Variation among four health indices in natural populations of the estuarine fish, *Fundulus heteroclitus* (Pisces, Cyprinodontidae), from five geographically proximate estuaries

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Synopsis

Variation among four commonly used health indices was examined in the mummichog, *Fundulus heteroclitus*. The four indices were liver glycogen content (LGC), liver-somatic index (LSI), condition index (K) and RNA–DNA ratio. Fish were collected from five coastal locations in southeastern Connecticut. Fish health, as determined by these four indices, varied considerably among estuaries and between sexes. The relationship between each index and specimen length was significantly different among estuaries for either sex. When regressed against length, the slopes for the indices ranged from positive to negative. For each index, significant differences existed among some of the length-centered means at each estuary for either sex. Estuary rank for one index did not necessarily correlate with the estuary rank for another index. The significance of this variability and its impact on the use of the indices as bioindicators of environmental perturbation is discussed.

Introduction

Estuaries are a crucial life history component for many marine fishes. The highly productive estuarine waters serve not only as feeding grounds for many sport fishes, but also as nursery grounds for species that comprise 75 percent of US commercial landings (Chambers 1991). River input to estuaries can contain municipal, agricultural and industrial wastewater, often high in solid waste and pollutants such as heavy metals, fertilizers and pesticides. Environmental monitoring is often used to enforce environmental regulations and protect the health of the estuary, commercially important species and their human consumers.

Fish health assessment is playing an increasing role in both fishery management and environmental monitoring policy. Useful health indicators are capable of detecting responses to synergistic, sublethal stress, which can affect the fitness of an organism with population level consequences. Various indicators cover a wide range of organism responses, and while no single measurement uniquely indicates a stress source, related measurements taken together indicate environment quality and organism health (Buckley 1985, Chung et al. 1993, Brightman et al. 1997).

A problem with use of these indicators is that the normal ranges for a particular fish species must be determined prior to use in a monitoring regime (Carr & Neff 1982). A comparison of the range and relationship of these indices between proximate but separate nonimpacted locations would present the natural range of values. Data obtained in such a study could function as a standard for evaluating other sites. Here, we report a study of normal variation in health indices of the mummichog, *Fundulus heteroclitus*, a species that is often used in pollution assessment (Eisler 1986).

The mummichog has several traits that make it an important species from both the environmental monitoring and ecological viewpoints. Mummichogs are resident in near shore sites that have sediments containing high levels of organic compounds, and are major sites for sorption of hydrophobic pollutants, such as aromatic hydrocarbons (PAHs) and chlorinated hydrocarbons (PCBs) (Beller & Simoneit 1986, Brownawell & Farrington 1985, Eisenreich et al. 1980, Hiraizumi et al. 1979, Karickhoff et al. 1979). As a hardy fish well suited to laboratory studies, the mummichog is the subject of a large body of physiological literature (reviewed in Atz 1986). Ecologically, the mummichog serves as an important link between benthic, detrital and planktonic food chains (Hughes & Sherr 1983) and larger commercially valuable predatory fishes (Valiela et al. 1977, Meredith & Lotrich 1979). The species has a wide geographical range; nonetheless, individuals show a high level of site fidelity (Lotrich 1975) and may be reliable indicators of the environmental quality of sites in which they are collected.

Health indices used in this study are currently used in biological monitoring applications (reviewed in Goede & Barton 1990, Wedemeyer & Yasutake 1977). Each of the indicators is representative of responses to secondary, rather than primary stress. Primary stress responses (hormonal changes) occur within seconds to minutes of the stress onset, and are effected by the stress of capture and handling. Secondary stress responses, such as changes in liver glycogen, stress protein levels, free fatty acids, and RNA/DNA ratios (Mazeaud et al. 1977) occur over a longer period of time, are less effected by capture and handling, and are thus more suited to field study. In addition, each of the indices can be determined relatively rapidly and inexpensively. All of the indicators are used to make estimations of the organism's energy reserves and growth rate. The variability in these indices is examined in several proximate populations of mummichogs from areas thought to be relatively free of pollution impact.

