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INDUCED THERMOTOLERANCE IN THE PACIFIC OYSTER, *CRASSOSTREA GIGAS*

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ABSTRACT Pacific oysters, *Crassostrea gigas,* were subjected to heat shock at various temperatures under controlled laboratory conditions. These experiments demonstrated that exposure to sublethal temperatures dramatically enhances thermotolerance. Oysters exposed to a single nonlethal heat shock (37°C for 1 h) acquired a transient tolerance to a subsequent exposure previously determined to be lethal (43°C for 1 h). The induced thermotolerance ("thermal memory") existed for at least 10 days after sublethal heat shock. Preliminary studies indicated that thermotolerance induction was correlated with the appearance of heat shock proteins in the 70-kD family (hsp-70), based on electrophoretic analysis of proteins from three different tissues, followed by immunoblot analysis with antibodies against hsp-70.

KEY WORDS: oyster, thermotolerance, heat shock protein, summer mortality

INTRODUCTION

Mass moralities among commercially important oyster species in the United States have become a recurring obstacle for oyster growers since the 1950s. For example, summer moralities of *Crassostrea gigas* in northern California have approached losses totaling 65% in recent years, (Friedman and Olin unpubl.), and along the East Coast, mortalities of *Crassostrea virginica,* due to documented parasitic infections, have drastically reduced commercial oyster fisheries (Ford 1996, Andrews 1996). Several hydrographic and biological factors have coincided with summer mortality in oyster populations; these include elevated water temperatures, salinity stress, pathogens, and reproductive stress (Perdue 1983, Newell 1985, Beattie et al. 1988, Littlewood and Ford 1990, Friedman et al. 1991, Friedman and Hedrick 1991, Newell et al. 1994).

The ability to adapt to changing environmental conditions is important in all organisms. One of the most studied phenomena is the capacity of different organisms to survive extreme temperatures developed by a short pretreatment at moderately elevated but sublethal temperature. This phenomenon, known as induced thermotolerance, induces resistance against high temperature conditions that would otherwise be lethal (Henle and Dethlefsen 1978, Li and Hahn 1980, Nover 1991, Parsell and Lindquist 1994). Thermotolerance is known to be a widespread phenomenon in organisms and is thought to be an important adaptation to survive changing environmental conditions. Such tolerance-inducing treatments also induce the synthesis of a small number of proteins known as the heat shock proteins (hsp) that play vital roles in allowing the organisms to survive subsequent more severe exposure to heat that would otherwise be lethal (Li and Laszlo 1985, Lindquist 1986). These proteins are involved in the protection, enhanced survival, and restoration of normal cellular activities in stressed cells and tissues (Subjeck and Shyy 1986, Schlesinger 1990, Hightower 1991, Welch 1991, Gething 1991, Craig et al. 1993, Schlesinger 1994). The synthesis of proteins in the 70-kD family (hsp 70) is correlated with the induction of thermotolerance (Bosch et al. 1988, Nover 1991, Solomon et al. 1991, Weber 1992, Sanders et al. 1994). These proteins are formed not only in response to heat but also are induced in cells and tissues of organisms by a variety of noxious stimuli including anoxia, heavy metal ions, ethanol, and viral agents (Nover 1991). In this study, we determined if the Pacific oyster, *C. gigas,* from two different geographic locations, could acquire thermotolerance. This was accomplished by heat shocking oysters at a predetermined sublethal temperature, followed by exposure to temperatures that were previously determined to be lethal. Evidence is presented that oysters acquired thermotolerance under laboratory conditions, and this induced thermotolerance existed for at least 10 days after the initial heat shock at a sublethal temperature. Preliminary studies indicated that this induced thermotolerance was associated with the expression of hsp-70.

