Vol. 596: 267–270, 2018
https://doi.org/10.3354/meps12631

MARINE ECOLOGY PROGRESS SERIES
Mar Ecol Prog Ser

Published May 28

REPLY COMMENT

Evidence for diverse responses to viscosity in suspension-feeding bivalves: Reply to Riisgård & Larsen (2018)

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ABSTRACT: In Specht & Fuchs (2018; Mar Ecol Prog Ser 589:129–140), we found that hard clams *Mercenaria mercenaria* modify their feeding rates in response to changes in temperature but not to changes in viscosity alone. These results differed from previous findings in the blue mussel *Mytilus edulis*, and we concluded that whereas *M. edulis* pumping rates appear to be driven by biomechanical effects of viscosity, *M. mercenaria* pumping rates appear to be driven by physiological effects of changing temperature, perhaps in response to changes in dissolved oxygen concentration. Riisgård & Larsen (2018; Mar Ecol Prog Ser 596:263–265) criticize several methodological details in our study and argue that no new explanation is needed for bivalve responses to temperature. Further investigation revealed that we did incorrectly identify laterofrontal cirri as lateral cilia in experiments on ciliary beat rate in Specht & Fuchs (2018). However, despite this error, our general conclusions are unchanged and are strongly supported by feeding experiments. Riisgård & Larsen (2018) overlook differences that make our feeding experiments on *M. mercenaria* more statistically robust than prior studies on *M. edulis.* Riisgård & Larsen (2018) also ignore the diversity of responses to viscosity in other members of the animal kingdom. Therefore, we stand by our previous conclusion that hard clams and mussels fundamentally differ in their responses to viscosity.

KEY WORDS: Clearance rate · Lateral cilia · *Mercenaria mercenaria* · *Mytilus edulis* · Temperature · Viscosity

INTRODUCTION

In Specht & Fuchs (2018), we conducted feeding experiments on live clams exposed to a range of temperatures and on live clams exposed to a range of temperature-equivalent viscosities manipulated by addition of polyvinylpyrrolidone (PVP). Feeding experiments quantified clearance rates (termed filtration rate by Riisgård & Larsen 2018), which are often used as a proxy for pumping rates. These experiments on live, intact clams were also supported by observations of the ciliary beat on excised gill filaments, submerged in seawater with viscosity manipulated by addition of PVP. Our results indicated that, although clearance rate depended strongly on temperature, neither clearance rate nor ciliary beat rate varied with viscosity at constant temperature.

In their Comment, Riisgård & Larsen (2018) criticize several aspects of our methods. As detailed below, we consulted independent experts about the identification of gill cilia in our videos of gill preparations. These investigations confirmed that the cilia were misidentified. However, Riisgård & Larsen's (2018) other comments have no bearing on the interpretation of our study, and our conclusions are unchanged. Although hard clams' lack of response to

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viscosity was unexpected, it is not unique. Moreover, a comparison of study designs reveals that the feeding experiments in Specht & Fuchs (2018) were more statistically robust than previous experiments on mussels. The feeding experiments provide not just an 'opposing view,' as stated by Riisgård & Larsen (2018), but strong empirical evidence that hard clams and mussels differ in their response to viscosity.

CILIARY BEAT RATE EXPERIMENTS

Riisgård & Larsen (2018) contend that the ciliary beat observations are invalid because we misidentified the laterofrontal cirri as water-pumping lateral cilia. Their argument is based on 3 points. First, the lateral cilia — but not laterofrontal cirri — of *M. edulis* are inactivated by gill excision, and Riisgård & Larsen (2018) expect cilia of *M. mercenaria* to react similarly. Second, we reported a cilium width of 0.8 µm for *M. mercenaria* (Specht & Fuchs 2018), but lateral cilia of *M. edulis* are 0.2 µm wide, and therefore our measurement must indicate compound, laterofrontal cirri. Third, we reported ciliary beat frequencies for *M. mercenaria* (8 Hz at 21°C, Specht & Fuchs 2018) that are lower than those of *M. edulis* lateral cilia (23 Hz at 21°C) but similar to those of *M. edulis* laterofrontal cirri (~12 Hz at 20°C, Riisgård et al. 1996). These arguments rest on the assumption that the gill cilia of all bivalve species should function identically. That assumption has not been rigorously tested, and the gills of hard clams and mussels differ not only in their structure but also in their innervation (Gainey et al. 2003). However, these points did raise doubt about our ciliary identification, and they required further investigation.

