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# Temperature modulates PFAS accumulation and energy allocation in sheepshead minnows

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## Abstract

Climate warming and chemical pollution shape aquatic ecosystems, yet the physiological mechanisms underlying their combined effects remain unclear. We investigated how projected increases in mean summer surface water temperature alter per- and polyfluoroalkyl substances (PFAS) toxicokinetics and their effects on sheepshead minnows (*Cyprinodon variegatus*) physiological performance. Adult fish were chronically exposed to an environmentally relevant PFAS mixture (perfluorooctane sulfonate (PFOS) + perfluorooctanoate (PFOA)) under current and projected mean-temperature scenarios. Tissue PFAS concentrations, whole-organism metabolic rates, swimming performance, reproductive parameters, somatic indices were assessed. Temperature modified PFAS tissue concentrations in a compound- and tissue-specific manner, promoting PFOA redistribution to eggs. Metabolic responses were temperature-dependent: at 26 °C, higher tissue PFAS concentrations were associated with elevated standard and maximum metabolic rates (SMR and MMR), maintaining aerobic scope (AS). At 28.5 °C, SMR remained stable while MMR and AS declined with rising PFAS, indicating less oxygen for energetically demanding activities. Despite unchanged swimming and reproductive outputs, an increased hepatosomatic index with increasing tissue PFAS concentrations and altered PFAS distribution suggest detoxification costs. These findings indicate that increases in mean water temperature are likely to exacerbate contaminant stress, with consequences for coastal fish population resilience and offspring development. PFAS risk assessment should consider co-stressors under projected warming.

**Keywords: PFAS, temperature, fish, accumulation, metabolism, reproduction, swimming performance**

## Synopsis

Limited research addresses how temperature affects PFAS toxicokinetics and toxicity. This study shows that warming reshapes tissue PFAS concentrations and distribution, and influences fish energy-allocation trade-offs.

## 1. Introduction

Multiple stressors shape ecosystems worldwide and pose a growing threat for aquatic organisms (1). In particular, chemical pollution and climate warming can interact to increase contaminant exposure and heighten organismal sensitivity (2,3), with consequences for growth, reproduction, and behavior that may promote nonlinear ecological responses and reduce ecosystem resilience (4,5,6). The physiological mechanisms driving these interactions remain poorly understood, especially for contaminants of emerging concern, despite their importance for anticipating contaminant risks under accelerating climate change.

Per- and polyfluoroalkyl substances (PFAS) represent a large family of synthetic contaminants for which temperature-dependent effects remain largely unknown. PFAS have been widely used since the 1950s for their unique chemical and physical properties (7). Strong carbon–fluorine bonds and fluorinated chains make them highly stable and water- and oil-repellent. Some PFAS, like perfluoroalkyl acids (PFAA), also have a hydrophilic group, giving them surfactant-like behavior (8). These characteristics make PFAS resistant to degradation in natural conditions and allow their widespread transport and accumulation, especially in aquatic ecosystems (9,10). Perfluorooctanoate (PFOA) and perfluorooctane sulfonate (PFOS) are among the most historically widely used long-chain PFAA and have attracted attention in the scientific, public, and regulatory communities (11). Due to their persistence, bioaccumulation, and toxicity, they are listed under Annexes A (Elimination) and B (Restriction), respectively, in the Stockholm Convention on Persistent Organic Pollutants (12,13) and are regulated in many countries (14). Although their production and use have been largely phased out since the early 2000s (11), their extreme stability and remaining sources continue to contaminate surface and groundwater. Concentrations typically range from a few ng $L^{-1}$ to low µg $L^{-1}$ (10), but much higher levels have been reported near industrial sites and legacy contamination areas; for example, up to 42,000 ng $L^{-1}$ of PFOA and 2,700 ng $L^{-1}$ of PFOS have been found in groundwater near 3M company disposal sites in Minnesota (15).

85 PFAA bioaccumulation and health risks in people and wildlife are well documented (9). The
86 amphiphilic structure of PFAA confers a high affinity for serum albumin, fatty acid–binding proteins
87 (FABPs), and other endogenous proteins that influence their partitioning within organisms (16). In fish,
88 PFAS primarily accumulate in protein-rich tissues such as blood, liver, and kidneys (17), and can also
89 bind to vitellogenin, contributing to deposition in eggs and posing potential multigenerational risks
90 (18). Generally, PFAS bioaccumulation increases with carbon chain length and hydrophobicity and
91 depends on the nature of the functional group (19). In fishes, PFOS and PFOA induce reprotoxicity
92 (20), cause developmental abnormalities (21), and disrupt energy metabolism (22).

93 Although the physiological impacts of PFAA are increasingly recognized, little is known about how
94 temperature may alter their uptake, distribution, and toxicity in fish (23, 24). Temperature governs
95 the environmental behavior of contaminants in aquatic environments, influencing solubility, binding
96 affinities, and partitioning (5). For PFAA specifically, non-covalent interactions with proteins, stabilized
97 by van der Waals forces and hydrogen bonding, are exothermic and weaken at higher temperatures,
98 potentially enhancing dissociation and elimination (25, 26). In parallel, temperature is a fundamental
99 driver of ectotherm performance, as it sets the pace of biochemical reactions and defines the
100 energetic capacity available for essential functions such as growth, locomotion, and reproduction (27,
101 28, 29, 30). Because warming can elevate metabolic rates in ectotherms (31), it may influence
102 contaminant uptake and internal exposure (32), although uptake is not a direct function of metabolic
103 rates and depends on multiple temperature-sensitive processes. Altogether, clarifying how projected
104 increases in mean water temperature and PFAS exposure interact to shape bioenergetic traits is
105 essential for predicting energy balance and organismal fitness (33). Whole-organism metabolic rates
106 provide a valuable integrative measure, reflecting oxygen consumption and the combined capacity for
107 oxygen delivery and energy conversion. However, research on PFAA (and, more broadly, PFAS) and
108 fish metabolic rates remains scarce (34). Critically, no studies have addressed PFAA mixtures, and only
109 a single study has examined temperature-dependent effects, reporting metabolic disruptions in
110 juvenile fish exposed solely to PFOS (35). This gap underscores the need to evaluate PFAA toxicity

111 through whole-organism metabolic traits that capture energy allocation trade-offs and ecological
112 function, particularly under varying thermal conditions.

113 The overarching aim of this study was to assess how projected increases in mean summer surface
114 water temperature may alter PFAA toxicokinetics and compromise physiological performance in
115 sheepshead minnows (*Cyprinodon variegatus*). *Sheepshead minnows* are widely used in toxicology
116 studies (36,37). They are a eurythermal species broadly distributed along the U.S. Atlantic coast and
117 Gulf of Mexico, including areas influenced by wastewater treatment plant (WWTPs) discharges, a PFAS
118 source due to low removal efficiencies (38). Consistent with this exposure context, our field sampling
119 downstream of WWTPs (Connecticut shore, Long Island Sound) detected PFOS/PFOA at all
120 investigated sites and documented sheepshead minnows, confirming exposure relevance
121 (unpublished data). In our study, we aimed (i) to characterize how temperature affects the tissue
122 accumulation and distribution of a PFAA mixture made of PFOS and PFOA, and (ii) to evaluate whether
123 temperature modulates the toxicity of the mixture on fish metabolic, swimming and reproductive
124 performance. We hypothesized that PFOS would accumulate to higher concentrations than PFOA
125 because of its known greater protein-binding affinity and slower elimination (19), and would therefore
126 primarily drive PFAA-associated toxicity. We further tested the hypothesis that increasing water
127 temperature would alter PFOS and PFOA tissue accumulation and distribution, as prior work suggests
128 temperature can affect PFAA binding to proteins (25). Lastly, building on evidence that PFOS exposure
129 can modify oxygen consumption in fish, and that these effects can vary with temperature (35), we
130 tested the hypothesis that exposure to a mixture of PFOS and PFOA would alter metabolic rates and
131 aerobic scope in a temperature-dependent manner, with potential consequences for swimming and
132 reproductive performance. To test these predictions, we conducted two complementary, laboratory-
133 based chronic aqueous exposure experiments with adult sheepshead minnows acclimated to three
134 surface water temperatures representative of current and projected mean surface water
135 temperatures in the northeastern USA. By integrating thermal and contaminant stressors within a

136 whole-organism framework, this study advances our understanding of how combined environmental

137 stressors influence fish physiological function and ecological fitness in the context of global change.

138 ## 2. Materials and Methods

139 ### 2.1 Fish husbandry and temperature acclimation

140 All fish husbandry and experimental procedures were in accordance with the University of
141 Connecticut's Institutional Animal Care and Use Committee protocols A22-049 & A23-049. Juvenile
142 sheepshead minnows (*Cyprinodon variegatus,* > 60 days post hatch (dph)) were obtained from Aquatic
143 BioSystems (Fort Collins, CO, USA), where they are reared at ~24 ppt. Upon arrival, fish were gradually
144 acclimated to 1 ppt by stepwise dilution over 12 days (- 4 ppt every 48 h, from 24 ppt to 1), using
145 dechlorinated freshwater mixed with Instant Ocean synthetic sea salt (Aquarium Systems, Inc, USA),
146 and then maintained at 1 ppt in two 380–1360 L recirculating aquaculture systems (Iwaki Aquatic
147 Systems and Services, Holliston, MA, USA) until adulthood prior to experimental exposures. Salinity
148 was held at 1 ppt to represent low-salinity estuarine conditions that can occur in coastal systems (e.g.,
149 during freshwater influx events).
150 Water conditions for housing were: temperature 24 °C, salinity 1 ppt, dissolved oxygen 8 mg/L and pH
151 7.5. Temperature in the recirculating aquaculture systems was maintained using heaters in a reservoir
152 controlled by thermostats (Figure 1). During husbandry, water quality (temperature, salinity, dissolved
153 oxygen, pH) was checked three times a week using a multiparameter YSI probe, and ammonia, nitrite,
154 and nitrate were checked weekly to ensure they remained within acceptable limits. Prior to
155 experiment onset, sexually mature fish were acclimated for at least three weeks to their assigned
156 temperature (Exp. 1: 24 °C, 26 °C, or 28.5 °C; Exp. 2: 24 °C or 28.5 °C) under the same monitoring
157 regime. Fish were fed once daily to satiation with standard commercial flaked food (TetraMin Tropical
158 Flakes, Tetra, Melle, Germany), until fish no longer surfaced to feed, and maintained under a 14 h
159 light:10 h dark photoperiod cycle during the holding and experimental periods.

