Impaired hatching exacerbates the high CO$_2$ sensitivity of embryonic sand lance *Ammodytes dubius*

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**ABSTRACT:** Rising oceanic partial pressure of CO$_2$ (pCO$_2$) could affect many traits in fish early life stages, but only few species to date have shown direct CO$_2$-induced survival reductions. This might partly be because species from less CO$_2$-variable, offshore environments in higher latitudes are currently underrepresented in the literature. We conducted new experimental work on northern sand lance *Ammodytes dubius*, a key forage fish on offshore Northwest Atlantic sand banks, which was recently suggested to be highly CO$_2$-sensitive. In 2 complementary trials, we produced embryos from wild, Gulf of Maine spawners and reared them at several pCO$_2$ levels (~400–2000 μatm) in combination with static (6, 7, 10°C) and dynamic (10→5°C) temperature treatments. Again, we consistently observed large, CO$_2$-induced reductions in hatching success (−23% at 1000 μatm, −61% at ~2000 μatm), and the effects were temperature-independent. To distinguish pCO$_2$ effects during development from potential impacts on hatching itself, some embryos were switched between high and control pCO$_2$ treatments just prior to hatch. This indeed altered hatching patterns, consistent with the CO$_2$-impaired hatching hypothesis. High CO$_2$ also delayed the day of first hatch in one trial and peak hatch in the other, where later-hatched larvae were of similar size but with progressively less endogenous energy reserves. For context, we extracted seasonal pCO$_2$ projections for Stellwagen Bank (Gulf of Maine) from regional ensemble simulations, which indicated a CO$_2$-induced reduction in sand lance hatching success to 71% of contemporary levels by 2100. The species’ unusual CO$_2$ sensitivity has large ecological and scientific ramifications that warrant future in-depth research.

**KEY WORDS:** Ocean acidification · CO$_2$-impaired hatching · Dynamic temperature · Endogenous energy reserves · Regional pCO$_2$ projections

1. **INTRODUCTION**

Anthropogenic ocean warming and acidification continue to accelerate globally (Garcia-Soto et al. 2021) and thus lend urgency to the question of how marine organisms will cope with novel emerging climates (Lotterhos et al. 2021). Over recent decades, CO$_2$ exposure experiments have provided first answers by aiming to distinguish CO$_2$-sensitive from CO$_2$-tolerant organisms and traits and by elucidating mechanisms and stressor interactions (Boyd et al. 2018, Baumann 2019). This revealed that many traits in a majority of taxa are potentially sensitive to changing CO$_2$ conditions (Harvey et al. 2013, Cat...
tano et al. 2018, Doney et al. 2020). It implies fundamental shifts to the fitness landscape in future high-CO2 oceans (Munday et al. 2019), but specific predictions are still beset by unresolved questions of adaptation (Sunday et al. 2014) and the notorious heterogeneity of experimental trait responses to high CO2 across all levels of taxonomic organization (Kroeker et al. 2013, Busch et al. 2015, Przeslawski et al. 2015).

Experiments on marine fish have shown that short-term high CO2 exposures can reduce survival in some but not most tested species and that such lethal effects occur almost exclusively at the earliest, least developed life stages (Baumann et al. 2012, Dahlke et al. 2020). Other responses to acidified conditions comprise non-lethal changes to e.g. gene expression (e.g. Porteus et al. 2018, Mazurais et al. 2020), metabolism (e.g. Munday et al. 2009, Pimentel et al. 2014, Crespel et al. 2019), growth and morphometry (e.g. Bignami et al. 2013, Perry et al. 2015, Murray & Baumann 2020), behavior (Ashur et al. 2017) or reproduction (e.g. Faria et al. 2018, Concannon et al. 2021), which are by-products of most fishes’ capacity for swift CO2 acclimation and effective acid-base regulation (Esbaugh 2018). Non-lethal CO2 effects may still inflict real fitness costs (e.g. aberrant behavior could increase predation mortality; Munday et al. 2012), but this is more often assumed than explicitly shown.

Interestingly, one of the more consistent experimental findings has been that parental CO2 environments modulate offspring CO2 sensitivities in most studied taxa (Murray et al. 2014, Donelson et al. 2018). Since aquatic habitats differ vastly in their degree of diel, seasonal, or ephemeral CO2 fluctuations even across small spatial scales (Hofmann et al. 2011), the complex mosaic of species’ CO2 sensitivities might therefore not be surprising. Indeed, recent analyses concluded exactly this for select echinoderm, mollusk, and copepod species, showing that the more CO2-tolerant groups were those sourced from more CO2-extreme and CO2-variable environments (Kelly et al. 2013, Vargas et al. 2017). The ocean variability hypothesis (Baumann 2019) arguably applies to fishes as well, but current data likely overrepresent CO2-tolerant species from more accessible nearshore, metabolically active environments (Franke & Clemmesen 2011, Depasquale et al. 2015, Lonthair et al. 2017). There, contemporary CO2 fluctuations already exceed average surface ocean projections decades and centuries from now (Baumann et al. 2015, Doney et al. 2020). In contrast, the fewer cases of direct CO2 sensitivity (i.e. reduced survival) appear to occur in fishes that are adapted to less CO2-variable, offshore habitats closer to atmospheric equilibrium (i.e. ~400 μatm; e.g. Chambers et al. 2014, Stiasny et al. 2016, Dahlke et al. 2017, Alter & Peck 2021).

High early life CO2 sensitivity in fishes could also depend on slow rates of early life development, as seen in most cold-water species from temperate to polar environments (Baumann 2019). This may allow CO2 effects to accumulate while embryos are still maturing the cellular structures for acid–base regulation and thus lead to acidosis, which is so far the presumed mechanism causing all CO2-induced survival effects in fish early life stages (Heuer & Grosell 2014). In contrast, most tropical and subtropical fishes develop into acid–base competent, active swimmers mere days after fertilization and therefore likely before detectable lethal CO2 effects can accrue (Pimentel et al. 2014, Munday et al. 2016). Albeit intuitive, these emergent patterns still require further empirical corroboration, particularly from candidate species chosen more strategically, i.e. from more offshore habitats at temperate to polar latitudes. Such fishes could prove more CO2-sensitive and thus more vulnerable to climate change than currently appreciated.

