# Activity, energy use and species identity affect eDNA shedding in freshwater fish

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**Abstract**

The quantitative measurement of eDNA form field-collected water samples is gaining importance for the monitoring of fish communities and populations. The interpretation of these signal strengths depends, among other factors, on the amount of target eDNA shed into the water. However, shedding rates are presumably associated with species-specific traits such as physiology and behavior. Although such differences between juvenile and adult fish have been previously detected, the general impact of movement and energy use in a resting state on eDNA release into the surrounding water remains hardly addressed.

In an aquarium experiment, we compared eDNA shedding between seven fish species occurring in European freshwaters. The investigated salmonids, cyprinids and sculpin exhibit distinct adaptions to microhabitats, diets, and either solitary or schooling behavior. The fish were housed in aquaria with constant water flow and their activity was measured by snapshots taken every 30 s. Water samples for eDNA analysis were taken every 3 h and energy use was determined in an intermittent flow respirometer. After controlling for the effect of fish mass, our results demonstrate a positive correlation between target eDNA quantities as measured with digital PCR, fish activity and energy use, as well as species-specific differences. For cyprinids, the model based on data from individual fish was only partly transferable to groups, which exhibited lower activity and higher energy use.

Our findings highlight the importance of fish physiology and behavior for the comparative interpretation of taxon-specific eDNA quantities. Species traits should therefore be incorporated into eDNA-based monitoring and conservation efforts.

## Introduction

The sensitivity, non-invasiveness and cost-efficiency of environmental DNA (eDNA) based methods has been proven for diverse habitats and species making them powerful new tools for conservation biology and biodiversity assessments (Barnes and Turner, 2016; Deiner et al., 2017; Huerlimann et al., 2020). Regarding the detection of fish species, eDNA-based monitoring outperforms traditional methods such as electrofishing: for example, for the detection of the endangered European weather loach, *Misgurnus fossilis* (Sigsgaard et al., 2015), the assessment of fish communities in Australian streams (McColl-Gausden et al., 2020), and the distribution of brook trout, *Salvelinus fontinalis* in a US watershed (Evans et al., 2017). The manifold successes of eDNA-based species detection lead to a call for more standardization and better reporting practices (Goldberg et al., 2016; Minamoto et al., 2020; Thalinger et al., 2020a) and to an international effort for implementing the technology into routine species monitoring (Leese et al., 2016; Pilliod et al., 2019). Although reporting the presence/absence of particular species is the starting point of these endeavors, a more quantitative interpretation of field-derived eDNA data is key for the broader applicability of this technology.

Different processes influence the distribution of eDNA in space and time and the detection probabilities of species from environmental samples, namely the origin, degradation, suspension, resuspension, and transport of eDNA (Barnes and Turner, 2016; Harrison et al., 2019). The latter processes are directly linked to local hydrology (e.g. flow and substrate type (Shogren et al., 2017; Pont et al., 2018; Thalinger et al., 2020b)) and environmental conditions (e.g. water temperature, pH, UV-radiation (Strickler et al., 2015; Lacoursière-Roussel et al., 2016; Tsuji et al., 2017)). The amount of eDNA in the water column is directly linked to fish biomass and originally, this was confirmed for common carp (*Cyprinus carpio*) in an aquarium trial and experimental ponds (Takahara et al., 2012). In subsequent experiments, this positive relationship was confirmed for a range of freshwater and marine fish species (Evans et al., 2016; Lacoursière-Roussel et al., 2016; Sassoubre et

al., 2016; Doi et al., 2017; Horiuchi et al., 2019; Jo et al., 2020). However, these results were primarily obtained for individuals at the same life stage.

eDNA is released into the environment in the form of mucus, feces, scales, and gametes (Merkes et al., 2014; Barnes and Turner, 2016; Sassoubre et al., 2016; Bylemans et al., 2017). Under natural conditions, differences in fish physiology, diet and behavior are likely to affect this process and confound the interpretation of eDNA-based results from a water body (Klymus et al., 2015). For perch and eel, Maruyama et al. (2014) and Takeuchi et al. (2019), respectively, found lower eDNA shedding rates for adults in comparison to juveniles, which is likely caused by the ontogenetic decrease in metabolic rates (Winberg, 1960). However, these findings could not be confirmed in an experiment with a salmonid species (Mizumoto et al., 2018). In general, the metabolic rate and activity differ between fish species due to distinct physiology and behavior with pelagic species being more active and displaying higher resting metabolic rates than benthic species (Johnston et al., 1988; Killen et al., 2010). These factors are frequently hypothesized as underlying causes for spiking eDNA levels at the beginning of aquarium experiments and mismatching quantitative results in studies comparing eDNA levels between species in the same water body (Takahara et al., 2012; Maruyama et al., 2014; Evans et al., 2016).

Here, we investigate the effect of fish activity (i.e. movement), energy use (i.e. oxygen use × oxycaloric factor), and species identity in an aquarium experiment with seven fish species commonly occurring in European rivers and streams. The examined species comprised four salmonids (*Salmo trutta, Salvelinus fontinalis, Oncorhynchus mykiss, Thymallus thymallus*), two cyprinids (*Phoxinus phoxinus*, *Squalius cephalus*) and one sculpin (*Cottus gobio*). *Salmo trutta* is a rhithral species, territorial in later life stages, and primarily feeds on benthic organisms and insects drift on the surface. *Salvelinus fontinalis* and *O. mykiss* were anthropogenically introduced into European freshwaters; they are omnivorous and less territorial than *S. trutta*. If possible, these three species choose areas with reduced current close to the main riverbed as preferential microhabitat. *Thymallus thymallus* is also a rhithral species, but its scales are larger and adults primarily use the main riverbed (Spindler,

1997; Freyhof and Kottelat, 2007). *Phoxinus phoxinus* is a schooling, small fish species in the rhithral. It feeds on a mixture of plant debris, algae and small invertebrates. The juveniles prefer vegetation-rich microhabitats without current, while adults switch to gravel substrate with low to intermediate flow. *Squalius cephalus* is eurytopic and can occur in habitats with strong to low current. Its juveniles are schooling and omnivorous with adults predominantly preying on fish. *Cottus gobio* is a rheophilic and benthic species primarily feeding on small bottom invertebrates. It has no swim bladder and mostly resides in interstices between large boulders or on coarse gravel characterized by low current (Spindler, 1997; Freyhof and Kottelat, 2007).

We hypothesized that higher activity (i.e. movement) leads to higher eDNA concentrations as there is more shearing between the fish surface and the surrounding water, and higher volumes are pumped through the gills due to the elevated oxygen demand. Independent of activity, fish species with higher energy use in a resting state potentially also emit more eDNA. Additionally, the species-specific composition of the constantly renewed cutaneous mucus layer (Ángeles Esteban, 2012) might lead to differences between individual taxa.

## Materials and Methods

### *Experimental setup*

The aquarium experiment was carried out between 2$^{nd}$ March and 17$^{th}$ July 2017 at the Research Department for Limnology Mondsee of the University of Innsbruck, Austria (further on “ILIM”). The salmonid species were purchased from commercial hatcheries, *P. phoxinus* and *S. cephalus* were caught with permission in Lake Mondsee and *C. gobio* were caught with permission in rivers in Tyrol (Austria). Fish individual sizes were chosen as similar as possible within and between species. Until the start of the experiment, the fish species were kept separately in aquaria fed with lake water at the ILIM.

In accordance with the regulations of the Austrian Animal Experiment Act (December 28, 2012) (Tierversuchsrechtsänderungsgesetz, part 1, section 1, §1, point 2), and with the Directive 2010/63/EU of the European Parliament and of the Council of the European Union (September 22, 2010) on the protection of animals used for scientific purposes (chapter 1, article 1, point 5a), all fish were reared according to regular agriculture (aquaculture) practice, including provision of appropriate tank size, sufficient rate of waterflow, natural photoperiod, ad libitum food supply, and temperatures within the species' thermal tolerance range. This ensured that no pain, suffering, distress or lasting harm was inflicted on the animals, confirmed by the fact that mortality rates were low and equal between rearing groups. Based on the legislative provisions above, no ethics approval and no IACUC protocol was required for the experiments performed. In particular the respirometry experiments were discussed with the legislative authorities (Austrian Federal Ministry of Education, Science and Research and University of Veterinary Medicine, Vienna) and the conclusion was that the assessment of basic metabolism under these conditions (small fish sizes in relatively large chambers) does not incur pain, suffering or distress to the fish and no formal animal experimentation protocol was required.