We asked independent experts to review our cilia video (Movie S1 in Specht & Fuchs 2018). Before publication, the video was reviewed by an expert on hard clam physiology, experienced at multiple types of gill observation, who indicated that the video showed lateral cilia beating in a metachronal wave. After publication, that expert was unwilling to state for certain that the lateral cilia were beating. We sought a second and third opinion from 2 others who routinely work with bivalve gill preparations. Dr. Ed Catapane (Medgar Evers College, New York, NY, USA), an expert in ciliary control, confirmed that the video shows lateral cilia beating, although they are less obvious than the laterofrontal cirri. Dr. Peter Beninger (University of Nantes, Nantes, France), an expert in bivalve gill function, disagreed and did not clearly see any movement except that of the laterofrontal cirri. This lack of consensus — even among experts — highlights the difficulty of identifying cilia from microscopy videos. Dr. Catapane also marked an image to help clarify the locations of lateral cilia in the video. These communications confirmed that we did incorrectly report the beat rates of laterofrontal cirri rather than lateral cilia in Specht & Fuchs (2018).

Despite the error in cilia identification, the fundamental result remains unchanged: When observed at a constant temperature, the ciliary beat was unaffected by changes in viscosity. Laterofrontal cirri are not considered part of the water-pumping system (Jørgensen et al. 1986), but they are still subject to viscous drag forces that vary with viscosity. It remains unclear how these isolated gill fragments could be unresponsive to changes in viscosity if ciliary beating was controlled solely by the biomechanical effects of viscosity, as Riisgård & Larsen (2007, 2018) contend. Our observations seem to support neurophysiological studies suggesting that the ciliary beat of hard clams is controlled at least partially within the gill (Gainey et al. 1999, 2003), although the mechanism is unclear.

FEEDING EXPERIMENTS

The main conclusion of Specht & Fuchs (2018) — that hard clam feeding is unaffected by viscosity alone — is primarily supported by the feeding experiments on intact clams. Those experiments provide strong evidence that clearance rates are dependent on temperature but independent of viscosity at constant temperature. The other 'methodological shortcomings' identified by Riisgård & Larsen (2018) are in fact merely aspects of the results and experimental design that differed from previous experiments on *M. edulis*.

Riisgård & Larsen (2018) express doubts about our feeding experiments based on the observation that clearance rates are higher in the 23.5°C treatment of the temperature experiments (Fig. 1A in Specht & Fuchs 2018) than in the 23.5°C control treatments (no PVP added) of the viscosity experiments (Fig. 1B in Specht & Fuchs 2018). The difference is 2.5-fold, not 5-fold as Riisgård & Larsen (2018) state. Riisgård & Larsen (2018) suggest that clearance rates may have been low in viscosity experiments because clams were not open and feeding, but most clams (>80%) had their siphons out simultaneously in all viscosity treatments, just as they did in all temperature treatments ≥12°C (Specht & Fuchs 2018). Riisgård & Larsen (2018) also argue that if we used the higher

clearance rate value from temperature experiments as the 23.5°C control in the viscosity experiments, the data would show the expected increase in clearance rates with decreasing viscosity.

This argument is flawed on 2 counts. First, Riisgård & Larsen (2018) overlook one purpose of an experimental control, which is to determine if responses differ between treated and untreated individuals from the same population or culture. Each of our controls was paired with temperature or viscosity treatments using clams from the same batch, so they were held in the laboratory for the same amount of time and under the same conditions. The controls are not interchangeable because the 2 feeding experiments were done in different months using different batches of clams: viscosity experiments were done in August using clams obtained in August, whereas temperature experiments were done in September and October using clams obtained in August and September. Therefore, it would be highly inappropriate to use the 23.5°C clearance rate data from the temperature experiment as a 23.5°C control for the viscosity experiment. Results may differ at 23.5°C due to seasonal effects, as was observed previously in *M. edulis* (Jørgensen et al. 1990). Second, even if we omit results of the control treatment from the viscosity experiment, there is no significant trend in clearance rate from the PVP treatments (Fig. 1B in Specht & Fuchs 2018), either including or excluding the data at temperature equivalents (T_e) < 12°C ($p = 0.68$ and $p = 0.88$, respectively). The data unequivocally fail to support the expectation of Riisgård & Larsen (2018) that clearance rates of *M. mercenaria* should depend on viscosity.