Consider thus the slender-bodied sand lances (Ammodytes dubius), which are key forage fishes in temperate to polar ecosystems in the northern hemisphere (Staudinger et al. 2020). In some areas, their high local abundance and nutritious quality alone appear to sustain local diversity hotspots of higher trophic piscivores, as for example on Stellwagen Bank in the southern Gulf of Maine (Fig. S1 in the Supplement at www.int-res.com/articles/suppl/m687p147_supp.pdf; see also Suca et al. 2021). Here, dense aggregations of northern sand lance (Ammodytes dubius, hereafter sand lance) attract predators such as cod, tuna, and sharks, in addition to foraging seabirds, seals, dolphins, and humpback whales (Silva et al. 2020, Staudinger et al. 2020). Sand lances are gonochoristic, iteroparous fish that mature at 1–2 yr of age and can live up to 8 yr, although most in the Gulf of Maine are ≤5 yr old (Staudinger et al. 2020). Important, sand lance are winter spawners, which causes their demersal embryos to develop very slowly over weeks and larvae to emerge in time to utilize the first productivity bloom of the new year (Robards et al. 2000). Larvae can remain pelagic for 2–3 mo, before settling as juveniles on offshore, coarse-grain sand banks across the Northwest Atlantic shelf (Suca et al. 2021).

For these reasons, we recently began studying the CO2 sensitivity of sand lance offspring in factorial
rearing experiments, with striking results (Murray et al. 2019). Embryos showed some of the strongest CO₂-induced survival reductions documented yet among fishes (up to ~90% at ~2000 versus 400 μatm CO₂). Intriguingly, we noticed that many embryos at high CO₂ treatments did not merely arrest their development (indicative of acidosis), but appeared fully developed and ‘ready’ to hatch but incapable of doing so. We therefore hypothesize that, in addition to acidosis, high CO₂ directly impairs hatching in sand lance, potentially via affecting the production or efficacy of choreolytic hatching enzymes, but this requires additional data and more targeted tests. Moreover, the species’ apparent CO₂ sensitivity alone demands further empirical evaluations (‘you better repeat it’; Murray & Baumann 2018) as well as context to specific partial pressure of CO₂ (pCO₂) projections for its natural habitat. We thus conducted further CO₂ × temperature experiments on sand lance from the same population in the southern Gulf of Maine (Stellwagen Bank National Marine Sanctuary, SBNMS). Beyond replication, we aimed to identify potential CO₂-sensitivity thresholds by testing additional CO₂ levels and employed 2 complementary approaches to explore the CO₂-impaired hatching hypothesis. Finally, we compared our experimental findings to recent, seasonally explicit pCO₂ projections for Stellwagen Bank in 2050 and 2100 based on Siedlecki et al. (2021) to begin constraining the species’ potential climate vulnerability.

2. MATERIALS AND METHODS

2.1. Experimental setup

Two complementary experiments were conducted in late 2018 (Expt 1) and 2020 (Expt 2), each rearing newly fertilized sand lance embryos to hatch over the course of 32–65 d. Founder adults were sampled at SBNMS (42° 9’ 58.26” N, 70° 18’ 44.19” W) at the peak of their narrow, local spawning window on 15 (Expt 1) or 27 November (Expt 2), using a 1.3 × 0.7 m beam trawl (6 mm mesh) towed over ground at 3 knots for 15 min. On deck, all flowing-ripe males and females were strip-spawned together (at 10°C, Expt 1: Nmale/female = 29/13; Expt 2: Nmale/female = 50/46) and their progeny were transported to the University of Connecticut’s Rankin Seawater Lab. There, exposure experiments commenced within 8 h post-fertilization by placing a volumetrically measured random sample of 600 (Expt 1) or 1200 embryos (Expt 2) into each replicate rearing container.

Rearing containers (750 ml plastic cups) were fitted with 100 μm mesh bottoms and received a gravity-fed flow of approximately 4 l h⁻¹, while floating in larger recirculating treatment tanks (600 l) controlled for temperature, pH, and oxygen conditions (Automated Larval Fish Rearing System, ALFiRiS). Briefly, ALFiRiS works by pumping treatment water past a central pH electrode (Hach pHD, cross-checked daily with an independent Hach Intellical PHC281 sensor on a HQ11D handheld pH/ORP meter, both being calibrated weekly with 2-point National Institute of Standards and Technology [NIST] pH standards) and an optical dissolved oxygen sensor (Hach LDO Model 2) to sequentially monitor experimental conditions in each of 9 independent units. Customized LabView (National Instruments) routines then control solenoid valves connected to pressurized CO₂ (bone-dry grade), N₂, and CO₂-stripped air (for details, see Murray et al. 2019). Between Expts 1 and 2, ALFiRiS’ temperature system was upgraded from manually set thermostats (Expt 1) to LabView control (Expt 2) over relay loops that activate heaters/chillers, to now allow dynamic, computer-controlled temperature treatments. Experimental seawater was drawn from subsurface eastern Long Island Sound (31 psu), filtered to 1 μm, and UV-sterilized before use. Oxygen levels were maintained at ~100% saturation, while the photoperiod was 11 h light:13 h dark. Ten percent of seawater in each unit was replaced weekly.

2.2. Seawater chemistry

Realized pCO₂ conditions and other seawater chemistry parameters (Table 1) were estimated in CO2SYS (V2.1, Pierrot et al. 2006) based on samples taken every 10 d and measured for temperature, pHNIST, salinity (refractometer, Cole-Parmer, ±0.3%) and total alkalinity (AT, μmol kg⁻¹). Seawater samples were filtered to 10 μm, stored in 300 ml borosilicate bottles at 3°C, and within days measured for AT using endpoint titration (Mettler Toledo G20 Potentiometric Titrator) with an accuracy of ±1% (Murray et al. 2019; verified and calibrated using Dr. Andrew Dickson’s certified reference material for AT in seawater; Scripps Institution of Oceanography, batch nos. 162 and 164).