160 ### 2.2 Experimental design

161 We conducted two complementary experiments to test how temperature affects the tissue
162 accumulation and distribution of PFOS and PFOA and the fitness of small-bodied fish. In the first
163 experiment, we assessed PFAS accumulation (whole-body of swimming fish, muscle tissue of both sex
164 and eggs) among temperature treatments as well as the coupled influences of PFAS and elevated
165 temperature exposure on fish metabolism, swim performance, and reproduction (Figure 1). The
166 second experiment, motivated by the results of the first, assessed the influence of temperature on
167 PFOS and PFOA distribution among muscle, liver, ovary, and blood (Figure 1). Three temperatures
168 were tested, representative of contemporary summer surface water temperatures (24°C) and those
169 projected 50 years and 100 years in the future (26 °C and 28.5 °C) for the Long Island Sound estuary
170 (39). We targeted a combined PFOS and PFOA exposure concentration of 100 $\mu g \cdot L^{-1}$, corresponding to
171 the combined U.S. EPA Freshwater Aquatic Life Water Quality Criteria for PFOA and PFOS (40). This
172 comprised equal concentrations of PFOS and PFOA (50 $\mu g \cdot L^{-1}$ each). Relative to compound-specific
173 criteria, PFOA was 2 times below its criterion (0.10 $mg \cdot L^{-1}$) whereas PFOS was 200 times above its
174 criterion (0.00025 $mg \cdot L^{-1}$, 40). Details of the exposure module including temperature control during
175 PFAS exposure, sample sizes by endpoint for each experiment, water-quality parameters during PFAS
176 exposure, PFAS stock-solution preparation, and PFAS concentration monitoring are provided in the
177 Supporting Information (Tables S1-S3).

178 Fish were randomly assigned to PFAS-exposed or non-exposed groups and one of three temperature
179 treatments, for six experimental conditions in experiment 1 and four conditions in experiment 2
180 (Figure 1). For the first experiment, 684 fish (mean lengths and weights ± standard errors (SE): 53.0 ±
181 0.39 mm, 3.26 ± 0.07 g) were distributed across 36 aquaria (6 treatments * 6 replicates with 19 fish
182 per aquaria, and at a ratio of 12 females for 7 males). Experimental exposures were initiated on a
183 rolling basis as aquaria became available, in batches of six aquaria at a time, with each batch
184 comprising all six treatment conditions. A pre-exposure period of 6 days was conducted to allow them
185 to acclimate to the system, establish baseline egg production, and to allocate fish to exposure groups,
186 ensuring that any changes in reproductive output during the exposure period could be attributed to

PFAS exposure rather than natural variation (41). Each aquarium included spawning boxes for daily egg quantification throughout the pre-exposure and exposure periods. Egg samples were collected for PFAS analysis from the three PFAS-exposed groups at day 0 (prior to exposure), day 14, and day 28, sampling three aquaria per treatment at each time point (n= 27 samples; 9 samples per PFAS-exposed treatment group). At the end of the 28-day exposure, two males from each aquarium were selected for swimming and metabolic performance trials (see below). Males were prioritized to limit variance under sample-size and logistical constraints, as metabolism varies by sex and reproductive status. All other fish were euthanized using tricaine methane sulphonate (MS-222; 0.5 $g \cdot L^{-1}$; Syndel, USA). Four fish (two males and two females) per aquarium were collected for biometric measurements (total length, height, width, and body weight) and dissected for skinless muscle for PFAS analysis. Liver and gonads were weighed to calculate somatic indices. Water, eggs, and muscle tissue were preserved at -20 °C for PFAS analyses.

The second experiment was performed using a new batch of fish. In total, 105 (49.2 ± 0.22 cm, 2.40 ± 0.04 g) fish were distributed across 12 aquaria (4 treatments * 3 replicates with 7-10 fish per aquaria and at a ratio of 6-9 females for 1 male). All fish were euthanized at the end of the 28-day exposure. Three females per aquarium were collected for biometric measurements (total length, height, width, and body weight) and dissected for skinless muscle, liver, blood, and ovary for PFAS analysis. Liver and gonads were weighed to calculate somatic indices. Water, muscle, liver, blood and ovary tissue were preserved at -20 °C for PFAS analyses.

In both experiments, tissues PFAS concentrations were analyzed from individual fish.

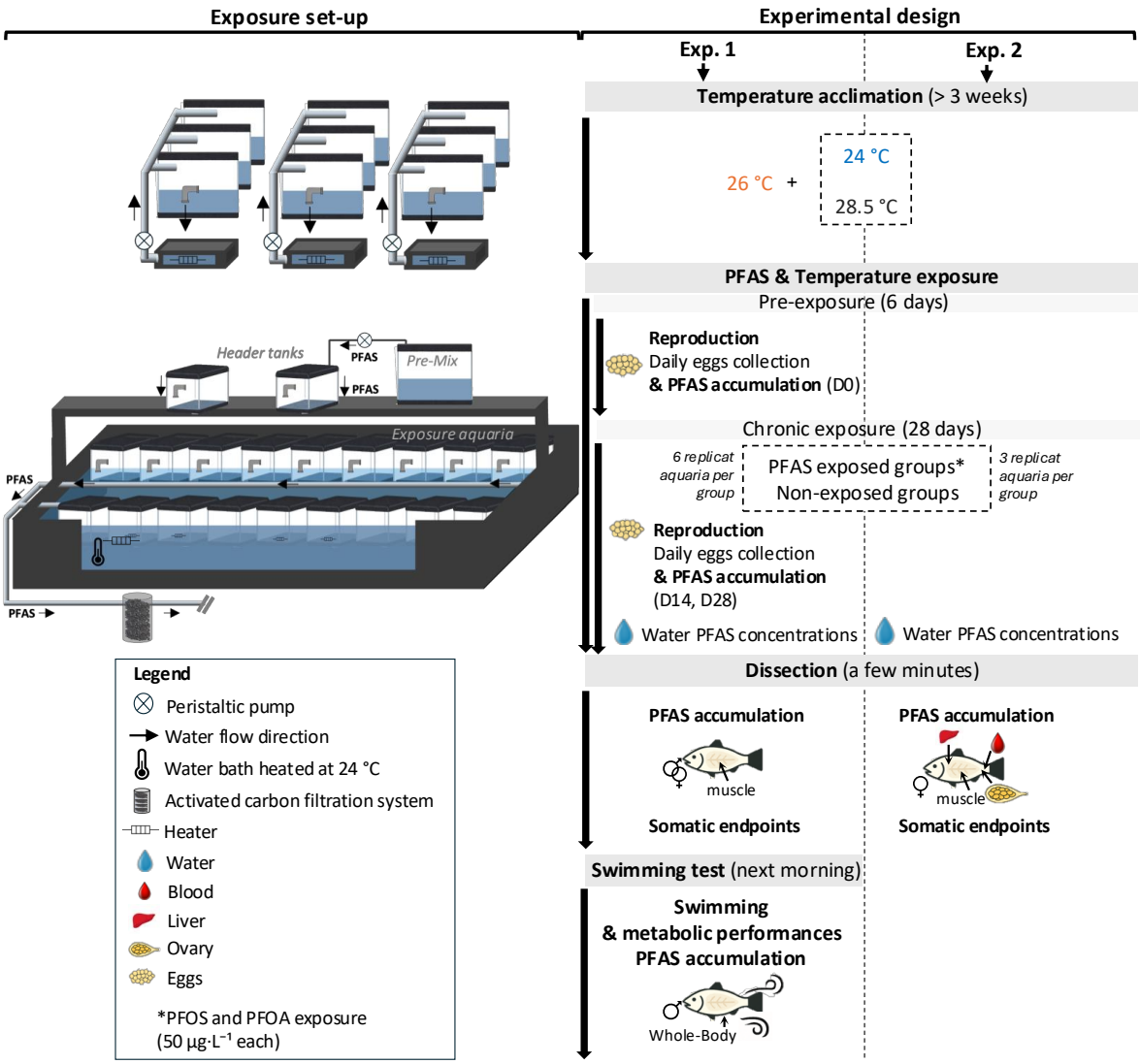



**Figure 1. Schematic representation of the exposure module and experimental design.** Temperature treatments were crossed with two PFAS treatments (PFAS-exposed and non-exposed control groups): 24 °C, 26 °C, and 28.5 °C in Experiment 1; and 24 °C and 28.5 °C in Experiment 2. **Experiment 1:** Daily egg collection was conducted during a 6-day pre-exposure period and a 28-day exposure period to quantify egg production; additional egg samples were collected for PFAS analysis on exposure days 0, 14, and 28. **Experiments 1–2:** Water samples were collected throughout the 28-day exposure period to monitor PFAS concentrations. At the end of exposure, skinless muscle was collected from both males and females for PFAS analysis in Experiment 1, whereas skinless muscle, liver, gonads, and blood were collected from females for PFAS analysis in Experiment 2. Somatic endpoints were assessed at termination in both experiments. In Experiment 1, additional males were collected and assessed for swimming and metabolic performance the morning following dissection, and whole-body samples were subsequently analyzed for PFAS analysis.

## 2.3 Somatic and reproductive endpoints

219 Hepatosomatic index (HSI) and gonadosomatic index (GSI) were calculated in both experiments as:

220 $$\text{Somatic index } (\%) = \frac{\text{organ weight (g)}}{\text{body weight (g)}} \times 100 \quad \textbf{equation (1)}$$

221 Daily (eggs·female$^{-1}$·day$^{-1}$) and cumulative (eggs·female$^{-1}$) egg production in experiment 1 were
222 calculated for each aquarium on each day over the 28-day exposure period by summing egg counts
223 and normalizing by the number of females. Egg production data were expressed per female to account
224 for any discrepancies in the initial 12:7 female-to-male ratio, including any differences due to mortality
225 during the exposure period. These daily values were then averaged by treatment group to visualize
226 temporal trends. To summarize reproductive output, mean daily egg production was calculated per
227 aquarium over the full period and then averaged across groups. Finally, linear regressions of daily and
228 cumulative egg production over time were used to estimate rates of change (slopes), which were also
229 averaged by treatment group.

## 230 2.4 Swimming and metabolic performances protocol

231 Swimming performance and aerobic metabolic rates of individual males were determined using 1500
232 mL intermittent-flow swim tunnels (Loligo Systems, Tjele, Denmark). Water flow was calibrated with
233 digital particle tracking velocimetry and controlled by a DAQ-M data acquisition device. Oxygen
234 consumption ($MO_2$, mg $O_2$ kg$^{-1}$ h$^{-1}$) was recorded by intermittent-flow respirometry (AutoResp®,
235 version 3.2.2) connected to a Witrox-4 oxygen meter (Loligo Systems). Probes were calibrated weekly
236 with a two-point, temperature-paired calibration (0% and 100% air saturation), and no signal drift was
237 detected during the experiment. All swimming trials were performed at the fish's respective treatment
238 temperature (24, 26, or 28.5 °C).
239 Fish were fasted for 24 h prior to the swimming test. Fish were individually transferred into the swim
240 chamber and acclimated by gradually increasing the water velocity over a 10-minute period. Each fish
241 then remained overnight in the swim chamber at a low water velocity (U = 0.7 Body Length per second
242 (BL s$^{-1}$)) for habituation. Swimming and metabolic performances were assessed using a critical

243 swimming speed ($U_{crit}$) protocol, in which each fish was exposed to a stepwise increase in water
244 velocity, all performed at the same time of each day (e.g.,42,43,44). Swimming speeds were increased
245 stepwise by 0.3 BL $s^{-1}$ after each complete respirometry cycle which consisted of a 3-minute flush
246 phase to maintain oxygen saturation (i.e., above 80 % of air saturation), followed by a 1-minute closed
247 wait phase to allow flow stabilization, and a 10-minute closed oxygen measurement phase at steady
248 swimming speed, with the flush pump turned off. Swimming trials were terminated when fatigue was
249 reached, indicated by the fish's inability to swim against the current and the adoption of a C-shaped
250 posture on the grid located at the back of the swim chamber.
251 At the end of the stepwise protocol, males were removed from the swim chamber and euthanized
252 using MS-222 (0.5 g $L^{-1}$). For each male, total length, height, width, and body weight were recorded,
253 and the whole body was then stored at -20 °C for subsequent PFAS analysis. Whole-body PFAS
254 concentrations were analyzed from individual fish. Swim respirometers were cleaned between trials
255 with bleach and rinsed three times with freshwater. Background respiration (e.g., microbial
256 respiration) was measured in the empty swim tunnel for at least 20 minutes before and after each
257 trial.

