Title: Temperature acclimation (tempering) of hard clams *Mercenaria mercenaria* does not alter levels of *Vibrio vulnificus*

Authors: Anita C. Wright[1]*, Ana Quevedo[1], Leslie Sturmer[2], and Steve Otwell[1]

Key Words: *Vibrio vulnificus*, hard clams, aquaculture, tempering

[1]University of Florida, Food Science and Human Nutrition Department, P.O. Box 110370 Gainesville, FL 32611 U.S.A.

[2]University of Florida, IFAS Shellfish Aquaculture Extension Program, P.O. Box 89, Cedar Key, FL 32625

*Corresponding author
Mailing address: University of Florida, Department of Food Science and Human Nutrition, P.O. Box 110370, Gainesville, FL 32611 U.S.A.
Phone: 352-392-1991 x311
E-mail: acwright@mail.ifas.ufl.edu

Abstract

Gradual step-wise temperature reduction or “tempering” has been applied to the processing of aquacultured hard clams (*Mercenaria mercenaria*) in order to reduce mortalities associated with cold temperature shock. Regulatory guidelines in Florida [Department of Agriculture and Consumer Services’ Comprehensive Shellfish Control Code, Chapter 5L-1.008(5), Florida Administrative Code originally required refrigeration of live clams at temperatures below 45ºF (7.2ºC) within 10 hours post harvest during months of June, July, August and September. However, recent changes in the state guidelines (Chapter 5L-1.013(3)(b), F.A.C.) allowed initial storage for 6 hours at ambient temperature, followed by 10 hours at 68ºF (20ºC) with subsequent refrigeration at 45ºF. Although this process increased post harvest survival in prior research, the microbial consequences in relationship to the human pathogen, *Vibrio vulnificus,* were not determined. This bacterium is the leading cause of mortalities associated with molluscan shellfish consumption, and thrives in warm coastal waters of the Gulf of Mexico. Vectors of disease primarily involve raw oysters, but occasional cases have been associated with clams. In order to determine that tempering protocols would not increase infectious disease risks, numbers of *V. vulnificus* in clams were examined before, during, and after tempering protocols. *V. vulnificus* levels ranged from <10 to 630 CFU/g immediately after harvest and did not change significantly during or after processing. Thus, these results demonstrated that tempering of live hard clam shellstock prior to refrigeration does not increase consumer risk of *V. vulnificus* exposure.

Introduction

Aquaculture of the hard clam, *Mercenaria mercenaria*, is a rapidly growing industry in Florida and has placed the state in the forefront of clam production nationwide. Production increased dramatically in the last decade as a result of successful job retraining programs for displaced workers in the commercial fishing industry and a progressive shellfish aquaculture leasing program. By 1999, Florida Agricultural Statistics Service reported over 134 million clams sold in the state at a value of $15.9 million, representing a fourteen-fold increase over survey results in 1991 (FASS, 2000). Currently, Florida is one of the largest producers of farm-raised clams in the U.S. with a total impact to the state's economy reported at $55 million in 1999 (Philippakos et al., 2001).

In order to reduce stress and mortality on shellstock, processing protocols have been introduced recently that use gradual temperature reduction or "tempering" of clams prior to refrigerated storage. Elevated water temperatures at harvest may cause clams to experience "cooling shock" upon refrigeration, resulting in physiological stress as a consequence of rapid changes in large thermal gradients (Hochachka, P.W. and G.N. Somero, 1984). The stress varies with the extent and rate of temperature change, and routine harvest practices may further exacerbate stress and increase mortalities. Tempering protocols allow clams to acclimate to the customary storage temperatures and decrease clam mortality, particularly during summer months. Previous work showed that immediate placement of wild clams (*M. campechiensis*) directly from harvest into refrigeration temperatures at or below 45ºF significantly decreased survival of shellstock (Menzel and Sims, 1962; Menzel, 1972; Menzel et al. 1975; and Otwell, et al. 1986). Several methods were effective in increasing survival, including dry storage and wet tempering in recirculated seawater (Otwell, et al. 1986). Similar results were reported for cultured hard clams, and survival of clams in refrigerated storage was influenced by the rate of cooling (Applewhite, et al. 1996). In the dry tempering regime, clams were stored 6 hours at ambient temperature, followed by 10 hours in an air-conditioned processing plant at 68ºF (20ºC), and 8 hours of refrigeration 45ºF (7.2ºC). Recent changes in the Florida shellfish regulatory guidelines (Chapter 5L-1.013(3)(b), Florida Administrative Code) have implemented this dry tempering practice.