2.3. Experimental design

During Expt 1, we tested factorial combinations of 2 static temperatures and 3 target pCO₂ levels,
Table 1. Experimental conditions and seawater chemistry during Expts 1 (2018) and 2 (2020). Mean (±SD) pHNIST and temperature from hourly records, total alkalinity ($A_T$) from replicated seawater samples, partial pressure and fugacity of CO$_2$ (pCO$_2$ and fCO$_2$), dissolved inorganic carbon (C$_T$), and carbonate ion concentration (CO$_3^{2−}$) calculated with CO2SYS (V2.1)

<table>
<thead>
<tr>
<th>Temperature ($°C$)</th>
<th>pH</th>
<th>$A_T$ (μmol kg$^{-1}$)</th>
<th>pCO$_2$ (μatm)</th>
<th>C$_T$ (μmol kg$^{-1}$)</th>
<th>fCO$_2$ (μatm)</th>
<th>CO$_3^{2−}$ (μmol kg$^{-1}$)</th>
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</thead>
<tbody>
<tr>
<td>Expt 1, 2018</td>
<td>5.8 ± 0.5</td>
<td>8.12 ± 0.02</td>
<td>2022 ± 13</td>
<td>391 ± 9</td>
<td>1914 ± 12</td>
<td>390 ± 9</td>
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<td></td>
<td>5.9 ± 0.2</td>
<td>7.75 ± 0.06</td>
<td>2019 ± 13</td>
<td>946 ± 68</td>
<td>2009 ± 20</td>
<td>942 ± 67</td>
</tr>
<tr>
<td>Expt 2, 2020</td>
<td>7.1 ± 0.8</td>
<td>8.10 ± 0.04</td>
<td>2152 ± 31</td>
<td>436 ± 35</td>
<td>2034 ± 31</td>
<td>434 ± 35</td>
</tr>
<tr>
<td></td>
<td>7.1 ± 0.8</td>
<td>7.76 ± 0.05</td>
<td>2152 ± 39</td>
<td>993 ± 44</td>
<td>2132 ± 41</td>
<td>989 ± 43</td>
</tr>
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<td></td>
<td>7.1 ± 1.0</td>
<td>7.64 ± 0.02</td>
<td>2139 ± 36</td>
<td>1344 ± 43</td>
<td>2154 ± 38</td>
<td>1338 ± 42</td>
</tr>
<tr>
<td></td>
<td>7.3 ± 1.0</td>
<td>7.54 ± 0.05</td>
<td>2149 ± 30</td>
<td>1674 ± 50</td>
<td>2189 ± 32</td>
<td>1667 ± 49</td>
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<tr>
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<td>7.2 ± 1.0</td>
<td>7.47 ± 0.04</td>
<td>2142 ± 28</td>
<td>1995 ± 70</td>
<td>2205 ± 32</td>
<td>1987 ± 70</td>
</tr>
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<td>Expt 1, 2018</td>
<td>10.0 ± 0.4</td>
<td>8.12 ± 0.02</td>
<td>2031 ± 32</td>
<td>437 ± 40</td>
<td>1908 ± 38</td>
<td>435 ± 40</td>
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<td>10.2 ± 0.3</td>
<td>8.04 ± 0.02</td>
<td>2025 ± 3</td>
<td>530 ± 49</td>
<td>1927 ± 12</td>
<td>528 ± 48</td>
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<td>10.4 ± 0.4</td>
<td>7.95 ± 0.03</td>
<td>2022 ± 9</td>
<td>663 ± 33</td>
<td>1951 ± 7</td>
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<td>2032 ± 19</td>
<td>839 ± 58</td>
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<td>2028 ± 16</td>
<td>1010 ± 17</td>
<td>2003 ± 15</td>
<td>1007 ± 17</td>
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<td>1992 ± 47</td>
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<td>8.11 ± 0.04</td>
<td>2154 ± 24</td>
<td>416 ± 40</td>
<td>2037 ± 18</td>
<td>414 ± 36</td>
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<td>2136 ± 23</td>
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<td>1336 ± 72</td>
<td>2179 ± 32</td>
<td>1331 ± 72</td>
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<tr>
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<td>7.48 ± 0.05</td>
<td>2149 ± 28</td>
<td>1928 ± 138</td>
<td>2213 ± 28</td>
<td>1921 ± 137</td>
</tr>
</tbody>
</table>

thereby encompassing contemporary thermal conditions on Stellwagen Bank between late fall ($10°C$) and early winter ($6°C$), as well as current ambient ($400 μatm, pH=8.12$), predicted end-of-century ($1000 μatm, pH=7.76$), and maximum open ocean pCO$_2$ benchmarks ($2000 μatm, pH=7.48$; Caldeira & Wickett 2003, Salisbury & Jönsson 2018). At $10°C$, 3 additional pCO$_2$ levels below 1000 μatm (570, 690, 890 μatm; Table 1) were included to better describe near-future CO$_2$ sensitivities of sand lance embryos. The replication level for each of the 9 treatments was $N = 5$. Prior to hatch, 50 embryos per replicate were subsampled (150–200 degree-days post-fertilization, ddpf) and preserved in RNAlater for future analyses (to be reported elsewhere). Another 50 embryos per replicate were subsampled at 90–190 ddpf and preserved in buffered (sodium tetraborate) 5% formaldehyde/freshwater solution. Embryos sampled just before hatching began (170 ddpf, one random replicate per treatment) were later submitted for sectioning and staining (H&E stain; Horus Scientific) and then imaged for analyses of chorionic thickness (Nikon SMZ-1000 with Luminera Infinity2-2 camera and ImagePro Premier V9.0, Media Cybernetics).

