This variability means that methods like portable analysers may not be directly applicable across different fish species and need to be first validated.

The portable Hb analyser HemoCue Hb 201+ (hereafter referred to as Hb 201+; HemoCue, Ängelholm, Sweden) was developed in 1982. It could measure human [Hb] within 10 min, using coated cuvettes. This device was proven accurate and validated quite rapidly for mammalian blood (Kutter et al., 2012; Lardi et al., 1998; Neufeld et al., 2002; Posner et al., 2005; Von Schenck et al., 1986). However, it was not until recently that this methodology was validated for teleost (Andrewartha et al., 2016; Clark et al., 2008) and elasmobranch (Schwieterman et al., 2019) blood. These studies revealed that a correction factor was needed to account for the differences in blood composition between fish and humans, as mentioned earlier. This correction factor was relatively similar between fish species of the same subclass (i.e., teleosts or elasmobranchs) but differed between the two subclasses (Andrewartha et al., 2016; Clark et al., 2008; Schwieterman et al., 2019). Over the range of four teleost species tested, the Hb 201+ overestimated [Hb] by 22%–50% (Clark et al., 2008). The authors further demonstrated that previously published articles using the Hb 201+ may have overestimated the [Hb] of their species by 21%–24% (Alvarez-Pellitero & Pintó, 1987; Ek et al., 2006; Schmitt et al., 2005, 2007).

In 2019, HemoCue launched a new Hb method, the HemoCue Hb 801 (HemoCue, Ängelholm, Sweden, hereafter referred to as Hb 801). Compared to the Hb 201+, this device uses uncoated cuvettes and takes under 1 s to analyse [Hb], making it even more efficient and suitable for fieldwork. However, as of 2024, the Hb 801 has not been validated nor used in peer-reviewed scientific literature to measure [Hb] in fish blood. Regarding other animals, only one study on birds has reported its use in house sparrows, *Passer domesticus*, but without prior apparent validation (Stierhoff et al., 2023). As the previous Hb 201+ also overestimated the [Hb] in bird blood (Harter et al., 2015), it is important to first test this device with a validated method before use in a new organism.

The aim of the present study was to validate the use of the Hb 801 portable device to measure [Hb] in fish blood. For this purpose, we have compared the results obtained from the Hb 801 analyser with the results obtained using the Drabkin method, used as a reference standard. Three important species for the Swedish aquaculture industry were examined: rainbow trout (*Onchorynchus mykiss*), Atlantic wolffish (*Anarhichas lupus*) and Nile tilapia (*Oreochromis niloticus*).

2 | MATERIALS AND METHODS

2.1 | Blood sampling

Blood sampling was conducted on experimental fish already used by colleagues at the Department of Biological and Environmental Sciences (University of Gothenburg, Sweden) and the Department of Applied Animal Science and Welfare (Swedish University of Agricultural Sciences, Sweden). This was to reduce the number of experimental animals by utilizing individuals already used in experimental settings, based on the 3Rs principles (replacement, reduction and refinement) and the EU directive 2010/63/EU promoting tissue and organ sharing in research (Díaz et al., 2021; Russell & Burch, 1959). In this way, no fish was sacrificed specifically for this experiment. Instead, blood samples were taken when fishes were sacrificed for terminal samplings in other experiments. All procedures were performed by trained and certified personnel in accordance with the ARRIVE guidelines, European legislation, and the ethical permits Dnr:5.8.18-15096/2018 (Henrik Sundh, rainbow trout, Atlantic wolffish) and Dnr:5.8.18-15311/2023 (Albin Gräns, Nile tilapia), approved by the Swedish ethical review authority.

Rainbow trout specimen ($N = 17$, mean weight [± SEM]: 381.5 ± 15.4 g, mean length [± SEM]: 31.6 ± 0.4 cm) from a freshwater recirculating aquaculture system (RAS) were used in a student practical course at the University of Gothenburg in December 2023.