The leading cause of fatal human infection in the U.S. as a result of seafood consumption is *Vibrio vulnificus* (Reviewed in Strom and Paranjpye, 2001). Although disease results almost exclusively from consumption of raw oysters and not clams, public health concerns for this pathogen have dictated guidelines for safe handling and storage practices of all molluscan shellfish. *Vibrio vulnificus* is indigenous to warm coastal waters approved for shellfish harvesting and recreational activity (Tamplin et al., 1982; Oliver et al., 1983; Wright et al 1996). Prolonged storage of oysters at temperatures above customary refrigeration can elevate levels of *Vibrio* spp. post-harvest (Cook and Ruple, 1989; Kasper & Tamplin, 1993), and lowering the storage temperature reduced growth of this bacterium (Cook & Ruple, 1989; Kaysner et al. 1989; and Cook and Ruple, 1992). Mandated time and temperature processing regimes for shellfish have reflected conditions that retard bacterial survival and growth during storage conditions; however, the effects of specific handling conditions related to tempering of hard clam during live storage have not been investigated.

Prior research indicated that tempering of clams prior to refrigerated storage did not alter fecal coliform or overall Vibrio content, as indicated by growth of selective medium (Applewhite et al., 1996). In the present study, three different temperature regimes for dry storage were compared specifically for their effects on the growth of *V. vulnificus* in aquacultured Florida clams. Protocols included the current standard processing practice with or without tempering, and also investigated a more prolonged tempering time prior to refrigeration that would allow wholesalers to more effectively integrate the tempering process into their daily operating schedules. Thus, the objectives of this study were to evaluate the microbial consequences of temperature acclimation protocols and provide data to support processing recommendations that will increase the survival of aquacultured clams.

Tempering protocols for clam storage. Studies were conducted to specifically examine the effects of tempering on the *V. vulnificus* content of clams. Clams were harvested from high-density clam leases in Cedar Key, FL on July 11, August 27, and Sept. 27, 2002**.** A flow diagram of the different temperature regimes over a 24 hour period is shown in Table 1 and included the following: Protocol 1 was the control sample without tempering and followed national (VIII@.03 OPTION 1.A.,B.,C., and D., Model Ordinance) and state (Chapter 5L-1.008(5), Florida Administrative Code) guidelines which permit maximum incubation at ambient temperature (about 80ºF) for 10 hours prior to refrigeration (45ºF/7.2ºC); Protocol 2 followed tempering procedures defined in national (VIII@.03 OPTION 1.E, Model Ordinance) and state (Chapter 5L-1.013(3)(b), Florida Administrative Code) guidelines, which permit 6 hours at ambient, followed sequentially by a maximum of 10 hours tempering at 68ºF (20ºC) with subsequent refrigeration. In Protocol 3 clams were incubated for 10 hours at ambient temperature, followed by 14 hours at tempering conditions. This protocol is not currently approved but would permit more convenient overnight tempering following harvest.

