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Electric Power Generation Association

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One Page Summary of EPGA Comments on Proposed Rulemaking: 25 Pa. Code Chapter 93: Triennial Review of Water Quality Standards

Possible Conflict with Statutes: EPGA questions whether the regulation is consistent with Section 5(a) of the Clean Streams Law (35 P.S. § 691.5(a)) in that it fails to properly analyze the economic impact on industry in Pennsylvania and appropriately consider the state of scientific and technological knowledge.

Economic Impact of the Regulations: EPGA has serious concerns with the potential economic impact of this regulation. The Preamble suggests that this rulemaking will only impact "persons expanding a discharge or adding a new discharge point." However, if adopted, this rulemaking will impact all existing discharges containing chlorides and sulfates as part of the NPDES permit renewal process including electric generating facilities.

The technology needed to remove chlorides and sulfates has not been developed for use in electric power industry applications and is not in commercial use in the United States at flows that commonly occur from many of the electric generation plants in PA.

The types of wastewater that could be impacted in our industry include, but are not limited to: Flue Gas Desulfurization (FGD) purge water, cooling tower blowdown, landfill leachate, demineralizer regeneration water, ash pond effluent, coal pile runoff effluent, and wetland mitigation water. Costs to retrofit these technologies to our systems are extremely high with no guarantee that the needed reductions will be obtained with that equipment.

EPGA's comments include recent examples of studies designed to evaluate emerging Zero Liquid Discharge (ZLD) technologies and processes and assessments of their feasibility as FGD wastewater treatment alternatives. The ZLD options evaluated had estimated capital costs ranging from $70.7 million to $111.9 million and annual operating expenses ranging from $3.59 million to $9.7 million.

Based on the experience of EPGA members the information in the preamble regarding the costs and the maturity of the available technology is wholly inaccurate.

Need and Possible Conflict with Existing Regulations: Discharges of sulfates and chlorides, which are primary sources of TDS, are already regulated under Title 25 PA Code Chapter 95 (Wastewater Treatment Requirements) that became effective on August 21, 2010. As a result, the rationale for the proposed chloride and sulfate rulemaking is flawed.

Dissolved Oxygen: EPGA supports the change from discrete minimum daily averages to 7-day averages as these standards are more representative and better capture the temporal variability in streams and water bodies.

Temperature: The existing rate of temperature change criterion (2°F during a 1-hour period) cannot even be met under naturally occurring conditions without any influence from a point source discharge. Several literature reviews do not support the existing standard. Since there is no available basis for the temperature criterion, it is appropriate that the Department review the limit. A report entitled, "Evaluating the Seasonal Effects of Short-Term Temperature Fluctuations on Macroinvertebrates and Fish in the Susquehanna River near the Brunner Island Steam Electric Station" should be considered by the Department in its evaluation of a revised temperature criterion.

Aquatic Life Standard and Human Health Standard for Molybdenum: It is our understanding that the aquatic life molybdenum standards were developed as a result of a request from one regional office for only one or two NPDES discharges. This is an inappropriate foundation for developing a statewide standard. Regarding the proposed human health criteria, our concerns are the same as those expressed by the Senate Environmental Resources and Energy Committee during the previous water quality standard triennial review. There is no drinking water standard or federal water quality standard for molybdenum.

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Environmental Quality Board
P. O. Box 8477
Harrisburg, PA 17105-8477

Comments on Proposed Rulemaking: 25 Pa. Code Chapter 93: Water Quality Standards Triennial Review

Following are the comments of the Electric Power Generation Association (EPGA) on proposed revisions to 25 Pa. Code Chapter 93 that were published in the July 7, 2012 Pennsylvania Bulletin.

EPGA is a trade association of electric generating companies with headquarters in Harrisburg, PA. Collectively, our members own and operate more than 150,000 megawatts of electric generating capacity, approximately half of which is located in Pennsylvania and surrounding states. Our members include AES Beaver Valley, LLC, Dynegy Inc., Edison Mission Group, Exelon Generation, FirstEnergy Generation Corp., GenOn Energy, PPL Generation, LLC, Sunbury Generation, LP, Tenaska, Inc., and UGI Development Company. These comments represent the views of EPGA as an association of electric generating companies, not necessarily the views of any individual member company with respect to any specific issue.

Possible Conflict with Statutes

According to the Environmental Quality Board (Board), this rulemaking is being promulgated, in part, under Sections 5(b)(l) and 402 of the Clean Streams Law (Law) (35 P.S. §§ 691.5(b)(l) and 691.402). While we do not question the Board's authority under these provisions, we do question whether the regulation is consistent with Section 5(a) of the Law (35 P.S. § 691.5(a)). That section of the Law requires the following factors to be considered, where applicable, when adopting rules and regulations:

1. Water quality management and pollution control in the watershed as a whole;
2. The present and possible future uses of particular waterways;
3. The feasibility of combined or joint treatment facilities;
4. ***The state of scientific and technological knowledge; and***
5. ***The immediate and long-range economic impact upon the Commonwealth and its citizens.*** (Emphasis added)

EPGA believes the rulemaking is inconsistent with the fourth and fifth provisions of Section (5) (a) of the Law. In regard to the fifth provision, we believe that the Board has failed to properly analyze the economic impact the regulation will have on industry in Pennsylvania. Additional information and comments regarding the state of scientific and technological knowledge and economic impact of the rulemaking is summarized below.

Economic Impact of the Regulations

EPGA members have serious concerns with the potential economic impact of this regulation throughout the Commonwealth. The Preamble provides little analysis on the impact the regulations will have on the regulated community. Specifically, the Preamble indicates that the proposed amendments to Chapter 93:

> *...may impose additional compliance costs on the regulated community. These regulatory changes are necessary to improve total pollution control. The expenditures necessary to meet new compliance requirements may exceed that which is required under existing regulations.*
>
> *Persons conducting or proposing activities or projects must comply with the regulatory requirements relating to designated and existing uses. Persons expanding a discharge or adding a new discharge point to a stream could be adversely affected if they need to provide a higher level of treatment to meet the more stringent criteria for selected parameters or there are changes in designated and existing uses of the stream. These increased costs may take the form of higher engineering, construction or operating cost for wastewater treatment facilities. Treatment costs are site-specific and depend upon the size of the discharge in relation to the size of the stream and many other factors. Therefore, it is not possible to precisely predict the actual change in costs. Economic impacts would primarily involve the potential for higher treatment costs for new or expanded discharges to streams that are redesignated. The initial costs from technologically improved treatments may be offset over time by potential savings from and increased value of improved water quality through these improved and possibly more effective or efficient treatments.*

First, the Preamble suggests that this rulemaking will only impact "persons expanding a discharge or adding a new discharge point." If adopted, this rulemaking will impact all existing discharges containing chlorides and sulfates as part of the NPDES permit renewal process. In determining the projected costs associated with the chloride and sulfate criteria a multitude of factors need to be considered. One key factor is to determine who may be impacted by the proposed rulemaking. Some of these facilities may include municipal wastewater treatment plants, municipal drinking water treatment plants, PennDOT garages, and industries such as electric generation, coal and natural gas production, food processors, canneries, and industries that discharge cooling water. No description as to how the criteria will be implemented (e.g., nonpoint source discharges from deicing, roadway storm water runoff, etc.) was considered.

As we explained during the Total Dissolved Solids (TDS) regulation process, the technology needed to remove chlorides and sulfates has not been developed for use in our industry's applications and is not in commercial use in the United States at flows that commonly occur from many of the electric generation plants in the State. The types of wastewater that could be impacted in our industry include, but are not limited to: Flue Gas Desulfurization (FGD) purge water, cooling tower blowdown, landfill leachate, demineralizer regeneration water, ash pond effluent, coal pile runoff effluent, and wetland mitigation water. Costs to retrofit these technologies to our systems are extremely high with no guarantee that the needed reductions will be obtained with that equipment. Further, annual operation and maintenance costs are very high because of the amount of energy, water and chemicals used by these systems. Also, the fact that these systems are not designed for FGD wastewater and cooling tower blowdown may create long periods of "down-time" for maintenance and overhaul.

For example in 2010, the Conemaugh Generating Station completed a study designed to evaluate currently available and emerging Zero Liquid Discharge (ZLD) technology and processes and assess their feasibility as FGD wastewater treatment alternatives. The FGD ZLD Technology Study Report was submitted to the Pennsylvania Department of Environmental Protection (the Department) in July 2010.

The Conemaugh Generating Station is a coal-fired power generating facility consisting of two 900 MW boilers and two steam, turbine-driven generators. The Station was built in the late 1960s and is located on a 2,500-acre site along the Conemaugh River, 13 miles northwest of Johnstown in New Florence, Pennsylvania. The station burns about 4.8 million tons of Pennsylvania coal per year and operates a wet FGD system to remove SO_2.

Purge water generated from the wet FGD process is currently treated in a wastewater treatment plant (WWTP). The existing FGD WWTP is equipped with flow equalization, pH adjustment, chemical precipitation, coagulation, clarification, neutralization, ion exchange (boron removal), anaerobic biological treatment (selenium removal), aerobic biological treatment, and filtration unit processes. Although this WWTP is equipped with the latest in treatment technologies, the system does not significantly remove dissolved chlorides or sulfates as proposed under this rulemaking.

The FGD ZLD Technology Study was initiated in August 2009 and completed in June 2010. The specific ZLD technologies are listed as follows with their final assessment:

1. ZLD Blending System (Pug Mill Blending)

   This alternative was an add-on process to the existing Conemaugh wet limestone FGD system. The process relied on a pug mill mixer to blend controlled portions of dewatered gypsum cake, fly ash, quicklime and FGD purge water. The dissolved solids from the FGD absorber system would be captured in the stabilization/fixation process resulting in a blended mixture byproduct suitable for land fill disposal.

The capital cost estimate for the ZLD Blending System was $70.7MM. The annual net operation and maintenance (O&M) cost estimate was $3.84MM.

2. ZLD1 (Softening+RO+Evaporation+Crystallization)

   ZLD1 included softening, reverse osmosis (RO), falling film evaporator (brine concentrator), and crystallization/dewatering centrifuge. A byproduct consisting of dry crystals would be produced for disposal or resale.

   An initial conceptual budgetary capital cost estimate for the ZLD1 process was $111.9MM. The conceptual budgetary annual net O&M cost estimate for the ZLD1 process was $7.3MM.

3. ZLD2 (1-StageEvaporation+BrineSolidification)

   This was a commercially available evaporation process that would produce an estimated 20 gallon per minute (gpm) brine stream that would be used in the existing fly ash wetting process and disposed of in the existing onsite landfill. The concept has been modified to include lime feed or Portland cement feed to a pug mill wetting process in order to ensure byproduct stabilization.

   The capital cost estimate for the ZLD2 process was $79.6MM. The annual net O&M cost estimate was $3.59MM.

4. ZLD3 (2-Stage Evaporation+Offsite Concentrated Brine Disposal)

   This was a commercially available two stage evaporation process that would produce a 10 gpm concentrated brine stream.

   The capital cost estimate for the ZLD3 process was $73.4MM. The annual net O&M cost estimate was $9.7MM.

5. Non-Fixated Gypsum Disposal

   This alternative required the FGD absorbers to operate at chloride concentrations between 50,000 ppm and 100,000 ppm in order to maximize chloride retention within the unwashed gypsum byproduct. The chlorides are retained in the gypsum by increasing the moisture content in the unwashed gypsum from approximately 10 percent to 20 percent. The gypsum byproduct would then be landfilled.