258 *Calculation of fish metabolic rates and swimming performance.*
259 Oxygen consumption (unscaled $MO_2$, mg $O_2$ $kg^{-1}$ $h^{-1}$) of the fish was calculated using the following
260 equation (29):

261 $$\text{Unscaled } MO_2 = \left[\left(\frac{\Delta[O_2]}{\Delta t}\right) - \left(\left(\frac{\Delta[O_2]}{\Delta t}\right)_{bact}\right)\right] \times \left(\frac{V_{resp}}{m}\right)$$ **equation (2)**

262 where $\Delta[O_2]/\Delta t$ (mg $O_2$ $L^{-1}$ $h^{-1}$) is the rate of oxygen concentration decrease in the swim respirometer
263 over time during each $MO_2$ measurement period. Only slopes with a regression coefficient greater
264 than 0.85 were considered valid. $(\Delta[O2] / \Delta t)_{bact}$ (mg $O_2$ $L^{-1}$ $h^{-1}$) represents the background respiration
265 slope, calculated as the mean of two measurements taken before and after the swimming test in the

empty chamber. $V_{resp}$ is the respirometer volume (1.5 L) minus the volume of the fish, and m (kg) is the fish body mass.

As fish respiration depends on animal body mass, metabolic rates were corrected to a standard body mass of 0.1 kg using an allometric exponent of 0.68, consistent with values reported for *C. variegatus* (e.g., 45, 46):

$$\mathrm{MO_2} = \text{Unscaled } \mathrm{MO_2} \times \left(\frac{\mathrm{m}}{\mathrm{m_{corr}}}\right)^{1-\mathrm{A}} \textbf{ equation (3)}$$

where $MO_2$ (mg $O_2$ $kg^{-1}$ $h^{-1}$) is the oxygen consumption standardized to a reference body mass $m_{corr}$ of 0.1 kg. Unscaled $MO_2$ (mg $O_2$ $kg^{-1}$ $h^{-1}$, equation 2) is the uncorrected oxygen consumption calculated for each fish with a body mass m (kg). A is the allometric exponent of the relationship between metabolic rate and fish mass.

The Standard Metabolic Rate (SMR, in mg $O_2$ $kg^{-1}$ $h^{-1}$) of each fish was estimated by plotting $MO_2$ against swimming speed (BL $s^{-1}$) and extrapolating the resulting linear regression to a null swimming velocity (e.g., 42,43,47). The highest $MO_2$ recorded during the $U_{crit}$ test was considered an estimate of the Maximum Metabolic Rate (MMR, in mg $O_2$ $kg^{-1}$ $h^{-1}$, e.g., 43, 48).

Aerobic scope (AS, mg $O_2$ $kg^{-1}$ $h^{-1}$) was then calculated for each individual as the difference between MMR and SMR (e.g., 29,42).

$U_{crit}$ (BL $s^{-1}$) was calculated according to the following formula (42, 44, 49):

$$\mathrm{U_{crit}} = \mathrm{U_t} + \frac{\mathrm{t_1}}{\mathrm{t}} \times \mathrm{U_1} \textbf{ equation (4)}$$

where $U_t$ (BL $s^{-1}$) is the highest velocity that the fish maintained for a complete swimming step, $t_1$ (min) is the time spent at the fatigue velocity step, t (min) is the time of a complete swimming step (i.e. 14 min) and $U_1$ is the last increase of the velocity before the fish fatigued (i.e., 0.3 BL $s^{-1}$).

## 2.5 Chemical analyses

288 All samples (water and tissues) were analyzed for PFOS and PFOA concentrations at the University of
289 Connecticut's Center for Environmental Sciences and Engineering (CESE). Water samples were
290 processed according to U.S. EPA Method 537.1, while tissue samples were extracted and analyzed
291 following the protocol described in Campbell et al. (50). Analyses were conducted using an ACQUITY
292 UPLC system coupled to a tandem mass spectrometer (UPLC–MS/MS; Waters, Milford, MA, USA).
293 Standard quality assurance/quality control (QA/QC) procedures were followed, including analysis of
294 method blanks, duplicate samples, pre-extraction matrix spikes, and laboratory control samples. The
295 method reporting limits were 1.8 $ng \cdot L^{-1}$ for PFOA and 2.3 $ng \cdot L^{-1}$ for PFOS in water samples, and 0.49
296 $ng \cdot g^{-1}$ for PFOA and 0.35 $ng \cdot g^{-1}$ for PFOS in tissue samples. Concentrations are reported as $ng \cdot g^{-1}$ wet
297 weight for tissue samples and $ng \cdot L^{-1}$ for water samples.

## 298 2.6 Statistical analysis

299 All statistical analyses were performed in R (version 4.3.1). Selection between linear mixed models
300 (LMMs) and linear models (LMs) was guided by Akaike Information Criterion, Bayesian Information
301 Criterion, and likelihood ratio tests (using the ANOVA function). In cases where (1) the inclusion of sex
302 did not improve model fit, or (2) sex-related terms and interactions were not statistically significant,
303 the simpler model excluding sex was retained for clarity and ease of interpretation. Diagnostic checks
304 included visual inspection of residual and Q–Q plots to assess linearity, homoscedasticity, and
305 normality, supported by Shapiro–Wilk tests. When assumptions were not met, variables were log-
306 transformed and models refit. Mixed models were fitted using the lme4 package (lmer), and fixed
307 effects were evaluated via Type III Wald chi-square tests using the car package. Post hoc tests of
308 interactions were performed with emmeans-tests with Tukey correction. Statistical significance was
309 defined as $p < 0.05$. The specific models used are described below.

### 310 2.6.1 Tissue concentrations, distribution, and temperature effects

311 To test whether tissue PFOS, PFOA, and ΣPFAS concentrations vary with temperature, LMMs were
312 used with temperature and PFAS treatments as fixed effects and aquarium identity as a random effect
313 to account for aquarium-level variation. In experiment 1, temperature was modeled either as a
314 continuous variable to assess linear trends (including both PFAS-exposed and non-exposed
315 individuals), or as a categorical factor to capture potential non-linear or threshold effects. In
316 experiment 2, temperature was modeled only as a categorical factor. PFAS concentrations in eggs
317 (Exp. 1) were not subjected to statistical analysis due to limited replication (n = 3 aquaria per PFAS
318 treatment group) and were considered exploratory.
319 To test whether temperature altered PFOS's proportional contribution to ΣPFAS within each tissue
320 among PFAS-exposed fish, LMMs were used in experiment 1 with temperature included as a fixed
321 effect and modeled either as a continuous variable or as a categorical factor, and aquarium was
322 included as a random effect. In experiment 2, comparisons between groups were conducted using
323 either Student's t-tests or Wilcoxon rank-sum tests, depending on the distribution of the data.
324 Temperature effects on PFAS distribution in PFAS-exposed fish (Exp. 2) were evaluated by analyzing
325 the percent share of PFAS concentrations by tissue and organ:blood concentration ratios. Percent
326 shares were modeled for each compound with LMs including temperature and tissue as fixed effects.
327 Organ:blood ratios were compared between temperatures using either Student's *t*-tests or Wilcoxon
328 rank-sum tests, depending on the distribution of the data.
329 Measured concentrations were analyzed as reported (i.e., concentrations in non-exposed fish were
330 not subtracted from PFAS-exposed fish and non-exposed values were not set to zero). Values below
331 the detection limit were considered as zero prior to analysis.

### 332 2.6.2 Temperature effects on PFAS toxicity

333 To test whether fish length and weight were different between treatment groups, LMMs were used
334 in both experiments with temperature and PFAS treatment as fixed effects, and aquarium identity as

335 a random effect, while mortality was analyzed using LMs, with temperature and PFAS treatments as
336 fixed effects.
337 For the somatic indices and metabolic and swimming performances, statistical analyses were
338 structured in two main components. First, the main and interactive effects of temperature and PFAS
339 exposure on SMR, MMR, AS, $U_{crit}$, HSI, and GSI were quantified using LMs with temperature (24, 26,
340 28.5 °C) and PFAS treatment (non-exposed vs. PFAS-exposed) as categorical factors (3*2 design).
341 Second, to test whether individual PFAS concentrations predicts somatic indices and metabolic and
342 swimming performances, and whether this depends on temperature, LMs or LMMs were used using
343 log-transformed PFOS, PFOA, and ΣPFAS as continuous factors, and temperature as a categorical
344 factor. Aquarium identity was included as a random factor when appropriate. Simple per-temperature
345 linear regressions were then fitted to describe the slopes within each temperature when the
346 PFAS*temperature interaction was significant, indicating temperature-dependent PFAS effects.
347 To test whether PFAS concentrations predicts daily egg production per female, and whether this
348 depends on temperature, LMMs were used. As a preliminary step, LMMs were fitted separately within
349 each temperature group during the pre-exposure period to assess potential baseline differences in
350 reproductive output prior to PFAS exposure. Since no significant differences were detected at
351 baseline, a LMM model was used during the exposure period to test the effects of temperature, PFAS
352 treatment (exposed vs. non-exposed), and their interaction. In addition, LMs were used to compare
353 the slopes of egg production over time across treatment groups, using both daily and cumulative egg
354 counts, to assess dynamic reproductive responses throughout the exposure.

# 3. Results and Discussion

In the context of rising multi-stressor pressures on aquatic ecosystems, this study assessed how projected increases in mean summer surface water temperature_may alter PFAS toxicokinetics and compromise physiological performance in sheepshead minnows, a coastal species of ecological relevance. Our central question was how temperature modulates PFAS effects on fish fitness. Accordingly, in the sections that follow, we first quantify PFAS levels and then evaluate how they vary with temperature. Experiments were conducted under environmentally relevant contaminant and temperature regimes, highlighting broader risks to aquatic health under global change.

## 3.1. Temperature modulation of PFAS toxicokinetics

The first objective of our study was to characterize how projected increases in mean summer surface water temperature_affects the tissue accumulation and distribution of PFOS and PFOA. In the first experiment, PFAS concentrations and distribution were assessed for eggs (Figure S1, Table S4), whole-body samples of swimming fish and muscle tissue (Table S5), and based on the observed patterns, a second experiment was designed to assess PFAS concentrations and distribution among muscle, blood, ovaries, and liver (Table S5).

