

## Blood Biochemistry of the Oyster Toadfish

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**Abstract.**—Blood biochemistry parameters were examined in the oyster toadfish *Opsanus tau* from the late spring through early fall for two consecutive seasons to establish baseline values and evaluate whether any of the parameters could be used as predictors of disease and mortality in this important experimental organism. The blood plasma was analyzed for alkaline phosphatase, gamma-glutamyl transpeptidase, glutamic oxalacetic transaminase, glutamic pyruvic transaminase, blood urea nitrogen (BUN), calcium, cholesterol, triglycerides, uric acid, creatine, bilirubin, total protein, and glucose. Elevated levels of BUN ( $28.5 \pm 4.1$  mg/dL) and depressed levels of cholesterol ( $83.0 \pm 6.3$  mg/dL) were strongly correlated with disease and subsequent death in the oyster toadfish population and thus may serve as useful indices by which to exclude fish from experimental studies.

The oyster toadfish *Opsanus tau* has been the focus of scientific research for over a century. Its development was first outlined by Ryder (1886), with further descriptions of its life history contributed by Gill (1907), Gudger (1910), and Dovel (1959). The discovery that the fish uses acoustical communication for mate attraction initiated a number of studies addressing the physical characteristics of the sound (Tavolga 1958), reproductive ecology (Gray and Winn 1961; Fish 1972; Winn 1972; Fine 1978), and anatomy of the toadfish acoustical system (Galeo et al. 1987; Edds-Walton and Popper 1995; Edds-Walton 1998a,b; Edds-Walton et al. 1999). Its readily accessible vestibular end organs have produced a number of anatomical (Highstein et al. 1992; Mensinger et al. 1997) and neurophysiological studies (Highstein

et al. 1996; Boyle et al. 2001; Rabbitt et al. 2001) that have furthered our understanding of the inner ear. The fish has also served as a model for nerve regeneration (Mensinger et al. 2000).

The popularity of the fish for scientific study can be traced to its historical abundance and robustness. However, in recent summers there has been a shortage of research animals due to a population decline in the Cape Cod area (Mensinger et al. 2003). Additionally, captive oyster toadfish have been decimated by numerous diseases such as bacterial pericarditis (Wenuganen et al. 1997; Smolowitz and Bullis 1997) and infections of *Pseudomonas putida* (Smolowitz et al. 1998), and *Edwardsiella* spp. (Baird et al. 2003), which have resulted in wide variation in fish health. Except for advanced terminal stages, the outward appearance of both moribund and healthy animals has been similar. Clinical changes in integument, feeding, swimming or social behavior, which have been used routinely to identify diseased teleosts, have remained elusive in the sedentary oyster toadfish. The lack of a clinical screen has resulted in the loss of time and animals because infection or disease was not revealed until invasive surgery, at which point the animal must be sacrificed. An accurate screening protocol would reduce the number of fish needed for research and subsequently reduce pressure on natural stocks by requiring fewer fish to be captured.

A mariculture program has been initiated at the Marine Biological Laboratory (MBL) to reduce reliance on wild stocks (Mensinger et al. 2003). However, the requirements for healthy maintenance of captive populations continue to be formulated. Seasonal changes in blood chemistry have been documented in the Lusitanian toadfish *Halobatrachus didactylus* (Rosety et al. 1992; Mu-

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ñoz-Cueto et al. 1996), and a series of extensive studies has delineated urogenesis in the congener Gulf toadfish *O. beta* (review in Walsh 1997). However, baseline blood values are not available for the oyster toadfish. Oyster toadfish blood samples were collected during two consecutive summers to establish baseline blood biochemistry and to screen for biochemical markers that would assist in identifying diseased animals. Initial blood concentrations were reported in a preliminary investigation (O'Neill et al. 1998).