MATERIALS AND METHODS

Collection and Maintenance of Animals

Pacific oysters, *C. gigas,* were obtained from two different seed sources: Kuiper Mariculture, Humboldt Bay, CA, and Dick Poole's Lummi Indian Shellfish Hatchery, Bellingham, WA. Live oysters from both sources were transported overnight on ice to Tomales Bay, CA, in April 1995 (Tomales Bay Oyster Company). Immediately after arrival, oysters were outplanted, on off-bottom

racks in Nytex 1/4-inch mesh bags at the +1.5-ft tide level. After an acclimation period of 4 mo, oysters from both sources were harvested and transported on ice to Bodega Marine Laboratory (BML) where the thermotolerance studies were conducted. On arrival at BML, oysters were transferred to aerated 135-L running seawater aquaria and maintained at ambient temperature (12 ± 1°C, monitored daily) until used in thermotolerance experiments within 7–8 days of collection. Oysters were measured (shell length), weighed, and placed in fiberglass screen bags (10 oysters per bag). Animals were fed *ad libitum* with a prepared algal diet, Diet C (Coast Seafood Co., Quilcene, WA), diluted to yield a suspension of 100,000 cells per oyster. Feeding was withheld for 24 h before the heat shock and thermotolerance experiments described below.

LT_{50} Determination

Oysters only from Humboldt Bay were used in the LT_{50} determination. These animals were not acclimated in Tomales Bay. Before any experiments on thermotolerance induction were conducted, we established: (1) the time taken for the core body temperature to reach the target temperature after immersion in a water bath; (2) the range of temperatures over which *C. gigas* survived under laboratory conditions, from which we determined the temperature that resulted in 50% mortality (LT_{50}).

The time for stabilization of the body temperature, after immersion into a water bath set at a desired temperature, was monitored with an Omega thermocoupler probe (Fisher Scientific). A 1-mm-diameter hole was drilled in the shell through which the thermocoupler probe was inserted into the body cavity. The hole was then sealed with Dow Corning high-vacuum grease, after which the animal with the inserted thermocoupler was immersed into a water bath (Masteline Forma Scientific, Model 2095) that contained 4 L of seawater previously heated to 44°C. Oysters were immersed so that the thermocoupler was not in contact with the water. The internal body temperature was recorded every 30 sec, and the time taken for the body temperature to reach the external (seawater) temperature was determined.

During a pilot study to determine the LT_{50}, oysters were exposed to elevated temperatures that ranged from 25 to 50°C in increments of 5°C. In order to minimize the drop in water temperature when oysters were immersed, a two-step heat shock protocol, using two water baths, was followed. The oysters were immersed for 10 sec in a water bath (Precision, Model 181) set at the desired temperature. After this, the animals were transferred to the second water bath (Masteline Forma Scientific, Model 2095) set at the desired temperature. Oysters were maintained at this temperature for 1 h, agitated for the first 10 min of immersion, and subsequently returned to ambient temperature for 7 days. Mortality was then assessed by examining valve closure and/or assessing the presence of decay. Valves that remained open after the shells were pinched together and then released indicated death.

During the pilot heat shock experiments described above, 100% mortality was observed at temperature greater than 40°C (1 h). In order to determine the temperature that induced 50% mortality (LT_{50}), oysters were exposed to a finer temperature range, 40–45°C in increments of 0.3°C, for 1 h as described above. From this study, the LT_{50} was determined to be 42.3°C. This formed the basis for subsequent studies on induced thermotolerance, as described below.

Induction of Thermotolerance

On the basis of results obtained from the LT_{50} studies described above, we selected 37°C for 1 h for the sublethal heat shock and 43°C for 1 h for the lethal shock. To examine acquired thermotolerance, oysters (maintained at ambient temperature of 12 ± 1°C) were exposed to a sublethal heat shock as described above and then returned to ambient temperature (12 ± 1°C) for recovery. The lengths of the recovery period varied: 5, 10, and 20 days postsublethal heat shock. At the end of each recovery period, oysters were heat shocked at 43°C for 1 h and then returned to ambient temperature and monitored daily. Control treatments included: (1) exposure of oysters to lethal temperature without prior exposure to sublethal temperature; (2) sublethal shock alone, and; (3) no heat shock. Mortality was assessed, as described above, 7 days after each treatment. All experiments were repeated three times, each using 10 oysters per treatment.