Methods and materials

Study species

Three hundred ninety-eight *Fundulus heteroclitus* were collected on a rising daylight tide using cylindrical wire mesh minnow traps baited with crushed mussels and set for approximately one to two hours. Three traps were placed within 100 meters of each other at each collection site. Collections occurred during the first three weeks in November, time periods were chosen to minimize drastic seasonal temperature swings, and to avoid variability induced by the spawning period, which extends from May to early August. The 'mussel watch' program also examined post-spawning animals to minimize reproduction-induced variability in the bio-indicator species *Mytilus edulis* (NOAA Technical Memorandum[1]). Fish were transferred to buckets and carried back to the laboratory alive. The maximum time spent in the buckets was approximately 1.5 h. In the laboratory, fish were sexed according to coloration, rapidly placed in plastic bags and frozen at −70°C. The maximum time any fish spent out of water before freezing was approximately two minutes. The first 25 males and 25 females from each trap were frozen, providing a maximum of 50 samples per trap, and 150 samples per site. For collections with fewer than 50 fish, the total catch was frozen. Samples were stored at −70°C until needed.

Study location

Fundulus heteroclitus were collected from five coastal locations in Southeastern Connecticut (Figure 1). The collection sites were Birch Pine Creek (BP, 41°20′15″, 72°03′31″, n = 56), Bakers Cove (BB, 41°19′59″, 72°03′20″, n = 50), Mamacoke Island (MM, 41°23′37″, 72°06′07″, n = 102), West Barn Island Marsh (HQ, 41°20′29″, 71°52′30″, n = 82) and East Barn Island Marsh (DM, 41°20′0″, 71°51′30″, n = 108).

In an effort to include data from a wide range of natural marsh habitats fish were collected from sites that appeared to be minimally anthropogenically impacted; there were no point sources of pollutants in the immediate vicinity of the collection sites but each was subject to runoff from surrounding developed and undeveloped land. Despite this similarity, the locations represented diverse environments. Collection sites were estuarine, and so were subjected to a range of salinities (3–36 ppt) and included ditched (HQ, BP, BB, and DM sites), non-ditched (MM site) and recently opened impounded areas (HQ site).
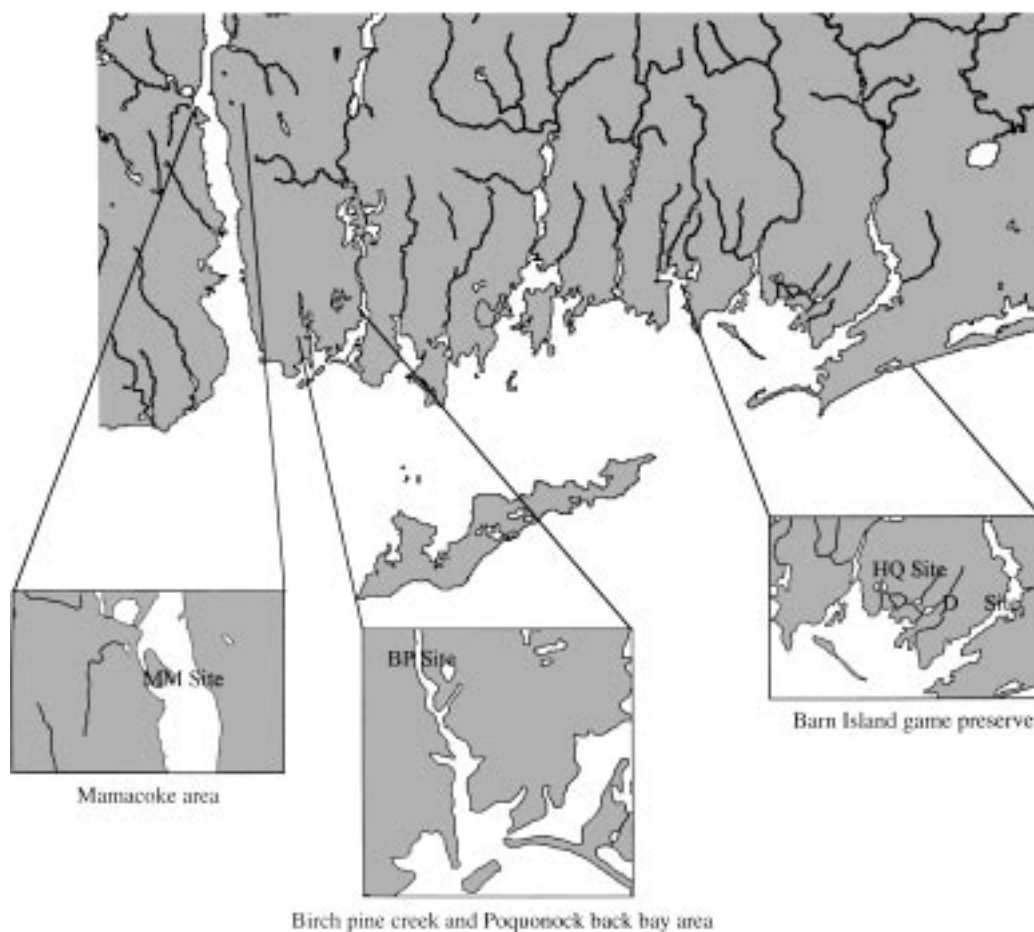
Figure 1. A map of the collection sites in southeastern Connecticut.