   This alternative was determined to be technically unattractive due to significant technical and operational issues and potential environmental risks related to the corrosive effects of the potentially high chloride levels in the leachate return system, FGD system, and other exposed systems. A qualitative development of a detailed capital cost and O&M cost was not pursued for this alternative because it did not provide the technical or environmental merits to warrant further evaluation.

The information contained in the preamble of the regulation does not consider accurate costs or the technology's maturity and practical application for the electric generation industry. Furthermore, a detailed analysis of the overall impact the rulemaking could have throughout Pennsylvania is also lacking. For example, what are the capital costs and annual operation and maintenance costs associated with installing the facilities needed to treat wastewater? What are the costs for the increased monitoring required by the rulemaking? What are the costs of dealing with treatment residuals?

Finally, EPGA would like to mention that the EPA is in the process of evaluating the Effluent Limitation Guidelines (ELG) for 40 CFR Part 423 Steam Electric Generating category. A proposed rulemaking may be released in November 2012. This EPA rulemaking will likely result in new or revised permit limitations for FGD and other wastewater. When determining chloride and/or sulfate standards, the Department should consider the EPA process and whether the PA standards are warranted.

We urge the Board to work with the regulated community to calculate the full impact the regulations will have throughout the Commonwealth.

Need and Possible Conflict with Existing Regulations

The proposed criteria will target new and existing industries and are based on Iowa Department of Natural Resources chloride and sulfate standards (Iowa DNR, 2009). Iowa DNR, 2009 based its sulfate standard on studies performed by the Illinois EPA (Illinois EPA, 2006), while the chloride standard was based on an EPA-contracted study with the Great Lakes Environmental Center and the Illinois Natural History Survey (GNEC, 2008). Iowa and Illinois adopted chloride and sulfate standards to replace existing TDS standards.

According to the Rationale document for chloride, the need for chloride criteria is clearly demonstrated by the characteristics of flow back water used in gas well drilling. The rationale for sulfate criteria was that there are currently no national ambient water quality criteria for sulfate which are designed to be protective of aquatic life.

Discharges of sulfates and chlorides, which are primary sources of TDS, are already regulated under Title 25 PA Code Chapter 95 (Wastewater Treatment Requirements) that became effective on August 21, 2010. According to the preamble, the Chapter 95 rules were created to ensure the continued protection of water resources from new and expanded sources of TDS and guarantee that waters of this Commonwealth will not exceed a threshold of 500 mg/l. As a result, the rationale for the currently proposed chloride and sulfate rulemaking is flawed.

Our concern is that the requirements to meet the proposed standards for these two parameters without an equivalent environmental need or Federal mandate places Pennsylvania at a significant economic disadvantage to neighboring states.

Dissolved Oxygen Conflict with Designated Use

The proposed changes to 25 Pa. Code Chapter 93 for dissolved oxygen standards to achieve consistency with EPA's risk level assessment and associated dissolved oxygen criteria are generally supported by EPGA. EPGA supports the change from discrete minimum daily averages to 7-day averages as these standards are more representative and better capture the temporal variability in streams and water bodies. However, EPGA requests that the Department consider potential implications of higher 7-day average and minimum dissolved oxygen requirements for the DO_2 and DO_3 specifically as it relates to water bodies throughout the Commonwealth where natural stream conditions, without anthropogenic point source discharges, exist that may not meet these revised criteria.

Temperature

For the triennial review of water quality standards and rulemaking, the Department is reviewing the rate of temperature change provision in the temperature criteria found in Table 3 – which states that "...these wastes may not result in a change by more than 2°F during a 1-hour period." As stated in the published rule, "...the Board is seeking technical and scientific information, data and studies regarding the rate of temperature change and its effect on aquatic organisms. Only peer-reviewed or site-specific collections of acceptable quality will be considered."

Several literature reviews on the scientific basis of the existing temperature criterion conclude that temperature fluctuations do not appear lethal unless minimum and maximum temperatures exceed lethal limits of a fish species and do not support the existing standard. Since there is no available basis for the temperature criterion, it is appropriate that the Department review the limit.

In May, 2009, PPL Generation submitted to the Department a report entitled, "Evaluating the Seasonal Effects of Short-Term Temperature Fluctuations on Macroinvertebrates and Fish in the Susquehanna River near the Brunner Island Steam Electric Station." The Department has had the report for review since that time, and it is enclosed with these comments. The report details a carefully controlled laboratory study of the effects of temperature change on aquatic organisms that has generated a body of scientific evidence that has not been available previously. EPGA requests that the report be considered by the Department in its evaluation of a revised temperature criterion. EPGA also requests that the Department provide clarification in the temperature standard that provides permittees with the opportunity to conduct a full 316(a) variance evaluation in order to address the 2°F temperature criteria.

Aquatic Life Standard and Human Health Standard for Molybdenum

EPGA has concerns with the addition of an aquatic life standard and a human health standard for molybdenum. The Department is proposing to add molybdenum aquatic life criteria of 1,900 mg/l (continuous – CCC) and 6,000 mg/l (maximum – CMC), and human health criteria of 210 mg/l. It is our understanding that the molybdenum standards were developed as a result of a request from one regional office for only one or two National Pollutant Discharge Elimination

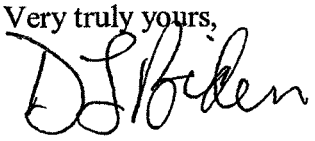
System (NPDES) discharges. This is an inappropriate foundation for developing a statewide standard.

Regarding the proposed human health criteria, our concerns are the same as those expressed by the Senate Environmental Resources and Energy Committee during the previous water quality standard triennial review. There is no drinking water standard or federal water quality standard for molybdenum. The Department is proposing the same human health criteria that were rejected by the Independent Regulatory Review Commission (IRRC) during the previous triennial review. The studies and sources cited by the Department are the same as those utilized to develop this proposed standard three years ago. Since nothing has changed regarding the sources and supporting information for this molybdenum human health standard, and because this standard was rejected by the IRRC, we question why it is being proposed again without any evaluation and inclusion of sound science.

The proposed aquatic life standards for molybdenum are based on a study performed in 2008 by Tetra Tech for the state of Nevada (Tetra Tech 2008). Since that report, a more recent aquatic life impact study of molybdenum (D.H. Heijerick, et al 2008) was published. The aquatic life criteria in the more recent Heijerick 2008 study were less restrictive than those reflected in the Tetra Tech 2008 study. The principal author of Tetra Tech 2008, Mr. Henry Latimer, reviewed Heijerick 2008 and concluded that the data provided in that report filled and completed data gaps that existed at the time of Tetra Tech 2008, and that the criteria of Heijerick 2008 would be the more appropriate criteria. Based on the lack of water quality impairment and that the proposed standard is based on a request from one regional office, the additional time for a more thorough sound scientific evaluation is very appropriate.

EPGA appreciates the opportunity to provide comments to the proposed revisions. Please contact me via telephone or email [(717) 909-3742, doug@epga.org] with any questions or concerns regarding these comments.

Very truly yours,



Douglas L. Biden, President
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Evaluating the Seasonal Effects of Short-Term Temperature Fluctuations on Macroinvertebrates and Fish in the Susquehanna River near the Brunner Island Steam Electric Station

Contribution No. 2009005

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STROUD
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A. Executive Summary

Macroinvertebrates (e.g., mayflies, stoneflies, caddisflies) and fish are poikilothermic (cold blooded) animals, meaning that an individual's internal temperature closely matches its external environment. Most, if not all, physiological, behavioral, and developmental aspects of their life history will be temperature sensitive, and respond to changes in surrounding water temperature including high or low temperature extremes that can be lethal. To protect macroinvertebrates from adverse effects of temperature changes, Pennsylvania has a water quality criterion codified in 25 Pa Code 96.6(b) and 93.7 that states: "heated wastewater discharges may not cause a change of surface water temperature of more than 2°F during any 1-hour period." The regulations allow for a site-specific alternative to this criterion if studies determine that an alternative criterion is still protective. The current NPDES permit for PPL Brunner Island, LLC (hereafter PPL) allows PPL to conduct an in-situ or laboratory study to determine a site-specific hourly temperature change criterion. PPL chose to conduct laboratory studies and contracted with the Stroud Water Research Center (SWRC) to design and implement a laboratory study focused on the hourly rate of temperature change (*i.e.*, °F/h) as the main study variable while contrasting season (cold vs. warm) into the design.

Water temperature in streams and rivers fluctuate greatly. For example, the Susquehanna River near the Brunner Island Steam Electric Station (BISES) naturally fluctuates in temperature on both a daily (~1.8 - 2.7°F per day) and seasonal (range: 32 - ~86 °F) basis. Obviously macroinvertebrates and fish that inhabit these reaches must tolerate a wide range of temperatures, and it was expected *a priori* that test parameters for each species would, in fact, change in response to diel (*i.e.*, in a 24-h period that includes a day and night) and seasonal changes in temperature that could be simulated in the laboratory.

Warm season experiments conducted by SWRC consisted of exposing macroinvertebrates and fish to a constant temperature treatment of 75°F and four fluctuating regimes ranging from 68 to 82°F on a diel basis while changing at rates of 1.5, 2, 4, and 8°F/h. The cold season experiments consisted of exposure to a constant temperature treatment of 46°F and four fluctuating regimes ranging from 39 to 53°F on a diel basis while changing at rates of 1.5, 2, 4, and 8°F/h. The SWRC study focused on sub-lethal responses in both fish and macroinvertebrates by avoiding high and low temperature extremes in the test conditions that might cause mortality.

The following seven species of fish, representing three major feeding groups in the Susquehanna River, were included in at least one aspect of the fish portion of the study: Predators [small mouth bass (*Micropterus dolomieu*), walleye (*Sander vitreum*)], bottom feeders [white sucker (*Catostomus commersonii*), channel catfish (*Ictalurus punctatus*)] and forage species [bluntnose minnow (*Pimephales notatus*), rosyside dace (*Clinostomus funduloides*), spotfin shiner (*Cyprinella spiloptera*)]. These species were also selected because they are widespread and abundant near the BISES. Stress, growth, and histopathology response for fish experiencing fluctuating and constant temperature regimes (as above) were examined for four species during the cool season (*i.e.*, bluntnose minnow, spotfin shiner, small mouth bass, white sucker), and for six species during the warm season (*i.e.*, channel catfish, rosyside dace, small mouth bass, spotfin shiner, walleye, white sucker).

1. Overall results for fish indicated that temperatures fluctuating at rates up to 8°F/h had no sublethal effect on the seven species examined.

2. Across 32 cool-season comparisons involving four fish species and eight stress-related variables only four comparisons (*i.e.*, 12.5%) conformed to our *a priori* definition of evidence for significant findings of thermal stress. Moreover, across 44 warm-season comparisons involving six fish species and eight stress-related variables, only five comparisons (*i.e.*, 11%) conformed to our *a priori* definition of evidence for significant findings of thermal stress (*i.e.*, the level of response differed significantly from responses observed in the control treatment).

3. Of the seven total species examined, two species (channel catfish and spotfin shiner) showed no indication of an increased stress response due to higher rates of temperature change across all parameters measured. A small percentage of the significant findings of thermal stress were found in four other species, where significant trends in glucose or triglycerides during the cold- or warm-season were found. One other species (white suckers) showed significant trends in cortisol and growth during the cold season.

4. However, no species showed more than two significant findings of thermal stress within a season, and the responses were consistently less than expected for severely stressed fish. Therefore temperature fluctuating at rates up to 8°F/h had no sublethal effect on the seven fish species examined.