Electrophoresis and Immunoblotting

In order to determine if a sublethal heat shock was associated with the induction of heat shock proteins, tissue samples were prepared for electrophoresis and immunoblotting as follows. After sublethal shock at 37°C for 1 h, oysters were maintained at ambient temperature (12 ± 1°C) for 24 h. At least 3 oysters from each trial (total of three trials, each using 10 oysters) were opened, and the tissues (mantle, gills, and adductor muscle) were excised, blotted with Whatman No. 1 filter paper, and weighed. Samples were homogenized in a buffer containing 5 mM $MgSO_4$, 5 mM NaH_2PO_4, 40 mM HEPES, 70 mM potassium gluconate, 150 mM sorbitol (pH 7.55), and were centrifuged at 1000 × g for 10 min. An aliquot of each sample (supernatant) was analyzed to determine total protein concentration with the Micro BCA Assay kit (Pierce, Rockford, IL). The remaining aliquots were combined with equal volumes of 2× sodium dodecyl sulfate–sample buffer (Laemmli 1970) and heated for 5 min at 100°C. Similar amounts of proteins from various tissue samples, predetermined on the basis of the Micro BSA assay described above, were loaded onto 12% polyacrylamide gels and electrophoresed. After electrophoresis, polypeptides were transferred to nitrocellulose membranes as described by Towbin et al. (1979). Blots were incubated with Tris-buffered saline containing 3% bovine serum albumin (''blocking solution,'' pH 7.4) and probed with mouse anti-hsp-70 (Affinity Bioreagents, MA3-006) for 90 min at room temperature. The blots were then rinsed in blocking solution (3×, 10 min each), incubated with goat anti-mouse immunoglobulin G (Sigma Chemical Co., St. Louis, MO; Product No. A4416) conjugated to horseradish peroxidase for 90 min, washed, and visualized with 4-chloronaphthol.

RESULTS AND DISCUSSION

Oysters, like other marine invertebrates, are ectotherms; their body temperature, is controlled by ambient temperatures that often change rapidly. In our studies, oysters became isothermic with the elevated ambient temperature up to 44°C (from an initial temperature of 12 ± 1°C) within 7–8 min. (Fig. 1). Changes in oyster body temperature in natural settings may be influenced by factors not present in our laboratory setting. It is known that behavioral regulation of body temperature is usually of most importance during short-term fluctuations in ambient temperature, as might occur on a diurnal cycle; however, long-duration temperature changes pro-