*PFOS dominance across tissues and maternal transfer.*

PFAS (primarily PFOS) was detected in 87 % of non-exposed fish tissues from both experiments with ΣPFAS concentrations between 8- and 70-fold lower than in PFAS-exposed fish, indicating a relatively minor source of PFAS (i.e., background contribution from source water or food; Table S5). After accounting for the low background, PFAS treatment significantly increased PFOS and PFOA concentrations in exposed vs. non-exposed fish across all tissues and temperatures (Treatment effect: all $p < 0.05$; Figure 2, Table S6), with no evidence of sex differences in muscle PFOS and PFOA concentrations (Exp. 1; Table S7). Compared with U.S. EPA monitoring data, muscle ΣPFAS in our study (Means 263–777 $ng \cdot g^{-1}$ ww; Table S5) were higher than national medians (11.8 $ng \cdot g^{-1}$ ww; PFOS-

379 dominated; 51) yet below hotspot maxima (up to 5,150 ng·g$^{-1}$ ww near historical fluorochemical
380 facilities; 52,53). Thus, our values reflect substantial accumulation while remaining within the range
381 observed in environmentally impacted systems. Mirroring the PFOS-dominated pattern reported in
382 national monitoring, our samples were PFOS-dominated: PFOS accounted for 94–99 % of ΣPFAS across
383 tissues and temperatures despite a 1:1 mixture (Figure 2), aligning with observations across taxa
384 including fish, birds and mammals (54). This dominance may be partly attributed to the stronger
385 protein-binding affinity of PFOS, as demonstrated in carp (*Cyprinus carpio*) by Zhong et al. (55), likely
386 due to stronger ionic interactions of the sulfonate vs. the carboxylate group, leading to slower
387 elimination and greater bioaccumulation. In line with the stronger protein-binding of PFOS described
388 above, the percent share of PFOS concentrations by tissue was highest in blood, next in liver, and
389 lower in ovary and muscle (Exp. 2; tissue effect, $p < 0.001$; Figure 3, Table S8), matching prior evidence
390 that PFOS accumulates preferentially in compartments rich in serum albumin and fatty acid-binding
391 proteins (17,56). By contrast, the share of PFOA concentrations in tissue was flatter, with the largest
392 share in blood and comparable shares across liver, muscle, and ovary (tissue effect: $p < 0.001$; Exp. 2,
393 Figure 3, Table S8). Although ovaries carried lower PFAS concentrations than blood or liver (Figures 2-
394 3), reproductive tissues still accumulated strongly over the exposure period. In eggs (Exp. 1), ΣPFAS
395 rose from 3.30–8.10 ng·g$^{-1}$ ww at day 0 to 54.5–113 ng·g$^{-1}$ ww at day 14 (13–34 fold higher), and
396 further to 388–543 ng·g$^{-1}$ ww at day 28 (48–165 fold higher from day 0; Figure S1, Table S4). In ovaries
397 (Exp. 2), ΣPFAS increased from 25.8–56.5 ng·g$^{-1}$ ww in non-exposed females to 1,470–1,530 ng·g$^{-1}$ ww
398 in exposed females at day 28 (26–59 fold higher, treatment effect: $p = 0.032$; Figure 2, Tables S5-6).
399 Together with prior reports (57), our findings reinforce maternal transfer through eggs/ovaries as a
400 key PFAS exposure route.

401 *Temperature shapes PFAS accumulation*.

402 Under warming, ΣPFAS varied in parallel with PFOS, reflecting PFOS dominance in tissues.

403 In Exp. 1, ΣPFAS and PFOS concentrations in PFAS-exposed fish declined with increasing water
404 temperature, moderately in whole-body (swimming males; ΣPFAS: estimate ± SE = −302 ± 150,

405 PFAS*temperature (continuous), $p$ = 0.052; PFOS: −307 ± 150, $p$ = 0.049; Figure 2, Table S6) and
406 significantly in muscle (both sexes; ΣPFAS: −97.7 ± 47, $p$ = 0.045; PFOS: −98.8 ± 47, $p$ = 0.041; Figure 2,
407 Table S6; models fit with sexes combined, as sex terms were non-significant in models that included
408 sex; Table S7). For muscle, the categorical-temperature model shows that the temperature effect is
409 driven by lower concentrations at 28.5 °C (PFAS*Temperature (factor), ΣPFAS: $p$ = 0.021; PFOS: $p$ =
410 0.019; Table S6): ΣPFAS and PFOS at 28.5 °C were lower than at 24.0 °C (post hoc tests, ΣPFAS: $p$ =
411 0.035; PFOS $p$ = 0.031) and 26.0 °C (ΣPFAS: $p$ = 0.012; PFOS: $p$ = 0.009), with no difference between 24
412 and 26 °C (both $p$ > 0.05). By contrast, for whole-body, the categorical model detected no
413 PFAS*temperature effects for ΣPFAS or PFOS (swimming males; all $p$ > 0.05; Table S6), although trends
414 mirrored the continuous analysis. The decrease in muscle and whole-body PFOS concentrations with
415 warming is consistent with temperature weakening of PFAS–protein binding (58) and suggests
416 enhanced elimination and/or redistribution of free PFOS to excretory organs. Experimental evidence
417 on temperature-modulated PFAS toxicokinetics in fish is extremely limited (26), though our results are
418 supported by observations in rainbow trout (*Oncorhynchus mykiss),* where elevated temperatures
419 increased PFOS clearance and hepatic redistribution, reducing muscle concentrations and decreasing
420 half-lives in multiple tissues (i.e., liver, brain, kidney; 26). In Exp. 2, the temperature-driven decline of
421 ΣPFAS and PFOS were not observed in muscle, blood, and liver of PFAS-exposed females, where ΣPFAS
422 and PFOS remained stable with warming (all temperature effect and PFAS*temperature interaction:
423 $p$ > 0.05; Figure 2, Table S6). The discrepancies in muscle PFOS temperature responses between
424 experiments likely reflect a plateau in muscle PFOS concentrations (maximum binding capacity or
425 steady state) reached at 24 °C in both experiments and maintained at 28.5 °C in Exp. 2. This
426 interpretation is supported by comparable muscle PFOS concentrations across Exp. 1 (24 °C) and Exp.
427 2 (24 °C, 28.5 °C; Table S5) and by unchanged organ-to-blood PFOS ratios across temperatures in Exp.
428 2 (Figure 4, Table S9), which may indicate a redistribution plateau in which uptake and elimination
429 scale proportionally. A higher whole-body PFAS burden in Exp. 2 (i.e., total mass per fish; not
430 measured) could have kept muscle near saturation at 28.5 °C, masking temperature effects. Further

431 investigations are needed to understand the temperature-related differences in muscle PFOS
432 concentrations, potentially including PFOS burden measurements.

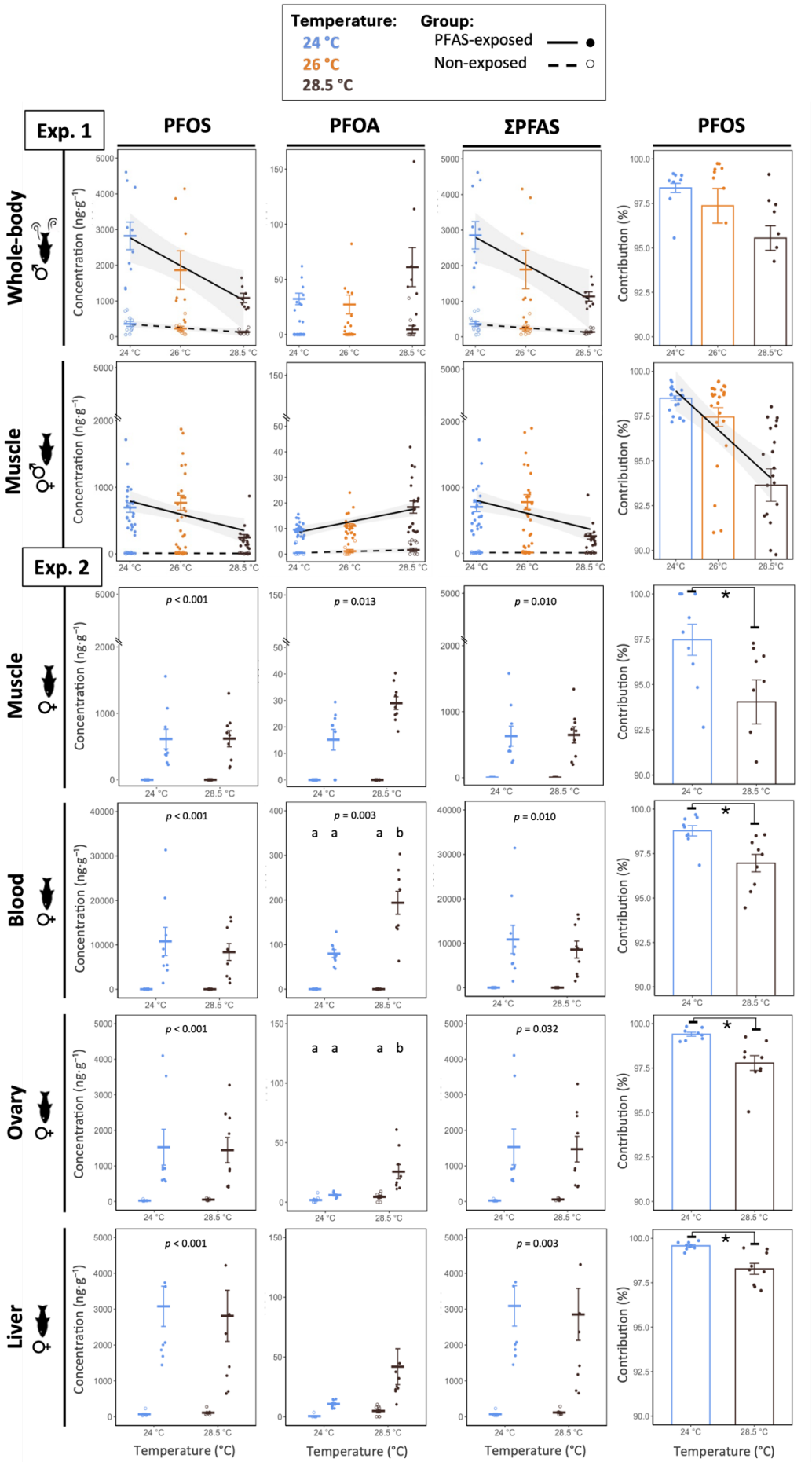
433 In parallel, warming increased tissue PFOA concentrations in PFAS-exposed fish from both
434 experiments. In Exp. 1, muscle PFOA increased with temperature (both sexes; estimate ± SE = 1.71 ±
435 0.70; PFAS*temperature (continuous): $p = 0.019$; Figure 2, Table S6; models fit with sexes combined,
436 as sex terms were non-significant in models that included sex; Table S7). For muscle, the categorical-
437 temperature model shows that the temperature effect is driven by higher concentrations at 28.5 °C
438 (PFAS*temperature (factor): $p = 0.029$; Table S6): PFOA at 28.5 °C exceeded 24 °C and 26 °C (post hoc
439 tests, both $p < 0.018$), with no difference between 24 and 26 °C ($p > 0.05$). By contrast, whole-body
440 PFOA showed no statistically significant temperature effect (swimming males; all $p > 0.05$; Figure 2,
441 Table S6). In Exp. 2 (females) warming from 24 °C to 28.5 °C significantly increased PFOA in blood (2.4-
442 fold; PFAS*temperature: $p = 0.003$), and showed non-significant increases in liver (~4.0-fold;
443 PFAS*temperature: $p = 0.139$) and muscle (~2.0-fold; PFAS*temperature: $p = 0.108$; Figure 2, Tables
444 S5–S6). This change in PFOA muscle concentrations with temperature may partly be explained by
445 PFOS–PFOA competition for shared and limited binding sites. While competition between PFOS and
446 PFOA (or between two long-chain PFAS) has not been experimentally demonstrated, such a hypothesis
447 is supported by prior evidence of antagonism between long- and short-chain PFAS compounds with
448 overlapping binding affinities (e.g., in zebrafish (*Danio rerio)*; 59) and by their different polar head
449 groups that likely influence their binding affinities and transport kinetics (60). In Exp. 1, warming likely
450 weakened PFOS-protein binding, reducing muscle PFOS concentrations. This could have released
451 common binding sites, enabling greater PFOA association with muscle, in line with the observed
452 increase in muscle PFOA concentrations with warming. Whole-body PFOA concentrations remained
453 stable, which is consistent with redistribution toward muscle (or other tissues) rather than net
454 accumulation. In Exp. 2, by contrast, evidence points to PFAS saturation of binding sites in muscle at
455 both temperatures (i.e., plateau conditions, see above), with PFOS occupying shared sites. This could
456 explain that PFOA did not increase significantly with temperature in muscle of PFAS-exposed females.