Five aquaria (60 l) and corresponding plastic lids were used in the experiment, each of which was thoroughly cleaned with sodium hypochlorite (5 %) and then rinsed with tap water (fish-DNA free) prior to each experimental run (i.e. changing the fish under investigation). The flow-through rate (aquaria were tap-water fed) was set to 5.45 l/min to mimic natural conditions and keep eDNA concentrations in the fish tanks constant based on previous test runs (data not shown). Water temperature was kept stable at 15 °C for inflowing water automatically stabilizing temperature in the aquaria at this level. Each tank was further equipped with an air-stone to ensure water mixing. At the start of each experimental run, a water sample (negative control) was taken from one of the aquaria and processed as described below. Then, five fish individuals of each species were selected aiming at similar sizes across all fish species. Each fish was placed individually in an aquarium using DNA-free fishnets (Fig. 1). For *P. phoxinus* and *S. cephalus*, the experiment was carried out twice:

once with individual fish, and once with groups of three fish per aquarium. The day before the experiment and for its duration, the respective fish were not fed to avoid contamination by fish feed and minimize effects of defecation. Each run started with one day of familiarization in the aquaria.

*Water sampling, filtration and pH*

All equipment used for this process was cleaned with sodium hypochlorite (5 %) and rinsed with tap water prior to each use; DNA-free gloves were always worn. On the second day, 2 l water samples were taken every three hours from 9 o'clock to midnight at the back end of each aquarium (opposite to the inflow) using flexible tubes and 2 l wide neck bottles (Fig. 1). The water samples were immediately filtered in an adjacent laboratory using glass microfiber filters (1.2 µm pore width, 47 mm diameter, Whatman GF/C) and one negative control consisting of 2 l MilliQ-water was included per sampling event. Thereafter, the filters were individually placed in 2 ml reaction tubes and stored at -28 °C until further processing in a special diagnostic molecular laboratory at the Department of Zoology, University of Innsbruck (Austria). After each sampling, pH was measured in three arbitrarily selected fish tanks using a Hach HQ40 device.

*Activity measurement*

During the familiarization time (day 1) and between water samplings, fish swimming activity was quantified using a custom-made activity monitoring system consisting of one high-definition USB camera (Ziggi HD Plus, IPEVO.COM, Great Britain) per aquarium. The cameras were placed at the front of each tank and the focus was set towards the back end (Fig. 1). To enable recordings during the night, aquaria were lighted throughout the two recording days. Additionally, white polystyrene plates were used to cover the bottom and the sides of each fish tank to exclude influences from neighboring aquaria and standardize reflections. The signals from the cameras were acquired with a frame rate of 2 fpm ("frames per minute") with a macro using the image analysis software FIJI (http://fiji.sc/, a distribution

of ImageJ) for MacOS (Schindelin et al., 2012; Rueden et al., 2017). For each aquarium, a region of interest ("ROI") excluding the inflow, air-stone and sidewalls was set manually (Supporting Information (SI) 1). Subsequent frames were arithmetically subtracted and the average gray scale within the region of interest, as a quantification of fish activity, was extracted from the difference-images. The dataset was manually checked to exclude artifacts produced by changes in illumination (light/dark illumination of the fish), water sampling, measurement of abiotic factors, fogged-up aquarium front and few other camera movements sometimes leading to a changing region of interest in the recordings (SI 1).

*Respirometry*

A custom made intermittent-flow respirometer was used (Forstner, 1983; Svendsen et al., 2016) including three measurement chambers placed in a larger tank (Fig. 1). The device was cleaned prior to each fish change using a mixture of 3 % hydrogen peroxide and 3 l of tap water. The volume of each chamber was determined prior to the experiment and oxygen saturation (100 %) and temperature (8 – 9 °C) were kept constant in the tank via an airstone and a laboratory heating/cooling device (Lauda DLK 10 and Alpha 1, Lauda Germany). The three chambers of the respirometer were connected to the respirometers' water circuit, constantly pumping $O_2$-saturated water from the large tank through the three chambers. For measurements of oxygen consumption, a chamber was cut off from this circuit and a short, closed loop for this chamber was established. Dissolved oxygen was measured in this chamber every 30 s for a period of 15 min. using a YSI ProODO probe (YSI Inc. Yellow Springs, USA) and logged to a computer before the system switched to the next chamber for a 15 min measuring period. On the third day of an experimental run, three of the five fish were placed individually in the chambers avoiding air bubbles and kept there for 24 h for familiarization. On the fourth day, respirometer measurements were carried out for 24 h. Thereafter, the remaining two fish individuals were placed in two measurement chambers for one day of familiarization followed by one day of measurements (day five and six; Fig. 1). The third chamber was left empty, but measured as well, to evaluate potential

213 microorganism-induced oxygen decrease. After the respirometer measurement day, the
214 mass [g] and total length [mm] of each fish was determined before placing them together in a
215 fish tank. For respirometer measurements of fish groups, the three individuals previously
216 sharing an aquarium, were put together in a respirometer chamber.
217
218 *Filter processing and molecular analysis*
219 After defrosting, each filter was soaked with 200 µl of lysis buffer consisting of TES-buffer
220 (0.1 M TRIS, 10 mM EDTA, 2 % sodium dodecyl sulphate; pH 8) and proteinase K (20 mg/m)
221 in a ratio of 19:1 and incubated at 56 °C over night in a rocking platform. On the next day,
222 filters were transferred with DNA-free forceps to a perforated inset which was repositioned in
223 the top half of the original 2 ml reaction tube and centrifuged for 10 min at 14 000 rpm.
224 Afterwards, filters were discarded and the lysate at the bottom of the reaction tube (300-
225 800 µl) used for DNA extraction. Insets were cleaned in sodium hypochlorite (2.5 %) for at
226 least 30 min, thoroughly washed with MilliQ-water (10 wash steps) and reused.
227 DNA extraction was carried out with the Biosprint 96 instrument (Qiagen) using the
228 Biosprint 96 DNA blood Kit (Qiagen) and the Biosprint 96 tissue extraction protocol in
229 accordance with the manufacturer's instructions except for using 100 µl of TE-buffer instead
230 of AE-buffer for DNA elution. Extractions were carried out in 96-well plates and four negative
231 controls (containing TES-buffer instead of lysate) were included per plate. To process the
232 whole lysate volume, a custom DNA-uptake program was set up: three uptake plates were
233 used and 300 µl of lysate, 300 µl AL-buffer and 300 µl isopropanol were mixed per well in
234 each plate. Missing lysate volumes (i.e. if only a total of 400 µl were available after
235 centrifugation) were replaced by TES-buffer. Additionally, 30 µl MagAttract was added per
236 well in the first plate. Using custom "binding" steps of the robotic platform, the DNA contained
237 in the first plate was transferred to the second one, next a binding step was carried out in the
238 second plate before transferring and releasing the entire collected DNA into the third plate,
239 which was then used for the Biosprint 96 tissue extraction protocol. After extraction, each
240 eluate was transferred to a 1.5 µl reaction tube for subsequent PCR.

All used primers (Table 1) have been previously published after extensive specificity and sensitivity testing (Thalinger et al., 2016, 2020b) and additional specificity tests were carried out on the digital PCR (dPCR) system (see below) confirming the specificity of the molecular assays under the following conditions: each 22 µl dPCR master mix for droplet generation on the QX200 AutoDG (Biorad) consisted of one-time EvaGreen Supermix (Biorad), 0.25 µM forward and reverse primer (Table 1) and up to 10.5 µl DNA extract. Depending on the results of initial tests with capillary electrophoresis PCR (i.e. the Relative Fluorescence Units (RFU) of the resulting band; see SI 2), extracts were diluted with molecular grade water for dPCR as follows: RFU < 0.2: undiluted; 0.2 ≤ RFU < 1.3: 1:1 dilution; 1.3 ≤ RFU < 2: 1:3 dilution; 2 ≤ RFU: 1:7 dilution. Optimized thermo-cycling conditions were 5 min at 95°C, 40 cycles of 30 s at 95°C, 1 min at 58°C (*O. mykiss*, *P. phoxinus,* and *S. cephalus*) or 60°C (*C. gobio*, *S. fontinalis*, *S. trutta*, and *T. thymallus*), 1 min at 72°C, followed by one step of 5 min at 4°C and 5 min at 90°C. dPCR results were analyzed on the QX200™ Droplet Reader and the corresponding QuantaSoftTM Analysis Pro Software (Version 1.7; Biorad). As target signal amplitude varied with the length of the amplified fragment, amplitude thresholds were set individually per primer pair (Table 1) prior to determining target copy numbers per µl for each DNA extract. Per primer pair, a positive (DNA extract from target species) and a negative control (molecular grade water) were included in dPCR, all of which resulted positive and negative, respectively.