### Methods

**Fish collection.**—The MBL has historically obtained oyster toadfish as a bycatch from the commercial eel fishery. Adult oyster toadfish were captured in eel traps or tin cans within Waquoit Bay, Massachusetts, and transported at irregular intervals during the late spring and early summer to the MBL. Although the water parameters were not measured by the commercial fishers, the water conditions near the collection sites in Waquoit Bay ranged from 15 to 22.4°C, 25.2‰ to 32.1‰ salinity, and 4.7 to 8.2 mg/L dissolved oxygen during the 1997 and 1998 collection period (Waquoit Bay National Estuarine Research Reserve, Hamblin Pond, unpublished water quality data). The fish were maintained by commercial fishers up to a week prior to transport. Based on their arrival date at the MBL, oyster toadfish were segregated into large fiberglass aquaria (2 m × 3 m, ~2,000 L), which were maintained at 15°C with flow-through seawater. Fish were fed fresh squid approximately three times per week.

**Sampling.**—Fish were acclimated for 1 week at the MBL prior to the experiments. Fish were chosen based on the selection criteria for neurophysiological experiments that historically have yielded the best subjects for both acute and chronic neurophysiological recording. Individual fish that were positioned off the substrate on extended pelvic fins and were exhibiting regular opercular movements (6–10 per min) were selected. These fish were individually netted by hand and examined externally for integument necrosis, infection, and parasites (primarily *Argulus* spp.). Fish that failed the external exam, as well as gravid females, were excluded from the study.

Selected fish were placed immediately in a 0.015% solution of MS-222 (tricaine methanesulfonate; Sigma, St. Louis, Missouri). Once the fish lost equilibrium, a 25-gauge hypodermic needle was inserted approximately halfway between the anus and the caudal fin origin, into the caudal

blood vessels but ventral to the spinal cord, and 1–3 mL of mixed arteriovenous blood was withdrawn into a syringe containing 0.1 mL of heparin (1,000 U.S. Pharmaceutical units/mL). The time from netting until blood sampling averaged less than 5 min. Each fish was then weighed, measured, and sexed. An identification tag (Avid Biomark) was inserted directly into the dorsal musculature anterior to the first dorsal spine. The fish was placed into a recovery aquarium, monitored until gill ventilation and equilibrium was restored (usually within 10 min), and then placed into the experimental aquaria. All animal protocols conformed to National Institutes of Health and MBL guidelines.

The experiments were performed during two consecutive summers (1997 and 1998) on recently captured fish. Each year, 20 fish were evenly divided between two 1,600-L fiberglass tanks and maintained in ambient (range 15–22°C), flow-through seawater with an initial density of 2 fish/m<sup>2</sup>. The fish were fed pieces of squid or live mummichog *Fundulus heteroclitus* three times per week. The tanks were maintained on the ambient light cycle of the region by means of overhead fluorescent lights and indirect sunlight. The conditions were identical for both years except that in 1998, when a fish expired, it was replaced by a similar-size fish from the same collection period to maintain density. The standard length of the oyster toadfish averaged 29.4 ± 0.3 cm (SE) and 27.7 ± 4.0 cm for 1997 and 1998, respectively. The average wet weight was 811 ± 29.3 g and 650 ± 25.1 g for 1997 and 1998, respectively.

Blood samples were taken between 1000 and 1300 hours from all experimental fish on the same day. In 1997, blood samples were taken from each fish on three dates: 3 July, 4 August, and 7 September. In 1998, blood was drawn from each fish five times: 20 June, 10 and 31 July, 28 August, and 25 September.

**Blood analysis.**—Each blood sample was transferred to microcentrifuge tubes and centrifuged (Eppendorf Centrifuge 5402) at 8,000 × gravity for 10 min at 4°C. In the event that the supernatant was contaminated by lysed blood cells, the sample was discarded. In 1997, all blood samples were analyzed using a Dade Analyst bench-top chemistry system. The blood supernatant was pipetted in 10 or 80 µL aliquots to a sample rotor. The blood was analyzed for the following components (acronym, if applicable, and instrument range in parentheses): alkaline phosphatase (10–500 units [U] per L), gamma-glutamyl transpeptidase (GGT;

5–500 U/L), glutamic oxalacetic transaminase (GOT; 5–500 U/L), glutamic pyruvic transaminase (GPT; 5–500 U/L), blood urea nitrogen (BUN; 2–60 mg/dL), calcium (5–15 mg/dL), cholesterol (50–500 mg/dL), triglyceride (10–500 mg/dL), uric acid (1–15 mg/dL), creatine (0.2–15 mg/dL), bilirubin (0.1–6.0 mg/dL), and total protein (2–12 g/dL).