The following 11 mayfly species were included in at least one aspect of the macroinvertebrate portion of the study: *Procloeon fragile*, *Procloeon viridoculare*, *Centroptilum triangulifer*, *Centroptilum minor, Acerpenna macdunnoughi, Cloeon cognatum, Callibaetis fluctuans, Leucrocuta hebe, Maccaffertium modestum, Ameletus ludens, and Ephemerella subvaria.* Mayflies were chosen because they are widespread and abundant in or near the Susquehanna near the BISES, they are important in the structure and function of streams and rivers, and they are proven biomonitoring tools that are moderately sensitive to environmental perturbation.

5. The results of these experiments clearly indicate that the mayflies studied generally did not respond negatively to the four rates of temperature change (*i.e.*, 1.5°F/h, 2°F/h, 4°F/h, and 8°F/h) examined, for either warm-season (68 to 82°F), or cold-season (39 to 53°F) thermal regimes.

6. Across 51 warm-season comparisons involving 5-9 mayfly species per variable measured, only five (*i.e.*, 10%) comparisons conformed to our *a priori* definition of evidence for significant findings of thermal stress. Moreover, across 20 cold-season comparisons involving four species per variable measured, only one (*i.e.*, 5%) comparison conformed to our *a priori* definition of evidence for significant findings of thermal stress (*i.e.*, the level of response differed significantly from responses observed in the control treatment).

7. Of the small percentage of significant findings of thermal stress, most involved only the 8°F/h rate. These findings were distributed among five different species – warm-season drift was *P. fragile*, cold-season drift was *C. cognatum*, warm-season egg development time was

A. macdunnoughi, warm-season growth was *L. hebe* and *M. modestum*, warm-season fecundity was *L. hebe*.

8. *L. hebe* and *M. modestum* exhibited a combination of significant and insignificant responses for larval survivorship, growth, and development time, and female fecundity that suggests these two species may be more sensitive than the other species examined to rate of temperature change or time at higher temperatures during the warm-season thermal regime.

Conclusion

The overall weight of evidence suggests that rates of temperature change at 1.5°F/h, 2°F/h, or 4°F/h did not have a general negative effect on the mayfly or fish species examined here, within the thermal ranges simulated (*i.e.*, 68 to 82°F or 39 to 53°F). Several significant responses were observed at the 8°F/h rate for certain parameters for both fish and macroinvertebrates, but the majority of these findings did not fit the *a priori* definition or pattern needed to interpret them as significant findings of thermal stress.

B. Introduction

The current NPDES permit for PPL Brunner Island, LLC (hereafter PPL) requires PPL to conduct an in-situ or laboratory study to determine a site-specific hourly temperature change criterion under 25 Pa Code Chapter 93.8(a) as an alternative to the criterion in 25 Pa Code 96.6(b) and 93.7 that states: "heated wastewater discharges may not cause a change of surface water temperature of more than 2°F during any 1-hour period." PPL chose to conduct laboratory studies and contracted with the Stroud Water Research Center to design and implement the study.

The laboratory studies focused on rate of temperature change (*i.e.*, °F/hour) as the main study variable while contrasting season (cold vs. warm) into the design. The study examined sub-lethal responses in macroinvertebrates and fish by avoiding temperature extremes in the test conditions that might cause mortality. The design acknowledged and built upon certain important facts: (i) the Susquehanna River near the Brunner Island Steam Electric Station (BISES) naturally fluctuates in temperature on both a diel (~1.8 - 2.7°F per day) and seasonal (range: 32 - ~86°F) basis; (ii) periods of temperature change are associated with thermal effluent from the plant during operation (usually a rising of temperature associated with increased power generation and a lowering of temperature due to a reduction in power generation); (iii) the timing of warming and cooling due to both natural and BISES processes is coincident with and thus exacerbates rather than reduces the diel magnitude of temperature change of the river; and (iv) macroinvertebrates and fish are poikilothermic (cold blooded) animals and most, if not all, physiological, behavioral, and developmental aspects of their life history will be sensitive and respond to any change in surrounding water temperature and certain temperature extremes will be lethal. Thus, it was expected *a priori* that test parameters for macroinvertebrate and fish species would, in fact, change in response to diel and seasonal changes in temperature. However, such changes would be deemed significant if and only if the level of response in treatments exceeding the currently permissible level of 2°F per hour differed significantly from the control treatment.

One of the major challenges in the study was to test the degree of sensitivity of macroinvertebrates and fish to different rates of diel temperature change by using a gradient of fluctuating temperature regimes whose diel minima and maxima, as well as overall diel heat accumulation (degree days > 32°F), did not result in mortality. This meant that the overall design needed to keep experimental temperature treatments slightly (*i.e.*, a few degrees Fahrenheit) cooler during warm season experiments and warmer during cold season experiments than might theoretically occur.

An important aspect of the study was the inclusion of species of fish and macroinvertebrates that were known to be widespread and abundant in the vicinity of the BISES (main river or tributaries) and that represented species sensitive to environmental change and, in the case of fish, represented the three major feeding groups (predator, bottom feeder, forage) in the Susquehanna River.

This report summarizes the experimental protocol, procedures, methods, and results of a series of experimental laboratory studies intended to test the response of several species of macroinvertebrates and fish collected from the Susquehanna River to various rates of diel temperature fluctuations.

C. Experimental Approach

I. Thermal regimes

Figures 1 and 2 schematically show the five experimental temperature treatments (over a 24 h period) that were used for the cold and warm season experiments, respectively. The five temperature treatments in a given season were designed such that animals reared in each regime accumulate the same amount of heat (degree hours > 32°F) on a daily basis. This is important because rates of function (physiological, developmental, growth, metabolism) are all temperature dependent and the experiments were designed to separate out the "rate of change" factor from the absolute "temperatures" per se in a given season. The design included four fluctuating temperature treatments in each season with the daily range set at 14°F. This magnitude was chosen for several important reasons: (i) 14°F is representative of observed diel changes in temperature level in the Susquehanna River downstream of BISES operation; (ii) a 14°F delta is wide enough to allow acclimation time at both ends of the fluctuation period each day (*i.e.*, at the upper end of a temperature rise as well as the lower end of a temperature decline) but still allow a complete cycle up and down over a 24 h period; (iii) a 14°F delta allows the maximum and minimum temperatures in both the warm and cold season to be close to but not at temperatures that would be considered lethal, limiting (below key thresholds), or impossible to maintain in a laboratory setting (e.g., minimum temperatures < 39°F). The rate of change of temperature for each of the four fluctuating temperature treatments was chosen to represent rates of change slightly below the current regulation (1.5°F/h), the current regulation (2°F/h), and two and four times the current regulation (*i.e.*, 4°F/h and 8°F/h). The constant temperature treatment for both seasons represents a control for laboratory conditions (*i.e.*, for non-temperature related factors such as flow, food, substrate, light, etc that might impact animal survivorship and/response) as well as non-linearity of rate-temperature relationships. The 1.5°F/h temperature treatment serves

as a secondary or pseudo-control in the sense that it is less than the level allowed by law and fluctuates on a diel basis much like the 2, 4, and 8°F/h treatments.

Experimental temperature treatments were maintained for several months during a given season. Specific experiments had beginning and endpoints within that experimental window. Various simulated streams containing experimental animals were fed water from reservoirs adhering to a specific temperature treatment. Warm season experiments consisted of a constant temperature treatment of 75°F and four fluctuating regimes ranging from 68 to 82°F on a diel basis while changing at a rate of 1.5, 2, 4, and 8°F/h. The cold season experiments consisted of a constant temperature treatment of 46°F and four fluctuating regimes ranging from 39 to 53°F on a diel basis while changing at a rate of 1.5, 2, 4, and 8°F/h.

II. Laboratory Rearing Systems

Five separate recirculating sytems (one for each temperature treatment) were constructed, each consisting of a rack containing 28 flow-through, (~ 20 liter) insulated plastic coolers (Coleman, Witchita, KS, USA) on the lower level for fish and 20 similar coolers plus six flow through troughs (10 cm wide, 6 cm deep and 1.5 m long) for macroinvertebrates on the upper level (see Figures 3 and 4). Each system was given its own reservoir complete with biofiltration, pump, bead filter, 6 kilowatt in-line electric water heater, heat exchanger (for cooling) and 50-watt UV sterilizer. The five heat exchangers shared a closed-loop recirculating system with a 5-ton chiller that maintained a 32°F reservoir of ethylene glycol. In-line water heaters were regulated via an SCR panel. Heating and cooling was controlled with an Allen-Bradley Programable Logic Controller (PLC) using a PC running RSLogix 5000 Enterprise Series software with a human-machine interface (HMI), InTouch-WindowViewer software. This system allowed us to control temperatures in all five systems independently to within plus or minus 0.1°F. A schematic representation of the controls is shown in Figure 5. Pop-up windows allowed for setting rate of temperature change, periods at constant maximum or minimum temperature (hereafter "soaks"), alarm values, etc. (Fig. 6). Another window enabled the user to monitor water temperature and setting trends over time (Fig. 6). Each rack system of microcosms had four platinum RTD thermoprobes to monitor temperature at various locations in the system (see Fig. 6). The probe located between the UV sterilizer and the manifold that delivered water to the microcosms was used by the PLC for control purposes. In addition, each system had one HOBO thermologger (Onset Computer Corporation) that was downloaded monthly throughout the experimental period.

For macroinvertebrate work, the water used in the experiments came from White Clay Creek (WCC), an "Exceptional Value" stream (designated by PA DEP) supporting a diverse assemblage of over 300 species of macroinvertebrates (e.g., >50 mayfly species) and 19 species of reproducing fish [including brown trout (*Salmo trutta*) and the American brook lamprey (*Lampetra appendix*)]. For fish experiments, there was concern that natural White Clay Creek stream water might introduce pathogens, so the water used in those experiments came from a well (approximately 150 feet deep, untreated) on the Stroud Water Research Center site. Fresh stream (or well) water was added to each thermal regime at a rate of about 5% per day.

Simulated daylight was provided to experimental systems by 8-foot fluorescent "gro-lites" that were controlled by a timer programmed to match the photoperiod experienced in the field for the season/dates associated with each experimental period.

Temperature was monitored continuously and free ammonia was monitored daily (Ammonia alert tag, Seachem, Madison, GA USA). Temperature, specific conductance, salinity, oxygen concentration, percent oxygen saturation, ammonia, pH, and calcium as calcium carbonate were measured weekly. Free ammonia concentrations greater than 0.2mg/L resulted in an immediate exchange of 50% of the water with fresh well water. High ammonia values were observed only in the quarantine system during the cool season. No other chemicals were ever found to be outside of the normal range in any of the experimental systems.

D. Experimental Protocol, Results, Discussion

I. Fish

A. Introduction

Most fishes are poikilotherms meaning that an individual's internal temperature closely matches its external environment (Brett 1956). Fish are sensitive to temperature change because a number of life history processes are driven by temperature including behavior, feeding, growth, and reproduction (Brett 1956). Because water temperatures fluctuate seasonally and daily, in order for fish to survive they must maintain homeostasis in a wide range of temperatures. The Susquehanna River near the Brunner Island Steam Electric Station (BISES) naturally fluctuates in temperature on both a diel (~ 1.8 - 2.7°F per day) and seasonal (range: 32 - ~86 °F) basis. Variation in the discharge of heated water from power generation can add additional levels of temperature fluctuations to that occurring naturally.

Rapid temperature changes can be stressful or even lethal to fish (Crawshaw 1979, Donaldson *et al.* 2008). To better understand the effects of temperature change on fish, it is necessary to determine the rate of temperature change that initiates a stress response. We expected, *a priori*, that test parameters for each species would change in response to diel and seasonal changes in temperature. However, such changes are significant if and only if the level of response differs significantly from responses observed in the control (constant temperature) treatment.