Riisgård & Larsen (2018) also comment that data from feeding experiments have large scatter (Figs. 1 & 2 in Specht & Fuchs 2018). We attribute this scatter to the robust experimental design, in which all treatments were replicated 6 times, and each treatment and replicate used a different, randomly chosen group of 10 clams. Each panel in those Figs. 1 & 2 represents the individual variation among N = 360 clams in temperature experiments and N = 360 clams in viscosity experiments. Each clam was exposed to only one treatment, so the experiments did not quantify response variability of individuals; instead, they were designed to estimate response variability of the population. Fig. 1A,B (in Specht & Fuchs 2018) shows a highly significant ($p < 0.0001$) trend in clearance rate versus temperature, whereas there is no trend ($p = 1$) in clearance rate versus viscosity. Significance (or lack thereof) is compelling when it persists in the presence of so much individual variation.

It is difficult to compare the variability in clearance rates of *M. mercenaria* from our study with that of *M. edulis* in previous studies, because different study designs were used to quantify responses to temperature and viscosity in *M. edulis*. For example, Riisgård & Larsen (2007) used a single group of intact mussels (N = 6) to test responses to all viscosity treatments, while Kittner & Riisgård (2005) and Jørgensen et al. (1990) used a single group of mussels (N = 6 or 20 to 30, respectively) in each test of mussel response to different temperature acclimation. Trends in individual experiments lack scatter partly because each figure shows the response of the same small group of individuals. Although these repeated-measures experiments can capture response variability of individuals, treatments should optimally be applied in random order to prevent carry-over effects. The mussel experiments used sequential treatments (e.g. steadily increasing viscosity; Riisgård & Larsen 2007) that may have confounded trends associated with treatments. Moreover, these experiments on *M. edulis* were unreplicated and provide no estimate of response variability within the population. This unknown variability becomes particularly relevant when the viscosity responses of *M. edulis* are assumed to be representative not just of the population, but of all bivalves, as Riisgård & Larsen (2018) assert. The clearance results for *M. mercenaria* should be considered more robust, because the feeding experiments of Specht & Fuchs (2018) were done with randomization and replication—2 fundamental elements of experimental design that were missing from previous experiments on *M. edulis*—and provide an estimate of response variability within the population.

Riisgård & Larsen (2018) rightly point out that many organisms change their ciliary beat rates as a biomechanical response to changes in viscosity, causing swimming speeds or feeding rates to vary with temperature (e.g. Larsen & Riisgård 2009, Humphries 2013). However, they overlook counterevidence from other organisms whose ciliary beat rates are unresponsive to viscosity over the small range (~1 to 2×10^{-6} m^2 s^{-1} or ~1 to 2 cP [centipoise]) associated with environmentally relevant temperatures. Vertebrate respiratory cilia are capable of maintaining a constant ciliary beat over a wide range of viscosities (Johnson et al. 1991), as are the cilia of some simpler organisms such as flatworms (Rompolas et al. 2010). In single-celled *Paramecia* spp., doubling the viscosity causes the beat rates of swimming cilia to drop by ~50%, whereas the beat rates of feeding cilia are barely altered (Jung et al. 2014). These

results suggest that even single cells can exert multiple mechanisms of ciliary control. There are several possible explanations for why *M. mercenaria* and *M. edulis* differ in their response to viscosity, including differences in ciliary control mechanisms and in muscular control of the canals between gill filaments (Specht & Fuchs 2018). Regardless of the mechanism, there is convincing evidence that *M. mercenaria* and *M. edulis* do differ in their responses to viscosity, and this difference cannot be denied based only on the expectation that all bivalves should function identically to blue mussels (Riisgård & Larsen 2018). Productive avenues for future research would include determining the underlying source of different responses to viscosity and investigating viscosity responses in other bivalve species, using experiments with appropriate randomization and replication.

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Editorial responsibility: Emily Carrington, Friday Harbor, Washington, USA & Christine Paetzold, Oldendorf/Luhe, Germany

Submitted: April 4, 2018; Accepted: May 9, 2018
Proofs received from author(s): May 16, 2018