During Expt 2, we again targeted pCO$_2$ levels of 400, 1000, and 2000 μatm, first at an intermediate static temperature of $7°C$ and second at a dynamic temperature of $10°C$ decreasing to $5°C$ at a rate of $0.2°C d^{−1}$ ($10–5°C$). The latter was chosen to approximate the seasonal decline in bottom temperatures experienced by sand lance embryos on Stellwagen Bank (Murray et al. 2019, Suca et al. 2021). The 2 treatments reached thermal equivalence at 32 ddpf (224 dd dp) – just after hatching had started. To better describe sand lance upper CO$_2$ sensitivities ($1000–2000 μatm$), we added 2 intermediate pCO$_2$ levels at $7°C$ ($~1300$ and $~1700 μatm$) and 1 at $10→5°C$ ($~1300 μatm$). The initial replication level for each of the 9 treatments was $N = 6$. However, to disentangle potential pCO$_2$ effects on embryonic development versus effects on hatching itself, we switched 3 random replicates from each extreme pCO$_2$ treatment per temperature with the opposite pCO$_2$ treatment (i.e. $3 × ~400→~2000 μatm$ and $3 × ~2000→~400 μatm$). The switch happened at 175 dd pf (i.e. 25 dpf at $7°C$; 22 dpf at $10→5°C$), just before hatching started.

2.4. Response traits

From 90 dd pf onwards, rear ing containers were monitored daily until hatching commenced; then, the number of hatchlings per replicate was recorded daily until hatching ceased. All hatchlings were immediately preserved in buffered 5% formaldehyde/freshwater solution for later morphological measurements. At the conclusion of Expt 1, unhatched remains were imaged at 4× magnification, allowing the later distinction between (a) early arrested embryos (no or only amorphous cell masses visible), (b) partially
developed embryos (unpigmented eyes visible, body not fully wrapped around the egg), and (c) fully developed embryos (pigmented eyes, body clearly visible and more than 1× wrapped around; Fig. S2). In Expt 2, we continued daily monitoring for 7 more days after hatching had ceased, then examined the remains microscopically for embryos still alive (i.e., with beating hearts). Absolute hatching numbers were transformed to daily relative frequencies by dividing by the initial number of embryos that was adjusted for subsampling (Expt 1, N = 500 per replicate) or reduced fertilization success (Expt 2, N = 873 per replicate, based on examining independent post-fertilization subsamples). Relative frequencies were then summed to yield cumulative hatching success (HS, %) for each replicate. For Expt 1, we additionally calculated the proportions of (a) fully developed but unhatched embryos and (b) all other arrested embryos combined. The latter also included decayed stages that were no longer detectable at the conclusion of Expt 1.

To characterize hatching phenology, we recorded the day of first hatch (dpf), day of peak hatch (=dpf with the highest relative hatch frequency), and the total hatching period (d) for each replicate. Following Murray et al. (2019), a large number of hatchlings were imaged at 4× magnification (Expt 1: N_total = 3923; Expt 2: N_total = 2659) and then individually measured (ImagePro) for 3 morphological traits, i.e., standard length (SL, to the nearest 0.01 mm), yolk sac area (to the nearest 0.001 mm²), and the size of the remaining oil globule inside the yolk sac (to the nearest 0.001 mm²). The latter 2 traits are proxies for endogenous energy reserves (EER) after hatching, but they were strongly correlated (N = 5552, R = 0.62, p < 0.001). Hence, we used principal component analysis to extract PC1 (explaining 73% [Expt 1] and 81% [Expt 2] of variability) and then used the PC1 scores as the new variable, hereafter referred to as EER. Histological sections of fully developed, pre-hatch embryos from Expt 1 were imaged at 20× magnification to measure the thickness of the egg envelope (chorion, ImagePro). Chorion thickness was measured at 10 randomly selected locations around the circumference of the sectioned embryo, with measurements averaged subsequently for each embryo. Unfortunately, fewer than expected embryos were sectioned well enough for quality measurements, ranging from 2 to 7 per treatment.

All husbandry and experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Connecticut (nos. A17-043, A20-046).

### 2.5. Statistical analysis

For each experiment (Expts 1 and 2), we first used a general linear model (GLM) with temperature as a categorical fixed factor and pCO₂ as a continuous fixed factor to test for pCO₂, temperature, and pCO₂ × temperature effects on logit-transformed HS [HSlogit = log (HS/1– HS)], hatching phenology (day of first, peak hatch, hatch period), mean chorionic thickness (Expt 1 only), as well as hatch SL and EER of the initial hatch peaks (first 4 d of hatching). For traits showing significant pCO₂ effects (p < 0.05), we then used linear regression to further explore their relationships to pCO₂, first separately by experiment and temperature and then across all data based on replicate means.

For HS, we further calculated effect sizes as log-transformed response ratios (R_1s):

\[
R_{1s}(i,j,k,l) = \ln(HS_{i,j,k,l}) - \ln(HS_{\text{cont},j,k,l})
\]

for each replicate i at pCO₂ level j, temperature k, and experiment l, based on the temperature- and experiment-specific mean HS at control pCO₂ (HS_\text{cont}, ~400 μatm). We then averaged R_1s and calculated bootstrapped 95% confidence intervals (95% CI) for each pCO₂ level, temperature, and experiment, and regressed these values linearly against pCO₂. This effectively standardized pCO₂ effects across temperatures and experiments with different HS baselines — an approach common in meta-analyses (Kroeker et al. 2010, Baumann et al. 2018, Cattano et al. 2018). Effects with 95% CIs excluding zero were considered significant. For Expt 2, we used a GLM for each temperature to test the null hypothesis that hatching success (HS_\text{logit}) did not differ between static (control or high) and switched pCO₂ groups (control→high; high→control, with group as a fixed factor, LSD post hoc tests). Rejection of the null hypothesis would suggest that pCO₂ influenced hatching itself, apart from effects on embryonic development. In addition, we used linear regression to test whether hatch SL and EER changed over the course of the extended hatching period during Expt 2. One treatment (Expt 2, 7°C, 1700 μatm pCO₂) showed consistent outlier values across several traits and all replicates (i.e., abnormally low HS, hatch SL, EER) and thus had to be excluded. Statistical analyses were computed using SPSS (V20 IBM).