B. Methods

To investigate the sub-lethal response of fish to change in temperature, seven fish species (Tables 1, 2) were exposed to a constant or one of four fluctuating temperature regimes (1.5, 2, 4, or 8°F/h) for up to six weeks under cool (39°-53°F, mean 46°F) or warm (68°-82°F, mean 75°F) season conditions (Figures 1, 2). The total degree hours were equalized among regimes by adjusting the time spent at the maximum and minimum temperatures. In both seasons, all fish were acclimated to the mean temperature for two weeks prior to initiating the experiment. Sub-

lethal response was assessed by measuring the primary, secondary, and tertiary stress response (cortisol, glucose or triglyceride, respectively) after one day and again after 4-6 weeks of temperature cycling; by periodically measuring growth over 4-6 weeks of temperature cycling; and by histopathology analysis of a number of tissues after 4-6 weeks of temperature cycling (Tables 3, 4).

1. Quarantine

Fish collection targeted wild fish in the Susquehanna River, but other sources were sometimes needed (Tables 1, 2). Upon delivery to the Stroud Center, each fish species was quarantined in a separate 600 liter fiberglass, circular, flat bottom, insulated tank connected to a shared recirculating biofiltration system filled with well water. Water from the tanks passed through a particulate biofilter, Sweetwater Linear II airpump, AquaDyne Bead Filter 2.2, Clearwater Low-Space Bioreactor, and UV sterilizer (120 W, puts out 30,000 $\mu Ws/cm^2$) before being returned to the tanks. The smallmouth bass and walleye were fed live guppies (*Poecilia* sp. Aquafeed, Avondale, PA, USA). All other fish were fed 1 or 1.5 mm pellets (Melick Aquafeed Inc., Catawissa, PA, 17820, USA), or Spectrum All Purpose Formula 1mm sinking pellets (New Life International Inc., Homestead, FL, USA). Feeding occurred every day and fish were fed until consumption visibly slowed, except no feeding occurred within 24 hours of fish being anesthetized or 24 hours before, during, or after formalin treatment.

In the cold season, fish were quarantined for three months at 48°F and 10% of the water was exchanged for fresh well water each day. During the quarantine period in the cold season, all species received one treatment of formalin for 24 hours because *Ichthyophthirius* spp. was observed on some of the spotfin shiners. In the warm season, fish were quarantined for one to six weeks at 68°F. *Saprolignian* sp. fungus was observed on the spotfin shiners and this prompted three 24 hour treatments of 25 mg/l formalin for all species. Individual species were treated again as needed when fungus reappeared. At the first sign of disease in the summer the dissolved salt level was elevated to 2.4-3ppt by adding sodium chloride. The elevated salt level was maintained for the duration of the warm season quarantine period by replenishing salt following a water exchange.

2. Acclimation to experimental system

Exposure of fish to experimental temperatures was conducted in one of five recirculating systems, one for each temperature treatment (see description above and Figures 3,4). Each of the flow through coolers on these systems could accomodate up to fifteen fish of a single species. The coolers were connected to the recirculating system by in-house modifications for water inflow and sediment removal via the outflow which regulated the water volume at 13 liters. Light was provided by fluorescent bulbs above the tanks and were timed to matched ambient light cycles. Five percent of the water was exchanged for fresh well water each day. In between winter and summer experiments, all five experimental recirculating systems were cleaned with bleach (10mg/l for 24 hours) and flushed three times with well water before fish were introduced for the next experiment.

Transfer and acclimation to the experimental system proceeded as follows: Fish from the quarantine system were anesthetized in 100mg/l MS-222 (tricaine methanesulfonate, Western Chemical, Ferndale WA, USA), measured for length and weight, given one of ten unique elastomer tag color-location configurations (Northwest Marine Technologies; Olympia, WA USA), and transferred to a cooler with up to fifteen uniquely identifiable fish of the same species (Tables 1, 2).

In the cool season, fish were acclimated in the coolers for 2 weeks at constant 48°F before temperature cycling commenced. In the warm season, fish were acclimated in coolers for 3 weeks at constant 68°F before temperature cycling commenced. Feeding occurred daily or every other day until consumption visibly slowed, except no feeding occurred within 24 hours of fish being anesthetized or euthanized. During both seasons fish were fed during the peak (maximum) of the temperature cycle, or in the middle of the day if temperature did not cycle.

3. Temperature exposure

Once in the coolers, fish had one of three dispositions: short-term body chemistry after one day of cycling; long-term growth and body chemistry after 4-6 weeks of temperature cycling; or long-term growth and histopathology after 4-6 weeks of temperature cycling. Two coolers were dedicated to each disposition for each species (total of six coolers per species per recirculating system), except in the summer when five species destined for short-term chemistry were all given the same elastomer color-tag combination and divided evenly among the remaining coolers of the same species to save on space (total of four coolers per species per rack). An exception was made for the catfish which were kept in separate coolers because of their larger size (total of six coolers per rack). Cooler order was randomized on each recirculating system by species and disposition.

In the cool season, temperature cycling commenced simultaneously in all recirculating systems on the afternoon of day 0. In the warm season, temperature cycling was staggered because of the increased handling required for the additional species. Constant temperature started on day 0, 1.5°F/h and 4°F/h started on day 1, and 2°F/h and 8°F/h started on day 2, and all measurements from then on followed the same staggered pattern. Within two hours of reaching the minimum temperature at the end of one day of temperature cycling, fish destined for short-term body chemistry were euthanized in 300 mg/l MS-222, snap frozen in liquid nitrogen, placed in a plastic sandwich bag, and stored at -80°C until stress hormone and chemical assay. Growth was tracked in the remaining fish by periodically measuring length and weight in anesthetized fish (100mg/l MS-222). After 6 weeks (cool season) or 4 weeks (warm season) of diel temperature fluctuations, fish destined for body chemistry were euthanized, snap frozen and stored at -80°C. Fish destined for histopathology were euthanized and fixed in 10% neutral buffered formalin for two days, then removed from the formalin, vacuum sealed, and shipped to North Carolina State University (NCSU) for analysis.

4. Stress response and growth

Traditionally stress hormones have been measured directly in blood. In small fish, however, collecting sufficient blood to measure more than one chemical would not be possible without pooling blood from multiple individuals. However, pooling individuals reduces the power to detect significant changes in the stress response. We chose, therefore, to assay chemicals directly in whole body homogenates, which allows for multiple chemicals to be assayed within the same individual (Pottinger *et al.* 2002).

Whole body homogenates of fish less than 8 grams were prepared as in Bennett *et al.* (2007). Briefly, partially thawed fish were minced with a razor, transferred to a 20 ml scintillation vial containing 2:1 (v:w) nanopure water, and homogenized with an Ultra-Turrax 18/60. Fish larger than 8 grams were partially thawed, minced with a razor and homogenized in 2:1 (v:w) nanopure water using an Omni Mixer (Sorvall, Newton, CT, USA 06470) reserving enough homogenate to fill a 20 ml scintillation vial and discarding the rest. Two 500 ul aliquots of the homogenate were taken in 1.5ml microtubes using a 1000 ul pipet tip trimmed to increase the bore. The aliquots were centrifuged at 8000 g for 10 min. One of the homogenate aliquots was used for cortisol determination and the other for glucose and triglyceride determination.

Cortisol was measured directly in whole body homogenate using a competitive immunoassay as in Sink et al. (2007), without extracting the homogenate (Cortisol Enzyme Immunoassay Kit #901-071, Assay Designs, Ann Arbor, MI USA). Preliminary comparisons between diethyl acetate extracted homogenate and unextracted homogenate yielded similar measures of cortisol concentration, and so the unextracted homogenate was used. Glucose concentration was measured directly in aliquots of whole body homogenate using the Trinder method as in Pottinger et al. (2002), an enzyme based colorimetric determination (Glucose Assay Kit #GAGO-20 by Sigma-Alridch). Triglyceride concentration was measured directly in unextracted homogenate using a two step enzyme based colorimetric determination as in Bennett et al. (2007), but without the addition of sodium citrate or heating the homogenate (Serum Triglyceride Determination Kit #TR0100, Sigma-Aldrich). True triglyceride concentration was measured by first measuring unincorporated glycerol prior to measuring the glycerol resulting from enzymatic digestion of triglycerides. We found that sodium citrate increased the coefficient of variation of the measurements and therefore did not use it. All samples were run in duplicate in 96 well plate format and compared to standards on the same plate. All three assays produced a color change proportional to concentration, and absorbance at the appropriate wavelength was measured using a Spectramax M2 spectrophotometer (Molecular Devices). Samples producing concentrations above the range of the standard curve were diluted and re-assayed. Samples with a coefficient of variation above 10% were also re-assayed.

All enzymatic assays had been previously validated in fish but we choose to conduct our own validation with our species and conditions. All assays were validated in advance in three fish species and all samples were run in triplicate. Validation followed the 'Validation of Analytical Procedures: Methodology' (FDA CVM 1999). Accuracy was tested by spiking samples with a known quantity of the appropriate target chemical. The precision was tested by comparing replicate samples and by serial dilutions (0, 25, 50, 75 and 100%) made by adding assay buffer (cortisol) or water (glucose and triglyceride) and calculating the coefficient of variation (%CV). Linearity was tested using the serial dilution extracts. Spiked and diluted sample recovery of 85-

115% were defined as meeting validation criteria. A %CV of ≤10% was set as the acceptable limit. An R^2 of ≥0.90 was set as the passing limit for the linearity test.

Growth was measured in each individual as the slope of the line fit to the percent change in weight or length from day 14 (cool season) or day 0 (warm season) to the end of the experiment.

We used analysis of variation (ANOVA) to test for significant differences among temperature treatments for each stress response or growth rate within each species and season. First, we used a two-level nested ANOVA treating temperature change as a factor and controlling for differences between replicate tanks in the same treatment (*i.e.,* tank effect). When tank effect was not significant we repeated the ANOVA dropping the tanks term and used a post hoc Tukey's test to identify significantly different stress responses among the treatments. When tank effect was significant we used a one-way ANOVA to compare tank means among treatments and then conducted a post hoc Tukey's test to identify significantly different stress responses among the treatments. Significant differences among treatments were investigated to determine if differences followed a pattern suggesting that higher rates of temperature change increased the stress response relative to constant or lower rates of temperature change.

5. Histopathology

Fish specimens for histopathology analysis were fixed in 10% buffered formalin, vacuum-packed and shipped to Dr. Mac Law at the North Carolina State University (NCSU). Fish were inventoried according to Chain of Custody, ordered, and divided into groups. Sealed packages were opened one at a time and the external condition of the fish was examined. Any lesions, discoloration, or injuries were noted on a data sheet and sampled for histology.

For smaller fish (less than about 6 cm), the tail was removed and the entire fish was placed in a histology cassette. For small but wide fish, one wall of the body cavity was removed (ribs and muscle tissue) and the muscle dorsal to the spine was sometimes removed. The entire cavity and head were always within the same cassette. Whole fish were then decalcified in 10% formic acid for 24-72 hours.

For midsized fish, the head was removed, split down the center, and placed in one cassette, and the remainder of the body was placed in another cassette after the tail had been removed.

For larger fish individual organs were removed and placed in cassettes. The body cavity was opened and all major tissues were sampled, if present. This includes anterior kidneys, heart, esophagus, gastrointestinal tract, pancreas, liver, spleen, and swim bladder. In some of the larger fish gall bladder, posterior kidneys, urinary bladder, ureters, and gonads were also removed. These organs were placed in a cassette together and stored in 10% neutral buffered formalin until processed by the histology lab. One gill arch was sampled and a cross-section of muscle and skin was excised from the caudal peduncle. These samples were decalcified in 10% formic acid for 24-72 hrs and then stored in 10% neutral buffered formalin until histology.