Atlantic wolffish specimen ($N = 21$, mean weight [± SEM]: 683.1 ± 57.4 g, mean length [± SEM]: 47.1 ± 1.0 cm) from a saltwater RAS were used in a stress experiment at the University of Gothenburg in April 2023 (Hedén, 2023).

Nile tilapia specimen ($N = 29$, mean weight [± SEM]: 725.7 ± 37.8 g, mean length [± SEM]: 31.7 ± 0.5 cm) were collected from a freshwater partial RAS from our industrial partner Gårdsfisk (Åhus, Sweden) subsequently subjected to hypoxia, and transport experiments conducted in May 2023 by Erika Sundell and Dr Albin Gräns (SLU, Sweden).

Blood samples (0.2–2 mL) were collected from fish euthanized with an anaesthetic overdose (MS-222, FINQUEL, Argent Chemical Laboratories, Redmond, USA) followed by a sharp blow to the head. Blood was drawn through a puncture of the caudal vein using a syringe rinsed and flushed with heparin (4000–5000 U mL^{-1}, Leo Pharma AB, Malmö, Sweden). Blood samples were immediately put on ice until analyses, which were performed within 1 h. [Hb] was measured on whole-blood samples of each species. Both Drabkin's method and the Hb 801 method were used to analyse each blood sample simultaneously.

2.2 | Drabkin's method

The Drabkin's reagent was either prepared locally or purchased from a manufacturer. When prepared locally (for the studies on Atlantic wolffish and Nile tilapia), 1 g of sodium bicarbonate ($NaHCO_3$) was combined with 50 mg of potassium cyanide (KCN) and 200 mg of

potassium ferricyanide ($K_3Fe[CN]_6$) in a 1-L vial containing distilled water. The bottle was then immediately covered with foil and stored at 4°C in a dark room to maintain stability (Clark et al., 2008). For the rainbow trout study, Drabkin's reagent (14 mg monopotassium phosphate [KH_2PO_4], 50 mg KCN, and 200 mg $K_3Fe[CN]_6$) was purchased from a commercial manufacturer (Chem-Lab NV, Zedelgem, Belgium). It was kept in a dark environment in its opaque container at room temperature, according to the manufacturer's recommendations.

Aliquots of 1-mL Drabkin's reagent were placed into 2-mL Eppendorf tubes, shortly before each sampling event; 10 μL of blood was subsequently mixed in the aliquots and immediately vortexed for about 30 s. Samples were then incubated at room temperature for 1 h with frequent agitation using a platform rotator at 50 rpm (PS-3D, Grant Bio, Cambridge, UK). Following this incubation period, samples were centrifuged at 10,000 rpm for 10 min to precipitate degraded proteins (Eppendorf, Centrifuge 5415R, Eppendorf, Hamburg, Germany). Then, the supernatant was carefully transferred into 10 × 4 × 45-mm spectrophotometer cuvettes (VWR international, Darmstadt, Germany), and the absorbance was read within 15 min at 540 nm using a spectrophotometer (BioRad SmartSpec Plus, BioRad, Hercules, USA). A cuvette filled with 1.01 mL of Drabkin's reagent was used as blank. The absorbance reading was converted to [Hb] (g L^{-1}) according to Clark et al. (2008), where $[Hb] = (A_{540} - A_{BLANK}) \times W_{Hb} \times F_D/(C_E \times D \times 1000)$, where A_{540} is the absorbance at 540 nm, A_{BLANK} is the absorbance of pure Drabkin's reagent at 540 nm, W_{Hb} is the molecular mass of the human Hb tetramer (64,458 g mol^{-1} or 64,458 Da), F_D is the dilution factor used (10 μL blood in 1 mL Drabkin's reagent, 101), C_E is the millimolar extinction coefficient of tetrameric cyanomethemoglobin at 540 nm (44), D is the vial light path in centimeter (1) and 1000 the factor used to convert from milligram to gram (Clark et al., 2008, adapted from ICSH, 1978, Dacie & Lewis, 1995). In short, $A_{540} - A_{BLANK}$ was multiplied by 148. For practical reasons, Hb measurements using the Drabkin methods were either conducted in duplicate (field sampling, Nile tilapia, Atlantic wolffish) or quadruplicate (laboratory sampling, rainbow trout).