Tissue preparation

Fish were removed from the freezer and allowed to thaw. Length and body weight were taken. The body cavity was opened, and the liver removed and weighed. The liver weight recorded for determining the liver-somatic index and the liver was stored frozen at $-70°C$ until the LGC assay was performed. A fillet, weighing approximately 300–400 mg, was cut from the side of the fish for RNA–DNA extraction. The fillet was stored in a labeled tube at $-70°C$ until the nucleic acids were extracted.

Liver glycogen content assay

Liver glycogen content was determined enzymati-cally as described in Carr & Neff (1984). In brief,

livers were thawed and individually homogenized using a glass/glass homogenization in a 1-in-10 dilution (w/v) of ice-cold 100 mM sodium citrate, pH 5.0. The homogenization was performed in an ice bath to limit enzymatic degradation of the glycogen.

The homogenate was boiled at $100°C$ for 5 min to denature existing enzymes, then stored at $-70°C$. When desired, the homogenate was thawed, re-homo-genized, and two 100 ml aliquots were removed. To one aliquot, 2.5 ml of 5% amyloglucosidase was added, no additions were made to the other. Both aliquots were then incubated for 24 h at $37°C$. After incu-bation, the samples were centrifuged at 11000 rpm for 30 min, and the supernatant decanted into sep-arate tubes. The tubes were frozen at $-70°C$ until analyzed.

Calibration standards were used to ensure accuracy and were incubated side by side with the samples. Powdered glycogen (50 mg) was dissolved in 1 ml pH 5.0, 100 mM sodium citrate achieving a final concentration of 500 mg dl^{-1}. A 100 ml aliquot was removed, and the reminder was diluted with the sodium citrate solution to provide a 400, 300, 200 and two 100 mg dl^{-1} aliquots. 2.5 ml of 5% amyloglucosidase was added to all of the aliquots, with the exception of one of the 100 mg dl^{-1} aliquots, which served as a 0 mg dl^{-1} standard. Standards were run through the incubation and centrifugation process with the current batch of homogenates.

Samples were analyzed with a YSI blood glucose analyzer (Yellow Springs, Ohio). The samples and standards were thawed, and the enzymatically treated tubes were diluted threefold. Samples were expressed in units of mg dl^{-1} glucose. The original concentration of glycogen was calculated as per Carr & Neff (1984).

RNA–DNA ratio

RNA and DNA were obtained using the Schmidt-Thannhauser method as modified by Munro & Fleck (1966) and Buckley (1979). The tissue, weighing roughly 100–200 mg, was mechanically glass–glass homogenized in 2.0 ml of ice-cold distilled H$_2$O. A 1.4 ml aliquot of the homogenate was placed in a 15 ml plastic screw top centrifuge tube, to which 0.7 ml of ice-cold 0.6 M perchloric acid (PCA) were added. The resultant mixture was incubated at 0 to 4°C for 15 min, and centrifuged at 6000 ×g for 10 min in a Sorvall RC-5 refrigerated centrifuge. The supernatant was discarded, and the sediment washed twice with ice-cold 0.2 M PCA, to remove free nucleotides and sugars. After each washing, the mixture was centrifuged as before, and the supernatant discarded. After the second wash, the sediment was treated with 1.12 ml of 0.3 M KOH, and incubated at 37°C for 60 min. Fifteen minutes into the incubation, the samples were removed and vortexed to ensure complete tissue digestion. After incubation, the solution was neutralized with 1.0 ml of 1.32 M PCA, the mixture was then placed on ice for 30 min, and re-centrifuged. The supernatant, containing the RNA was decanted into a separate tube. The pellet, containing the DNA and protein, was washed with 0.2 ml of ice-cold PCA as before, and the supernatant discarded. Ice-cold 0.6 M PCA (2.2 ml) was added to the pellet and incubated at 85°C for 15 min. The mixture was then placed on ice for 15 min, and centrifuged.