Once samples were in cassettes and decalcified if necessary, they were transferred to the in-house histology lab on the NCSU campus of the College of Veterinary Medicine. They were

routinely processed in an automated tissue processor, embedded in paraffin blocks, sectioned at approximately 5 microns, mounted onto glass slides, and stained routinely with hematoxylin and eosin. Slides were ordered and cataloged, then examined microscopically by Dr. Law.

Microscopic examination was performed by Dr. Law using a light microscope. Each season five fish of each species taken just before the experiment began were examined to set a baseline range of "normal" for each species. All major organs and tissues were examined for abnormalities. Parasites found in microscopic sections were identified to general category (nematode, trematode, etc.). Observations were made on each fish and entered into the project data sheets. These were qualitative or descriptive assessments of lesions on each individual animal for any variations from normal.

C. RESULTS

Fish survival was very high during the experiments with a few exceptions (Tables 1, 2). There were isolated incidents of cannibalism among the smallmouth bass during the warm season despite feeding them to satiation every day. The white suckers also suffered uniformly high rates of mortality during the warm season. Observations made by Dr. Law showed the presence of tremotode or protozoan parasites which left too few fish to conduct the short term experiments. It is clear that these parasites were present when the fish were collected as mortality began during quarantine and was found across all temperature regimes and the control.

1. Stress response

All assays fell within our validation criteria for percent recovery, linearity and coefficient of variation (Tables 5 to 13).

We expected that stressful temperature changes would lead to an increase in cortisol concentrations compared to the control temperature regime (Wendelaar Bonga 1977). Short term and long term cortisol concentrations differed significantly among treatments in 11 out of 19 tests in both the cool season (Figure 7) and warm season (Figure 8), however only the short term cortisol response in white suckers during the cool season appear to be affected by higher rates of temperature change. In no other species did cortisol appear to increase in response to the treatment. In all cases, though, the cortisol concentrations were low during both seasons (<9 ng/ml).

Glucose is a secondary stress chemical that increases in response to most stresses (Silbergeld 1974), other than starvation (Pottinger *et al.* 2002). We expected that stressful temperature changes would increase metabolism, which would lead to an associated increase in glucose concentrations, compared to the control temperature regime (Wendelaar Bonga 1977). Short term and long term glucose concentrations differed significantly among treatments in 11 out of 19 tests in both the cool season (Figure 9) and warm season (Figure 10), but only in 6 instances did the differences appear to be related to the treatment. During the cool season, it appeared that increasing the rate of temperature change to 8°F/h increased glucose concentrations in

smallmouth bass and bluntnose minnow in the long term compared with the control (Figure 9). During the warm season it appeared that higher rates of temperature change (*i.e.,* 8°F/h) had increased glucose concentrations in walleye in the short and long terms and in smallmouth bass and rosyside dace in the long term (Figure 10). Significant differences in glucose concentrations in other species may have been a statistical artifact or were caused by something other than temperature change given that they did not fit our *a priori* definition of evidence for a significant finding of thermal stress.

Triglycerides are used as an energy reserve that decreases in response to prolonged stress (Weber *et al.* 2003). We expected that stressful temperature changes would lead to a decrease in triglyceride concentrations compared to the control temperature regime. Short-term and long-term triglyceride concentrations differed significantly among treatments in 7 out of 19 tests in both the cool season (Figure 11) and warm season (Figure 12), but only in one instance did the differences appear to be related to treatment. During the cool season there was no indication that increasing rates of temperature change affected triglyceride concentration (Figure 11). During the warm season the rate of temperature change appeared to significantly decrease triglyceride concentrations in smallmouth bass in the long term (Figure 12). Significant differences in triglyceride concentrations in other species may have been a statistical artifact or were caused by something other than temperature change.

2. Growth

Growth inhibition is a sensitive indicator of fish health suggesting that stress adaptation has reallocated energy toward activities required to restore homeostasis (Wendelaar Bonga 1997). In the cool season temperature cycling increased the growth rate compared with the constant temperature control for white suckers, bluntnose minnow and spotfin shiners (Figure 13). One exception was for white suckers, where growth rate at the highest rate of temperature change (8°F/h) did not differ significantly from the control and was significantly lower than for the other fluctuating temperature regimes (1.5, 2, and 4°F/h). In the warm season temperature cycling had no effect on the growth rate for any species, although our inability to mark white suckers and mortality biased towards smaller fish prevented tracking this species (Figure 14).

3. Histopathology

Histopathology analysis revealed that the fish used in these experiments were generally healthy. During the cool season all species contained some granulomas, parasites, or pigmented macrophage aggregates, but there was no apparent treatment-related lesion trend (Figure 15). In the warm season there were incidences of parasites in the viscera, parasites in the gills, and granulomas observed in the viscera of smallmouth bass and channel catfish (Figure 16), but again there was no apparent treatment-related lesion trend. In the warm season, walleye, white sucker, rosyside dace and spotfin shiner were found to have only very low instances of lesions, which were not graphed.

Most fish had normal tissues such as head, gill, liver, intestinal tract, etc (Figures 17, 18). Some fish from both control and treated groups had granulomas caused by parasites, which is a

common finding in wild source fish and is not considered a confounding factor in this study. There were additional incidences of liver vacuolation, possibly due to diet (Figure 19), deep tissue parasites encysted in the bile ducts (Figure 20), intestinal flukes (Figure 21), and multiple granulomas in the same organ (Figure 22). These observations were of low incidence and were not graphed. They were not considered to have affected the experiment.

II. Aquatic Insect Studies

A. Introduction

Based on results of a field trip to Susquehanna River near the BISES in June 2007, as well as field records from previous studies near there, we settled on the following eleven species for the macroinvertebrate portion of the study: *Procloeon fragile*, *Procloeon viridoculare*, *Centroptilum triangulifer*, *Centroptilum minor, Acerpenna macdunnoughi, Cloeon cognatum, Callibaetis fluctuans, Leucrocuta hebe*, *Maccaffertium modestum, Ameletus ludens, and Ephemerella subvaria*. These eleven mayflies were chosen because, as a group, they: (i) are widespread and abundant in or near the Susquehanna near the BISES; (ii) are moderately sensitive to environmental perturbation; (iii) span the entire gamut of habitat types in the river (marginal [edge], pool, riffle); (iv) represent at least four different phylogenetic lineages; (v) exhibit an array of life history characteristics (growth rates, emergence times, adult sizes, fecundity, etc); (vi) have hemimetabolous life cycles which facilitates whole life cycle testing with a definitive end point (*i.e.*, adult emergence); and (vii) unlike most other macroinvertebrate groups, measurements of adult size and fecundity completely reflect environmental conditions (e.g., thermal regime) of the larval stage. Other non-mayfly taxa were considered but rejected because either: (i) we could not identify them with certainty to species at all sizes and life stages; (ii) they were univoltine and hence available only during one season for experiments; (iii) they do not do well in respirometer experiments (based on personal experience); (iv) they are holometabolous —so pupation marks the end of the larval growth period (stage whose onset is difficult to monitor experimentally); or (v) adult size and fecundity reflect conditions in both the larval, pupal, and adult stages which confounds the use of those parameters as response variables to the various treatments.

Tables 14 and 15 show all eleven species and the range of studies performed for each during both warm and cold season. For species that were reared to the adult, we also collected data for the timing of adult emergence, adult size and fecundity.

B. Methods

1. Larval Drift

Macroinvertebrate drift is a natural phenomenon that can be stimulated or induced to unnatural levels by disturbance. Here we used the magnitude of drift as an indication of stress and/or disturbance. Laboratory experiments were carried out to test the mayflies' propensity to drift when exposed for the first time to various rates of fluctuating temperatures. Drift was measured in small simulated streams (troughs, 10 cm wide, 6 cm deep and 1.5 m long) provided

continuously with stream water (average rate of discharge = 170 ml/s) that were subjected to various diel rates of temperature change as described earlier. At least two (in most cases three) replicate experimental troughs for each of the five temperature treatments (one constant and four fluctuating) were tested for each species/season combination. Streams were fitted with acrylic plates coated with stream periphyton as food for the test animals and 500 μm mesh bags over the outflows to capture drifting insects.

For all warm season experiments, animals were reared from eggs in the laboratory at a constant 68°F for several weeks prior to the experiment until they reached a suitable size (about 2/3 to nearly full grown larvae). At 1800 h on day 1 of the experiment 100 to 125 individual mayflies (depending on species) were transferred to the head of each trough. Individuals destined for the constant 75°F treatment were gradually stepped up from 68 to 75°F over several hours first. Animals were then allowed to acclimate overnight as temperatures in the experimental system were held constant (75°F for the control, 68°F for the treatments) (Fig. 23). At 0400 h on day 2 drift nets were emptied, drifting larvae enumerated, and living larvae replaced at the head end of each trough. At 0600 h all fluctuating thermal regimes began their temperature rise and another drift sample was taken. Larvae in this and all subsequent samples were enumerated and removed from the experiment. At 1800 h on day 2 temperatures began their fall in the fluctuating treatments. Drift was sampled from all troughs at 11 points during the experiment corresponding with the onset and completion of temperature rise and fall for each treatment, with the last sample being taken at 0320 h on day 3 (Fig. 23)

Cold season drift experiments were done in a similar fashion for two species (*C. cognatum* and *C. triangulifer*), except that larvae were initially reared at 60°F and then stepped down to 39°F (46°F for the control group) over the 24 h prior to the drift experiment. For cold season experiments on *E. subvaria* and *A. ludens*, larvae were field collected and transferred to troughs the same day. Figure 24 shows the sampling schedule for cold season drift experiments.

2. Egg Development Time and Hatch Success

Egg masses were obtained from females allowed to oviposit after artificial mating under laboratory conditions (or without mating, in the case of parthenogenetic species). The duration of egg development and % hatch was quantified by incubating eggs in glass museum jars (4.5cm deep; 3.5 cm diameter) containing about 10 ml of filtered (0.45 μm), sterilized stream water (Sweeney and Vannote 1984). Jars were submerged in the water kept at the various experimental regimes. Replicate egg masses were incubated at each thermal regime. Eggs were inspected daily for hatching. Newly hatched larvae were removed and counted daily to determine the frequency distribution of egg hatch for each mass. At the end of the experiment hatched vs. unhatched eggs were enumerated under the microsope for each jar.

3. Larval Survivorship, Growth, Timing of Adult Emergence, Adult Size and Fecundity

Two different larval rearing chambers were used for these experiments depending on the species. For species with faster water flow requirements, we used flow-through coolers [28 x 45 x 28 cm

deep; 13-L (3.5 gal)] provided continuously with stream water kept at the various specific temperature regimes (*sensu* Sweeney *et al.* 1986). For species without flow requirements, we used 1.9-L glass jars containing 1.5-L of aerated stream water and partially submerged in water baths associated with the various temperature regimes (*sensu* Sweeney *et al.* 1993). All test species were herbivores and were fed algae (mainly diatoms). Algae were cultured on the surface of clear acrylic plates [64 x 228 x 1.6mm (2.5 x 9.0 x 0.06 inches) thick] in an artificial stream system enclosed in a greenhouse at the Stroud Center. One or more of these plates were placed in the rearing vessels as needed during all growth experiments to keep food levels non-limiting.