### 2.6. Stellwagen Bank seasonal pCO₂ projections

To contextualize sand lance CO₂ sensitivities under future pCO₂ conditions, we utilized recent, high-res-
olution projections for the Gulf of Maine for the years 2050 and 2100 under the RCP 8.5 climate change scenario (Alexander et al. 2020). Three global projections (HadGEM2-ES: Hadley Center Earth System; GFDL: Geophysical Fluid Dynamics Lab; IPSL: Institut Pierre Simon Laplace) were downscaled using the Regional Ocean Modeling System (ROMS) with a 7 km horizontal resolution and 40 terrain-following vertical levels. The simulations were performed using the ‘delta method’, which provided estimates of future conditions for the average of a 30-yr period (2070–2100) centered on 2085. To obtain values for the year 2050, Brickman et al. (2021) uniformly scaled the temperature and salinity values by 0.546, based on the difference in the radiative forcing between 2085 and 2050. Similarly, we scaled the temperature (T) and salinity (S) values by 1.183 to represent the 2100 climate. Using an empirical model for dissolved inorganic carbon (DIC) and $A_T$ (McGarry et al. 2021), the projected hydrography (T, S) was subsequently used to calculate DIC and $A_T$. Then, a slug of additional anthropogenic DIC was added assuming equilibrium with the projected atmospheric carbon dioxide in the future according to the emissions pathway, with further details described in Siedlecki et al. (2021). This approach has been shown to reproduce the seasonal cycle of aragonite saturation state ($\Omega$) at the surface in the Gulf of Maine (Siedlecki et al. 2021). Monthly temperature, salinity,DIC, and $A_T$ values in 2050 and 2100 were extracted from the simulations for the upper 40 m on Stellwagen Bank (bounded by 42.13°–42.5°N, 70.5°–70.12°W). DIC and $A_T$ values were then used to calculate pCO$_2$ (μatm) using CO2SYS (V2.1). Values were also averaged for the 3 winter months (December to February), when sand lance embryos actually occur on the bank.

3. RESULTS

Mean HS under control pCO$_2$ conditions ranged from 49 to 58% in Expt 1 and 35 to 40% in Expt 2 (Fig. 1A). In both experiments, GLMs showed strong negative pCO$_2$ effects on HS regardless of thermal conditions (pCO$_2$: p < 0.001; temperature: p > 0.3; Table 2). Indeed, the 6°C and 10°C treatments during Expt 1, as well as the 7°C static and the 10→5°C dynamic treatments during Expt 2, showed consistent HS declines with pCO$_2$ (Fig. 1A). No temperature × pCO$_2$ interaction occurred. Thus, the linear regression model across all HS replicate means ($R^2 = 0.59$, p < 0.001) suggested an overall CO$_2$-induced HS decline from 47 ± 7% (95%CI) at control pCO$_2$ levels (400 μatm) to 18 ± 15% at 1000 μatm and 18 ±
9% at 2000 μatm, which are reductions of −23% and −61%, respectively (Fig. 1A). Controlling for different baseline levels of HS via response ratios ($R_{HS}$) strengthened the linear relationship ($R^2 = 0.79$, $p < 0.001$) and suggested that the negative effect on mean $R_{HS}$ grew with each 500 μatm pCO$_2$ increase by −0.25 (Fig. 1B).

While HS declined with pCO$_2$, the proportion of fully developed but unhatched embryos at the conclusion of Expt 1 increased from 2−3% at control pCO$_2$ to 19−22% at ~2000 μatm pCO$_2$ (linear regression, $R^2 = 0.99$). Similarly, the proportion of arrested or decayed embryos increased from 40−49% at pCO$_2$ controls to 53−58% at ~2000 μatm pCO$_2$ ($R^2 = 0.69$; Fig. 2). During Expt 2, a negligible, pCO$_2$-independent proportion of unhatched embryos (0−0.6%) remained alive 7 d after hatching had ceased. The switch of ready-to-hatch embryos from high to control pCO$_2$ increased HS compared to static high pCO$_2$ treatments at 7°C (E2; GLM, $p = 0.09$) and 10→5°C (GLM, $p = 0.002$; Fig. 3A,B). Conversely, the switch from control to high pCO$_2$ reduced HS in the 7°C treatment (GLM, $p = 0.014$, Fig. 3C), but had no effect in the 10→5°C treatment (GLM, $p = 0.88$; Fig. 3D).

During Expt 1, the 6°C hatchlings were significantly larger (mean SL$_{6°C} = 5.23$ mm) than those at 10°C (mean SL$_{10°C} = 5.02$ mm, GLM, $p = 0.001$; Fig. 4A). A weak positive pCO$_2$ effect (GLM, $p = 0.021$) was driven mostly by small SL values in just one treatment (10°C, control pCO$_2$), and there was no CO$_2$ × temperature interaction (GLM, $p = 0.12$). During Expt 2, pCO$_2$ did not affect hatch SL ($p = 0.88$; Table 2, Fig. 4A), and there were also no temperature or interactive effects. However, if the interaction term was omitted from the GLM, the temperature term became significant ($p = 0.004$), because embryos experiencing dynamic 10→5°C temperatures hatched with a greater mean (±SD) SL than their conspecifics in static 7°C treatments (first 4 d: 5.20 ± 0.16 mm versus 5.02 ± 0.19 mm, Fig. 4A; entire hatching period: 5.31 ± 0.09 mm versus 5.06 ± 0.22 mm, Fig. S3). Within Expts 1 and 2, EER varied independently of pCO$_2$ ($p > 0.4$) and temperature ($p > 0.06$, no interaction), but differed across experiments, because Expt 1 hatchlings had greater EER than those during Expt 2 (Fig.4B). Overall, we found EER to be