Evaluation of *V. vulnificus* in tempered clams. Clams were assayed at harvest, during tempering, and after refrigerated storage over a 24-hour period. Numbers of *V. vulnificus* were determined immediately post harvest at 0 hour, as well as at 6, 10, and 24 hours post-harvest. Three independent experiments used duplicate aliquots of clam meats (n=12) at each time point. Incubations at ambient temperatures were done in a shaded area with adequate ventilation. The internal clam and external ambient air temperatures were monitored (Ellab, Tracksense II recorder, Arvada, CO) throughout the study. Continuous temperature monitoring indicated that ambient temperature ranged from 78-86ºF, and stated incubation temperatures were ±2ºF. *V. vulnificus* content in clams was monitored using species-specific DNA probe and colony hybridization method as previously described (Wright et al., 1993) and recommended by the FDA Bacteriological Analytical Manual (2004). This probe is derived from the cytolysin gene *vvhA* and will theoretically detect as few as 10 CFU g^{-1} of oyster (Wright et al., 1996).

Clam meats were diluted 1:2 (wt:wt) in phosphate buffer saline (PBS) and homogenized for 90 seconds. Clam homogenates were serially diluted in PBS, and spread plated (100 µl) to T1N1 agar (1% NaCl, 1% Tryptone, 1.5% agar), using 6 plates at each dilution. Plates were incubated for 24 hours at 37°C, and colonies were transferred to filters for DNA colony blot hybridization studies as previously described (Wright et al.,

1993). Briefly, bacterial colonies were transferred from spread plates by overlaying filter papers (85 mm Whatman #541). The filters were microwaved in lysis solution (0.5 M NaOH and 1.5 M NaCl) for 1-6 min. and rinsed successively in ammonium acetate neutralization buffer (Fisher Scientific, Pittsburgh, PA) and standard saline citrate buffer (SSC). Filters were treated with proteinase K (20 µg/ml) to remove background enzymatic activity and washed extensively in SSC. Filters were hybridized under stringent conditions (56ºC) for 1h with alkaline phosphatase-labeled oligonucleotide probe (DNA Technologies, Denmark) derived from the species–specific *V. vulnificus vvhA* gene (VVAP). Filters were rinsed in SSC with 1% SDS at hybridization temperature, followed by additional rinsing with 1X SSC at room temperature. NBT/BCIP substrate (Roche Diagnostics, Indianapolis, IN) was used for detection of alkaline phosphatase label while filters developed in the dark. Appropriate control filters with colonies of *V. vulnificus, V. parahaemolyticus, V. cholerae,* and *Escherichia coli* were developed concurrently for all time points.

Tempering does not alter *V. vulnificus* content in clams. No mortalities were observed in any clams during this study. The effects of tempering (step-wise decrease in temperature) on *V. vulnificus* contamination during acclimation of clam shellstock prior to cold storage were examined for clams collected at three different periods during summer months. Initial levels of *V. vulnificus* in clams ranged from 50 to 400 CFU/g for the three sampling periods with a mean of 203 CFU/g (Table 2). The highest level of *V. vulnificus* observed was 790 CFU/g, which was recorded during incubation in the refrigerated control group without tempering. After 24 hours, the mean values for *V. vulnificus* in clams from either group receiving tempering were 154 and 52 CFU/g for the approved or extended tempering protocols, respectively. These values were similar to or less than mean initial values. In comparison, both values for tempered samples were less than the mean number observed in refrigerated control samples, which was 277 CFU/g. The large standard deviations for the mean values probably reflected differences in the initial values for numbers of *V. vulnificus* in clams that were observed among the different experiments. However, no significant differences in the numbers of *V. vulnificus* in clams were observed as a result of any of the different temperature regime protocols in this study, using Student T-test, 2 tailed.

Conclusions. Environmental distribution of *V. vulnificus* in oysters is ubiquitous throughout most temperate estuaries, and numbers in oysters may exceed >10,000 CFU/g during summer months (Oliver et al., 1983; Tamplin et al., 1982; Wright et al., 1996). In contrast, levels of *V. vulnificus* in clams from Cedar Key were exponentially lower than those generally observed in oysters from the Gulf of Mexico in summer months. Hard clams are cultured in waters with salinity that is frequently higher (>20 ppt) than the moderate levels (5-15 ppt) typically associated with oysters (Menzel, 1990; Rice and Pechenik, 1992). Salinities above 25 ppt are not tolerated by *V. vulnificus,* and therefore less likely to support growth (Kelly et al., 1982). Although salinity associated with clam aquaculture may reduce human exposure and risk relative to oysters, the growth and survival of *V. vulnificus* is influenced by the combined effects of both temperature and salinity (Hood et al, 1983; Kaysner et al, 1987; Kasper and Tamplin, 1993). Therefore,

the present study examined the consequences of different temperature storage protocols on levels of *V. vulnificus* in clams.