The supernatant, containing DNA, was decanted into a separate tube.

RNA–DNA ratios were obtained using UV spectroscopy. The RNA samples were zeroed with the supernatant of 0.3 M KOH/1.32 M PCA mixture, after centrifugation to remove the resultant salts. The DNA samples were zeroed with 0.6 M PCA. Samples were read at 260 nm in a Perkin Elmer Lambda 2 spectrophotometer.

Liver-somatic index and condition index

The LSI was determined by dividing the wet weight of the excised liver (in mg) by the total wet weight of the entire fish (in mg). Condition index was obtained by dividing the total wet weight of the fish (in mg) multiplied by 10^5, by the total length (in mm).

Statistical analysis

Statistical analysis was conducted using PC-SAS, consisting of analyses of covariance, with separate analyses for each index and sex (preliminary analysis revealed significant differences between sexes). The predictors in each analysis were estuary, length and the estuary by length interaction as a test for homogeneity of slopes. Following any significant interaction effects, Tukey-Kramer multiple comparison analyses were conducted to test if the estuaries' regression slopes were significantly different. Similarly, Tukey-Kramer analyses were conducted on any sex index datasets with significant estuary effects, to test which of the estuaries' regression intercepts were significantly different. The length of each individual, and all predictors with the exception of liver glycogen content, were log transformed to achieve linearity and eliminate variance–mean relationships. Following transformation the length of each individual was centered (i.e. expressed as the residual from the overall mean length). The intercept of each regression is then the predicted value for the index at the mean length, and is independent of the regression slope.

Results

Three hundred ninety eight individuals, 203 female and 195 male, were captured at five estuaries. The total morphological and physiological data from combined sites and sexes are summarized in Table 1.

Table 1. Total morphological and physiological data for combined sexes, and collection sites.

Parameter	Mean	SD	SE	Minimum	Maximum	Sample n
Length (mm)	62	11	0.53	37	90	398
Body weight (g)	3.1	1.8	0.09	0.59	11	398
Liver weight (g)	0.13	0.1	0.01	0.01	0.53	398
[Glycogen] per body weight (mg g^{-1})	4.5	2.1	0.10	0.19	12	396
Liver-somatic index (LSI)	3.5	1.3	0.07	0.65	7.4	398
Condition index (K)	1.2	0.11	0.01	0.83	1.6	398
RNA–DNA ratio	2.2	0.65	0.03	0.80	5.0	371

Table 2a. Slope of condition index regression on log length for *Fundulus heteroclitus*.

Log liver glycogen					
Site	HQ	MM	DM	BB	BP
Intercept value	−64.2	−23.0	5.92	24.0	48.5
SD from HQ	—	NS	NS	S	S
Log liver weight					
Site	MM	BB	HQ	DM	BP
Intercept value	2.53	2.95	3.07	3.37	3.84
No SD	NS	NS	NS	NS	NS
Log RNA–DNA ratio					
Site	DM	MM	BB	HQ	BP
Intercept value	−0.38	−0.17	0.35	0.35	0.61
No SD	NS	NS	NS	NS	NS
Log body weight					
Site	MM	BB	HQ	DM	BP
Intercept value	3.02	3.03	3.28	3.32	3.43
SD from MM	—	NS	NS	S	S

NS = Difference is not statistically significant.
S = Difference is statistically significant (p < 0.05).

Table 2b. Slope of condition index regression on log length for *Fundulus heteroclitus*.

Log liver glycogen					
Site	MM	HQ	BB	BP	DM
Intercept value	−103.5	−28.2	16.9	26.8	35.5
SD from MM	—	NS	NS	S	S
Log liver weight					
Site	MM	BB	HQ	DM	BP
Intercept value	2.49	3.13	3.34	3.48	3.56
No SD	NS	NS	NS	NS	NS
Log RNA–DNA ratio					
Site	DM	MM	BB	HQ	BP
Intercept value	−0.44	0.22	0.60	0.77	1.11
SD from DM	—	NS	NS	NS	S
Log body weight					
Site	BB	BP	MM	HQ	DM
Intercept value	3.11	3.27	3.29	3.38	3.46
No SD	NS	NS	NS	NS	NS

NS = Difference is not statistically significant.
S = Difference is statistically significant (p < 0.05).