<table>
<thead>
<tr>
<th>Response trait</th>
<th>Expt 1</th>
<th>2018</th>
<th>CO2 × Temperature</th>
<th>Expt 2</th>
<th>2020</th>
<th>CO2 × Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hatching success</td>
<td>&lt;0.001</td>
<td>0.33</td>
<td>0.20</td>
<td>&lt;0.001</td>
<td>0.42</td>
<td>0.54</td>
</tr>
<tr>
<td>Hatch length</td>
<td>0.021</td>
<td>0.001</td>
<td>0.12</td>
<td>0.88</td>
<td>0.45</td>
<td>0.72</td>
</tr>
<tr>
<td>Endogenous reserves</td>
<td>0.41</td>
<td>0.06</td>
<td>0.65</td>
<td>0.94</td>
<td>0.06</td>
<td>0.80</td>
</tr>
<tr>
<td>First hatch</td>
<td>0.001</td>
<td>&lt;0.001</td>
<td>0.65</td>
<td>Not computed, uniform</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak hatch</td>
<td>0.13</td>
<td>&lt;0.001</td>
<td>0.99</td>
<td>&lt;0.001</td>
<td>0.26</td>
<td>0.19</td>
</tr>
<tr>
<td>Hatch period</td>
<td>0.39</td>
<td>0.001</td>
<td>0.95</td>
<td>0.97</td>
<td>0.5</td>
<td>0.97</td>
</tr>
<tr>
<td>Chorionic thickness</td>
<td>0.035</td>
<td>0.54</td>
<td>0.33</td>
<td>Not assessed</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2. General linear model (GLM) p-values by experiment, testing for effects of temperature (fixed factor), CO$_2$ (continuous fixed factor), and their interaction on hatching success (logit-transformed), hatch length, endogenous reserves (=PC1, yolk sac, oil globule size), day of first and peak hatch, hatch period, and chorionic thickness (Expt 1). Significant effects ($p < 0.05$) are in bold ($^*p = 0.004$, if the CO$_2$ × Temperature term is omitted from the GLM).
significantly negatively related to hatch SL (linear regression, \( R^2 = 0.11, p = 0.003 \)), i.e. larger hatchlings tended to have lower EER as approximated by their yolk and oil globule sizes (Fig. 4B). During Expt 2, mean hatch SL across all replicates increased over the course of the hatching period (linear regression, \( R = 0.23, p = 0.008 \)), while EER declined steeply (\( R = -0.50, p < 0.001 \)); hence, later hatched fish were only slightly larger but had severely reduced EERs compared to earlier hatchlings (Fig. 5).
As expected, hatching phenology was strongly temperature-dependent during Expt 1 (GLM, \( p \leq 0.001 \); Table 2), because first and peak hatch occurred approximately 2 wk earlier at 10°C (19 and 21 dpf, respectively) than at 6°C (34 and 36 dpf, respectively), with the latter also having a significantly longer hatch period (6°C = 22 d, 10°C = 14 d; Fig. 6A). High pCO2 slightly delayed the day of first hatch (\( p = 0.001 \)) by approximately 1 d regardless of temperature (Fig. 6B). During Expt 2, static versus dynamic temperatures had no effect on first hatch (27 ± 0 dpf), peak hatch (33 ± 6.9 dpf) or hatching period (33 ± 1 d, GLM; Table 2), but increasing pCO2 conditions significantly delayed peak hatch regardless of thermal conditions (\( p < 0.001 \); Fig. 6C).

Measurements of embryonic chorion thickness at 170 ddpf showed a significant positive pCO2 effect regardless of temperature (Expt 1: GLM, pCO2: \( p = 0.035 \), temperature: \( p = 0.54 \), no interaction), thus suggesting that embryos developing at high pCO2 conditions had to hatch out of thicker chorions than their conspecifics at control pCO2 levels (Fig. 7).

Regional simulations using RCP 8.5 (Siedlecki et al. 2021) predicted average seasonal pCO2 fluctuations on Stellwagen Bank in 2050 between 526 μatm in October and 614 μatm in March (0–40 m, Fig. 8A). The range was provided by the 3 models used to project future conditions and reflects intra-model differences, as well as natural variability in the system. By 2100, pCO2 concentrations were projected to double and seasonal fluctuations to triple, reaching 1084–1365 μatm (September, March; Fig. 8C). Mean winter projections (December–January) were 572 μatm (2050) and 1255 μatm pCO2 (2100). After controlling for the different experiment- and temperature-specific baseline levels of HS in our study (HSN, by normalizing to 100% HS at ~400 μatm pCO2), the fitted linear relationship HSN (%) = 116 − 0.036 × pCO2 (\( R^2 = 0.77 \), \( p < 0.001 \), \( N = 17 \); Fig. 8B) implied a 7% decrease in HS for every 200 μatm increase in pCO2. Hence, by the year 2100, sand lance HS would be reduced to 71% relative to contemporary HS levels (Fig. 8C).

4. DISCUSSION

Our study re-evaluated early-life CO2 sensitivities in northern sand lance, a key forage fish and non-model species potentially vulnerable to future high CO2 oceans. In both new experiments, we again observed large CO2-induced reductions in hatching success (~23% and ~61% at 1000 and 2000 μatm pCO2, respectively), which are consistent with the main conclusion in Murray et al. (2019) that sand lance embryos are highly sensitive to CO2. This has now been demonstrated by 4 independent trials in as many years (2016–2017: Murray et al. 2019; 2018–2020: present study), conducted by different primary experimenters (albeit in the same facility) on off-
spring produced from wild, genetically diverse spawners. Thereby, the added empirical heterogeneity reduces the likelihood of spurious attribution to CO₂, which is the purpose of serial experimentation (Baumann et al. 2018). Conversely, while the initial data had suggested an increase in sand lance CO₂ sensitivity with temperature (10°C; Murray et al. 2019), CO₂-dependent hatching success in the present study was surprisingly similar at 6°C versus 10°C (Expt 1) or at static 7°C versus dynamic 10→5°C treatments (Expt 2). The reason for the different outcomes is unknown; however, the present study used twice as many CO₂ treatments at 10°C, higher levels of replication, and improved rearing protocols, which make the present findings more robust. In addition, a more eurythermic response is more realistic, because sand lance embryos must be adapted to the large seasonal temperature decline that they experience in their natural habitat (Suca et al. 2021). Indeed, during each year of experimentation, sand lance spawning on Stellwagen Bank occurred at approximately 10°C (H. Baumann pers. obs.), which is therefore unlikely to be a stressful temperature for the embryonic development of this species.