The results herein demonstrated that tempering prior to refrigerated storage did not increase the levels of *V. vulnificus* in clams. Even an extended tempering regime prior to storage of aquacultured clams did not alter the *V. vulnificus* content. Thus, this study supports the use of tempering practices for aquacultured clams and showed these protocols could be safely employed without increasing the risk of the shellfish-associated pathogen, *V. vulnificus.*

References

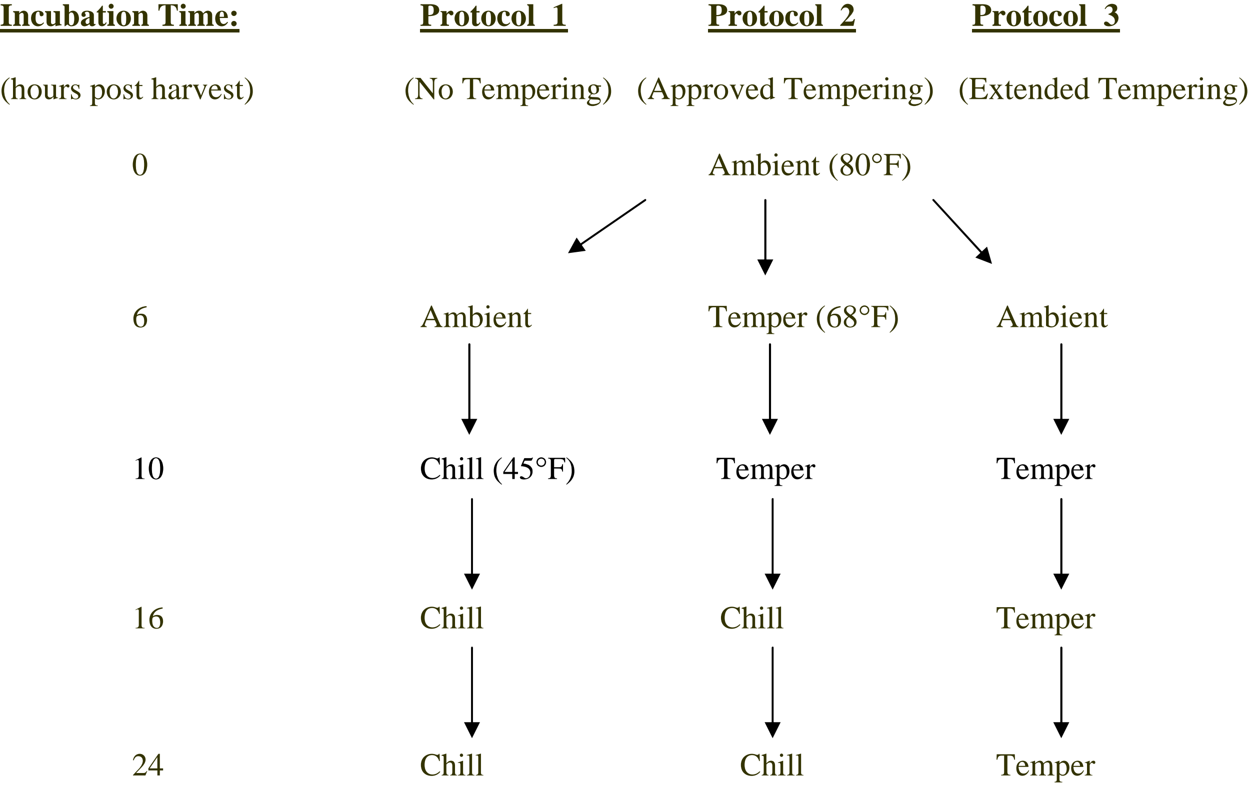
1. Applewhite, L., W.S. Otwell and L. Sturmer. 1996. Survival of Florida aquacultured clams in refrigerated storage. Proc. 21st Ann. Seafood Sci & Technol.. Soc.. of the
2. Cook, D.W. and A.D. Ruple. 1989. Indicator bacteria and *Vibrionaceae* multiplication in post-harvest shellstock oysters. J. Food Prot. 52:343-349.
3. Cook, D.W. and A.D. Ruple. 1992. Cold storage and mild heat treatment as processing aids to reduce the numbers of *Vibrio vulnificus* in raw oysters. J. Food. Prot. 55:985-989.
4. Florida Agriculture Statistics Service (FASS). 2000. Florida Agriculture: Aquaculture. United States Department of Agriculture, National Agricultural Statistics Service, Orlando, Florida. 4 pp.
5. Food and Drug Administration. 1998. Bacteriological analytical manual. 8th ed. AOAC International. Arlington, VA.
6. Hochachka, P.W. and G.N. Somero. 1984. Biochemical Adaptation. Princeton University Press. Princeton, NJ
7. Hood, M.A., G.E. Ness. G.E. Rodrick and N.J. Blake. 1983. Effects of storage on microbial loads of two commercially important shellfish species, *Crassostrea virginica* and *Mercenaria campechiensis*. Appl. Environ. Microbiol. 45:1221-1228.