457 Overall, warming altered PFAS accumulation in a compound-specific manner and shifted mixture
458 composition toward a higher PFOA contribution to ΣPFAS concentration, as indicated by a decline in
459 the PFOS fraction of ΣPFAS with increasing temperature (both experiments, all $p < 0.05$; Figure 2, Table
460 S6). Nevertheless, PFOS remained the dominant compound across temperatures. This shift in PFAS
461 composition reflected consistent increases in PFOA with warming in both experiments, whereas PFOS
462 showed condition-specific temperature patterns.

463 *Temperature shapes maternal transfer of PFOA.*

464 Temperature did not alter ovarian PFOS or ΣPFAS concentrations in PFAS-exposed females (Exp. 2; all
465 temperature main effects and PFAS * temperature interactions: $p > 0.050$; Figure 2, Table S6), and
466 ovary:blood PFOS and ΣPFAS ratios were unchanged (Exp. 2; Figure 4; Table S9). In contrast, warming
467 from 24 °C to 28.5 °C significantly increased ovarian PFOA (4.3-fold; PFAS * temperature: $p = 0.042$;
468 Tables S5-S6). Specifically, in PFAS-exposed females, the ovary:blood PFOA ratio was higher at 28.5 °C
469 than at 24 °C ($p = 0.034$; Figure 4; Table S9), whereas liver:blood and muscle:blood ratios were
470 unchanged. Together, these results indicate a compound-specific response to temperature in
471 reproductive tissues: PFOS (and thus ΣPFAS) remained stable, while PFOA increased, shifting ovarian
472 mixture composition toward a higher PFOA share despite PFOS remaining the dominant contributor
473 to ΣPFAS ($p = 0.004$; Figure 2, Table S6). This is consistent with the muscle patterns discussed above,
474 where results suggest PFOS approaches a binding plateau under these exposure conditions, while
475 PFOA shows a stronger temperature sensitivity. To our knowledge, these data provide novel
476 experimental evidence that maternal PFOA transfer is temperature-sensitive, adding to a growing
477 body of work linking maternal PFAS transfer to adverse developmental outcomes in offspring (61).
478 Recent work in zebrafish, for example, demonstrated transgenerational effects of PFAS exposure,
479 where PFOA, PFOS, and their mixture altered larval behavior and induced widespread changes in gene
480 expression in the F1 generation (61). These findings underscore the importance of understanding
481 maternal transfer mechanisms under warming conditions and their potential consequences for
482 reproductive success, early-life health, and long-term ecological impacts.

483 Evidence on temperature-dependent PFAS toxicokinetics in fish is scarce, and to our knowledge, our
484 work provides the first experimental evidence that warming reconfigures tissue-specific accumulation
485 under co-exposure to a PFAS mixture (PFOS + PFOA) in fish, including reproductive tissue, reshaping
486 mixture composition toward a greater PFOA share.


Temperature:
24 °C
26 °C
28.5 °C
Group:
PFAS-exposed
Non-exposed
Exp. 1
Exp. 2
PFOS
PFOA
ΣPFAS
PFOS
Whole-body
Muscle
Blood
Ovary
Liver
Concentration (ng·g⁻¹)
Contribution (%)
Temperature (°C)
p < 0.001
p = 0.013
p = 0.010
p = 0.003
p = 0.032
a a a b

487

**Figure 2: PFAS concentrations (PFOS, PFOA, ΣPFAS; ng·g⁻¹) and PFOS contribution to ΣPFAS (%) in fish tissues across temperatures (both experiments).** Panels are arranged left to right by compound (PFOS, PFOA, ΣPFAS) plus the PFOS fraction of ΣPFAS; and top to bottom by tissue: Experiment 1 (top) includes whole-body concentrations in swimming males and muscle concentrations summarized across male and female fish; Experiment 2 (bottom) includes female muscle, blood, ovary, and liver tissues. Crossbars represent group means ± SE. Circles represent individual fish. In experiment 1, lines show linear mixed models (LMMs) fits with PFAS and temperature as continuous predictors. Lines are plotted only when the interaction is significant with shaded 95% confidence intervals. In experiment 2, PFAS concentrations were analyzed by LMMs with temperature as a factor. Where interactions are significant, letters indicate pairwise differences (emmeans-tests with Tukey correction), and *p-values* for the main effect of PFAS treatment are annotated when significant. For PFOS contribution panels, asterisks indicate significant differences from Student's t-test or Wilcoxon test. Sample size: Exp. 1 - non-exposed fish: *n* = 9–12, PFAS-exposed fish: *n* = 17–24 (muscle) or 7–10 (whole-body); Exp. 2 - *n* = 8–9 fish per group. *Note:* the y-axis is scaled higher for blood and truncated for muscle to enhance visibility.

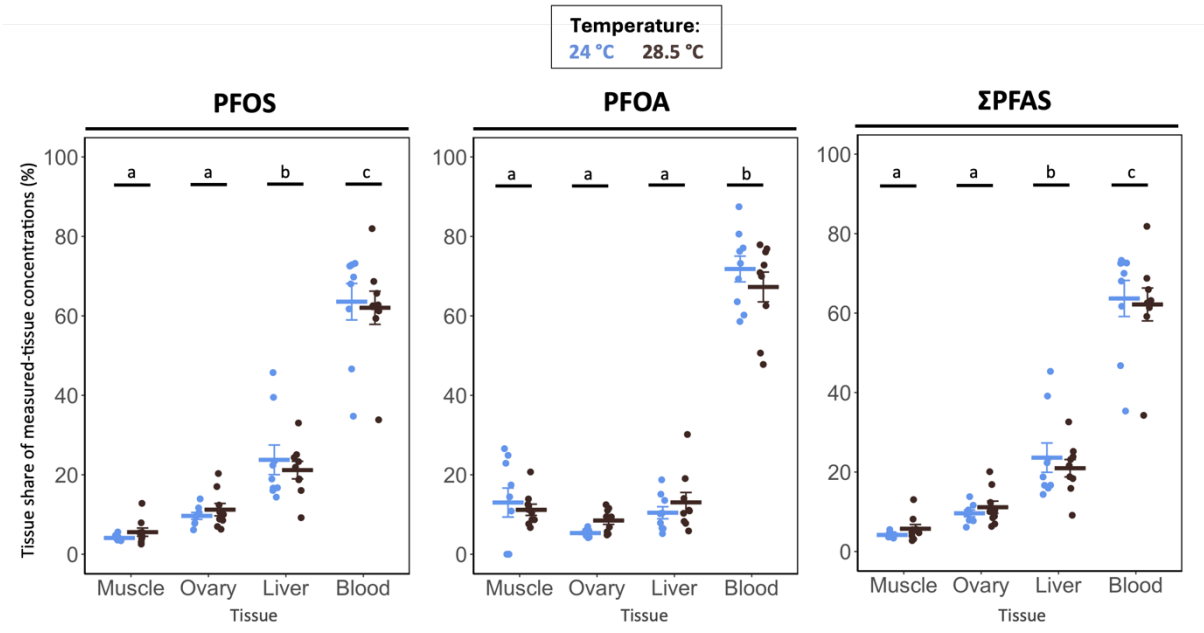



**Figure 3: Percent share of PFAS concentrations by tissue (PFOS, PFOA, ΣPFAS) in PFAS-exposed female fish across temperatures (experiment 2).** Percent shares were calculated as the concentration in a given tissue divided by the sum of concentrations in blood, liver, muscle, and ovary from the same fish. Crossbars represent group means ± SE. Circles represent individual fish. Letters indicate pairwise differences among tissues (emmeans-tests). Sample size: n = 8–9 fish per group.

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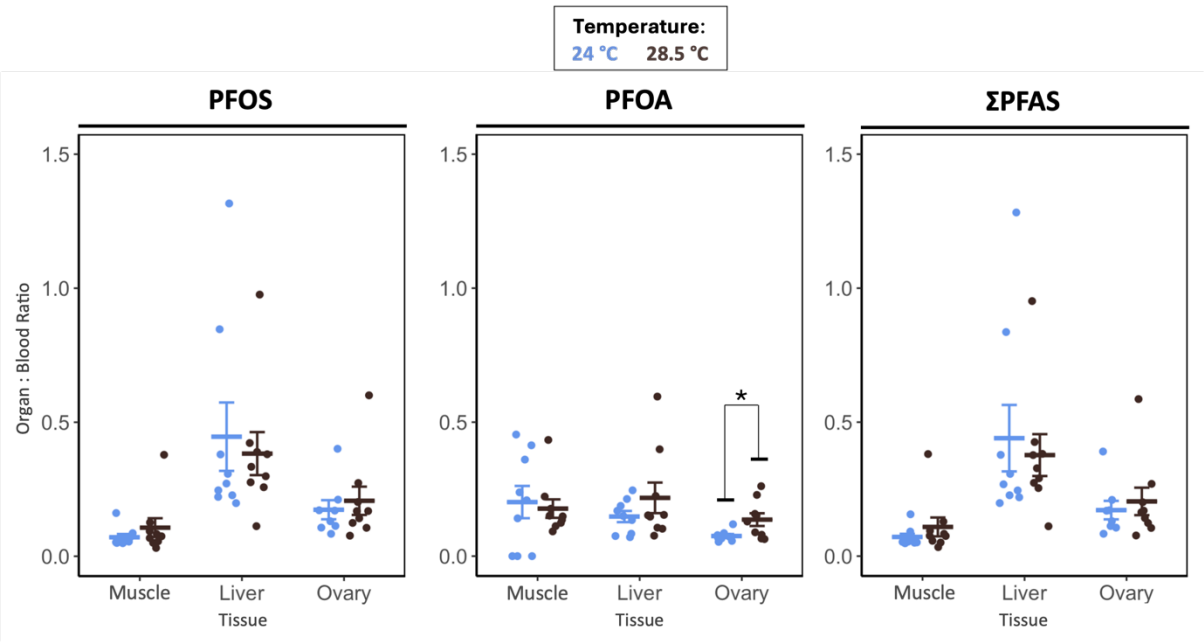



**Figure 4: PFAS organ:blood concentration ratios (PFOS, PFOA, ΣPFAS) in female fish from PFAS-exposed groups across temperatures (experiment 2).** Ratios were calculated for muscle, liver and ovary relative to blood concentrations. Crossbars represent group means ± SE. Circles represent individual fish. Asterisk indicates a significant difference between groups (Student's t-test). Sample size: n = 8–9 fish per group.