For all experiments, we introduced 35 – 50 or more (depending on species and availability) small larvae (first instars in the case of laboratory-cultured species) into each rearing vessel. There were three to five replicate tanks (or jars) of larvae per temperature regime, depending on species. In the case of field-collected larvae, the initial size distribution in each vessel was characterized by removal of a random subsample of about 10 larvae to be dried and weighed (nearest μg). For warm season experiments, larvae were fed and maintained at the various test temperatures until they emerged as adults (generally from 30 – 50 days). For cold season experiments, larvae were either reared to the adult stage (in the case of *A. ludens*) or the experiment was terminated after a minimum of 30 days (the other cold season species) by removing, counting, and dry-weighing all remaining larvae. All species in the warm season experiments were reared from first instar larvae (hatchlings) to the final adult stage. Survivorship was measured as the proportion of individuals surviving to adulthood (or for the duration of the experiment in the case of most cold season experiments). Growth was measured as average adult or larval dry mass at the end of the experiment. The time of emergence (and thus the duration of larval development) was also recorded for each individual mayfly reared to adult.

The relationship between adult dry mass and fecundity of females of species reared to adult was determined for *L. hebe, C. triangulifer, C. minor, P. fragile, P. viridoculare* and *A. macdunnoughi* by dissection and enumeration of eggs from females whose dry mass had already been determined. For *A. ludens* and *M. modestum* we used existing data from our laboratory on this relationship. Fecundity of *C. cognatum* and *C. fluctuans* females was not determined because unlike most other mayfly species, females of these two species emerge with their eggs in and early stage of development and do not complete egg development for another 9 (in the case of *C. fluctuans*) or 19 (*C. cognatum*) days after emergence. However, like other mayflies, adults of these species do not feed, and thus the relationship between dry mass at emergence and their ultimate fecundity is likely similar to the other mayfly species tested here.

4. Larval Metabolism (Respiration Rate)

Metabolism was measured as oxygen uptake of larvae in a differential respirometer (Gilson 1963). Larvae spent 3-4 weeks in their respective fluctuating or constant thermal regimes prior to respiration experiments. Larvae were removed for experiments at a point in the fluctuating diel cycle where they reached the mean (or control) temperature, *i.e.*, 75°F in the warm season; 46°F in the cold season. Respiration runs were then conducted at these two temperatures.

For a given test at a specific temperature, individual larvae or small groups (n = 2-4) of similar sized small larvae were placed in individual test vessels with 7 ml of filtered (0.45µl) stream water. KOH (~0.5 ml) was placed on crinkled filter paper in a side arm to absorb carbon dioxide. About 20 test vessels containing a wide range of larval sizes for the species were used in each experiment. Larvae were allowed to acclimate to vessel conditions for 1 hour before measurements are taken. Oxygen uptake (µl O_2) was measured at 30 min intervals for two to three hours to characterize respiration rate at a given temperature. At the end of the experiment, larvae were sacrificed, dried at 122°F for 24 hours, and weighed (nearest µg). Regressions of weight specific respiration rate versus larval dry mass were used to describe the level of respiration for the species at a given temperature.

5. Data Analysis

Data analysis varied somewhat among study parameters as described in (Table 16). Additional statistical information is given below in the foreword of each results section.

C. Results

1. Drift

a. Foreword

It is well known that aquatic insect larvae in streams and rivers often periodically release their grasp or attachment to bottom substrates and "drift" (*i.e.*, allow the current to take them downstream some distance before reattaching to the substrate). This behavior can be part of a natural diel cycle (e.g., species often exhibit drift at sunrise or sunset) or in response to environmental change (e.g., change in temperature, presence of a predator).

In this study, we used drift as a behavioral response that was a short-term indicator of thermal stress. Stress was indicated if the levels of drifting larvae increased when diel temperatures were rising from the low temperature or falling from the high temperature (*i.e.*, exclusive of times such as sunrise or sunset). The sequence of analysis was as follows. We first tested with ANOVA if there was significant change in the "total cumulative number of drifting individuals" (hereafter "total drift") among the five temperature treatments (constant, 1.5, 2, 4, or 8°F/h). Here the total refers to combined drift for sample periods 1 to 10 in Figure 25. If that ANOVA was not significant, then the overall experiment was classified as "Not Significant" and the immediate conclusion was that there was no "significant finding of thermal stress" or SFTS. If the ANOVA was significant, then we performed the following Post Hoc Tukey multiple range tests (hereafter "Tukey"): (i) Tukey test of total drift (samples 1 to 10 of Figure 25) across all five experimental temperature treatments to see if total drift changed significantly in one or more of the fluctuating thermal treatments (1.5, 2, 4, or 8 °F/h) relative to the control (constant); (ii) Tukey test on drift levels during sample periods 2-5 and 6-10 of Figure 25 to determine whether, in fact, the observed change in total drift for a given treatment was caused by a higher number of drifting individuals during broad periods of either rising or falling temperatures (as opposed to

low soak periods at sunrise or sunset); (iii) Tukey test on drift levels during the first sampling period after temperatures began to rise (sample 2 of Figure 25) or fall (sample 7 of Figure 25) in the fluctuating temperature treatments. If the change in drift was associated with a period of rising or falling temperatures, then it was interpreted as a "significant finding of thermal stress" or SFTS if, and only if, the following criteria were met: (i) the finding was due to increased drift (rather than a decrease); and (ii) the finding was associated with the 8°F/h fluctuating regime alone or represented a consistent / gradient response that included either the 4 and 8°F/h or the 2, 4, and 8°F/h regimes relative to the constant temperature.

b. Summer (Warm Season) Drift

We studied drift behavior in five mayfly species during the summer (warm season): *C. triangulifer, C. minor, C. cognatum, C. fluctuans,* and *P. fragile*. One SFTS was observed (*P. fragile;* Figure 26) where drift levels increased significantly at the outset of falling temperatures in the 8°F/h treatment relative to the constant (as well as the 1.5°F/h) treatment. For two species, *C. triangulifer* (Figure 27) and *C. minor* (Figure 28), drift levels were very low throughout the experiments. For *C. cognatum*, drift levels were high (Figure 29) but reflected a natural response to sunrise (lights on). Drift levels were also high for *C. fluctuans* (Figure 30) but reflected a natural response to both sunrise and the fact that food (algae) became limiting (as reflected by algal samples) in the experimental system near the end of the experiment for the constant, 1.5, and 2°F/h treatments.

c. Winter (Cold Season) Drift

We studied drift behavior in four mayfly species during the winter (cold season): *C. cognatum, E. subvaria, A. ludens,* and *C. triangulifer* (Figures 31, 32, 33, and 34 respectively). One SFTS was observed (*C. cognatum*) where drift levels increased significantly at the outset of falling temperatures in the 8°F/h treatment relative to the constant (although not the 1.5°F/h) treatment. For the other three species, significant increases in drift was observed for certain fluctuating temperature treatments for two of the species (*E. subvaria* and *A. ludens* but not *C. triangulifer*) but was either associated with sunrise (*E. subvaria*) or the constant period of high temperatures (*A. ludens*) as opposed to periods of rising of falling temperatures.

2. Egg Development Time and Hatch Success

a. Foreword

Egg development time and hatch success are important life history characteristics for each species of aquatic insect. Both parameters are known to be sensitive to changes in ambient temperature. Moreover, egg development time and hatch success can only be studied during the summer (warm season) because aquatic insects do not reproduce during the winter. We studied five species: *C. minor, C. triangulifer, A. macdunnoughi, P. fragile,* and *P. viridoculare*. Four of them were parthenogenetic (all except *P. fragile*) which is advantageous because degree of fertilization becomes a non-issue across treatments. For *P. fragile*, females were mated artificially in the laboratory.

For both development time and egg hatch success, ANOVA was performed on the data to determine if either development time or hatch success changed significantly in the fluctuating temperature treatments relative to the constant treatment. If that ANOVA was not significant, then the overall experiment was classified as "Not Significant" or NS and the immediate conclusion was that there was no "significant finding of thermal stress" or SFTS. If the ANOVA was significant, then we performed a Post Hoc Tukey multiple range test across all five experimental temperature treatments to see if development time or hatch success changed significantly in one or more of the fluctuating thermal treatments (1.5, 2, 4, or 8°F/h) relative to the control (constant). A significant change was interpreted as a "significant finding of thermal stress" or SFTS if, and only if, the following criteria were met: (i) the finding was an increase or decrease in development time or a decrease in hatch success; and (ii) the finding was associated with the 8°F/h fluctuating regime alone or represented a consistent/gradient response that included either the 4 and 8°F/h or the 2, 4, and 8°F/h regimes relative to the constant temperature.

b. Summer (Warm Season)

For hatch success, none of the five study species exhibited a significant finding of thermal stress (SFTS) (Figure 35). For four of the species (*C. minor, C. triangulifer, A. macdunnoughi,* and *P. fragile*), hatch success was nearly 100% across all treatments and the initial ANOVA was not significant. For one species (*P. viridoculare*), the ANOVA was significant but the Tukey test revealed that egg hatch decreased significantly in the 4°F/h treatment but not in the 8°F/h treatment relative to the constant treatment. Hence, we concluded that it was not a SFTS.

For egg development time, one SFTS was observed (*A. macdunnoughi*; Figure 35) where median egg hatch time increased significantly (by about 1.5 days) in the 8°F/h treatment relative to the constant (as well as the 1.5°F/h) treatment. For three of the other species (*C. minor, C. triangulifer,* and *P. viridoculare*), median egg hatch time varied little across the various tempertature treatments. We did observe a significant (ANOVA) variation across treatments for *P. fragile* with the 8°F/h eggs taking significantly longer to develop than in the 1.5°F/h treatment but not the constant treatment. Hence, we concluded that it was not a significant finding of thermal stress (SFTS).

3. Larval Survivorship, Growth, Timing of Adult Emergence, Adult Size and Fecundity

a. Foreword

Larval Survivorship, Growth, Timing of Adult Emergence, Adult Size and Fecundity are all critical life history parameters for aquatic insect populations. All parameters are known to be sensitive to changes in ambient temperature. Eleven species were involved overall in studies of these parameters but not all parameters were studied on each species or for each season (Tables 14, 15). The eleven species were: *M. modestum, C. fluctuans, C. minor, C. triangulifer, C. cognatum, P. fragile, P viridoculare, A. macdunnoughi, L. hebe, A. ludens, E. subvaria.* For two

of the species involved in the winter experiments (*A. ludens* and *E. subvaria*), we used previously developed linear regressions to relate fecundity and adult female size (Sweeney unpublished data). Also, because of the slow growth of larvae and difficulty completing adult emergence during the winter, we were unable to grow any species from newly hatched first instar larvae through to the adult and thus do not have data for larval development time analogous to the summer experiments.

For all parameters, an ANOVA was performed on the data to determine if the parameter changed significantly in the fluctuating temperature treatments relative to the constant treatment. If that ANOVA was not significant, then the overall experiment was classified as "Not Significant" or NS and the immediate conclusion was that there was no "significant finding of thermal stress" or SFTS. If the ANOVA was significant, then we performed a Post Hoc Tukey multiple range test across all five experimental temperature treatments to see if development time or hatch success changed significantly in one or more of the fluctuating thermal treatments (1.5, 2, 4, or 8°F/h) relative to the control (constant). A significant change was interpreted as a "significant finding of thermal stress" or SFTS if, and only if, the following criteria were met: (i) the finding was an increase or decrease in survivorship or duration of the larval growth period (aka timing of adult emergence) or a decrease in adult size or fecundity; and (ii) the finding was associated with the 8°F/h fluctuating regime alone or represented a consistent/gradient response that included either the 4 and 8°F/h or the 2, 4, and 8°F/h regimes relative to the constant temperature.

b. Summer (Warm Season)

(1) Larval Survivorship

For larval survivorship, differences were observed (*M. modestum*; Figure 36) where larval survivorship decreased significantly (by about 40%) in the 2 and 8°F/h treatments (but not the 4°F/h) relative to the constant treatment. This inconsistent response was not considered evidence of a SFTS. For the other eight test species, larval survivorship did not change significantly (ANOVA) across the five temperature treatments (Figure 37).