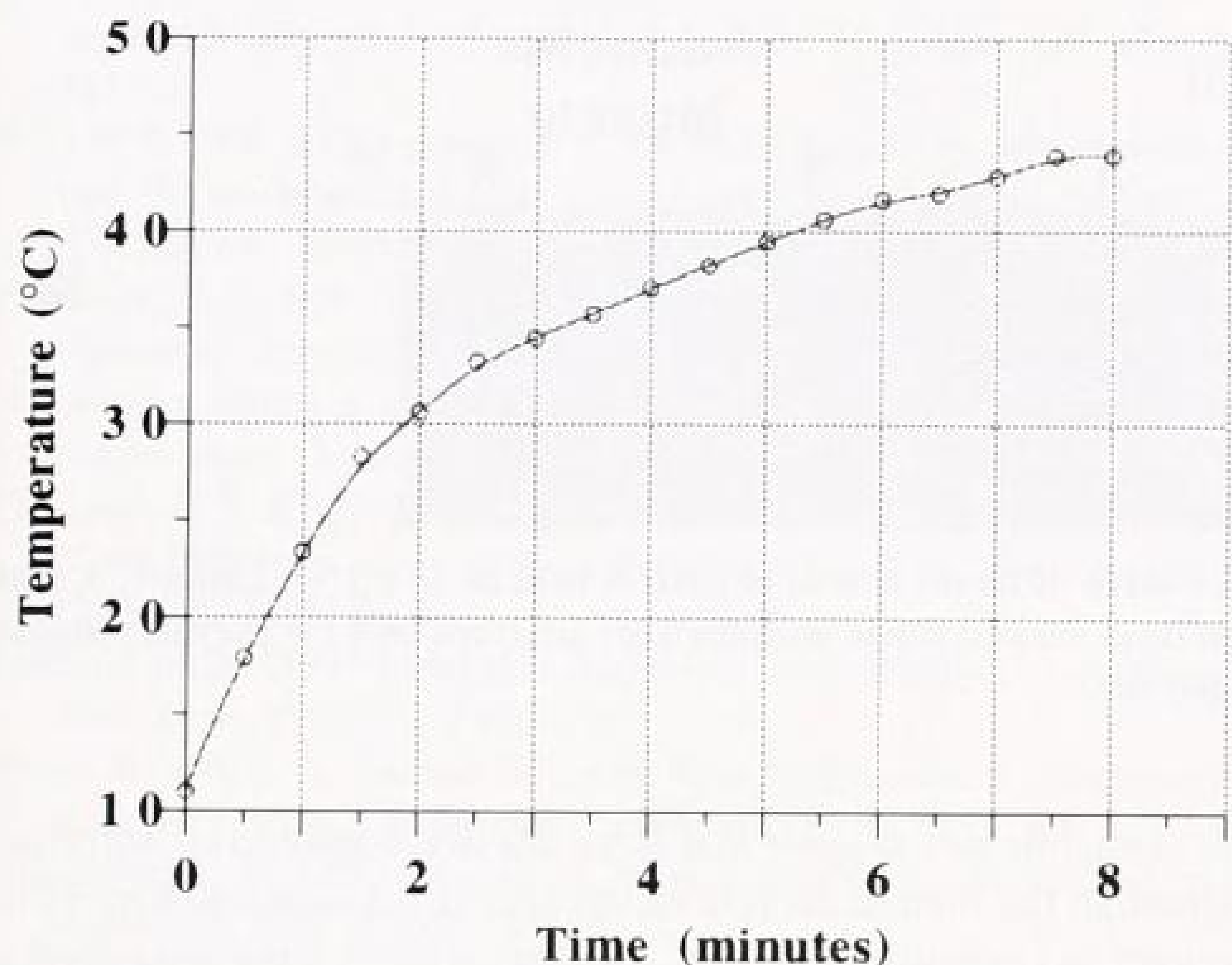

Figure 1. Time taken for the core body temperature to reach the ambient temperature after immersion in a water bath set at 44°C.

vide organisms with enough time for modifications in their biochemical systems (Hochachka and Somero 1984).

Because thermotolerance involves the capability of surviving extreme temperatures developed by a short pretreatment at moderately elevated but sublethal temperature, we first established lethal and sublethal temperatures (Fig. 2). No mortality was observed when oysters were exposed for 1 h to temperatures below 40°C. Direct heat treatments above 43°C, however, always resulted in 100% mortality. The LT_{50} (42.3°C) provided us with the baseline data for further studies on thermotolerance.

Although reared in Tomales Bay for several months, oysters from the two seed sources (Humboldt Bay and Washington State) responded to thermal regimens slightly differently from one another. All oysters from Humboldt Bay that were heat shocked for 1 h at 43°C, without prior sublethal temperature shock, did not survive (Fig. 3). When oysters were exposed to the lethal temperature (43°C) 5 days after a sublethal shock at 37°C, no mortality was observed. Only slightly higher mortality (7%) was observed among oysters exposed to sublethal shock 10 days before lethal shock. The mortality rates of oysters exposed directly to the lethal temperature and of those exposed to lethal temperature 10 days after a sublethal shock were significantly different ($p < 0.001$). Twenty days after sublethal shock, however, oysters were not able to withstand the lethal shock and approximately 80% of the oysters died; mortality rate was not significantly different from those exposed directly to lethal temperature ($0.1 < p < 0.25$). In all cases, 100% survival rates were observed among oysters that were either left at ambient temperature (12 ± 1°C) (data not shown) or shocked at 37°C for 1 h without a subsequent lethal shock. These data indicate that exposure to a sublethal shock followed by a recovery period enhances thermotolerance in these organisms. In addition, the 10-day duration of the enhanced thermotolerance observed in this study exceeds that reported in other organisms under *in vivo* conditions (see Nover 1991 for a review).