2.3 | Hb 801 method

The Hb 801 analyser system (www.hemocue.com) consists of the instrument together with uncoated microcuvettes. The Hb 801 method measures optic absorbance of whole blood at an Hb/HbO_2 isosbestic point of 506 nm and simultaneously at a wavelength of 880 nm to compensate for possible turbidity. The system is calibrated for human blood against the HiCN method (i.e., the Drabkin's method), which is the international reference method recommended by the International Council for Standardization in Haematology (ICSH) when measuring [Hb]. According to the manufacturer, the detection range of [Hb] by the Hb 801 is 10–256 g L^{-1}. To measure [Hb], 10 μL of blood was drawn into the microcuvette cavity using capillarity. The microcuvettes were placed in the Hb 801 analyser where the measurements of [Hb] were completed within 1 s. Duplicates were measured straight after blood was drawn into the microcuvette (within 15 s), as the manufacturer recommends reading the sample within 40 s after filling up the cuvette. [Hb] values of each blood sample were calculated as the mean of duplicate samples. In addition, for some samples, the blood-filled cuvette was read again after 2, 4, 6, 8, 10, 15, and 20 min to monitor any potential change in [Hb] over time.

3 | STATISTICS

Mean values (± SEM) are used in this study. For the comparison between the Drabkin method and the Hb 801 analyser, 17 rainbow trout, 21 Atlantic wolfish, and 29 Nile tilapia were used. For the time series, 19 Hb samples were used for rainbow trout. Here, samples from the first two fish were analysed in duplicates, whereas one Hb sample from each fish was analysed for the subsequent 15 trout; three unique Hb samples were used for Atlantic wolfish; and 14 unique Hb samples were analysed for Nile tilapia. Statistical analyses were performed using IBM SPSS Statistics 29 (IBM Corporation, New York, USA). Significance was assumed at $p < 0.05$.

Least-squares regressions were used to formulate a relationship between the Drabkin and Hb 801 methods. When the values were normally distributed (Shapiro–Wilk test $p > 0.05$; rainbow trout, Atlantic wolffish), a paired *t*-test was used to compare [Hb] obtained for each sample between the two methods. In the case of Nile tilapia, where the data were not normally distributed, a related-samples Wilcoxon signed-rank test was performed.

For the time series, a multiple comparison analysis (repeated one-way ANOVA with the Greenhouse–Geisser correction and Bonferroni's post hoc test) was used to compare the relative changes overtime during the 20 min following the initial reading for rainbow trout and Nile tilapia. As for Atlantic wolffish ($N = 3$), a non-parametric related-samples Kendall's coefficient of concordance was used.

4 | RESULTS

Overall, the variance in the replicate samples measured using the Hb 801 ranged from 0.0% to 8.5% (mean 1.3%). For the Drabkin method, the variance for the replicate samples ranged from 0.2% to 36.9% (mean 6.7%).

There were no differences between the two methods evaluated for any of the fish species tested (paired *t*-test, $p = 0.71$ for rainbow trout and $p = 0.36$ for Atlantic wolffish; related-samples Wilcoxon signed-rank test, $p = 0.36$ for Nile tilapia). There was a strong linear relationship between the [Hb] results measured using the two techniques ($R^2 = 0.978$ for rainbow trout, $R^2 = 0.706$ for Atlantic wolfish, $R^2 = 0.919$ for Nile tilapia, and $R^2 = 0.971$ for all fish) (Figure 1).