## 3.2. Temperature modulates PFAS exposure effects on fish fitness

The second objective of our study was to evaluate whether projected increases in mean summer surface water temperature modulates the toxicity of the mixture on fish metabolic, swimming and reproductive performance. To this end, we used two complementary approaches: (i) full-factorial analyses of PFAS*temperature effects (results for metabolic rates, somatic indices, body length/mass, and $U_{crit}$ are provided in the SI; Figures S2-S3-S5, Tables S10-S13-S16-S17), and (ii) analyses of the relationships between internal PFAS concentrations and physiological traits across temperatures (Figure S4, Figures 5-6, Tables S11-S12-S14-S15-S18). Here, we focus on the internal concentration–trait relationships, as these most directly inform temperature-dependent toxicity.

*End-century warming does not constrain aerobic capacity in unexposed minnows.*

Aerobic scope represents the portion of oxygen supply that an individual can allocate to support energy-demanding activities beyond basic maintenance and is widely considered ecologically relevant (27, 30, 42, 62). In our study, we found no significant difference in AS (405.2 to 482.2 $mg \cdot kg^{-1} \cdot h^{-1}$), as well as in SMR (101.5 to 108.7 $mg \cdot kg^{-1} \cdot h^{-1}$) and in MMR (512.3 to 572.6 $mg \cdot kg^{-1} \cdot h^{-1}$) between 24 °C, 26 °C, and 28.5 °C in unexposed fish, indicating stable metabolic performance across this temperature range (Figure S2, Table S10). The temperatures tested in our study fall within an intermediate thermal range for *S. minnows*, between the cooler (21 °C) and warmer (32 °C) conditions explored in previous studies. For instance, Kirby et al. (49) found a significant increase of MMR and AS at 21 °C compared to 32 °C, suggesting that aerobic capacity improves toward the upper end of this tested range, although no intermediate temperatures were tested to assess where the peak occurs. By contrast, Jung et al. (63) found no significant difference in RMR, MMR, or AS between 25 °C and 30 °C, indicating a performance plateau over this range. Our lack of significant differences between 24 °C and 28.5 °C aligns with this plateau pattern, implying that these temperatures fall within or near the species' optimal thermal window for maintaining aerobic scope. Note that the values reported in the present study are lower than those in Kirby et al. (49) and Jung et al. (63) because our data were allometrically

536 scaled to account for individual body mass, a step not applied in the comparative studies. Our results
537 therefore highlight that projected end-century warming within 24–28.5 °C is unlikely to constrain
538 aerobic performance in unexposed fish; this conclusion does not account for the multiple co-
539 exposures typical of natural environments (1).

540 *Temperature modifies PFAS impacts on metabolic performance.*

541 Whole-body PFOA concentrations showed no association with AS, SMR, or MMR and no interactions
542 with temperature (all $p > 0.05$; Figure 5, Table S11). By contrast, metabolic traits were associated with
543 whole-body ΣPFAS and PFOS, and these associations were temperature-dependent (all
544 PFAS*temperature: $p < 0.003$ across AS, SMR, MMR; Figure 5, Table S11). At 24 °C, PFOS and ΣPFAS
545 were not related to AS, SMR, or MMR (all $p > 0.475$; Table S12). At 26 °C, SMR and MMR increased
546 with PFOS and with ΣPFAS (all $p < 0.037$, all $R^2 > 0.34$; Table S12), such as AS showed no association
547 with PFAS concentrations (PFOS: $p = 0.386$; ΣPFAS: $p = 0.400$). These patterns indicate a PFAS-related
548 rise in oxygen demand at 26 °C that was matched by greater oxygen-supply capacity (higher
549 SMR/MMR), thereby maintaining aerobic scope. This compensatory response, along with the slightly
550 lower whole-body PFAS concentration relative to 24 °C (Figure 2), is consistent with higher energetic
551 demand associated with active elimination, even though AS was maintained. Indeed, in fish, PFAS are
552 primarily eliminated via renal and biliary excretion, similar to other vertebrates (56). In mammals, ATP-
553 binding cassette (ABC) transporters actively mediate PFAS excretion (64), and these transporters are
554 highly conserved and expressed in fish detoxification organs, including liver, kidney, and gills (65,66).
555 Recent work in Atlantic cod (*Gadus morhua*) shows that exposure to single PFOS, PFOA, and PFNA, or
556 their mixture (total ~138 $ug.L^{-1}$), significantly modulates ABC transporter gene expression in liver
557 tissue (67). Together, these findings suggest that active, ATP-dependent transport likely contributes
558 to PFAS elimination in fish and may add to the energetic cost of detoxification, although this cost
559 remains to be quantified. Notably, elevated oxygen consumption (SMR/MMR) does not necessarily
560 imply increased ATP production and could instead reflect reduced mitochondrial coupling efficiency,
561 resulting in higher $O_2$ consumption per unit ATP (68).

562 At 28.5 °C, PFAS concentrations were not related to SMR (PFOS: $p$ = 0.934; ΣPFAS: $p$ = 0.993; Table
563 S12), while both MMR and AS declined with increasing PFOS (MMR: $p$ = 0.005, $R^2$ = 0.46; AS: $p$ = 0.008,
564 $R^2$ = 0.43) and ΣPFAS (MMR: $p$ = 0.006, $R^2$ = 0.46; AS: $p$ = 0.008, $R^2$ = 0.43). These patterns indicate that
565 the compensatory capacity observed at 26 °C failed at 28.5 °C, resulting in a mismatch between oxygen
566 demand and supply, as reflected by the decrease in MMR and aerobic scope, despite a reduced whole-
567 body ΣPFAS concentration (Figure 2). Physiological constraints that were likely present but
568 compensated for at 26 °C may have become critical, preventing fish from maintaining sufficient
569 oxygen uptake and delivery. PFOS exposure has been shown to alter mitochondrial enzyme activity
570 (e.g., citrate synthase, cytochrome c oxidase; 69), downregulate key quality control genes (*pink1*, *fis1*),
571 and reduce basal and ATP-linked oxygen consumption rates (70) in fish species exposed to 0.1-1 $mgL^{-1}$.
572 Such mitochondrial impairments could limit how much oxygen can be used to support high metabolic
573 demands at the whole-organism level, helping to explain the decline in MMR and aerobic scope
574 observed at 28.5 °C in our study. Furthermore, PFOS and PFOA have been shown to cause structural
575 damage to gill tissue, reducing oxygen diffusion efficiency (69,71), and to induce cardiac oxidative
576 stress and dysfunction that may constrain oxygen transport capacity (72). Because MMR reflects peak
577 oxygen-transport capacity whereas SMR uses a fraction of that capacity, such impairments are
578 expected to reduce MMR/AS before SMR. This expectation is supported by Duthie & Hughes (73), who
579 showed that reducing functional gill area in Rainbow trout, lowered maximum oxygen consumption
580 without affecting resting oxygen consumption. Together, these mechanisms provide a plausible basis
581 for our observation at 28.5 °C, where SMR was maintained but MMR and aerobic scope declined.
582 Finally, lower ΣPFAS concentrations at 28.5 °C (Figure 2) may reflect reduced uptake and/or enhanced
583 passive loss rather than more efficient active elimination, given the limited oxygen supply, and
584 demonstrates that thermal stress reduces physiological tolerance, amplifying the impact of lower
585 contaminant concentrations.

586 To our knowledge, few studies have quantified whole-organism metabolic rates in PFAS-exposed fish
587 (34, 35), and prior to ours none have tested the effects of a PFAS mixture on metabolic rates. Xia et
588 al. (34) reported no significant effects on MMR (SMR not assessed) in juvenile goldfish (*Carassius*
589 *auratus*) exposed for 48 h to 0.5 $mg.L^{-1}$ of PFOS, with effects only at the highest doses tested (32.0
590 $mg.L^{-1}$). Building on this, Xia et al. (35) demonstrated that temperature can change the dose-response
591 threshold for PFOS effects on resting metabolic rate in juvenile *Spinibarbus sinensis*: at higher
592 temperatures, lower doses triggered an increase in RMR (lowest observed effect concentration:
593 5 $mg.L^{-1}$ at 18 °C vs. 0.8 $mg.L^{-1}$ at 28 °C after 40 days). However, no significant effect of PFOS on MMR
594 or aerobic scope was observed across temperature. In accordance with Xia et al. (35), our results also
595 demonstrate that a lower PFAS concentrations can become more metabolically costly at higher
596 temperatures. However, we found that thermal stress did not increase SMR at higher temperature
597 but instead constrained the ability to maintain peak oxygen supply suggesting that specific metabolic
598 endpoints and sensitivity thresholds may vary across species.