In 1998, commercial blood tests kits (Sigma Diagnostics, St. Louis, Missouri) were used to measure all 1998 blood samples; the Dade Analyst was used to test only the second series of blood samples, which were collected in early July. The 1998 samples were analyzed for BUN (0–75 mg/dL), cholesterol (0–600 mg/dL), and glucose (1–750 mg/dL) with the blood test kits in conjunction with a DU-460 Beckman spectrophotometer.

**Data analysis.**—Only blood samples from the original cohort of oyster toadfish that survived all sampling procedures were used to calculate average blood concentrations. The last blood sample from each nonsurviving fish was classified as the terminal sample. Preceding samples from these fish, if any, were categorized as compromised samples.

All statistical analysis was performed using version 3.05 of GraphPad InStat (GraphPad Software, San Diego, California). A one-way analysis of variance (ANOVA) was performed for each blood parameter based on data from surviving fish to determine if there was temporal variation during the course of the study. If there was no significant temporal variation, the results were averaged to obtain the mean blood concentration for the experimental period. To test for differences between the two tanks each year, *t*-tests were used; *t*-tests were used as well as to compare the two analytical methods used for the second 1998 sampling (BUN and cholesterol). Linear regressions were calculated to determine if there was correlation between blood chemistry and fish wet weight. All data represent the mean plus or minus one standard error.

## Results

There was no significant difference (unpaired *t*-test;  $P > 0.60$ ) between any of the blood parameters in the two aquaria in either year, and therefore the surviving fish for each year were treated as a single group. There was no correlation ( $r^2 < 0.10$ ) between fish size and any of the blood parameters as illustrated in Figure 1 for the BUN and cholesterol tests. There were also no significant differences (unpaired *t*-test;  $P > 0.60$ ) in either BUN or cholesterol concentrations for July 1998 sam-

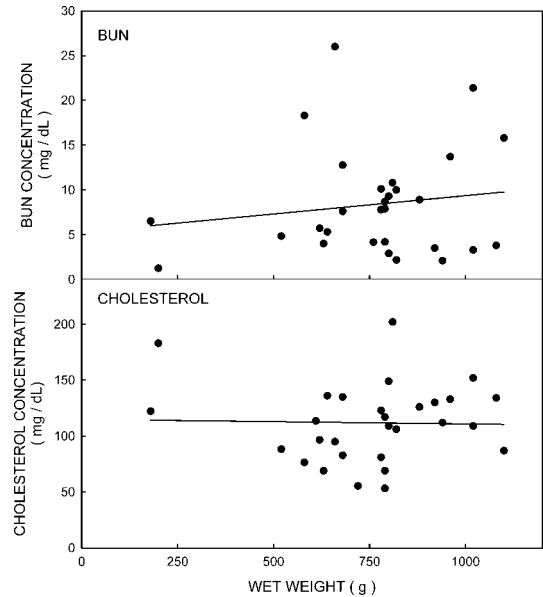


FIGURE 1.—Blood urea nitrogen (BUN) and cholesterol concentrations versus oyster toadfish wet weight. Each point corresponds to the blood taken during the initial sampling of all fish that survived the entire sampling period; 1997 and 1998 data are combined. The solid lines are linear regressions through the data and correspond to the following equations:  $BUN = 0.004 \cdot \text{wet weight} + 5.26$ ,  $r^2 = 0.02$ ;  $\text{cholesterol} = -0.004 \cdot \text{wet weight} + 115$ ,  $r^2 = 0.0006$ .

ples between the Dade Analyst and the commercial blood test kit used in conjunction with the spectrophotometer.