8. Kasper, C.W. and M.L. Tamplin. 1993. Effects of temperature and salinity on the survival of *Vibrio vulnificus* in seawater and shellfish. Appl. Environ. Microbiol. 59:2425-2429.
9. Kaysner, C.A., M.L. Tamplin, M.M. Wekell, R.F. Stott and J.M. Leitch. 1987. Virulent strains of *Vibrio vulnificus* isolated from estuaries of the United States West Coast. Appl. Enivron. Microbiol. 53:1349-1351.
10. Kaysner, C.A., M.L. Tamplin, M.M. Wekell, R.F. Stott and K.G. Colburn. 1989. Survival of *Vibrio vulnificus* in shellstock and shucked oysters (*Crassostrea gigas* and *Crassostrea virginica*) and effects of isolation medium on recovery. Appl. Environ. Microbiol. 55:3072-3079.
11. Kelly, M.T. 1982. Effect of temperature and salinity on *Vibrio (Beneckea) vulnificus* occurrence in a Gulf coast environment. Appl. Environ. Microbiol. 44:820-824.
12. Menzel, R.W. and H.W. Sims. 1962. Experimental farming of hard clams, *Mercenaria mercenaria*, in Florida. Proc. Natl. Shellfish Assoc. 53:103-109.
13. Menzel, R.W. 1972. Selection and hybridization in the mariculture of oyster and clams. Proc. 3rd Annual Meeting World Mariculture Soc., 309-31
14. Menzel, R.W., E.W. Cake, M.L. Haines. R.E. Marine and L.A. Olsen. 1975. Clam mariculture in Northwest Florida: Field study on predation. Proc. Natl. Shellfish Assoc. 65:59-62.
15. Menzel, W. 1990. Quahog clams in the United States. In: W. Menzel (editor), Estuarine and Marine Bivalve Mollusk Culture. CRC Press, Boca Raton, FL pp. 47-56.
16. Oliver, J. D., R. A. Warner, and D. R. Cleland. 1983. Distribution of *Vibrio vulnificus* and other lactose-fermenting vibrios in the marine environment. Appl. Environ. Microbiol. 45, 985-98.
17. Otwell, W.S., J.A. Koberger and S. Andree. 1986. Survival of Southern Hard Clams in Refrigerated Storage. FL Sea Grant College Program Report-83, 17 pages.