*Statistical analysis*

All calculations and visualizations were carried out in R Version 4.0.2 (R Core Team, 2020) using the packages “ggplot2” (Wickham, 2016), “ggpubr” (Kassambara, 2019), “nlme” (Pinheiro et al., 2020), "AICcmodavg" (Mazerolle, 2020), “rsq” (Zhang, 2020) and “sjPlot” (Lüdecke, 2020). As pH was not measured in all aquaria after each water sampling, missing values were estimated by averaging measurements taken at the respective fish tank before and after the skipped time step. If measurements at the first or last water sampling were missing, the values of the following or previous time step, respectively, were carried over.

The cleared activity dataset was visually inspected and summarized for each time step: for example, data obtained during the preceding day were associated with the first eDNA sampling event at 9 AM and measurements between 9 AM and 12 noon were considered relevant for the second water sampling at 12 noon. Mean activity was calculated per time interval. No cleared activity data was available for one *S. trutta* and *S. fontinalis* individual, respectively, and for one *P. phoxinus* and *T. thymallus* individual at a single time step each.

The total respirometry dataset was cleared of all 15 min measurement series showing an increase in dissolved $O_2$. As this value is expected to decrease linearly over the course of a measurement, a linear regression for the oxygen decrease in a measurement chamber over time was calculated for each measurement series. All intervals for which the obtained values showed a bad fit to a linear decrease ($R^2 < 0.8$) were also excluded from further analyses. For each of the remaining measurement intervals, oxygen consumption (OC) in mg / h was calculated as $OC = -s \times 60 \times vol$ where "s" denotes the slope of the linear regression and "vol" the volume of the respective measurement chamber minus the mass of the fish. Per fish species, the obtained value was corrected for the mean oxygen consumption in the empty chamber before calculating total energy use (oxygen consumption × 13.6 J/mg (oxycaloric factor (Brett and Groves, 1979)) per fish. Finally, energy use [J/h] was averaged across the values obtained from individual measurement intervals for each fish and fish group. Due to data clearing, this was not possible for one individual and one group of *C. gobio* and *S. cephalus*, two individuals of *S. fontinalis* and *S. trutta* and three individuals of *T. thymallus*. For these fish, energy use was estimated as the mean of the available values.

Concerning the fish-eDNA copy numbers obtained from dPCR, 21 filtered water samples did not lead to an amplification. They were removed from the dataset, as other fish individuals of comparable size and other samplings reliably produced positive results and hence, errors in sample processing might have occurred. One group of *P. phoxinus* had to be excluded from further analyses, as two of three individuals were accidentally chosen

297 *S. cephalus*. To determine whether the pH measurements, mean activity and eDNA copy
298 numbers were significantly influenced by sampling (i.e. time of the day), a one-way repeated
299 measurements ANOVA with rank transformation was calculated for each variable using a
300 combination of fish species and aquarium as random factor. A significant trend could not be
301 detected (Table 2). Despite efforts to standardize the mass of the chosen fish individuals
302 within and between species, fish mass was identified as confounding variable (SI 3). Hence,
303 eDNA copies, mean activity, and energy use were all normalized by the mass of the
304 respective fish individual prior to all further analyses.

305 Generalized Linear Models (GLM) for a Gamma-distributed dependent variable (i.e.
306 eDNA copies; positive continuous variable) and a log-link function were set up to investigate
307 the effects of fish species, energy use, mean activity, and pH (Faraway, 2016). Data
308 obtained from fish groups were excluded from the comparison of model performance. Due to
309 the small number of tested fish individuals and species examined, no models with random
310 factors were considered, but sampling and fish individuals were included in several models to
311 show their ineffectiveness in explaining the obtained data (Table 3). The variable "fish
312 species" was entered via dummy coding into the models using "*C. gobio*" as base category.
313 Corresponding with the focus of this study to investigate the effect of species identity, fish
314 physiology and activity on eDNA shedding, a set of six candidate models was chosen (Table
315 3). AICc, ΔAICc, and AICc weights (ω) were used to evaluate the strength of the six models
316 for describing the data including Nagelkerke, Cragg & Uhler's pseudo-$R^2$ values (Burnham
317 and Anderson, 2002). Simulated, scaled residuals were calculated based on the best-
318 performing candidate model, (package: DHARMa (Florian Hartig, 2020); function:
319 "simulateResiduals"; n = 1000); the best performing model passed the consecutive check for
320 outliers and overdispersion.

321 To test the differences between single and grouped fish in the different stages of the
322 experiment, a data subset containing only values obtained from single and grouped
323 *P. phoxinus* and *S. cephalus* was analyzed. Target eDNA copies, energy use, and mean
324 activity (all normalized by fish mass) of the four distinct fish categories were tested for

normality and homogeneity of variance with Shapiro-Wilk and Bartlett tests. Then, differences between groups were examined via Kruskal-Wallis tests followed by Wilcoxon rank sum tests with Benjamini-Hochberg-corrected p-values. In a final step, target eDNA copies for groups of *P. phoxinus* and *S. cephalus* were predicted using the model previously established for single fish. Pairwise Wilcoxon tests were used to verify whether there was a significant difference between predicted and measured target eDNA copy numbers for both species separately and combined.

## Results

The mean mass of individually housed fish was 3.06 g ± 1.56 g (SD) and *C. gobio* individuals had the highest mass (5 g ± 2.1 g (SD)). Water samples from *P. phoxinus* and *T. thymallus* aquaria had the highest eDNA copy numbers per µl extract and gram fish mass (31.13 ± 53.23 (SD) and 47.68 ± 41.13 (SD), respectively; Fig. 2). The normalized mean activity was highest for *S. fontinalis* (1.08 ± 0.33 (SD)) and lowest for *C. gobio* (0.34 ± 0.10 (SD); Fig. 2). The energy use per gram fish mass was highest for *O. mykiss* (1.81 J/h ± 0.91 J/h (SD)), while *S. fontinalis* and *S. trutta* aquaria had the lowest pH.

The ΔAICc-based comparison of model weight (single fish only) resulted in model #3 outperforming five other candidate models (Table 3 and Table 4). Therein, mean activity, energy use and fish species were contained as explanatory variables (pseudo-$R^2_{NCU}$ = 0.60; dispersion parameter = 1.02). Increased activity had a significantly positive effect on eDNA copy numbers ($p < 0.01$) and *P. phoxinus*, *S. cephalus* and *T. thymallus* displayed significantly higher copy numbers compared to *C. gobio* (base group), after controlling for the effect of fish mass. The relationship between energy use and copy numbers was also found to be positive, but not significant ($p = 0.05$; Table 5 and Fig. 3).

For single and grouped individuals of *P. phoxinus* and *S. cephalus*, significant differences were detected between the four groups regarding target eDNA copies

353 ($Chi^2 = 10.59$; $p < 0.05$), mean activity ($Chi^2 = 80.95$; $p < 0.001$) and energy use
354 ($Chi^2 = 36.77$; $p < 0.001$): mean activity was significantly higher when fish were kept solitary
355 compared to having them in groups for both species ($p < 0.001$). Contrastingly, energy use
356 was significantly higher for grouped individuals in both species ($p < 0.01$). Target eDNA
357 copies were significantly higher for grouped *P. phoxinus* (42.61 ± 48.04 (SD)) compared to
358 single and grouped *S. cephalus* and characterized by few outliers with particularly high eDNA
359 concentration (Fig. 2 and Fig. 4)

360 To test the suitability of model #3 for describing eDNA shedding also for grouped fish,
361 model #3-predicted eDNA copies were compared to the measured copy numbers in the
362 group treatments. For the two species combined, there was no significant difference between
363 predicted and measured copy numbers ($W = 0.98$, $p = 0.34$). For *P. phoxinus* alone, no such
364 difference was detected either ($W = 270$; $p = 0.72$; Fig. 5), while predicted and measured
365 copy numbers of *S. cephalus* showed a significant difference ($W = 614$; $p < 0.05$; Fig. 5).

366

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368

369 **Discussion**

370 This experiment confirms the hypothesized positive relationship between eDNA shedding,
371 fish activity and energy use. Additionally, species identity and thereby associated
372 physiological differences were found to influence the amount of released eDNA.
373 Furthermore, our data show that models of eDNA shedding cannot always be generalized
374 from experiments with individual fish to fish groups. For a conclusive habitat-scale estimation
375 of fish communities with eDNA-based methods it is therefore necessary to incorporate
376 species physiology and behavior into the analysis.