Table 1 summarizes the 1997 blood biochemistry from the Dade Analyst and Table 2 the 1998 data from the commercial blood kits. There was no difference (ANOVA;  $P > 0.05$ ) in the blood variables throughout the summer months except for a decrease in calcium (ANOVA;  $P < 0.01$ ) toward the end of 1997. The tables display mean values of surviving fish throughout the sampling period (except for calcium in 1997) and the mean of the terminal samples from the nonsurviving fish. Sixty percent of the original sample of toadfish ( $N = 20$ ) survived the study each year.

Based on combined data from 1997 and 1998, the terminal BUN concentrations ( $28.5 \pm 4.1$  mg/dL) were elevated significantly (ANOVA;  $P < 0.001$ ) compared with concentrations in surviving fish ( $7.7 \pm 0.6$  mg/dL; Figure 2A). All fish with BUN concentrations in excess of 40 mg/dL died. Cholesterol levels were significantly lower (ANOVA;  $P < 0.001$ ) in compromised ( $88.2 \pm 8.4$  mg/dL) and terminal samples ( $83.0 \pm 6.3$  mg/

TABLE 1.—Oyster toadfish blood biochemistry based on Dade Analyt data for three sampling dates in 1997. The means  $\pm$  SEs are shown, with the ranges in parentheses. Samples from nonsurviving fish are the last blood samples taken from fish that died. Previous samples from these fish are not included in the table. Abbreviations are units (U), gamma-glutamyl transpeptidase (GGT), glutamic oxalacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), and blood urea nitrogen (BUN). Significantly different means based on unpaired *t*-test ( $P < 0.01$ ) are denoted by asterisks.

Parameter (unit)	Surviving fish ( $N = 12$ )	Nonsurviving fish ( $N = 8$ )
Alkaline phosphatase (U/L)	123.2 $\pm$ 9.7 (45–215)	167.4 $\pm$ 27.1 (91–263)
GGT (U/L)	6.6 $\pm$ 0.5 (5.0–9.0)	7.4 $\pm$ 1.3 (5.0–11.0)
GOT (U/L)	41.1 $\pm$ 5.2 (22–84)	73 $\pm$ 4.7 (13–55)
GPT (U/L)	19.1 $\pm$ 1.8 (12–29)	17.2 $\pm$ 1.5 (13–22)
BUN (mg/dL)	5.8 $\pm$ 0.5* (3.5–8.1)	16.1 $\pm$ 2.7* (6.7–27.8)
Calcium (mg/dL)	9.2 $\pm$ 0.2 <sup>a</sup> (8.4–10.1)	9.2 $\pm$ 0.6 (6.4–13.3)
Cholesterol (mg/dL)	131.3 $\pm$ 8.1* (84–219)	91.0 $\pm$ 5.4* (69–120)
Triglyceride (mg/dL)	49.1 $\pm$ 4.8 (21–76)	39.0 $\pm$ 5.7 (16–71)
Uric acid (mg/dL)	1.3 $\pm$ 0.03 (1.1–1.4)	1.3 $\pm$ 0.06 (1.2–1.6)
Creatine (mg/dL)	0.21 $\pm$ 0.01 (0.2–0.25)	0.21 $\pm$ 0.01 (0.2–0.3)
Bilirubin (mg/dL)	0.15 $\pm$ 0.01 (0.1–0.2)	0.17 $\pm$ 0.02 (0.1–0.2)
Total protein (g/dL)	4.9 $\pm$ 0.5 (4.0–5.4)	4.4 $\pm$ 0.3 (3.1–5.3)

<sup>a</sup> Mean of July and August only.

dL) compared with levels in the surviving fish (124.6  $\pm$  4.3 mg/dL; Figure 2B). Cholesterol levels below 50 mg/dL had lethal consequences.

### Discussion

Previous studies have reported great variation in fish blood chemistry (Stoskopf 1993). To reduce intraspecific variability, collection dates and site-specific data (e.g., water temperature, water chemistry, and depth) could allow correlation of blood chemistry with environmental conditions. However, the current collection method is a bycatch of the commercial eel industry, and commercial fishers transport the animals whenever sufficient numbers warrant. As this method may continue into the foreseeable future, any clinical prescreening must factor this inherent variability. However, to minimize transport stress and standardize captive conditions, fish were acclimated in large holding tanks for 1 week prior to experimentation. This was sufficient time for the Gulf toadfish to return to baseline levels of cortisol and other stress indicators (Wood et al. 2003).