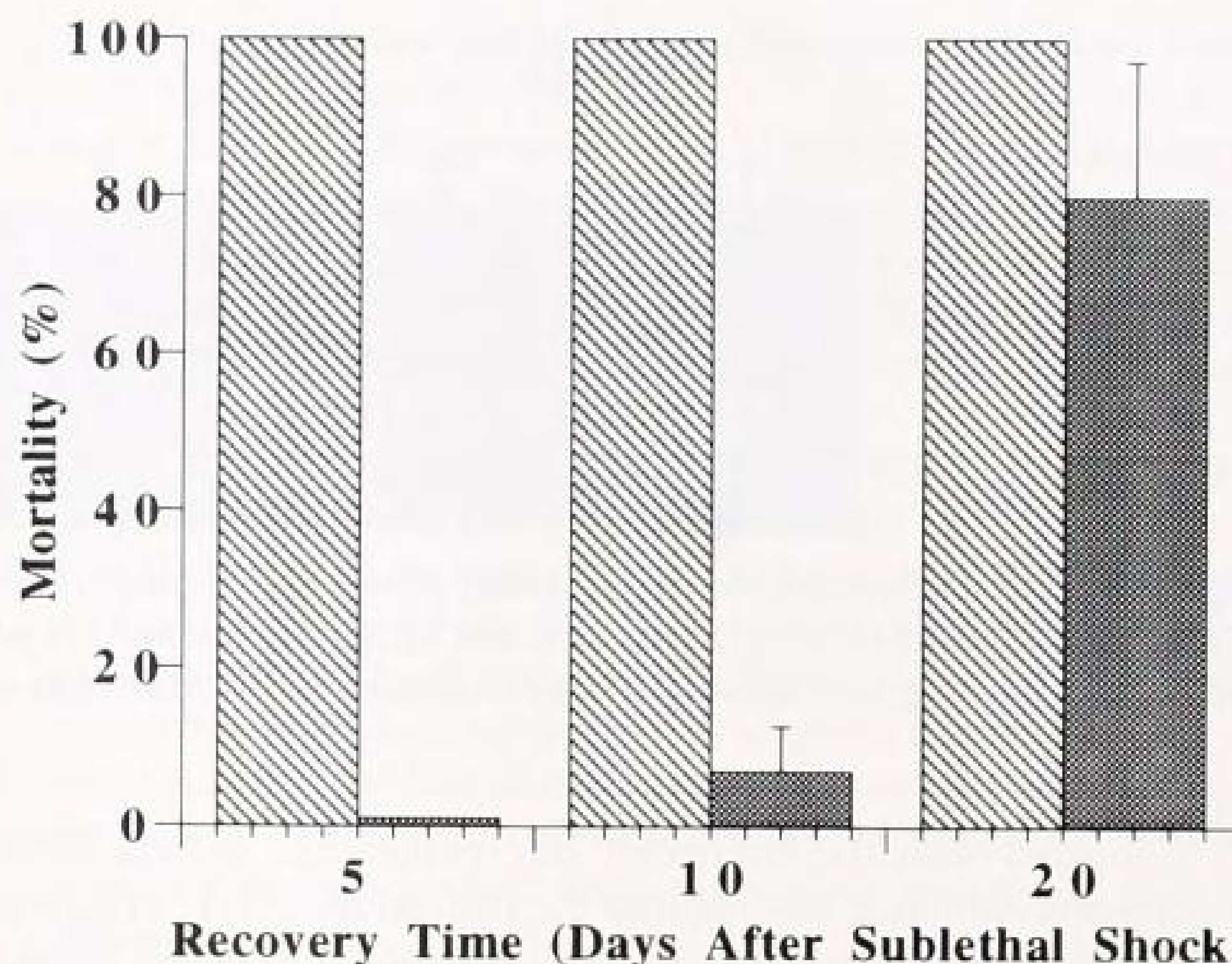

Figure 3. Induced thermotolerance of *C. gigas* from Humboldt Bay. Mortality rates of oysters exposed directly to a lethal temperature of 43°C (▨) or a sublethal temperature of 37°C for 1 h, allowed to recover for 5, 10, and 20 days (at 12 ± 1°C), and shocked at 43°C for 1 h (▩). Data represent mean ± SD for three different experiments, each using 10 oysters per treatment.