377 In early aquarium experiments, the strongest eDNA signals were found right after the
378 introduction of fish into tanks without water circulation and often explained by elevated stress
379 levels through handling and adaption to the new environment (Takahara et al., 2012; Klymus
380 et al., 2015; Maruyama et al., 2019). Hence, many recent studies allow for one or several

381 days of accommodation prior to eDNA sampling (Lacoursière-Roussel et al., 2016; Jo et al.,
382 2019; Takeuchi et al., 2019). We can confirm the positive relationship between fish activity
383 (i.e. movement) and eDNA shedding independent of the introductory phase of an
384 experiment. However, it was not possible to determine the actual reason for the elevated
385 eDNA levels associated with higher activity, as both higher metabolic rates during movement
386 and higher water volumes shearing against the fish body could be responsible for this effect.
387 For eDNA-based field studies this result indicates that signals emitted by highly active fish
388 (e.g. during spawning or predatory behavior) potentially mimic higher levels of fish biomass.
389 Energy use in a resting state as measured with an intermittent-flow respirometer, was
390 also positively correlated with eDNA shedding, albeit with a smaller effect size. Potentially,
391 this finding can be attributed to the higher metabolic rate and larger gill size of active species
392 in combination with higher water volumes pumped through them (Wegner et al., 2009).
393 However, the elevated eDNA signals could also stem from other physiological processes
394 (e.g. defecation), which are known to positively influence eDNA shedding rates (Klymus et
395 al., 2015). As fish were not fed during the entire experiment, the latter factor is potentially
396 negligible. Except for *T. thymallus* the energy use of the species preferring microhabitats with
397 strong currents and preying on fish as adults (primarily *O. mykiss* and *S. cephalus*) was
398 higher than for *C. gobio* and *P. phoxinus*. This is in concordance with general differences in
399 resting metabolic rates between these ecological guilds (Roberts, 1975; Johnston et al.,
400 1988; Killen et al., 2010).
401 There were distinct differences in eDNA shedding between the species, with
402 *T. thymallus*, *P. phoxinus* and *S. cephalus* emitting the most eDNA. The adaptation to
403 habitats with stronger currents (Freyhof and Kottelat, 2007), namely an increased mucus
404 production in combination with comparably large scales, might explain this result for
405 *T. thymallus* and *S. cephalus*. The underlying taxonomy could also contribute to this pattern if
406 cyprinids (*P. phoxinus* and *S. cephalus*) generally pass more DNA into the surrounding water
407 via their gills, feces or mucus. Another explanation for the high eDNA shedding of cyprinids
408 in this experiment could be the stress induced by solitary housing. The model estimating

409 eDNA concentrations for individual fish could not fully explain the findings obtained for
410 grouped fish: the activity of both *P. phoxinus* and *S. cephalus* was significantly lower when
411 fish were held in groups, while their energy use was significantly higher. However, the
412 opposing direction of these two effects potentially explains why differences between
413 predicted and measured eDNA quantities were only significant for *S. cephalus*.

414 Generally, the measured eDNA concentrations per µl DNA extract were right-skewed
415 and a few exceptionally high values showed considerable influence on the size of standard
416 deviations. These results were independent of fish handling and stress during the
417 introduction phase as eDNA sampling started only after 24 h and the aquaria had constant
418 flow with the entire volume being renewed every 11 min. Such “outliers” were also detected
419 in other aquarium experiments (Klymus et al., 2015; Wilcox et al., 2016) and cell-
420 conglomerates released into the surrounding water were previously deemed responsible for
421 this pattern (Wilcox et al., 2016). Additionally, the size distribution of eDNA particles (from
422 < 0.2 µm to >180 µm) and commonly detected fragment sizes suggest intact cells or
423 organelles as the primary source of eDNA in the water column (reviewed by Harrison et al.,
424 2019). Our data support the hypothesis of constant eDNA shedding rates at constant
425 environmental conditions. We could not observe any effects of sampling time, possibly due to
426 the constant illumination of the aquaria. Hence, this aspect is not necessarily transferable to
427 natural environments where fish are known to exhibit distinct diurnal movement patterns
428 (Helfman, 1986).

429 The influence of fish mass on eDNA concentrations was not in the focus of this
430 experiment and fish individuals were as similar in size/mass as possible. However, adult fish
431 of *P. phoxinus* and *C. gobio* are considerably smaller in comparison to the other species
432 (Freyhof and Kottelat, 2007) and the respective juveniles were thus in a later life stage.
433 Based on the ontogenetic decrease in metabolic rate (Winberg, 1960), this could be an
434 alternative explanation for the low energy use of these two species. For studies investigating
435 eDNA shedding directly from live animals, biomass will always be an influential and
436 potentially confounding variable and should thus be considered carefully already during

437 experimental design. Depending on the actual main source of eDNA (feces, mucus…) it is
438 furthermore questionable, if fish mass is the best index variable to describe study animals.
439 Fish length should also be considered as it is a good approximator for activity measurements
440 via videotaping, an estimator of fish surface, and at least for eels a good estimator of eDNA
441 shedding (Takeuchi et al., 2019). Since body shapes and fins differ a lot between taxa
442 (Freyhof and Kottelat, 2007), it could also be beneficial to factor body shape (fusiform,
443 elongated, flat,…) into future experiments in case the fish surface is the primary source of
444 eDNA. Finally, individual differences are well documented for fish behavior and metabolic
445 rates (Metcalfe et al., 2016). The number or study animals in future experiments should thus
446 be increased to better control for such effects within the same species.

447 Our results demonstrate that for the successful application of eDNA-based methods
448 on a habitat scale it is necessary to incorporate fish physiology and behavior not only in the
449 study design and sampling process (e.g. by sampling at different depths and in different
450 micro-habitats (Littlefair et al., 2020)), but also during data analysis (Barnes and Turner,
451 2016; Thalinger et al., 2020a). Seasonal patterns could have a much stronger effect on
452 eDNA concentrations in the water column as previously assumed: for instance, many
453 cyprinids in European freshwaters seek calm areas without current during the winter. Their
454 eDNA is less likely to spread through the water column and additionally, their decreased
455 activity and energy use lower the detection probability even further. In the future, the eDNA
456 shedding of manifold fish species and families in relation to their biomass, activity and energy
457 use should be investigated to deepen our understanding of taxon-specific effects. Until then,
458 estimations of fish biomass from eDNA quantities in field-collected samples should at least
459 take distinct physiology and behavior into account, especially for comparative analyses
460 between species or seasons.

461

462

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**Conflict of interest**

MT is the co-founder of Sinsoma GmbH, a for profit company dedicated to DNA analyses in environmental studies.

**Author contribution statement**

MT and JW, conceived the study; the experiment was designed by BT, MT, TS and JW, and carried out by AR and AT under the supervision of BT and JW. TS was responsible for the processing of activity data; AR, AT and YP were responsible for laboratory processing of the eDNA samples under the supervision of BT who also carried out statistical analysis and wrote the first draft of the manuscript which was revised by all co-authors.

**Data Availability Statement**

All data on eDNA signals, fish activity, energy use, fish mass and pH have been uploaded to Figshare and are available at https://doi.org/10.6084/m9.figshare.13151180.v1

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# Tables and Figures

**Table 1:** PCR assays used to amplify fish-eDNA: columns denote the target taxon of each primer combination, primer names, sequences, their respective concentration in PCR, target gene, amplicon sizes, and threshold value for positive droplets in dPCR. Additionally, the source column states whether a primer pair has been previously published. Please note that the *Salvelinus* sp. primer pair was designed to amplify both *S. fontinalis* and *Salvelinus umbla*.

**Table 2:** The results of one-way repeated measures ANOVA with rank transformation examining a potential effect of sampling on pH, mean activity and target eDNA copy numbers.

**Table 3:** Covariate structures of the candidate models compared for their potential to explain the target eDNA copy numbers per gram fish and µl extract obtained from single-fish aquaria with the following parameters: fish species identity (seven species), mean activity (per gram fish), energy use (per gram fish) and pH. Sampling event and fish individual number were included primarily to show their insignificance.

**Table 4:** Results of the ordinal ranking based on ΔAICc for the Generalized Linear Models (Table 3). Models are ordered from high to low weight and K denotes for the number of estimable parameters, AICc for the second-order variant of Akaike's Information Criterion, ΔAICc for AICc difference and ω for Akaike weight.

**Table 5:** The highest weight (ω = 0.90) Generalized Linear Model (#3) describing the measured eDNA copy numbers via mean activity, energy use and fish species identity. Significant p-values of fish species in the model refer to a significant difference between *Cottus gobio* (used as base category for dummy coding) and the respective fish species.