The Dade Analyt was used in 1997 to analyze all the blood samples because it allowed rapid screening for a wide variety of compounds. How-

ever, the Dade Analyt was designed for human serum, and its sensitivity was incompatible with some teleost parameters (e.g., glucose). The 1997 results revealed only two variables (BUN and cholesterol) with potential as clinical markers. Commercial blood test kits replaced the Dade Analyt in 1998 as the kits were more economical and allowed testing for glucose, which has been implicated in conjunction with cortisol as a marker of both acute and chronic stress in teleosts (Iwama et al. 1997). To ensure that the procedural change did not affect analysis, blood from a single sampling period in 1998 was analyzed with both the Dade Analyt and blood test kits, and no difference was found for BUN and cholesterol between the two techniques.

In studies of freshly caught Gulf toadfish there was very little annual variation in a number of blood parameters; in these studies only blood calcium was seen to have a peak during reproductive season in females (presumably as an indicator of vitellogenin synthesis; Hopkins et al. 1997). The same held true with the surviving oyster toadfish: all of the variables except calcium showed no significant temporal variation through the two (1997) or three (1998) month sampling periods.

TABLE 2.—Oyster toadfish blood biochemistry based on commercial blood kit data for five sampling dates in 1998. The means  $\pm$  SEs are shown, with the ranges in parentheses. See Table 1 for a full explanation of samples and abbreviations. Significantly different means based on, unpaired *t*-test ( $P < 0.01$ ) are denoted by asterisks.

Parameter (unit)	Surviving fish ( $N = 12$ )	Nonsurviving fish ( $N = 8$ )
BUN (mg/dL)	12.0 $\pm$ 2.3* (1.3–29.2)	37.4 $\pm$ 8.7* (4.6–89.9)
Cholesterol (mg/dL)	124.0 $\pm$ 10.9* (87–183)	82.3 $\pm$ 10.9* (47–146)
Glucose (mg/dL)	25.1 $\pm$ 1.1 (19.0–33.7)	24.2 $\pm$ 4.4 (6.1–49.7)

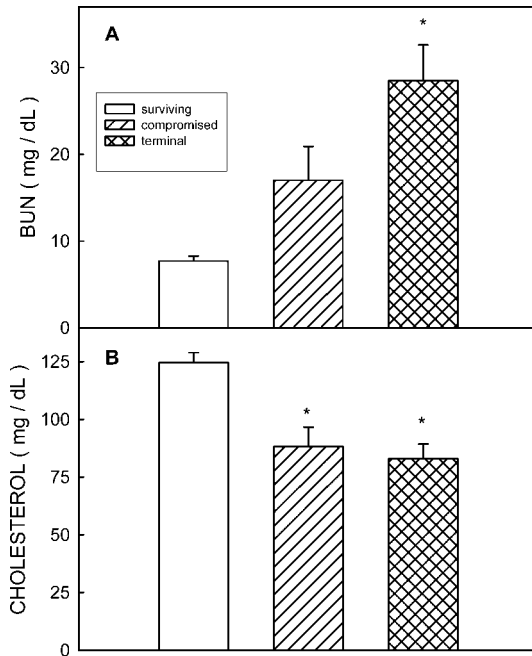


FIGURE 2.—Histograms of the mean concentration of (A) blood urea nitrogen (BUN) and (B) cholesterol for surviving, compromised, and terminal oyster toadfish from 1997 and 1998 data. The terminal sample is the last sample taken before the fish expired; the compromised sample includes any previous samples from terminal fish. Asterisks indicate significantly different means (ANOVA:  $P < 0.001$  in panel [A];  $P < 0.01$  in panel [B]). Error bars equal 1 SE.