18. Philippakos, E., C. Adams, A. Hodges, D. Mulkey, D. Comer & L. Sturmer. 2001. Economic Impact of the Florida Cultured Hard Clam Industry. Florida Sea Grant Publication SGR 123, University of Florida, Gainesville, FL. 23 pp.
19. Rice, M.A. and J.A. Pechenik. 1992. A review of the factors influencing the growth of the northern quahog, *Mercenaria mercenaria* (Linnaeus, 1758). J. Shellfish Research 11(2)279-287.
20. Strom, M.S., and R.N. Paranjpye. 2000. Epidemiology and pathogenesis of *Vibrio vulnificus*. Microbes Infect. 2: 177-188.
21. Tamplin, M.L., G.E. Rodrick. N.J. Blake and T. Cuba. 1982. Isolation and characterization of *Vibrio vulnificus* from two Florida estuaries. Appl. Environ. Microbiol. 44:1466-1470.\
22. Wright, A.C., G.A. Miceli, W.L. Landry, J.B. Christy, W.D. Watkins, and J.G. Morris. 1993. Rapid identification of *Vibrio vulnificus* on nonselective media with an alkaline phosphatase-labeled oligonucleotide probe. Appl. Environ. Microbiol. 59: 541-546.
23. Wright, A.C., R.T. Hill, J.A. Johnson, R.R. Colwell, and J.G. Morris, Jr. 1996. Distribution of *Vibrio vulnificus* in the Chesapeake Bay. Appl. Environ. Microbiol. 62: 717-724.

Acknowledgements

This work was supported and advised by the Florida Department of Agriculture and Consumer Services (FDACS), and the Florida Sea Grant College Program working in the Aquatic Food Products Lab in the Food Science and Human Nutrition Department at the University of Florida in Gainesville. This work was funded by the University of Florida under a USDA Grant No. 2002-34453-11946, entitled "Aquaculture, Florida Research Project", from the U.S. Department of Agriculture's Cooperative State Research, Education, and Extension Service. All work was made possible through generous cooperation with numerous representatives from the Florida hard clam industry, including Cedar Key Aquaculture Farms, Harriet Smith Clams and Big T Clam Farm. Commercial liaison was provided through the University of Florida, Institute of Food and Agricultural Sciences (IFAS), Cooperative Extension Services, Shellfish Aquaculture Extension Program based in Cedar Key, Florida.

Table 1. Summary of temperature regimes for clam storage.



Incubation Time: (hours post harvest)	**Protocol 1** (No Tempering)	**Protocol 2** (Approved Tempering)	**Protocol 3** (Extended Tempering)
0		Ambient (80°F)	
6	Ambient	Temper (68°F)	Ambient
10	Chill (45°F)	Temper	Temper
16	Chill	Chill	Temper
24	Chill	Chill	Temper

Table 2. Effects of tempering on *V. vulnificus* survival in clams.

Treatment:	***V. vulnificus* Content as a Function of Storage Temperature (Mean CFU/g±SD)[a]**				
	Time Post-Harvest				
	0 hour	6 hour	10 hour	16 hour	24 hour
Protocol 1: No Tempering	203 ± 148.5	211 ± 184.2	253 ± 160.0	360 ± 309.2	277 ± 261.2
Protocol 2: Approved Tempering	N.A.[b]	N.A.	123 ± 52.3	180 ± 160.3	154 ± 180.2
Protocol 3: Extended Tempering	N.A.	N.A.	253 ± 160.0	N.A.	52 ± 56.8[c]

a) Numbers of *V. vulnificus* in clams are shown as mean CFU/g ± standard deviation (SD) from three independent experiments using triplicate samples at each time point samples. Numbers were determined by colony counts identified by DNA probe, as described in text.
b) For samples incubated in the same manner, only one representative sample was examined, and other samples were not assessed (N.A.).
c) Samples that were below detection level of assay (<10 CFU/g) and were averaged as 10 CFU/g.