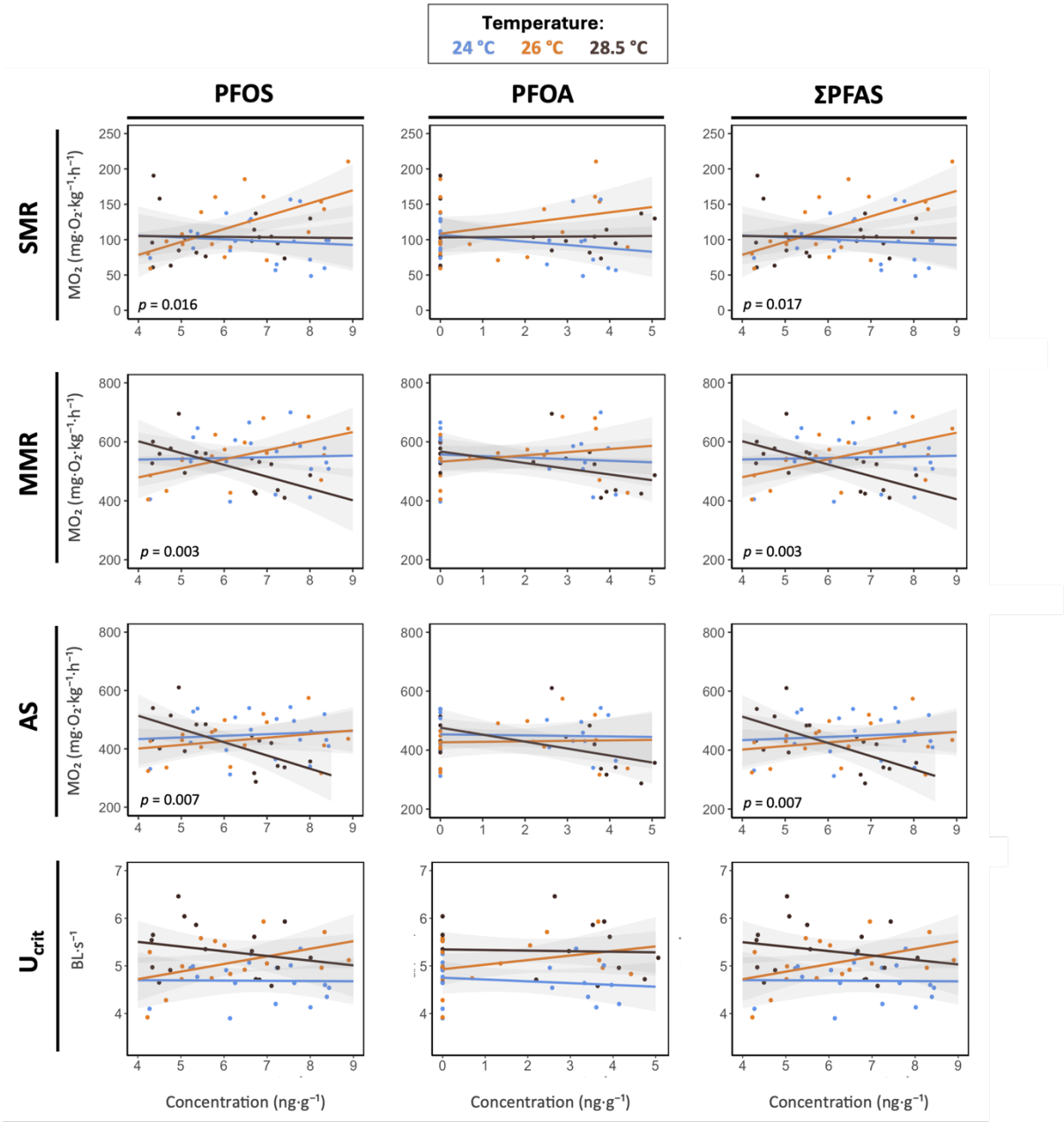
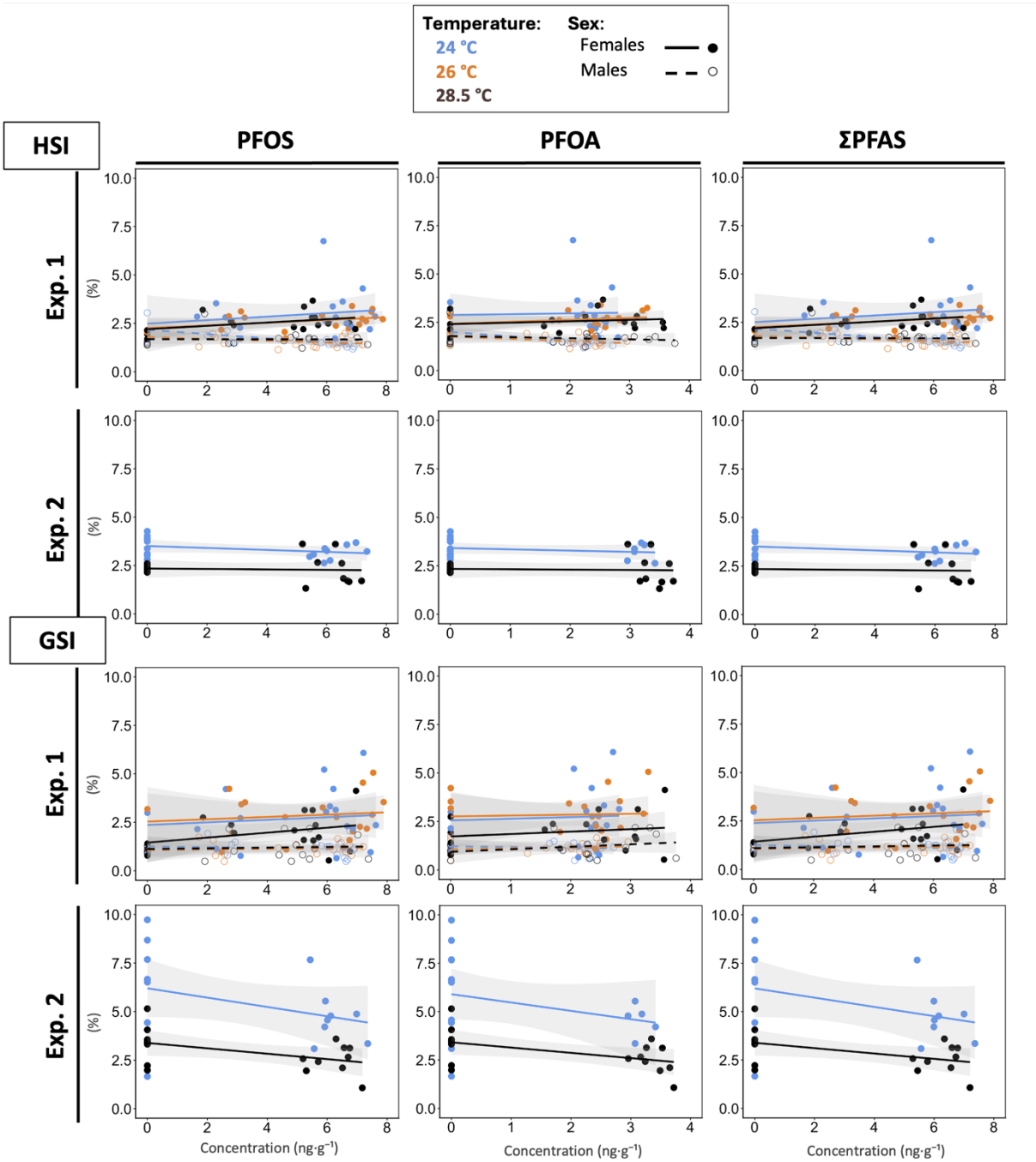



**Figure 5: Relationships between whole-body PFAS concentrations (log-transformed PFOS, PFOA, ΣPFAS; ng·g$^{-1}$) and metabolic traits (SMR, MMR, and AS; mg $O_2$ kg$^{-1}$ h$^{-1}$) or critical swimming speed ($U_{crit}$; BL s$^{-1}$) across temperatures in swimming male fish (experiment 1).** Panels are arranged left to right by compound (PFOS, PFOA, ΣPFAS) and top to bottom by trait (SMR, MMR, AS, $U_{crit}$). Circles represent individual fish. Lines show linear mixed models (LMMs) fits with shaded 95% confidence intervals and with PFAS as a continuous predictor and temperature as a factor. Where interactions are significant, *p-values* are reported. Sample size: n = 16-19 fish per temperature group.

606 *Hepatic somatic index reveals sex- and context-dependent PFAS effects.*

607 HSI showed context-dependent responses to PFAS. In experiment 1, the relationships between muscle
608 PFAS concentrations and HSI differed by sex (ΣPFAS*sex: $p$ = 0.033; PFOS*sex: $p$ = 0.029; Figure 6,
609 Table S14): females exhibited higher HSI with increasing muscle ΣPFAS concentrations, driven by PFOS
610 (ΣPFAS: slope = 0.081 ± 0.04; PFOS: slope = 0.082 ± 0.04, $p$ < 0.05), whereas males showed no
611 association. PFOA was unrelated to HSI (all PFOA*sex: $p$ = 0.185; Table S14). There was no evidence
612 that temperature modulated these relationships (PFAS*temperature, all $p$ > 0.05). The PFOS-driven
613 HSI increase in females in Exp. 1 mirrors field observations: Piva et al. (74), reported higher HSI in
614 *Squalius cephalus* from highly contaminated freshwater sites compared to less polluted locations,
615 attributing it to hepatic hyperplasia and hypertrophy, likely reflecting an adaptive response to
616 elevated PFAS accumulation in the liver. The liver may increase in mass to enhance detoxification
617 capacity. Similar structural liver changes (vacuolization, steatosis, and cell proliferation) have been
618 well documented in mammals and zebrafish chronically exposed to PFAS (75,76). In experiment 2
619 (females only), HSI was not related to muscle ΣPFAS, PFOS, or PFOA (all $p$ > 0.065; Figure 6, Table S14).
620 We did not observe PFAS*temperature interactions, though temperature had an overall effect (lower
621 HSI at 28.5 °C than at 24 °C; all $p$ < 0.002 across PFOS, PFOA, ΣPFAS). The lack of an HSI–PFOS (ΣPFAS)
622 association in experiment 2, despite a positive relationship in experiment 1, may partly reflect
623 differences in exposure-data structure. In experiment 2, 50 % of PFOS measurements were below the
624 detection limit (8 of 17 females per temperature group) and HSI values at PFOS = 0 $ng \cdot g^{-1}$ (below the
625 detection limit) were highly variable, which can obscure a PFOS–HSI relationship. Sensitivity analyses
626 restricted to PFOS detections (PFOS > 0; Figure S4) influenced slope estimation. The visual pattern was
627 altered with a positive PFOS–HSI trend at 24 °C (consistent with experiment 1) but an opposite trend
628 at 28.5 °C. However, these temperature-specific slopes were not statistically supported (all $p$ > 0.131;
629 Table S15) and were sensitive to influential observations (Table S15). Taken together, our results
630 suggests that PFAS, particularly PFOS, can induce liver enlargement in females. This may reflect
631 compensatory detoxification responses, although hepatic alterations remain possible.




632

633 **Figure 6: Relationships between muscle PFAS concentrations (log-transformed PFOS, PFOA, ΣPFAS; ng·g$^{-1}$) and somatic**
634 **indices (HSI and GSI; %) across temperatures (both experiments).** Panels are arranged left to right by compound (PFOS,
635 PFOA, ΣPFAS); and top to bottom by somatic index (HSI, GSI), with experiment 1 on the upper row and experiment 2 on the
636 lower row for each index. Circles represent individual fish. Experiment 1 includes both sexes; Experiment 2 includes females
637 only. Lines show linear models (LMs) fits with shaded 95 % confidence intervals and with PFAS as a continuous predictor and
638 temperature as a factor. Sample size: Exp. 1 - n = 23-24 fish per sex and per temperature group; Exp. 2 - n = 17-18 females
639 per temperature group.

640 *PFAS and temperature did not elevate mortality.*

641 Mortality was low overall (Exp. 1: 41/684, 6%; Exp. 2: 15/105, 14%) and did not differ by PFAS
642 treatment across temperatures in either experiment (all $p > 0.251$), indicating no PFAS- or
643 temperature-related increase in mortality. Most deaths involved females (> 70%), consistent with
644 aggressive male-female interactions reported during the experiment, this pattern was accentuated in
645 Exp. 2 by greater size asymmetry between sexes, increasing female susceptibility to male aggression.