There was a general tendency in all the three fish species to have an increase in [Hb] measured in the same cuvette over time (Figure 2). For rainbow trout and Nile tilapia, there was an initial increase in [Hb] that plateaued after 6 and 10 min, reaching 2% and 3% change

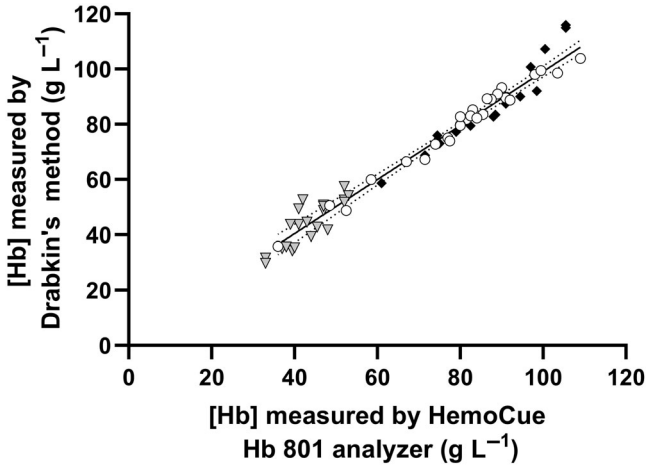

FIGURE 1 Comparison of blood [Hb] measured using the Drabkin's method (*y*-axis) and the Hb 801 analyser (*x*-axis) for rainbow trout (◆), Atlantic wolffish (▽), and Nile tilapia (○). Data for the Drabkin method were obtained after 1 h incubation at room temperature; data from the Hb 801 were measured within 15 s after the cuvette was filled with blood. No significant differences were observed for any of the fish species (paired *t*-test, $p = 0.71$ for rainbow trout and $p = 0.36$ for Atlantic wolffish; related-samples Wilcoxon signed-rank test, $p = 0.36$ for Nile tilapia). There was a strong linear relationship observed between the [Hb] results measured using the two techniques. The curve for the combined fish species (solid line) is described by $y = 0.9961x + 0.1012$, $R^2 = 0.971$. The dashed lines at either side of the regression are the 99% CI.

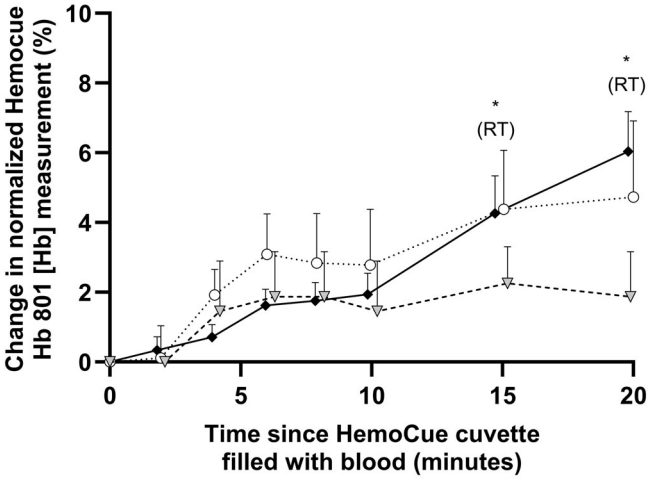

FIGURE 2 Relative changes in [Hb] readings from the Hb 801 analyser from the first reading at minute 0 and every 2 min until 10 min, and then at 15 and 20 min (mean ± SEM) for the blood of rainbow trout (◆), Atlantic wolffish (▽), and Nile tilapia (○). Asterisks (*) indicate significant differences from the initial value at minute 0 for rainbow trout (RT) (repeated one-way ANOVA with the Greenhouse–Geisser correction and Bonferroni's post hoc test, $p < 0.01$).

in [Hb], respectively, before the [Hb] continued to increase again to reach 6% and 5%, respectively, after 20 min. The difference with the initial measurement was only significantly different for rainbow trout and from 15 min onward (repeated one-way ANOVA with the Greenhouse–Geisser correction and Bonferroni's post hoc test, $p < 0.01$ for rainbow trout). For Nile tilapia (repeated one-way ANOVA with the Greenhouse–Geisser correction, $p = 0.031$), the Bonferroni post hoc test did not show any significant difference between time points. For Atlantic wolffish, there were no significant differences (related-samples Kendall's coefficient of concordance, $p = 0.08$). After 6 min, the samples reached an approximate 2% change and a plateau, whereafter no further change was observed.