4.1. How exceptional is the CO₂ sensitivity of sand lance?

Our ability to compare sand lance to other high latitude fishes is unfortunately limited by the scarcity of tested species and differences between studies re-
Regarding focal life stages, endpoints, pCO$_2$ levels, or sources of experimental fish. The clearest parallels exist for Barents Sea cod *Gadus morhua*, given the reported reductions in embryo survival of 11−47% in response to ~1100 μatm pCO$_2$ (Dahlke et al. 2017) and apparently similar post-hatch CO$_2$ sensitivities as demonstrated by Stiasny et al. (2016; showing a doubling of larval mortality rates in response to ~1200 μatm pCO$_2$). In Antarctic dragonfish *Gymnodraco acuticeps* embryos, survival was found to be sensitive to ~1000 μatm pCO$_2$ and warming, but the trials ended prior to hatch (Flynn et al. 2015). Conversely, experiments on summer flounder *Paralichthys dentatus* (offshore spawner) suggested large reductions in hatching success (−50%) at ~1800 relative to ~750 μatm pCO$_2$; however, this was based on offspring from just 3 females (Chambers et al. 2014). Poor genetic diversity or drift from wild populations may also underlie first reports of lethal CO$_2$ sensitivity in inland silverside *Menidia beryllina* embryos (−50% at ~1000 μatm; Baumann et al. 2012), which were sourced from a closed, commercial brood stock. We know today that co-occurring, congeneric Atlantic silversides *M. menidia*, when produced from wild spawners, are far more CO$_2$-tolerant (Baumann et al. 2018). Overall, however, fish early-life survival appears mostly robust to high pCO$_2$, as recently suggested by a meta-analysis (Cattano et al. 2018) that revealed no significant pCO$_2$ effects on embryo mortality (>1300 μatm) across all available species and contrasts (n = 13). Hence, the collective empirical evidence suggests that sand lance embryos are indeed unusual among fishes for their high CO$_2$ sensitivity, but whether this extends beyond the embryo stage has yet to be determined.

### 4.2. Why are sand lance embryos so CO$_2$ sensitive?

When CO$_2$-induced survival reductions occur in fish early life stages, the assumed cause of death is acidosis, a shorthand for the likely failure of pH-sensitive metabolic enzymes leading to arrested development due to ineffective acid–base regulation (Kikkawa et al. 2004, Esbaugh 2018). Yet most fishes appear to develop acid–base competency surprisingly early in life. For example, Dahlke et al. (2017, 2020) found that cod embryos remained CO$_2$-vulnerable only through the cleavage and gastrula stages (<50 ddpf), after which their ionocytes were sufficiently functional to tolerate CO$_2$ levels of 1100 μatm. Given that sand lance are likely adapted to offshore, low-CO$_2$ environments, their acid–base competency might develop more slowly, which therefore could have caused some of the CO$_2$-induced mortality that we observed in our experiments.

However, acidosis is unlikely to explain our observation that approximately one-fifth of sand lance embryos proceeded at high CO$_2$ conditions to fully pigmented, seemingly ready-to-hatch stages only to emerge delayed or not at all. This suggested that hatching itself was affected, perhaps because high CO$_2$ conditions reduced the efficiency of pH-sensitive hatching enzymes. Such proteolytic enzymes (chorionases) are ubiquitous in fish, produced by unicellular hatching glands and released into the perivitelline fluid to weaken the chorion before it can rupture and release the hatchling (Korwin-Kossakowski 2012). In most studied fishes, choreolytic enzymes work best at weakly alkaline conditions (e.g. Luberda et al. 1993, but see Shi et al. 2006), which implies that CO$_2$-induced acidification of the perivitelline fluid could impair enzyme activity and thus delay or impede hatching.

Our study provided 4 additional observations in support of the CO$_2$-impaired hatching hypothesis. First, when embryos were switched from high to control CO$_2$ conditions at 175 ddpf, their hatching success 2 d later improved in both 7°C and 10→5°C treatments, eventually reaching intermediate levels compared to unchanged embryos. This means that
many embryos held at high CO₂ may have still been able to hatch at that point. The reverse was true, too, because the switch from control to high CO₂ immediately worsened hatching success, at least for the 7°C treatment. Given the larger hatch size in the 10→5°C treatments, it is possible that these more developed embryos had already initiated the hatching process at the time of the switch; too late for their hatching to suffer from the pCO₂ change. Second, high CO₂ conditions appeared to coincide with increased chorionic thickness (Expt 1, 170 ddpf), which presumably makes it harder for embryos to digest and rupture the chorion. However, more high-quality histological sections of individual embryos are needed to corroborate this finding in the future. Third, high CO₂ slightly delayed the day of first hatch in Expt 1, but led to significantly protracted hatching in Expt 2 (later peak hatch), which is consistent with observations by Murray et al. (2019). Delayed hatching implies that high CO₂ embryos struggled longer to rupture their chorion and hatch than their control CO₂ conspecifics. Fourth, sand lance embryos that hatched later during Expt 2 were of similar length but had progressively smaller endogenous energy reserves (i.e. the size of the yolk sac and oil globule). Hence, sand lance embryos struggling to hatch continued to expend energy without benefitting in terms of somatic growth. In nature, fish larvae with smaller yolk reserves have less time to successfully transition to external feeding (Houde 1997). Therefore, even if sand lance embryos eventually manage to hatch under high CO₂ conditions, they would suffer higher post-hatch mortalities from starvation or predation (Anderson 1988, Bailey & Houde 1989).
4.3. How soon could sand lance hatching be impacted in the wild?