(2) Larval Development Time

For larval development time, *L. hebe* (Figure 38) was observed to have decreased development time (by about 6 days) in the 1.5 and 8°F/h (but not the 2 or 4°F/h) treatments relative to the constant treatment. This inconsistent response was not considered evidence of a SFTS. For one species (*C. cognatum*), development time varied significantly (ANOVA) across the five treatments and although development time for the 8°F/h treatment was significantly longer than for the 1.5, 2, and 4°F/h treatments, it did not differ significantly from the constant treatment. Moreover, the amount of variation across all treatments in development time was less than one day. Hence, we concluded that it was not a significant finding of thermal stress (SFTS). For the remaining six species tested (*A. macdunnoughi, C. minor, C. triangulifer, C. fluctuans, P. fragile,* and *M. modestum*), larval development did not change significantly (ANOVA) across the five temperature treatments (Figure 39).

(3) Larval Growth, Adult Size, and Fecundity

Two species exhibited an SFTS for larval growth (*L. hebe* and *M. modestum*; Figure 40). For *L. hebe*, growth decreased significantly as indicated by significantly smaller adults (by about 50%) in the 8°F/h treatment relative to the constant (and 1.5°F/h) treatment. Moreover, for *M. modestum*, all larvae died in the 8°F/h and adult females were about 50% smaller in the 4°F/h resulting in an SFTS for that treatment. For the remaining six species (*C. cognatum, P. fragile C. fluctuans, A. macdunnoughi, C. minor,* and *C. triangulifer*), three of which are parthenogenetic (*A. macdunnoughi, C. minor,* and *C. triangulifer*) and thus lack males, neither female adult size (hence female larval growth) nor male adult size (where applicable) changed significantly (ANOVA) across the five temperature treatments (Figures 41 and 42).

We quantified the relationship between fecundity and female adult size (dry mass) for six of the eight species included in the growth experiments (*L. hebe, P. viridoculare, P. fragile, C. minor, C. triangulifer, A. macdunnoughi*). The relationship was significant and linear for all species (Figures 43 and 44). For five of the species, there was no difference in adult female size across the five temperature treatments and so there is no difference for fecundity as well. Similarly, fecundity (as well as adult female size) for *L. hebe* was significantly lower in the 8°F/h treatment relative to the constant temperature treatment (Figure 43). Hence, one out of six species exhibited a SFTS and it was associated with the 8 °F/h treatment.

c. Winter (Cold Season)

(1) Larval Survivorship

For larval survivorship, none of the four species (*C. triangulifer, C. cognatum, A. ludens,* or *E. subvaria*) tested exhibited a significant (ANOVA) change across the five temperature treatments (Figures 45 and 46). For two of the species (*C. triangulifer and A. ludens*), we performed the experiments starting with small as well as large (or half grown) larvae. In either case, the results were the same (not significant).

(2) Larval Growth, Adult Size, and Fecundity

Larval growth (as indicated by final adult size) varied significantly (ANOVA) for two species across the five temperature treatments during the winter (*C. cognatum and C. triangulifer*; Figure 47). However, the Tukey tests revealed that, although the growth changed significantly in the 4 and 8°F/h treatment for *C. triangulifer* and the 8°F/h treatement for *C. cognatum* relative to the constant temperature treatment, in both cases the fluctuating temperature treatments had a positive effect on growth (hence adult size). Hence, we concluded that neither was a significant finding of thermal stress (SFTS). For the other two species, *A. ludens* and *E. subvaria*, no significant change in larval growth (adult size) was observed across the five treatments (Figure 48).

For fecundity, the relationship is already shown for *C. triangulifer* (Figure 44). For *C. cognatum*, it is a live bearer (as opposed to an egg layer) and hence fecundity was not measured.

For the others, we used the following linear regressions developed previously: *A. ludens* [No. eggs per female = 251.3 (female dry wt in mg) – 169.7] and *E. subvaria* [No. eggs per female = 59 (female dry wt in mg) + 143]. Given the linear relationship between fecundity and female size, there was no SFTS for fecundity for any of the three species tested (*i.e.*, *C. triangulifer, A. ludens*, and *E. subvaria*).

4. Larval Metabolism (Respiration Rate)

a. Foreword

Larval metabolism, as measured by weight specific respiration rate, is one of the major components in the energy budget of every aquatic insect. Metabolism is highly sensitive to stress in most animals, with higher metabolic rates associated with more stressful conditions. Metabolism is also temperature sensitive in all aquatic insects due to their poikilothermic (cold blooded) nature. It is also known that cold blooded animals can acclimate to temperature (and stress) such that metabolism gradually adjusts to compensate for changes in environmental temperature (or condition). Within this project, we are not interested in quantifying to what extent aquatic insects can or cannot acclimate. However, we are interested in whether, after exposure to repeated temperature fluctuations of various magnitudes, individuals of the same species exhibit the same basal metabolism at a given temperature. In this context, we interpret any elevation of basal metabolic rate at a given temperature over rates associated with control conditions as an indication of stress.

We studied basal matabolism for seven species: *C. minor, C. triangulifer, C. cognatum, P. fragile, P. viridoculare, A. ludens, and E. invaria. C. triangulifer* and *C. cognatum* were the only species studied during both the summer and winter season (Tables 14 and 15). For each species, we selected small, medium, and large larvae from on-going larval growth experiments in each of the five temperature treatments during a given study season (summer, winter). Regardless of the location of origin of a given group of study larvae (*i.e.*, the temperature treatment), metabolism was measured on all larvae at the constant temperature for that particular season (e.g., at 46 and 75°F for the winter and summer seasons). Using a Gilson Differential Respirometer, respiration rates would be measured over a 3-5 hour period for each group of larvae of a given species removed from a specific temperature treatment. The raw data for a given experiment would be the weight specific respiration rate (μg l O_2/mg dry mass / hour) and the total dry mass for each of the individuals involved. The raw data were log transformed and individual respiration rate was regressed as a function of individual dry mass. For each species studied in a given season, the experiments would produce five individual regressions (one for each temperature treatment; e.g., see Figure 49 for *A. ludens* during the winter season). For each species, the five regressions were compared via analysis of covariance to determine if slopes and intercepts differed significantly. A significant deviation of slope or intercept for the 8°F/h or both the 8 and 4°F/h temperature treatment relative to the constant treatment was taken as a "significant finding of thermal stress" or SFTS.

b. Summer (Warm Season)

There was one significant finding of thermal stress during the summer season. One of the five species (*P. viridoculare*) tested exhibited a significant (ANCOVA) across the five temperature treatments (Table 17). Both the slope and the intercept for this species in the 8°F/h treatment were significantly different from the constant temperature treatment. This means that, for this species, larvae in the 8°F/h rearing system exhibited a significant change in both the relationship between metabolism and size as well as the absolute level of metabolism. The other four species tested during the summer (*C. minor, C. triangulifer, C. cognatum,* and *P. fragile*) did not exhibit significant differences among any of the fluctuating temperature treatments relative to the controls.

c. Winter (Cold Season)

There were no significant findings of thermal stress during the winter season. One of the five species (*E. invaria*) tested exhibited a significant (ANCOVA) across the five temperature treatments (Table 17), with the intercept for this species in the 1.5°F/h treatment being significantly different from the constant temperature treatment. However, a 1.5°F/h treatment effect does not fit our criteria for a SFTS. The other three species tested during the winter (*A. ludens, C. triangulifer,* and *C. cognatum*) did not exhibit significant differences among any of the fluctuating temperature treatments relative to the controls.

E. Overall Discussion

A summary of the stress responses, growth and histopathology findings for fish experiencing diurnal temperature cycling are presented for four species during the cool season (Table 18) and for six species during the warm season (Table 19). Of the seven total species examined, two species (channel catfish and spotfin shiner) showed no indication of an increased stress response due to higher rates of temperature change (*i.e.*, 8°F/h) across all parameters measured. Four other species showed significant trends in glucose or triglycerides during the cold- or warm-season, and one species (white suckers) showed significant trends in cortisol and growth during the cold season. More specifically, across 32 cool-season comparisons involving 4 fish species and 8 stress-related variables only four comparisons (*i.e.*, 12.5%) conformed to our *a priori* definition of evidence for significant findings of thermal stress. Similarly, across 44 warm-season comparisons involving 6 fish species and 8 stress-related variables, only five comparisons (*i.e.*, 11%) conformed to our *a priori* definition of evidence for significant findings of thermal stress.

During the cold season the control fish lost weight during the study for three out of the four species (*i.e.* bluntnose minnows, spotfin shiners and white suckers). In contrast, cycling temperatures at 1.5, 2 and 4°F/h stimulated growth in these same species. The stimulatory effect of temperature cycling at low temperatures may be because some temperate freshwater fish species have a threshold temperature at which individuals drastically reduce or cease feeding (Fullerton *et al.* 2000). The threshold temperature appears to be species specific, and this threshold was exceeded during diel temperature cycling for only three of four species during the

cold season (*i.e.* not smallmouth bass). Bluntnose minnows and spotfin shiners exposed to 8°F/h also gained weight but white suckers grew little if at all, similar to the constant temperature control. The difference in growth rate of white suckers at 1.5, 2 and 4°F/h versus 8°F/h suggests that the rate of temperature change may have had a significant effect on growth. It is important to note that the growth response was not accompanied by a long-term stress response for glucose, triglycerides or histopathology at 8°F/h (or 1.5, 2, 4°F/h). Therefore, it cannot be concluded that the reduced growth rate observed in the white suckers would result in reduced performance or survival.

In all of the experiments, the observed cortisol levels were lower than expected for severely stressed fish. The concentrations we observed were similar to unstressed baseline values observed in the whole animal homogenate of another freshwater fish species (three-spined stickleback, 5 ng/ml), for whom five to ten fold increases in cortisol concentration are not uncommon in severely stressed individuals (Pottinger *et al.* 2002). Moreover, some of the significant differences observed for cortisol levels may have been an artifact of the assay and not an indication of a severe stress response. For example, white sucker exhibited short-term elevated levels of cortisol in the 2, 4, and 8°F/h treatments but only the 2 and 8 °F/h treatments were significantly higher than the control.

For fish histopathology, the analysis found no remarkable treatment-related lesions in skin, gills, or skeletal muscle (direct or stress-related effects), and no remarkable inflammatory or degenerative lesions found in internal organs suggesting secondary or immune system that can be correlated to treatments. Since well water used in the experiments is pathogen free, the fish most likely arrived with the observed parasites. In addition, the initial formalin treatment in our laboratory probably removed most surface parasites, but deep tissue parasites usually cannot be treated by a topical treatment.

The general lack of a sub-lethal effect in response to temperature change for fish may not be surprising in light of what is known about temperature cycling. Rapid temperature changes may not be stressful if the change occurs over a relatively narrow range of temperatures (in our case ±7°F from the acclimation temperature). Fish can detect a change in temperature of as little as 0.1°C (Brett 1956, Cooke and Scheer 2003), but will not actively avoid a temperature until the change is quite high (Brett 1956). Swimming activity can be stimulated by changing temperatures, independent of the magnitude of the change (Cooke and Scheer 2003), however it appears that the range of temperature change is more important than the rate in determining the magnitude of the stress response (Crawshaw *et al.* 1979).