646 *PFAS-temperature interactions on ecological performance.*

647 A lower aerobic scope should constrain energetically demanding activities. Accordingly, we examined
648 ecologically relevant performance metrics, starting with swimming performance, quantified in males
649 as critical swimming speed ($U_{crit}$, Figure 5). The stepwise $U_{crit}$ test recruits aerobic slow muscle fibers
650 and often covaries with aerobic metabolic performance in teleosts (e.g., mahi-mahi *Coryphaena*
651 *hippurus* (77); multiple damselfish species (78); golden grey mullet *Chelon auratus* (44)). In our study,
652 $U_{crit}$ were not associated with whole-body PFAS concentrations, or with temperature, and there were
653 no significant interactions (all $p > 0.05$ across PFOS, PFOA and ΣPFAS; Figure 5, Table S18).
654 Nonetheless, $U_{crit}$ showed a trend that visually mirrored MMR and AS, increasing with PFOS (and
655 ΣPFAS) at 26 °C and decreasing at 28.5 °C, although these slopes were not statistically supported (Table
656 S18). The absence of statistically $U_{crit}$ responses despite variation in aerobic capacity metrics is
657 consistent with prior work indicating that swimming performance can be decoupled from whole-
658 animal metabolic traits in sheepshead minnows. Kirby et al. (49) found that acclimation to 32 °C
659 increased MMR and AS relative to 21 °C, yet $U_{crit}$ did not increase. They hypothesized that sheepshead
660 minnow's skeletal muscle exhibits high thermal plasticity (including flexible nerve stimulation,
661 contractile kinetics, force generation, and enzyme activity), that may partly explain why this species
662 shows consistent swimming performance across a wide range of temperatures. Under PFAS exposure,
663 Xia et al. (34) similarly reported unchanged $U_{crit}$ in goldfish despite elevated MMR at 32 $mg.L^{-1}$ of PFOS,
664 likely due to reduced swimming efficiency caused by gill damage or disrupted glucose metabolism.

665 Thus, $U_{crit}$ may be relatively robust to PFAS concentrations and temperatures tested in our study, any
666 effects on aerobic capacity could have been partially offset by compensatory locomotor strategies and
667 do not translate into detectable differences in critical swimming performance.

668 As reproduction is one such energy-demanding function and key component of fitness, both GSI
669 (Figure 6) and egg production (Figure 7) were also assessed to evaluate potential sublethal impacts on
670 reproductive investment. Mean daily egg production per female was not influenced by PFAS exposure
671 and temperature, and there were no significant interactions (all $p > 0.095$; Figure 7C, Table S19).
672 Similarly, egg production remained stable over time, with no acceleration or decline attributable to
673 PFAS exposure or temperature (slopes of daily and cumulative eggs production: all $p > 0.05$; Figure
674 7D-E, Table S19). Note that the 24 °C non-exposed group had the highest mean cumulative egg
675 production at day 28; however, total egg production did not differ significantly among
676 PFAS*temperature groups (PFAS and temperature: $p = 0.113–0.144$; interaction: $p = 0.551$; Figure 7F,
677 Table S19). Because our temperature treatments closely bracket the ~25 °C commonly used to
678 standardize spawning conditions in EPA protocols for sheepshead minnows (79), this elevation in the
679 24 °C non-exposed group alone is more plausibly explained by tank-level variability and limited
680 replication (5–6 tanks per group) than by a consistent temperature effect across our tested range. GSI
681 was not associated with muscle PFAS concentration, and no PFAS*temperature interactions were
682 detected (both experiments; all $p \geq 0.065$ across PFOS, PFOA, and ΣPFAS; Figure 6, Table S14). In
683 females from Exp. 2, however, GSI varied with temperature overall (all $p \leq 0.002$; Figure 6, Table S14),
684 being lower at 28.5 °C than at 24 °C. In Exp. 1, GSI did not differ between sexes (note that females had
685 an overall greater GSI in the factorial model, Sex: $p = 0.005$; Table S13) and showed no overall
686 temperature effect (Table S13). These findings align with those of Gust et al. (80), who found no
687 significant effect of PFOS (0.1 to 100 $\mu g \cdot L^{-1}$) on parental or first-generation egg production in zebrafish.
688 Conversely, other studies have reported adverse reproductive outcomes. For instance, Suski et al. (81)
689 exposed sexually mature fathead minnows (*Pimephales promelas*) to PFOS for 42 days, reporting a
690 reduction in male GSI at 44 $\mu g \cdot L^{-1}$ and decreased fecundity in females at 140 $\mu g \cdot L^{-1}$. Similarly, Kang et

al. (82) reported reduced egg production in Japanese medaka (*Oryzias latipes)* following co-exposure to 10 mg·L$^{-1}$ PFOA and 1 mg·L$^{-1}$ PFOS for 21 days. These discrepancies likely reflect differences in PFAS concentrations, exposure duration, compound identity (single vs. mixture), and species-specific sensitivity. Although we detected no measurable effects on reproductive output or GSI in our fish, we cannot exclude the possibility of delayed or transgenerational effects. For instance, Lee et al. (22) exposed *O. latipes* to a mixture of PFOS, PFOA, PFBS, and PFNA at 0.5 µg·L$^{-1}$ over three generations (238 days), and reported significant reproductive alterations (e.g., inhibition of hatchability, induction of VTG expression).

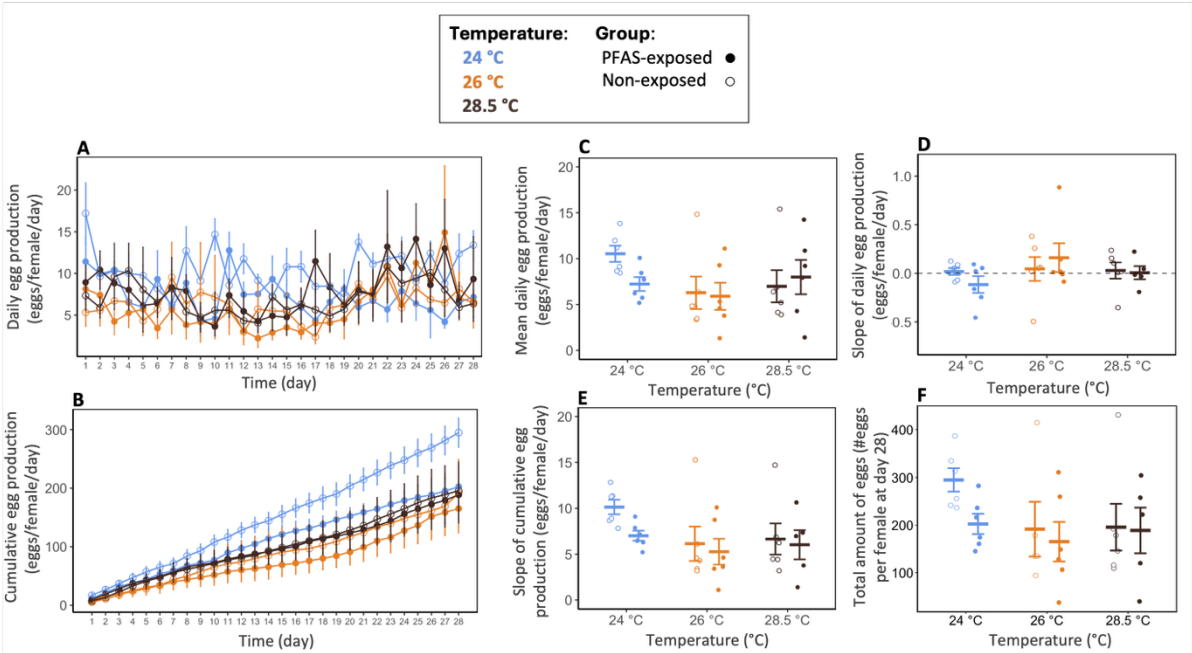



**Figure 7: Egg production across temperatures and PFAS exposure conditions in experiment 1.** (A) Daily egg production (eggs·female$^{-1}$·day$^{-1}$) and (B) cumulative egg production (eggs·female$^{-1}$) per female over the 28-day exposure period. (C) Mean daily egg production per female (eggs·female$^{-1}$·day$^{-1}$). Rate of (D) daily and (E) cumulative egg production per female, estimated as the slope of daily and cumulative egg counts over time (Δ eggs·female$^{-1}$·day$^{-1}$). (F) Total egg production at day 28 (eggs·female$^{-1}$). Points and crossbars represent group means ± SE. Circles represent individual fish. Sample size: n = 5-6 aquaria per group.

By combining thermal stress and chronic PFAS exposure, this study demonstrates that projected increases in mean water temperature can profoundly reshape contaminant dynamics and alter physiological performance in a key coastal fish species. To our knowledge, this is the first experimental demonstration that warming modifies both the toxicokinetics (internal distribution and maternal transfer) and the physiological toxicity of a PFAS mixture composed of PFOS and PFOA. Temperature altered PFAS accumulation in a compound- and tissue-specific manner, shifting the mixture toward PFOA. Notably, warming promoted PFOA redistribution toward reproductive tissues and increased maternal transfer to eggs. Importantly**,** at the highest temperature tested**,** fish lose their ability to fully compensate for the metabolic cost of contamination, resulting in reduced aerobic capacity. Although swimming and reproductive outputs were not impaired under the tested conditions, the observed physiological alterations, such as liver enlargement and changes in tissue distribution, point to hidden energetic costs associated with detoxification. These results indicate that even exposure conditions under environmentally relevant concentrations combined with warming temperatures may increase the vulnerability of estuarine fish to contaminant stress, with implications for the resilience and ecological fitness of coastal fish populations and potentially for the development and survival of their offspring.

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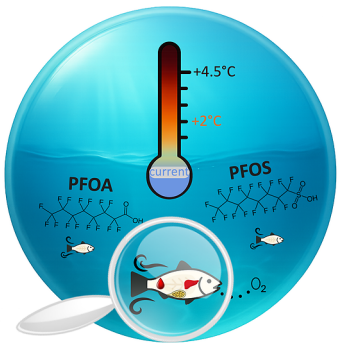

## Supporting Information

PFAS exposure methods and concentrations, endpoint sample sizes by experiment, statistical analysis results, and supporting figures.


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## CRediT authorship contribution statement

**Margot Grimmelpont**: Methodology – Conceptualization, Investigation, Formal analysis, Writing – original draft; **Maria Rodgers:** Methodology – Conceptualization, Writing – review & editing, Funding acquisition, Project administration; **Milton Levin**: Investigation, Writing – review & editing; **Sylvain De Guise:** Methodology – Conceptualization, Writing – review & editing; **Anika Agrawal**: Investigation, Writing – review & editing; Jacqueline Baron: Investigation; **Daniel Bolnick:** Formal analysis, Writing – review & editing; **Kathryn Milligan-McClellan:** Writing – review & editing; **Anthony Provotas:** Chemical analysis; **Jessica Brandt:** Methodology – Conceptualization, Writing – review & editing, Funding acquisition, Project administration.

## Data Availability Statement

The dataset underlying this study has been deposited in Zenodo (DOI: https://doi.org/10.5281/zenodo.17429806). The files are currently under restricted access for peer review and will be made publicly available upon publication.

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