5 | DISCUSSION

For the first time, the Hb 801 analyser has been tested on fish blood. Using the Drabkin's method as a reference, the Hb 801 analyser was validated as a reliable method for measuring blood [Hb] in all three species of fish assessed. Compared to its precursor, the Hb 201+, the Hb 801 does not need any correction factor for fish blood, and the cuvette containing the sample can be read immediately once it is filled with blood. The main difference between the two methodologies is that the cuvettes in the Hb 201+ device are coated with a reagent, which react differently between fish erythrocytes (nucleated) and human erythrocytes (unnucleated), whereas the cuvettes for the Hb 801 meter are uncoated (Andrewartha et al., 2016; Clark et al., 2008; Schwieterman et al., 2019). The variance between replicated samples was very low (below 1.3% on average), making this technique very reliable in comparison with the Drabkin method (with an average variance between replicates of 6.7%). The higher variation in the Drabkin method could be explained by the numerous steps (reagent preparation, pipetting, mixing, incubation, centrifugation, pipetting, and reading) in comparison with the Hb 801 where there were only two steps (filling the cuvette with blood and reading it). When evaluating the precursor, the Hb 201+ analyser, Clark et al. (2008) also noted a higher variance in the Drabkin method (4.4%) in comparison with the Hb 201+ meter (2.4%).

The Atlantic wolffish displayed the lowest and more narrow range in [Hb] among the three fish species (33–53 g L^{-1}). These low values, regardless of their stress status, could be imputable to their benthic and calm lifestyle (Foss et al., 2004). The two other species' [Hb] values were in a more comparable range of 61–109 g L^{-1} and 36–105.5 g L^{-1} for rainbow trout and Nile tilapia, respectively. Atlantic wolffish displayed the lowest R^2 (0.7055 for Atlantic wolffish, 0.978 for rainbow trout, and 0.919 for Nile tilapia) between the two methods, yet there were no significant differences for any species.

Both the Hb 201+ and the Hb 801 have been developed for measuring [Hb] in human blood. Consequently, the [Hb] calculation for both the Drabkin method and the HemoCue devices, whose internal calibration is based on the Drabkin method, relies on the molecular mass of human Hb. In normal human adults, three types of Hb are commonly found from which the HbA (composed of four monomers: two alpha and two beta globin chains) is the predominant Hb form, accounting for 96%–98% of all Hb. In the calculations, the molecular weight of Hb used is 64.5 kDa.

Recent studies have suggested that fish Hb might be more complex, where the composition is not universal but rather varies between and within species. Among vertebrates, fish display the highest diversity and the highest Hb polymorphism. Although there is no universal consensus on the biological significance of this diversity, the great heterogeneity and variability in fish Hb is thought to play a role in the ability of fishes to adapt to spatial and/or temporal variations in the environment (e.g., regarding oxygen availability, temperature, or acid–base balance) (De Souza & Bonilla-Rodriguez, 2007; Giardina et al., 2004).

More research has been carried out in rainbow trout Hb, where a high polymorphism in both Hb and their subunits was first described. This polymorphism could allow a better pH buffering within the erythrocyte and a higher Hb concentration. In rainbow trout, four to eight Hb isoforms have been identified (Binotti et al., 1971; Bossa et al., 1978; De Souza & Bonilla-Rodriguez, 2007; Fago et al., 2001; Iuchi, 1973; Petruzzelli et al., 1984, 1989; Weber et al., 1976). Based on those studies, the average molecular weight of trout Hb would be ca. 63.6 kDa, which is 0.9 kDa lighter than human Hb. There are no data on Hb molecular weight for Atlantic wolffish, but a study on its close relative, the spotted wolffish, found that there were three isoforms of Hb (Giordano et al., 2007), with an average Hb molecular weight of 63.5 kDa, 1 kDa lighter than human Hb. The first studies on Nile tilapia Hb suggested a molecular weight of 67–69 kDa for the tetrameric Hb molecules (Falk et al., 1998), about 2.5–4.5 kDa heavier than the human Hb. However, more recent studies, using cellulose acetate electrophoresis, suggested a molecular weight of Hb closer to the other two species studied, ca. 63 kDa (1.5 kDa lighter than human Hb) (Thongsarn et al., 2006).