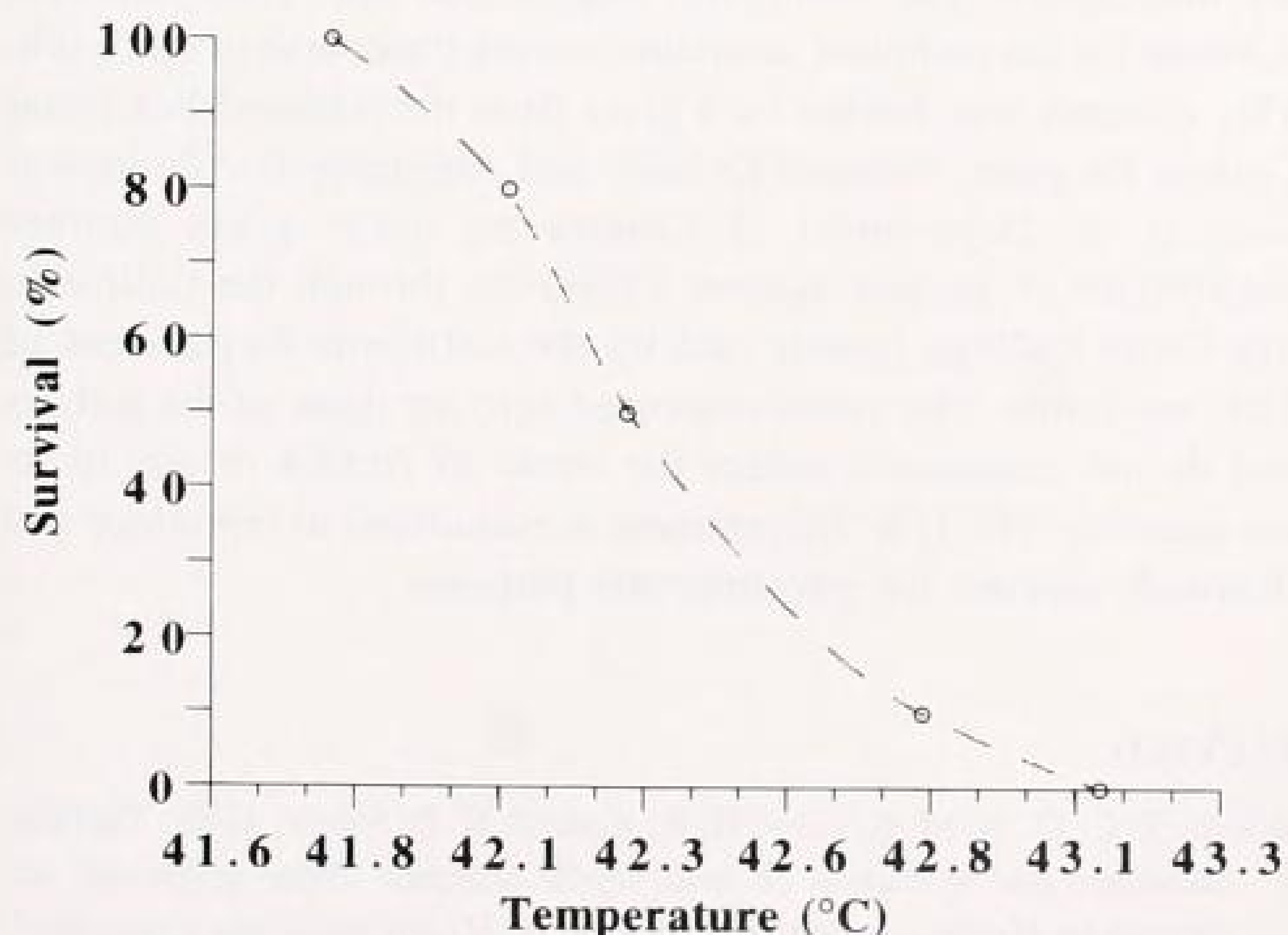

Figure 2. LT_{50} curve for *C. gigas* illustrates the percent survival of oysters 1 wk after exposure to elevated water temperatures.