**Figure 1:** The setup of the aquarium experiment carried out with seven fish species: five individual fish were put in fish tanks for water sampling (eDNA) and activity recordings (day 1 and 2) followed by respirometer measurements (three individuals on days 3 and 4; two individuals plus empty control chamber on days 5 and 6). For *Phoxinus phoxinus* and *Squalius cephalus* the experiment was repeated using groups of three individuals.

**Figure 2:** Key parameters obtained during the experiment for single fish. Boxplots display target eDNA copies per µl extract, energy use [J/h], mean activity, and pH per fish species. Fish species are abbreviated: "Cot gob": *Cottus gobio*; "Onc myk": *Oncorhynchus mykiss*; "Pho pho": *Phoxinus phoxinus*; "Sal fon": *Salvelinus fontinalis*; "Sal tru": *Salmo trutta*; "Squ cep": *Squalius cephalus*; "Thy thy": *Thymallus thymallus*. The variables target eDNA copies, mean activity and energy use were normalized by fish mass to control for the effect of this confounding variable.

**Figure 3:** Graphic representation of the GLM model coefficients (model #3) best describing the obtained target eDNA copy numbers. Significance codes of denoted fish species indicate differences in comparison to the base category *Cottus gobio*, whiskers denote the 95%-CI.

**Figure 4:** Comparison of target eDNA copies, mean activity and energy use (normalized by fish mass) in aquaria obtained from single and grouped individuals of *Phoxinus phoxinus* and *Squalius cephalus*. Different lower case letters above boxplots code for significant differences ($p < 0.05$) between categories, which are abbreviated as: “Pho pho”: *Phoxinus phoxinus* (single fish); “Pho pho g”: *Phoxinus phoxinus* grouped fish; “Squ cep”: *Squalius cephalus* (single fish); “Squ cep g”: *Squalius cephalus* grouped fish.

**Figure 5:** For groups of *Phoxinus phoxinus* (Pho pho g) and *Squalius cephalus* (Squ cep g) measured and predicted copy numbers are plotted: left: against each other; middle: predicted copy numbers are compared between species; right: comparison of measured copy numbers between the two species. The measured copy numbers were log-transformed to enable a direct comparison with the values predicted by the Gamma GLM with log-link function. For *S. cephalus* a significant difference between measured and predicted copy numbers was detected ($t = 2.37$; $p = 0.02$).

**Table 1**

| target taxon | primer name | primer sequence (5‘ - 3‘) | primer conc. in PCR (µM) | target gene | fragment length (bp) | amplitude threshold (dPCR) | source |
|---|---|---|---|---|---|---|---|
| *Cottus gobio* | Cot-gob-S632 | GAATAAAGGACTAAACCAAGTGGG | 0.25 | 16S | 118 | 13,500 | Thalinger et al. 2016 |
| | Cot-gob-A641 | GCTGTAGCTCTCAGTTGTAGGAAAA | 0.25 | | | | |
| *Salmo trutta* | Sal-tru-S1002 | TCTCTTGATTCGGGCAGAACTC | 0.25 | COI | 89 | 8,400 | Thalinger et al. 2020 |
| | Sal-tru-A1002 | CGAAGGCATGGGCTGTAACA | 0.25 | | | | |
| *Oncorhynchus mykiss* | Onc-myk-S655 | TCTCCCTTCATTTAGCTGGAATC | 0.25 | COI | 82 | 12,500 | Thalinger et al. 2016 |
| | Onc-myk-S655 | GCTGGAGGTTTTATGTTAATAATGGTC | 0.25 | | | | |
| *Salvelinus* spp. | Sal-vel-S651 | ATAGTCGGCACCGCCCTT | 0.25 | COI | 112 | 14,000 | Thalinger et al. 2016 |
| | Sal-vel-A651 | TAACGAAGGCATGGGCTGTT | 0.25 | | | | |
| *Thymallus thymallus* | Thy-thy-S653 | ATCAAATTTATAATGTGATCGTCACG | 0.25 | COI | 179 | 14,000 | Thalinger et al. 2016 |
| | Thy-thy-A653 | AAGAAAGGACGGGGGAAGC | 0.25 | | | | |
| *Phoxinus phoxinus* | Pho-pho-S639 | CGTGCAGAAGCGGATATAAATAC | 0.25 | 16S | 128 | 15,750 | Thalinger et al. 2016 |
| | Pho-pho-A648 | CCAACCGAAGGTAAAGTCTTATTG | 0.25 | | | | |
| *Squalius cephalus* | Squ-cep-S669 | CAGTATACCCACCGCTTGCG | 0.25 | COI | 130 | 14,250 | Thalinger et al. 2016 |
| | Squ-cep-A669 | TTAATAATTGTGGTAATGAAGTTGACC | 0.25 | | | | |

**Table 2**

| | | F-value | p-value |
|---|---|---|---|
| pH | (intercept) | 34.52 | < 0.001 |
| | sampling | 2.22 | 0.053 |
| mean activity | (intercept) | 78.78 | <0.001 |
| | sampling | 0.76 | 0.57 |
| target copies per µl | (intercept) | 39.00 | <0.001 |
| | sampling | 0.38 | 0.86 |

**Table 3**

| model # | covariate structures |
|---|---|
| 1 | mean activity + energy use + fish species + pH + sampling + fish individual |
| 2 | mean activity + energy use + fish species + sampling + fish individual |
| 3 | mean activity + energy use + fish species |
| 4 | mean activity + energy use |
| 5 | mean activity + fish species |
| 6 | fish species |

**Table 4**

| model # | K | AICc | ΔAICc | ω | pseudo-$R^2_{NCU}$ |
|---|---|---|---|---|---|
| 3 | 10 | 1208.32 | 0 | 0.90 | 0.60 |
| 5 | 9 | 1212.76 | 4.44 | 0.10 | 0.58 |
| 2 | 19 | 1221.36 | 13.04 | 0 | 0.62 |
| 6 | 8 | 1223.20 | 14.88 | 0 | 0.55 |
| 1 | 20 | 1223.64 | 15.32 | 0 | 0.62 |
| 4 | 4 | 1346.99 | 138.67 | 0 | 0.04 |

**Table 5**

| predictor variable | parameter estimate | standard error | lower 95% CI | upper 95% CI | t-value | p-value |
|---|---|---|---|---|---|---|
| intercept | 0.26 | 0.26 | -0.23 | 0.77 | 0.97 | 0.33 |
| mean activity | 1.05 | 0.36 | 0.36 | 1.75 | 2.91 | < 0.01** |
| energy use [J/h] | 0.40 | 0.20 | 0.04 | 0.77 | 1.97 | 0.05 |
| *Oncorhynchus mykiss* | 0.72 | 0.37 | 0.06 | 1.41 | 1.96 | 0.05 |
| *Phoxinus phoxinus* | 2.33 | 0.29 | 1.76 | 2.91 | 7.90 | < 0.001*** |
| *Salvelinus fontinalis* | 0.53 | 0.39 | -0.23 | 1.31 | 1.34 | 0.18 |
| *Salmo trutta* | 0.28 | 0.31 | -0.34 | 0.92 | 0.91 | 0.36 |
| *Squalius cephalus* | 1.16 | 0.32 | 0.54 | 1.80 | 3.65 | < 0.001** |
| *Thymallus thymallus* | 2.96 | 0.28 | 2.43 | 3.49 | 10.51 | < 0.001*** |