Taking into account these slight differences in molecular weight between the Hb of humans and the Hb of different fish species, as well as the complexity and high diversity of fish Hb compared to human Hb, there is a risk of a slight margin of error when measuring [Hb] in fish blood. That is, unless a species-specific standard or correct Hb weight is used in the calculation formula, there is a risk of a slight under- (or over-) estimation of the actual [Hb] in fish blood. For example, the calculation for the Drabkin method, in both the present study and previous one (Clark et al., 2008), uses a human Hb molecular weight of 64.5 kDa. This results in a factor of 148 for multiplying the absorbance readings. With all the species in the current study having a potentially lighter Hb molecular weight (e.g., roughly 1 kDa lighter), the multiplying factor for these species should be closer to 145–146. This means that the Drabkin method, as well as the HemoCue methods (internally calibrated using the human Drabkin method), could be overestimated by ca. 0.5%–1%. This difference could be considered marginal and negligible, but for other fish species, the difference from human Hb might be even larger in which case the effect of using human molecular weight for Hb to calculate [Hb] might result in larger consequences. Therefore, we encourage researchers to make sure that the molecular weight of their species' Hb is compatible with the method prior to usage.

On the one hand, considering the large heterogeneity and variability in fish Hb, it is recommended to know the Hb molecular weight for the fish species of interest before applying any of the methods for measuring blood [Hb] that are based on the calculation formula containing the human Hb molecular weight. On the other hand, the three fish species chosen in the present study were diverse in terms of Hb molecular weight, lifestyle, and habitat (e.g., one pelagic temperate carnivore, one benthic, and one pelagic tropical omnivore) and could, therefore, be considered sufficiently representative. This, together with the fact that there were no clear interspecies variability in the present study, suggests that the Hb 801 meter could be appropriate for most fish species, assuming they do not deviate largely from the molecular weights of the three representative fishes examined here.

When leaving blood for incubation in the Hb 801 cuvettes, we observed a minor increase in [Hb] over time for all species tested. However, the only statistically significant result was found for rainbow trout, where the [Hb] at minute 15 and onward were significantly higher compared to the value at minute 0. Commonly for all species, there seems to be a small, non-significant trend for an initial increase to reach a first plateau between 6 and 10 min of blood incubation time. Therefore, although the manufacturer recommends reading the samples within 40 s after the cuvette has been filled, this study suggests that the values could still be read for up to 10 min post-filling. Nevertheless, we would still recommend reading the cuvettes as soon as possible, following the manufacturer's instructions, and if possible, within the first 4 min of incubation.

6 | CONCLUSIONS

The present study validates the use of the new Hb 801 analyser to measure [Hb] in fish blood. In fact, the method allows for a quicker measurement compared to previously validated [Hb] readers. The authors, therefore, encourage the use of the Hb 801 meter for detection of fish Hb, especially in field settings where a robust, rapid, and user-friendly portable device can be the key for successful sampling. The Hb 801 analyser also has the advantage that there is no need to further calibrate and/or correct for fish blood as there is no biochemical reaction in the cuvette, which could be influenced by differences in fish blood property, compared to human blood. The three fish species chosen in the present study were diverse in terms of lifestyle and habitat (e.g., one pelagic temperate carnivore, one benthic, and one pelagic tropical omnivore) and could, therefore, be considered sufficiently representative. This, together with the fact that there was no clear interspecies variability in the present study, suggests that the Hb 801 meter could be appropriate for most fish species.

AUTHOR CONTRIBUTIONS

Investigation, methodology, writing—review and editing: Joana Henze, Sofia Kamperin, Eric Metsmaa, Lizeth Alcocer Arboleda, and M. Iranzu Calocany Aramendia. Investigation, writing—review and editing: Erika Sundell, Ida Hedén, and Niklas Warwas. Conceptualization, methodology, formal analysis, investigation, visualization, writing—original draft, supervision, funding acquisition, project administration, writing—review and editing: Jonathan A. C. Roques.

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