The study was conducted during two seasons when the captive adult oyster toadfish at the MBL were suffering from infections of epidemic proportions with mortality rates of 50–100% in many tanks. Most of the deaths in both the experimental and general captive populations were attributed to bacterial pericarditis (Smolowitz and Bullis 1997) or *Pseudomonas putida* infections (Smolowitz et al. 1998). Although it is uncertain whether these conditions are present in freshly caught fish or are a byproduct of captivity, certainly the stress of capture and high-density holding conditions at the MBL exacerbated the disease. The mortality experienced in the experimental population (approximately 40%) remained less than that in the general MBL oyster toadfish population, indicating that probably neither handling nor sampling the fish was a main contributor to the deaths.

The percentage of diseased fish raised the possibility of contamination of the baseline data. To prevent potentially diseased fish from affecting these values, data were excluded from any fish that

expired either during the course of the study or during an extended observation of the experimental fish into the early winter. All fatalities were recorded within 45 d of initial sampling. Additionally, other than calcium, there was no temporal variation in blood parameters in the surviving fish, indicating that they were probably not succumbing or recovering from disease. Therefore, we are confident that any sick or diseased animals were excluded from the baseline values. Thus the blood concentrations should be considered average values for the oyster toadfish for the summer season and could be used to screen future fish.

Urea is present in all fish, the liver being the primary organ of production and the gills appearing to be the main organ of excretion (Walsh et al. 2003). Therefore an elevated BUN is probably not indicative of renal disease as it might be in humans but is more likely associated with gill or liver disease. Diminished liver function would likely result in a decrease of urea production, as these pathways are energetically expensive. However, increasing urea content in the plasma is likely an indicator of failing gill osmoregulatory capability (as a marine species, the oyster toadfish would be expected to be “dehydrated” by the higher osmolarity of the surrounding seawater). Osmoregulatory failure has often been demonstrated to be an important contributor to death in fish (e.g., Wood et al. 2003). In contrast, a declining BUN may indicate liver disease or starvation. A low of 10 mg/dL is common in healthy freshwater fish and a low of approximately 5 mg/dL in marine fishes (Stoskopf 1993). In both 1997 and 1998, diseased fish showed significantly elevated BUN levels prior to dying, likely due to osmoregulatory failure. Given the likely link to osmoregulatory stress, in future studies a simpler indicator of disease worth exploring might be plasma osmolarity.

Marine fish tend to have higher cholesterol levels than other vertebrates (Larsson and Fänge, 1977). As cholesterol is an important component of cell membranes and functions as a precursor for the synthesis of sexual hormones, a number of fish species have seasonal variation in cholesterol with decreased levels reported during spawning in brown trout *Salmo trutta* (McCartney 1966), walking catfish *Clarias batrachus* (Tandon and Chandra 1976), and the female Lusitanian toadfish *Halobatrachus didactylus* (Muñoz-Cueto et al. 1996). Although many factors have been implicated in raising cholesterol levels (review Larsson and Fänge, 1977), few studies have documented decreased concentrations outside spawning season.

However, the common carp *Cyprinus carpio* did show depressed levels of cholesterol following *Aeromonas hydrophila* infection (Hari Krishnan et al. 2003) and during food restriction (Shimeno et al. 1997). Cholesterol levels can indicate disorders of lipid and lipoprotein metabolism and especially liver disease. Necropsies showed that fish suffering from bacterial pericarditis had severe edema in the liver and atrophy of the hepatic cords. Fish infected with *Pseudomonas putida* had widespread systemic infection (Smolowitz and Bullis 1997; Smolowitz et al. 1998), which could have compromised liver function and contributed to the decreased cholesterol levels.

In contrast with those of commercial aquacultural operations, the needs of the MBL investigators are modest. Although during past summers several thousand oyster toadfish were needed for research, the current demand is approximately 500 adult fish per year. This number could be reduced further by accurate screening that would remove compromised fish prior to surgery. As natural stocks appear to be declining, it is important to optimize the captive population and subsequently reduce the number needed to be collected for investigators. An oyster toadfish mariculture project has been initiated at the MBL to lessen the pressure on wild stocks.

In summary, we have established the summer baseline blood biochemistry for oyster toadfish. Elevated BUN levels, depressed cholesterol concentrations, or a combination of both were correlated with disease and death in oyster toadfish. This information will be valuable to screen future classes of oyster toadfish and possibly other benthic marine species.

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