Figure 1

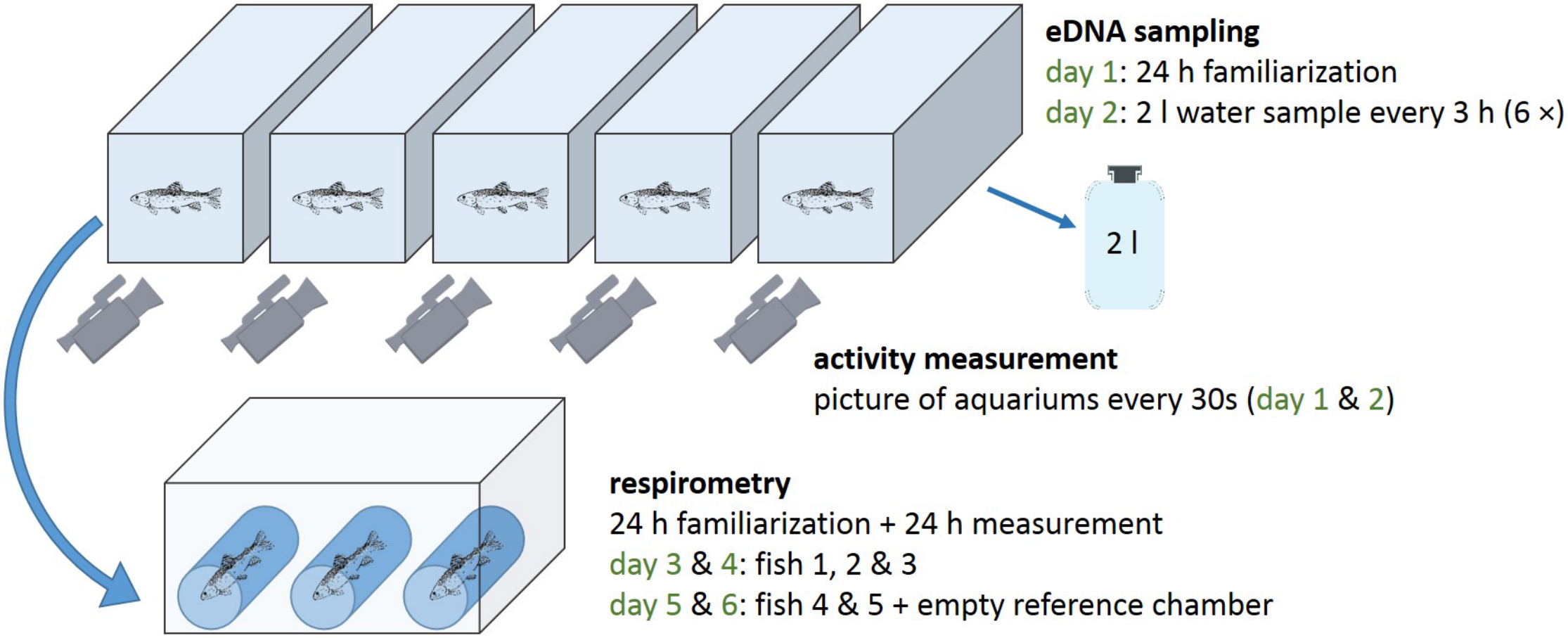
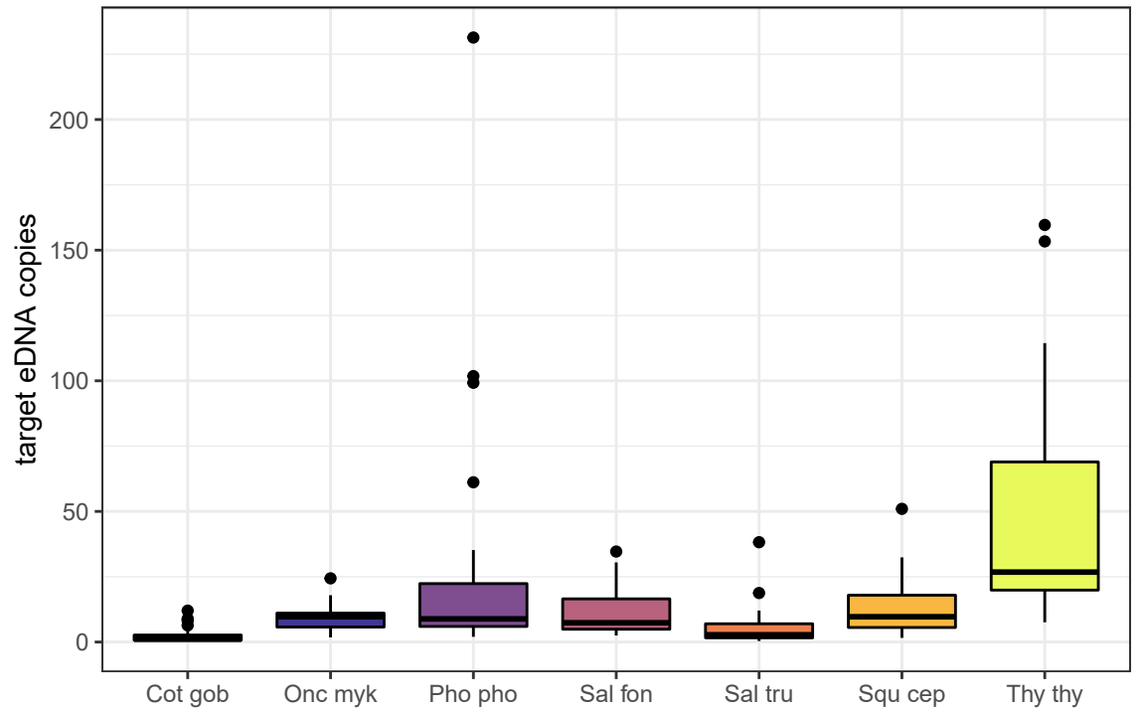
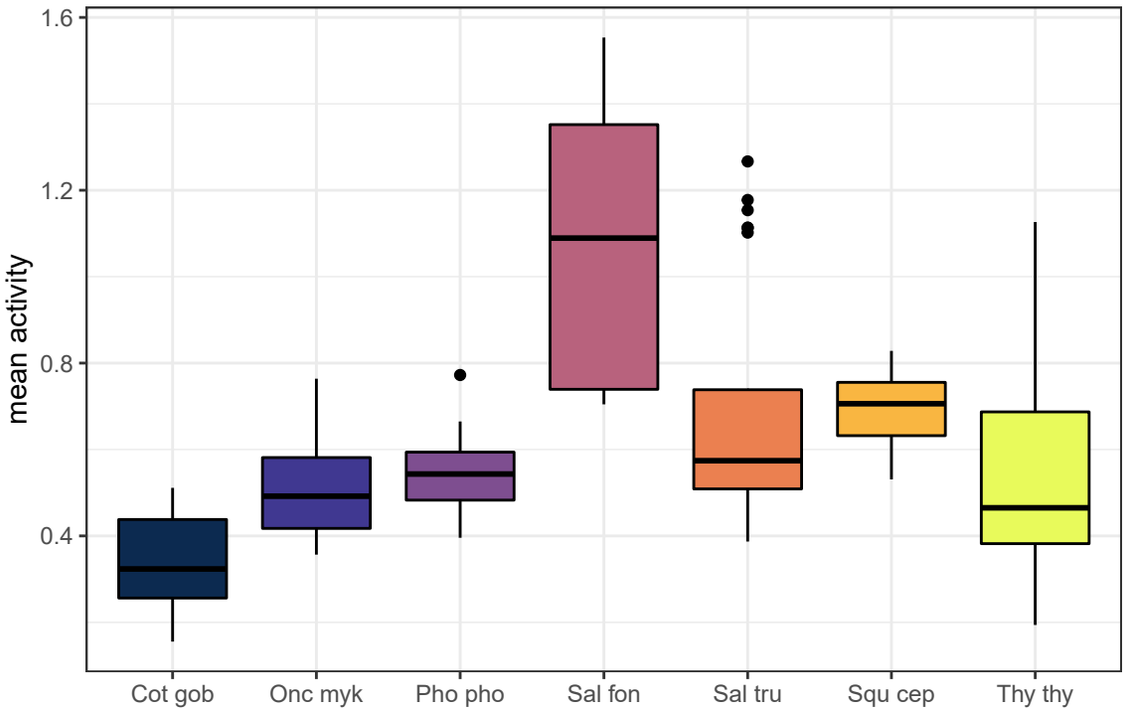
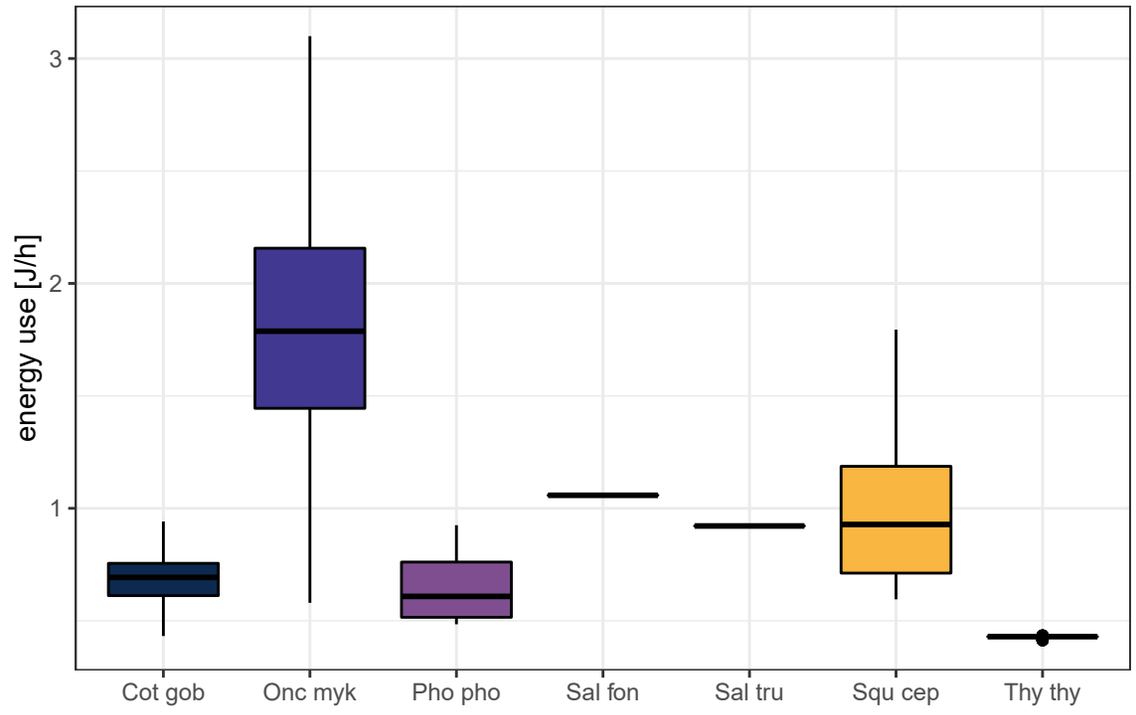