Most experiments evaluating species CO2 sensitivities employ common pCO2 benchmarks derived from global models of average surface ocean acidification (Caldeira & Wickett 2003, Riebesell et al. 2011). To move beyond general implications, regional differences in future acidification trajectories can be considered when available (Bopp et al. 2013, Vargas et al. 2017). In the Gulf of Maine, for example, past decades of acidification appear to have been masked by stronger inflows of warm, high-salinity slope water (Salisbury & Jönsson 2018), with recent ensemble simulations diverging on how this process will continue (Siedlecki et al. 2021). Regardless, the seasonally explicit pCO2 projections for Stellwagen Bank added valuable context to our experimental work. First, they suggest that sand lance hatching success should be robust to mid-century levels of predicted pCO2 (500–650 μatm in 2050). Second, they reveal that seasonal pCO2 fluctuations, which resemble northern hemisphere productivity cycles and thus attain their minima/maxima at the end of summer/winter, are likely to triple within this century (seasonal ΔpCO2,2050 = 88 μatm; ΔpCO2,2100 = 281 μatm), consistent with other modeling work (McNeil & Sasse 2016). The seasonality also entails that sand lance embryos actually experience slightly higher than annual average pCO2 conditions, because they develop during winter months (December–February). Third, the predicted rise in winter pCO2 to >1250 μatm by 2100 is more concerning, because adults on Stellwagen Bank appear to use a ~10°C threshold as a cue (H. Baumann pers. obs.). On the other hand, embryo development and therefore hatching will accelerate at warmer conditions, as quantified in Expt 1 of this study, where first and peak hatch occurred 15 d earlier at 10°C compared to 6°C treatments (-3.75 d °C−1 warming). This is consistent with a 16 d earlier first and peak hatch at 10°C versus 5°C (-3.2 d °C−1 warming) measured by Murray et al. (2019) and pioneering experimental work by Smigielski et al. (1984) on American sand lance A. americanus (-4.3 d °C−1 warming). Depending on the net effect on hatching phenology, this could result in mismatches between emerging larvae and the first plankton bloom of the year (Dam & Baumann 2017). Furthermore, there was some empirical evidence (Fig. 2) that warming may reduce larval size at hatch, which implies lower first-feeding success and thus higher post-hatch mortalities in the future (Pepin 1991). Survival may also be sensitive to warming-related reductions in oxygen conditions (Keeling et al. 2010, Breitburg et al. 2018), but this has yet to be quantified experimentally for this species. Similarly, top-down effects could be altered by sand lance predators suffering unrelated declines and, therefore, relaxing predation pressure. Lastly, the relatively short generation time of sand lance (1–2 yr) may allow for evolutionary responses to warmer, high CO2 oceans, but how fast fish and other metazoans may adapt to marine climate change is not yet well understood (Munday et al. 2019, Dam et al. 2021).

4.4. What other factors may impact sand lance embryos?

At first glance, the projected reductions in sand lance hatching success may appear small, but it is worth recalling that much smaller relative changes in early-life survival are known to cause order-of-magnitude fluctuations in many marine fish populations (Sissenwine 1984). Moreover, many additional factors will ultimately modulate the climate vulnerability of sand lance, exerting direct and indirect, potentially positive and negative pressures on these important forage fish. For example, Suca et al. (2021) suggested that further warming in the Gulf of Maine could directly reduce sand lance overwinter survival and negatively affect recruitment via potential declines in the cold-water, lipid-rich copepod Calanus finmarchicus (Ji et al. 2017). Warming will also have multiple, potentially antagonistic effects on spawning phenology, as suggested by field observations and our experimental data. On the one hand, warmer autumn temperatures may delay the onset of spawning, because adults on Stellwagen Bank appear to use a ~10°C threshold as a cue (H. Baumann pers. obs.). On the other hand, embryo development and therefore hatching will accelerate at warmer conditions, as quantified in Expt 1 of this study, where first and peak hatch occurred 15 d earlier at 10°C compared to 6°C treatments (-3.75 d °C−1 warming). This is consistent with a 16 d earlier first and peak hatch at 10°C versus 5°C (-3.2 d °C−1 warming) measured by Murray et al. (2019) and pioneering experimental work by Smigielski et al. (1984) on American sand lance A. americanus (-4.3 d °C−1 warming). Depending on the net effect on hatching phenology, this could result in mismatches between emerging larvae and the first plankton bloom of the year (Dam & Baumann 2017). Furthermore, there was some empirical evidence (Fig. 2) that warming may reduce larval size at hatch, which implies lower first-feeding success and thus higher post-hatch mortalities in the future (Pepin 1991). Survival may also be sensitive to warming-related reductions in oxygen conditions (Keeling et al. 2010, Breitburg et al. 2018), but this has yet to be quantified experimentally for this species. Similarly, top-down effects could be altered by sand lance predators suffering unrelated declines and, therefore, relaxing predation pressure. Lastly, the relatively short generation time of sand lance (1–2 yr) may allow for evolutionary responses to warmer, high CO2 oceans, but how fast fish and other metazoans may adapt to marine climate change is not yet well understood (Munday et al. 2019, Dam et al. 2021).

4.5. What are the critical knowledge gaps?

We argue that the discovery of CO2-sensitive embryo survival in sand lance has far-reaching ecological and scientific implications and thus warrants further in-depth research on several fronts. First, we need empirical data on post-hatch CO2 × tempera-
ture sensitivities in this species, which requires adjusted rearing methods and larger starting numbers of offspring. Second, it is imperative that we begin assessing other sand lance populations across the species’ large geographic distribution, as well as congener from Northeast Pacific and Northeast Atlantic ecosystems. For example, the congeneric American sand lance A. americanus, which occurs in nearshore US Atlantic habitats (Smigielski et al. 1984), might provide an important scientific contrast to the more offshore A. dubius—given the general decline in CO2 variability from nearshore to offshore habitats and the expectation of concomitant declines in CO2 tolerance (Vargas et al. 2017, Baumann 2019; ocean variability hypothesis). Third, targeted physiological assays should aim to better understand CO2-impaired hatching in sand lance, specifically the question of whether CO2 affects the amount made or the activity of the hatching enzyme, or may have other unrelated mechanisms. Given the ubiquity of pH-sensitive hatching enzymes in fishes, their potential CO2-related impairment also deserves a broader look in other taxa. Finally, targeted crosses or genomic analyses could begin to resolve the evolutionary potential (=heritability) of CO2-sensitive traits in sand lance (Johnson et al. 2010, MalveZZ et al. 2015), which would answer the question of whether evolutionary rescue is a possibility for fish species with highly CO2-sensitive embryo stages.

Data availability: Citable source data are available from the BCO-DMO database [DOIs: 10.26008/1912/bco-dmo.867401.1; 10.26008/1912/bco-dmo.867447.1; 10.26008/1912/bco-dmo.867707.1; 10.26008/1912/bco-dmo.867837.1; 10.26008/1912/bco-dmo.867931.1]. Output from the three ROMS physical simulations used for the projections as well as the control run is provided on a website (www.psl.noaa.gov/ipcc/roms/) and described further in Alexander et al. (2020).

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