The interactions between log length and index values varied widely depending on estuary, sex and index. General trends were examined by regressing the value for each estuary and sex on log length. Slopes between log liver weight and length, and log body weight and length were positive and uniform for both males and females at every location. The relationships between log liver glycogen and length, and log RNA–DNA ratio and length were more complex for both sexes, with substantial variation among estuaries and sexes. Values for log RNA–DNA and log liver glycogen seem to converge between log lengths of 4.4 and 4.6. This convergence was visible across estuaries and sexes. The relationship between index slope and location varied as well. An ANCOVA with Tukey-Kramer post hoc testing revealed significant differences in slope in some, but not all instances (Tables 2a, b). Interactions between the value of the condition index at mean length and estuary were significant in many, but not all cases

(Tables 3a, b). Condition index values were length centered so the Y intercept of the regression became the value at mean length, and analyzed with ANCOVA with a Tukey-Kramer post-hoc test.

Discussion

A great deal of attention has recently been placed on the use of secondary biomarkers or bioindicators as predictors of estuarine finfish health (Liao et al. 1995, Plisetskaya et al. 1994, Johansen et al. 1994). Before these bioindicators can be routinely used to assess organism health, the natural variability among populations from non-polluted sites must be assessed.

Data analysis revealed highly variable relationships between the four-health indices and log length for both sexes at each location. Significant differences between condition index values at mean length (Y intercept)

Table 3a. Y intercepts of condition index regression on log length for *Fundulus heteroclitus*.

Log liver glycogen

Site	BP	BB	MM	DM	HQ
Intercept value	93	100	116	119	138
SD from BP	—	NS	S	S	S
SD from BB	—	—	NS	NS	S
SD from MM	—	—	—	NS	S
SD from DM	—	—	—	—	S

Log liver weight

Site	BB	BP	DM	MM	HQ
Intercept value	−2.74	−2.65	−2.35	−2.34	−2.09
SD from BB	—	NS	S	S	S
SD from BP	—	—	S	S	S
SD from DM	—	—	—	S	S
SD from MM	—	—	—	—	S

Log RNA–DNA ratio

Site	HQ	BP	BB	MM	DM
Intercept value	0.638	0.641	0.666	0.784	0.996
SD from HQ	—	NS	NS	NS	S
SD from BP	—	—	NS	NS	S
SD from BB	—	—	—	NS	S
SD from MM	—	—	—	—	S

Log body weight

Site	BP	BB	DM	MM	HQ
Intercept value	0.78	0.80	0.84	0.89	0.90
SD from BP	—	NS	NS	S	S
SD from BB	—	—	NS	S	S
SD from DM	—	—	—	NS	S
SD from MM	—	—	—	—	NS

NS = Difference is not statistically significant. S = Difference is statistically significant ($p < 0.05$).

Table 3b. Y intercepts of condition index regression on log length for *Fundulus heteroclitus*.

Log liver glycogen

Site	MM	BB	BP	DM	HQ
Intercept value	98	100	102	118	121
SD from MM	—	NS	NS	S	S
SD from BB	—	—	NS	S	S
SD from BP	—	—	—	S	S
SD from DM	—	—	—	—	NS

Log liver weight

Site	BB	BP	MM	DM	HQ
Intercept value	−2.49	−2.28	−2.20	−2.18	−2.00
SD from BB	—	S	S	S	S
SD from BP	—	—	NS	NS	S
SD from MM	—	—	—	NS	S
SD from DM	—	—	—	—	S

Log RNA–DNA ratio

Site	HQ	BB	BP	MM	DM
Intercept value	0.588	0.706	0.718	0.765	0.898
SD from HQ	—	NS	NS	NS	S
SD from BB	—	—	NS	NS	S
SD from BP	—	—	—	NS	S
SD from MM	—	—	—	—	NS

Log body weight

Site	BB	DM	BP	HQ	MM
Intercept value	1.06	1.08	1.10	1.13	1.17
SD from BB	—	NS	NS	S	S
SD from DM	—	—	NS	NS	S
SD from BP	—	—	—	NS	S
SD from HQ	—	—	—	—	NS

NS = Difference is not statistically significant. S = Difference is statistically significant ($p < 0.05$).

were present for each index, but not between all estuaries. Although not statistically correlated, the relative rank of the estuaries for a given index for one sex closely resembles the rank for the other sex. It is important to note that estuary rank for one index does not appear to be a good indicator of estuary rank for any other index.