eDNA sampling
day 1: 24 h familiarization
day 2: 2 l water sample every 3 h (6 ×)
2 l
activity measurement
picture of aquariums every 30s (day 1 & 2)
respirometry
24 h familiarization + 24 h measurement
day 3 & 4: fish 1, 2 & 3
day 5 & 6: fish 4 & 5 + empty reference chamber

Figure 2


target eDNA copies
200
150
100
50
0
Cot gob
Onc myk
Pho pho
Sal fon
Sal tru
Squ cep
Thy thy


mean activity
1.6
1.2
0.8
0.4
Cot gob
Onc myk
Pho pho
Sal fon
Sal tru
Squ cep
Thy thy


energy use [J/h]
3
2
1
Cot gob
Onc myk
Pho pho
Sal fon
Sal tru
Squ cep
Thy thy

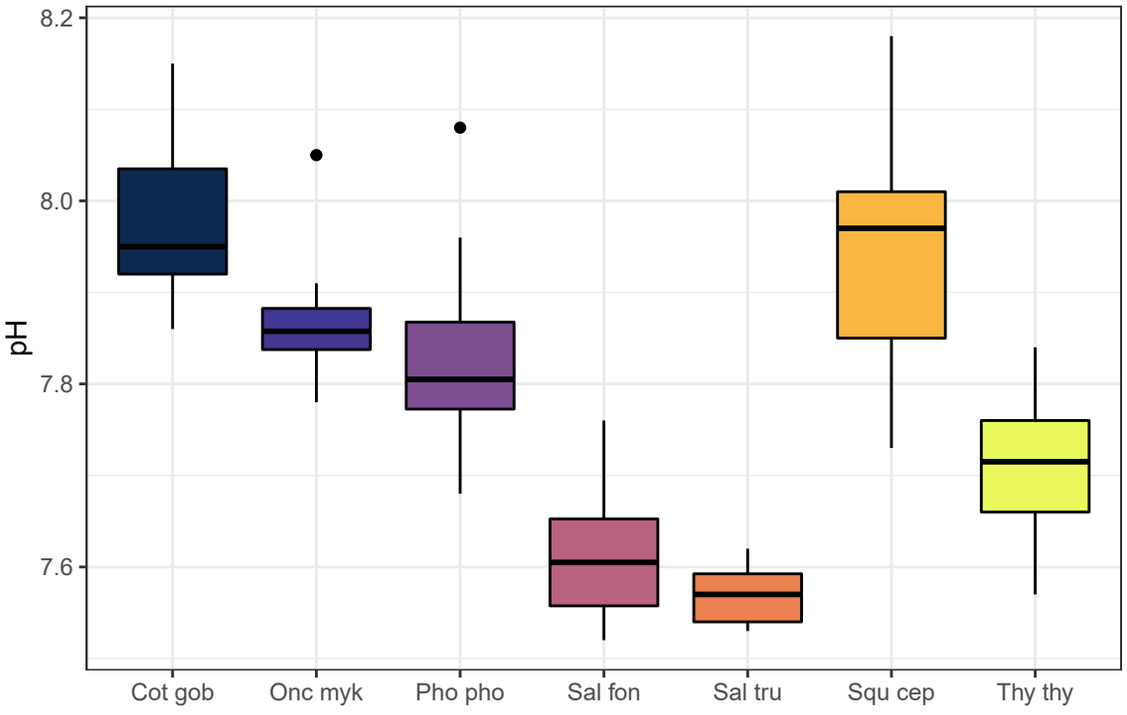

pH
8.2
8.0
7.8
7.6
Cot gob
Onc myk
Pho pho
Sal fon
Sal tru
Squ cep
Thy thy

Figure 3

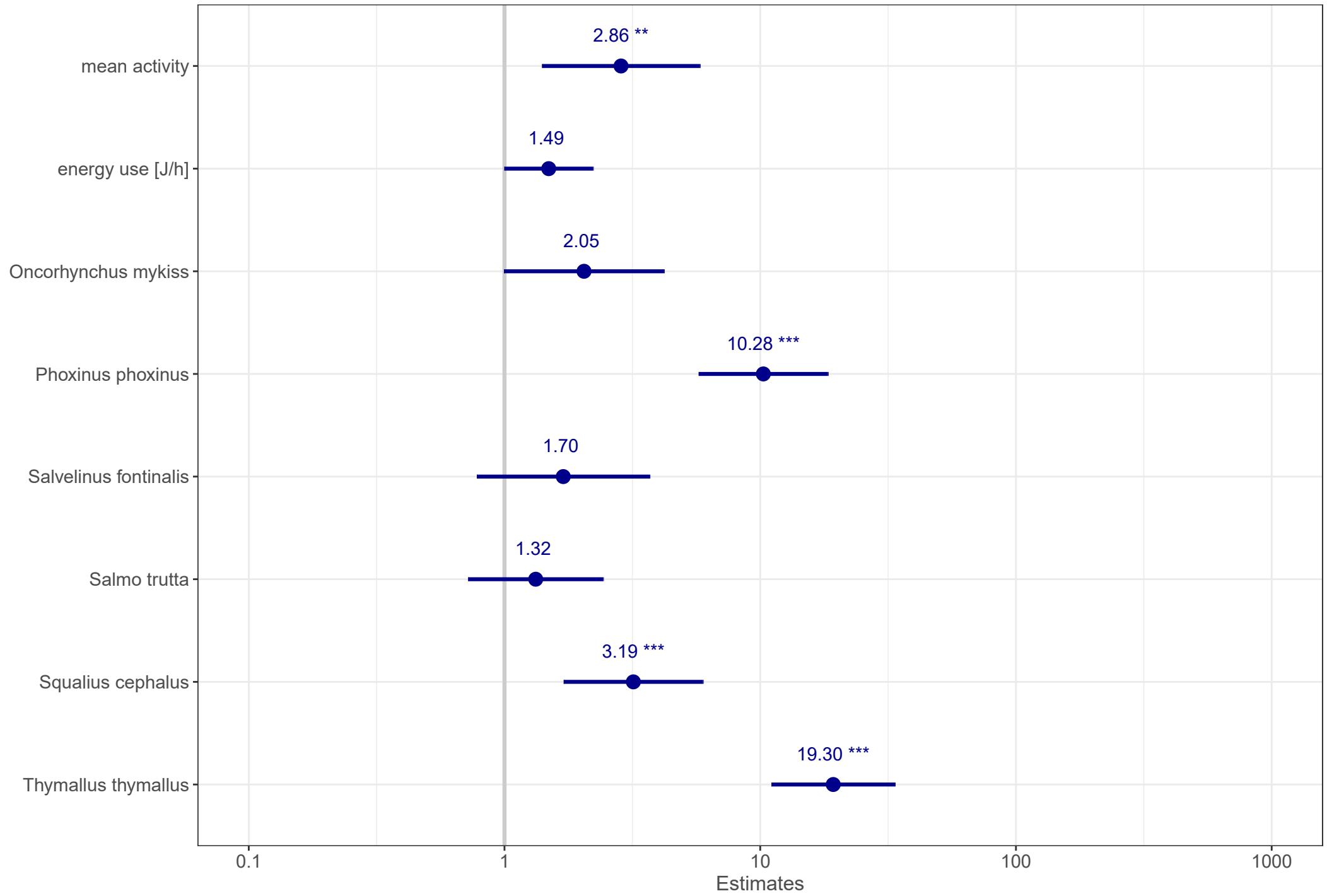

2.86 **
mean activity
1.49
energy use [J/h]
2.05
Oncorhynchus mykiss
10.28 ***
Phoxinus phoxinus
1.70
Salvelinus fontinalis
1.32
Salmo trutta
3.19 ***
Squalius cephalus
19.30 ***
Thymallus thymallus
0.1
1
10
100
1000
Estimates