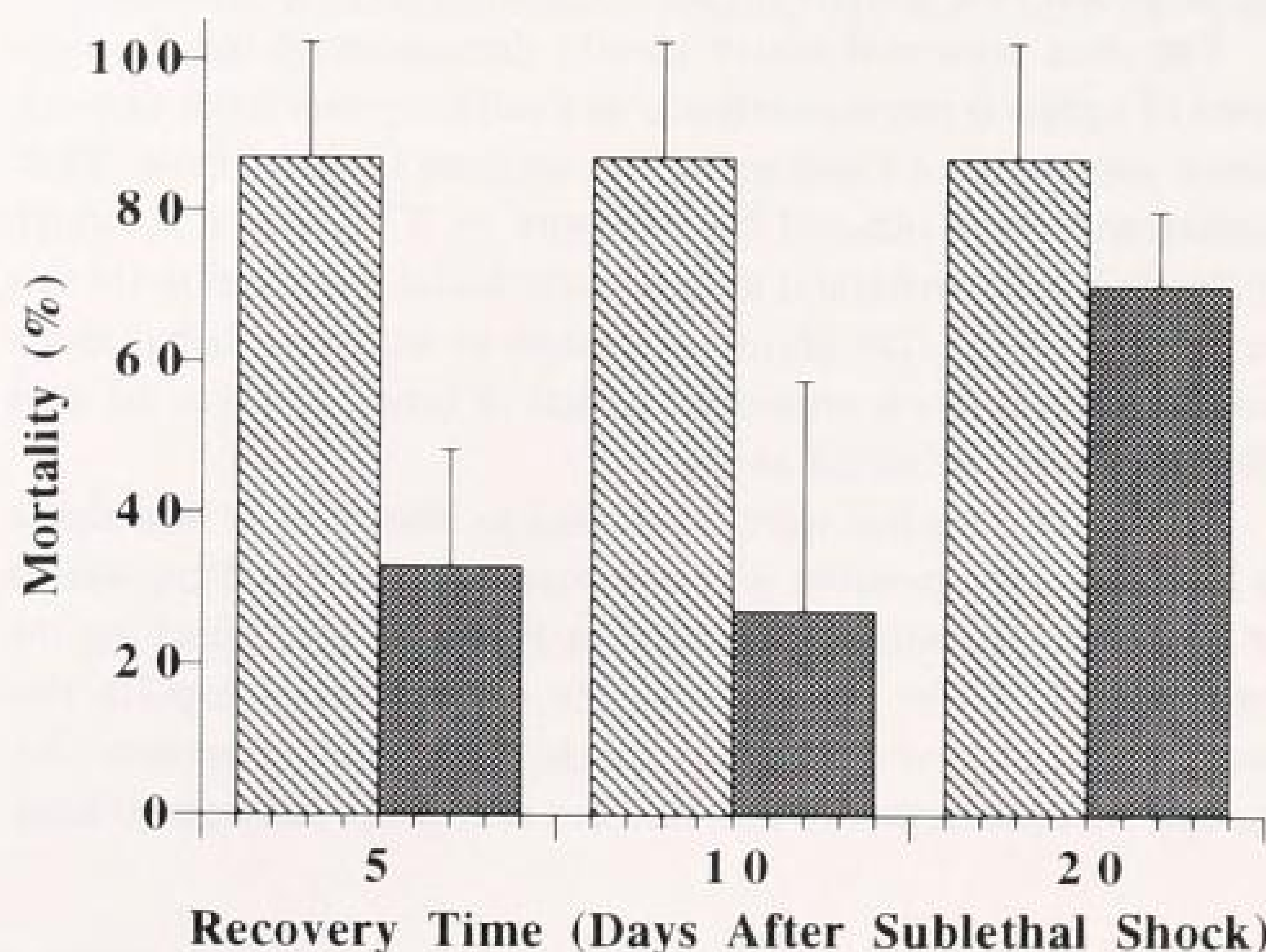

Figure 4. Induced thermotolerance of *C. gigas* from Washington State. Mortality rates of oysters exposed directly to a lethal temperature of 43°C (▨) or a sublethal temperature of 37°C for 1 h, allowed to recover for 5, 10, and 20 days (at 12 ± 1°C), and shocked at 43°C for 1 h (▩). Data represent mean ± SD for three different experiments, each using 10 oysters per treatment.