The variability of index means may be due to the fact that no two estuaries are identical. Habitat variations may result in plentiful food and lower stress in one location, but reduced food and higher stress in another (Liao et al. 1995). Differences in habitat such as these could account for differences in index means between estuaries. These results contrast the study conducted by Balk et al. (1996) who recorded baseline biomarkers including liver-somatic index and condition factor in female perch, *Perca fluviatilis*. Balk et al. (op. cit.) found that the biomarkers, with the exception of leukocyte composition, were stable both from station to station and year to year. However, when compared to the perch's habitat, *Fundulus heteroclitus*' shallow water habitat is characterized by rapid swings in temperature, dissolved oxygen and salinity; this dynamic environment may impart similar variability to the health index values.

The differences in the index slope between estuaries are not simply a question of degree, but slope direction (positive or negative). Slope differences are either the result of an actual individual health change with increasing length from estuary to estuary, or a difference in resource partitioning (Jurss et al. 1987). The fish from some estuaries increase in index value as they grow larger (positive slope), whereas fish from other estuaries decrease in index value as they grow (negative slope). This would seem to indicate that the index value changes with size, but whether it increases

or decreases with size depends on the estuary. This could be due to any number of factors as environmental considerations could influence different sizes of fish differently. For example, mummichogs have been shown to exhibit size-specific foraging patterns; in general, larger mummichogs are more omnivorous, eating more algae and detritus, while smaller mummichogs are more carnivorous (Kneib & Stiven 1978). In this case, the relative abundance of these size-specific food sources in each estuary could influence size classes differently. Grecay & Targett (1996) found that condition, gut fullness and dietary composition vary spatially in juvenile weakfish, *Cynoscion regalis*, which they suggested may result from different feeding efficiencies due to water clarity. Size also influences the manner in which the fish navigate the marsh. Butner & Brattstrom (1960) reported that larger mummichogs move up the center of tidal creeks, while smaller mummichogs stay close to the edges and banks. When the tide begins to ebb, smaller fish are more likely to spend the tidal cycle in tidal pools on the marsh surface, while larger fish tend to retreat with the ebbing tide to ditches and creeks. As a result, differences in quality and amount of high marsh available for exploitation could influence relative health.

Additionally, although anthropogenic impacts are assumed to be a minor consideration at these sites, pollutants have been known to have wide ranging effects depending upon size and age. Some pollutants, such as genotoxic and mutagenic substances have the greatest effect while organisms are young and rapidly growing. Other bio-accumulated pollutants such as PCBs and PAHs might have the greatest impact on older individuals, as a longer life would lead to more accumulation of the toxin. Thus it is possible, although unlikely, that non-point source pollutant runoff may be directly influencing fish health in these estuaries.

However, it is also possible to interpret these data as the result of different resource partitioning. It is possible that negative slopes in some estuaries are caused by individuals forgoing resource storage (in terms of liver glycogen, etc.) in favor of additional somatic growth. Large individuals may have lower indices because they have sacrificed resource to achieve the larger size. Smaller individuals may have allocated more resources for energy storage, and less toward growth. Any number of explanations could account for these diverse styles, such as the relative prevalence of size specific predation, size specific competition, etc.

Another possible basis for the difference in health indices is infectious agents (viral, bacterial, fungal and parasitic infections) which have been shown to affect health indices in fish (Goede & Barton 1990). An empirical autopsy-based system of organ and tissue indices can be used to assess the impact of disease(s) on natural fish populations. Though such an analysis was not carried out on these fish, the fish did not have any obvious external or internal indications of infections.

This study indicates that wide variations in health indices exist between geographically proximate estuaries, not only in mean index values, but also in certain indices' relationship with length. Regardless of whether these variations are caused by variable microhabitats or differences in resource partitioning, these results council for detailed baseline research and cautious interpretation of health index studies. Furthermore, it is likely that for inherently variable species, such as *Fundulus heteroclitus*, health index comparisons may be meaningless outside the given population.

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