Figure 4

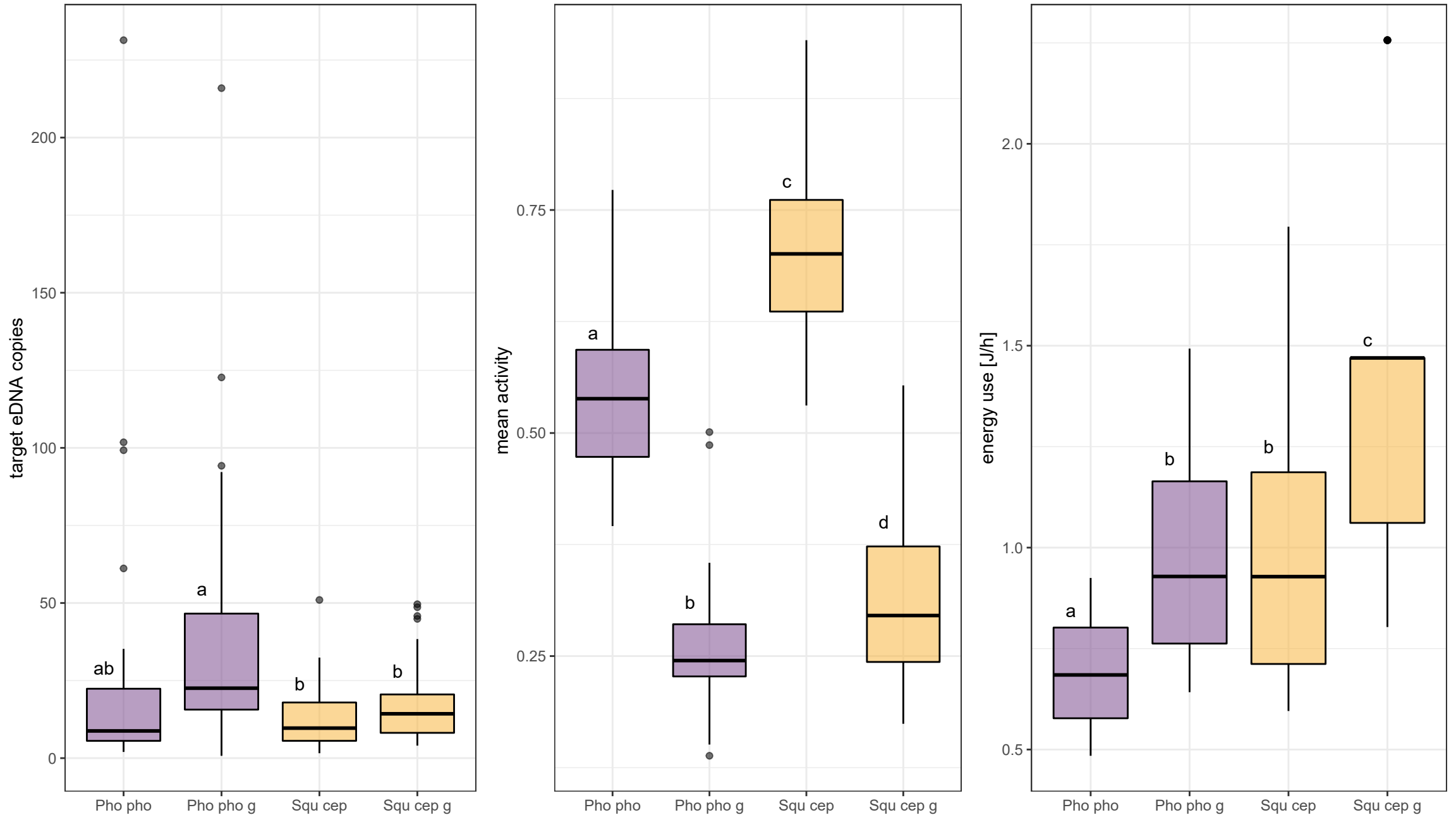
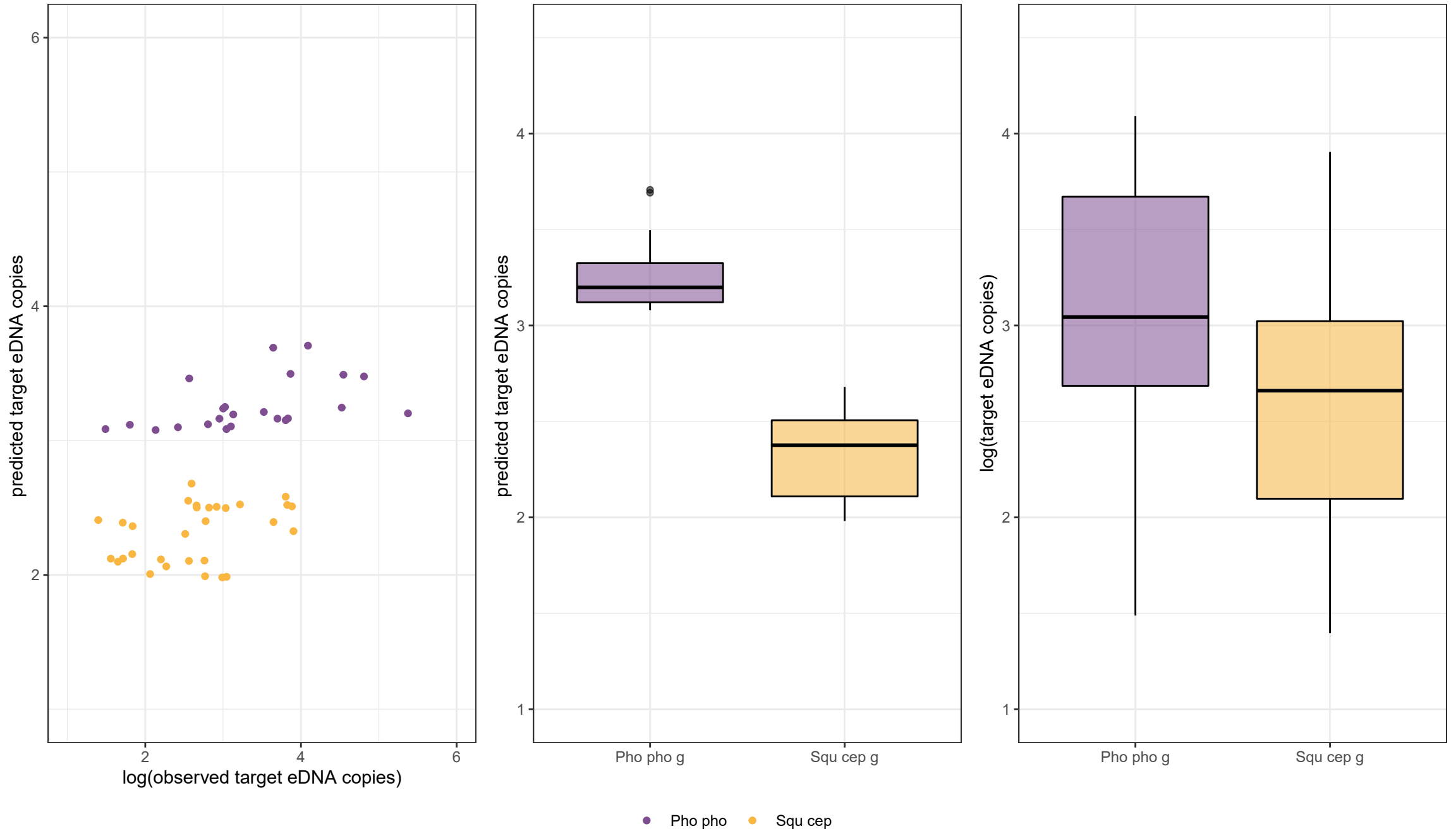

target eDNA copies
200
150
100
50
0
ab
a
b
b
Pho pho
Pho pho g
Squ cep
Squ cep g
mean activity
0.75
0.50
0.25
a
b
c
d
Pho pho
Pho pho g
Squ cep
Squ cep g
energy use [J/h]
2.0
1.5
1.0
0.5
a
b
b
c
Pho pho
Pho pho g
Squ cep
Squ cep g

Figure 5


predicted target eDNA copies
log(observed target eDNA copies)
predicted target eDNA copies
Pho pho g
Squ cep g
log(target eDNA copies)
Pho pho g
Squ cep g
Pho pho
Squ cep