In nature, fish may not be stressed by rapid temperature changes within a narrow range because they passively or actively move between different temperatures during the course of a day for feeding, breeding, predator avoidance, or possibly to avoid stressful temperatures (Brett *et al.* 1956). Maintaining homeostasis despite rapidly changing temperatures has obvious benefits (Wendelaar Bonga 1997). Diel temperature cycling (*i.e.*, <1°C/h) may have no effect on fish at moderate temperatures (Hartwell and Hoss 1979) and it can actually stimulate growth at low temperatures (Hubbs 1964, Hokanson *et al.* 1977, Spigarelli *et al.* 1982). Alternatively, the stress response increases as the range of temperature change increases (Tanck *et al.* 2000, Thomas *et al.* 1986), ultimately leading to death if the temperature change is too great

(Agersborg 1930). In this study, however, it appears that temperature changes up to 8 °F/h had no sublethal effect on the response parameters measured in this study.

In contrast to fish, macroinvertebrates do not typically move significant distances during the course of a given day, although they may drift downstream periodically to disperse or avoid predation or some other environmental irregularity (Allan and Castillo 2009). Regardless, it has been known for along time that most, if not all, of their life history characteristics are highly temperature sensitive (Sweeney 1984). This is especially true for mayflies which, as a group, are known to exhibit many fold differences in rates of larval growth, development, and metabolism as well as overall size and fecundity at maturity in response to small changes in temperature (Vannote and Sweeney 1980, Sweeney and Vannote 1981, 1984). Despite their temperature sensitivity to absolute levels of temperature, the results of these experiments clearly indicate that the mayflies studied here generally did not respond negatively to the four rates of temperature change (*i.e.*, 1.5°F/h, 2°F/h, 4°F/h, and 8°F/h) examined, for either warm-season (68 to 82°F), or cold-season (39 to 53°F) thermal regimes (Table 20). Across 51 warm-season comparisons involving 5-9 species per variable measured, only five comparisons (*i.e.*, 10%) conformed to our *a priori* definition of evidence for significant findings of thermal stress (SFTS). Moreover, across 20 cold-season comparisons involving 4 species per variable measured, only one comparison (*i.e.*, 5%) conformed to our *a priori* definition of evidence for significant findings of thermal stress (SFTS). The significant findings were distributed among five different species – warm-season drift was *P. fragile*, cold-season drift was *C. cognatum*, warm-season egg development time was *A. macdunnoughi*, warm-season growth was *L. hebe* and *M. modestum*, warm-season fecundity was *L. hebe* (Table 20). All but one significant finding of thermal stress involved the 8°F/h rate. The one exception was for *M. modestum* where warm-season female size was significantly smaller at 4°F/h (and the 8°F/h treatment resulted in 100% mortality for the species).

It seems important to note that *L. hebe* and *M. modestum* exhibited a combination of significant and insignificant responses for larval survivorship, growth, and development time, and female fecundity that suggests these two species may be more sensitive than the other species examined to rate of temperature change or time at higher temperatures during the warm-season thermal regime. For example, for *M. modestum*, larval survivorship was 0-10% for rates of change between 1.5°F/h, 2°F/h, 4°F/h, and 8°F/h, versus 38% for the constant control. Low survivorship for *M. modestum* was accompanied by smaller females at both 1.5 and 4°F/h (no females survived at 2 and 8°F/h). Similarly, for *L. hebe*, larval survivorship at 2 and 8°F/h (but not 4°F/h) was less than half that observed at the constant control or 1.5°F/h, both males and females decreased in size as the rate increased, and females had fewer eggs as their size decreased with increasing rate. The challenge in interpreting these observations for *L. hebe* and *M. modestum* as negative responses to the rate of temperature change (or time at higher temperatures) is the responses are inconsistent among the four fluctuating temperature treatments (1.5°F/h, 2°F/h, 4°F/h, and 8°F/h).

F. Conclusion

The overall weight of evidence suggests that rates of temperature change at 1.5°F/h, 2°F/h, or 4°F/h did not have a general negative effect on the mayfly or fish species examined here, within

the thermal ranges simulated (*i.e.*, 68 to 82°F or 39 to 53°F). Several significant responses were observed at the 8°F/h rate for certain parameters for both fish and macroinvertebrates, but the majority of these findings did not fit the *a priori* definition or pattern to interpret them as significant findings of thermal stress.

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Table 1. Cold season fish species, source, date of arrival to SWRC, initial size at acclimation, initial number of fish per tank (6 tanks per treatment, 5 treatments) and percent survival to endpoint over all tanks and treatments.

Species	*Source*	*Arrival date*	*Initial size (± 1 SD)*	*N per tank*	*Survival % Short term*	*Survival % Long term*	*Survival % Histopath.*
Smallmouth bass (*Micropterus dolomieu*)	Mixed origin Hicklins Fish Farm, New York	Nov. 9, 2007 (N=250)	12.9 ± 8.2 gm 92.5 ± 18.2 mm	7	99	83	94
White sucker (*Catostomus commersonii*)	Susquehana River, PA Peters Cr., Pequea Cr.	Oct. 30, 2007 (N=200) Oct. 31, 2007 (N=50)	2.9 ± 2.2 gm 61.5±12.5 mm	8	96	95	95
Bluntnose minnow (*Pimephales notatus*)	Susquehana River, PA Fishing Cr.	Oct. 24, 2007 (N=380) Oct. 29, 2007 (N=360)	2.4 ± 1.4 gm 56.4 ± 9.6 mm	8	100	98	94
Spotfin shiner (*Cyprinella spiloptera*)	Susquehana River, PA Fishing Cr., Peters Cr.	Oct. 23, 2007 (N=60) Oct. 24, 2007 (N=150) Oct. 31, 2007 (N=175)	2.8 ± 1.6 gm 62.7 ± 10.5 mm	10	100	98	97

Table 2. Warm season fish species, source, date of arrival to SWRC, initial size at acclimation, initial number of fish per tank [4 tanks short term (ST), 2 tanks long term (LT) and 2 tanks histopathology (Hist.) per treatment, 5 treatments] and percent survival to endpoint over all tanks and treatments.

Species	*Source*	*Arrival date*	*Initial size (± 1 SD)*	*N per tank (ST/LT/Hist.)*	*Survival % Short term*	*Survival % Long term*	*Survival % Hist.*
Smallmouth bass (*Micropterus dolomieu*)	Schuylkill River, PA Perkiomen Cr.& Mainstem	Jul. 8, 2008 (N=183) Jul. 9, 2008 (N=217)	3.1 ± 1.7 gm 56.5 ± 13.5 mm	5/10/10	72	64	81
Walleye (*Sander vitreum*)	Pymatuning Reservoir via PA Fish and Boat Commission Linesville State Fish Hatchery	Jun. 12, 2008 (N=500)	4.5 ± 1.2 gm 78.8 ± 7.1 mm	2/6/6	100	100	98
Channel catfish (*Ictalurus punctatus*)	Mixed origin domesticated line from Arkansas (Hopper Stevens) via a Delaware hatchery	Jul. 28, 2008 (N=400)	17.2 ± 6.1 gm 117.0 ± 14.2 mm	10/10/10	100	98	98
White sucker (*Catostomus commersonii*)	Susquehana River, PA Lake Aldred, Holtwood Pool	Jun. 11, 2008 (N=318)	0.7 ± 0.3 gm 39.8 ± 10.0 mm	0/6/6	0	58	72
Rosyside dace (*Clinostomus funduloides*)	Susquehana River, PA Fishing Cr.	Jul. 15, 2008 (N=400)	1.7 ± 0.9 gm 52.1 ± 6.9 mm	5/10/10	100	99	100
Spotfin shiner (*Cyprinella spiloptera*)	Susquehana River, PA Fishing Cr.	Jun. 16, 2008 (N=109) Jun. 23, 2008 (N=152) Jun. 26, 2008 (N=108)	2.6 ± 1.7 gm 59.9 ± 10.4 mm	5/10/10	95	84	74

Table 3. Variables measured for four fish species during cold season thermal regimes (i.e., constant at 46°F, and variable between 68 and 82°F with a temperature change rate of 1.5°F/h, 2.0°F/h, 4.0°F/h, 8.0°F/h).

	Primary Stressors		Secondary Stressors		Tertiary Stressors			
	Stress Hormones		Glucose/IGF1/ Osmolarity		Growth	Reprod. Status	Triglycerides	Disease
Fish	Acute	Long-term	Acute	Long-term				
a. Predator Smallmouth bass (*Micropterus domieui*)	X	X	X	X	X	X	X	X
b. Bottom Feeder White sucker (*Catastomas commersomi*)	X	X	X	X	X	X	X	X
c. Forage species Bluntnose minnow (*Pimephales notatus*)	X	X	X	X	X	X	X	X
Spotfin shiner (*Cyprinella spiloptera*)	X	X	X	X	X	X	X	X

Table 4. Variables measured for six fish species during warm season thermal regimes (constant at 75°F, and variable between 39 and 53°F with a temperature change rate of 1.5°F/h, 2.0°F/h, 4.0°F/h, 8.0°F/h).

	Primary Stressors		Secondary Stressors		Tertiary Stressors			
	Stress Hormones		Glucose/IGF1/ Osmolarity		Growth	Reprod. Status	Trigly-cerides	Disease
Fish	**Acute**	**Long-term**	**Acute**	**Long-term**				
a. Predator								
Smallmouth bass (*Micropterus domieui*)	X	X	X	X	X	X	X	X
Walleye (*Stizostedion vitreum*)	X	X	X	X	X	X	X	X
b. Bottom Feeder								
White sucker (*Catastomas commersomi*)		X		X	X	X	X	X
Channel catfish (*Ictalurus punctatus*)	X	X	X	X	X	X	X	X
c. Forage species								
Rosyside dace (*Clinostomus funduloides*)	X	X	X	X	X	X	X	X
Spotfin shiner (*Cyprinella spiloptera*)	X	X	X	X	X	X	X	X

Table 5. Cortisol assay validation by spike and recovery of cortisol in whole fish homogenates from three species, one individual per species. All samples were run in triplicate.

Species	*Cortisol spike level*	*Expected (ng/mL)*	*Observed (ng/mL)*	*Recovery %*
Smallmouth Bass	Low (2.5 ng /mL)	4.86	3.99	82.1
	Med (5 ng/mL)	7.36	5.12	69.5
	High (10 ng/mL)	12.36	8.93	72.3
Spotfin Shiner	Low (2.5 ng /mL)	3.19	2.93	91.7
	Med (5 ng/mL)	5.69	4.73	83.1
	High (10 ng/mL)	10.69	8.01	74.9
White Sucker	Low (2.5 ng /mL)	7.34	7.99	108.8
	Med (5 ng/mL)	9.84	9.18	93.3
	High (10 ng/mL)	14.84	13.28	89.5
				Mean=85.0

Table 6. Cortisol assay validation by measuring recovery and linearity of dilution of cortisol in whole fish homogenate from three species, one individual per species, diluted in assay buffer from the cortisol EIA kit. All samples were run in triplicate.

Species	*Dilution factor*	*Expected (ng/mL)*	*Observed (ng/mL)*	*Recovery %*	*Linearity (R^2)*
Smallmouth Bass	No dilution	2.38			0.87
	0.75	1.78	1.28	72.0	
	0.5	1.19	3.25	273.4	
	0.25	0.59	0.70	117.3	
Spotfin Shiner	No dilution	1.26			0.94
	0.75	0.94	1.06	112.1	
	0.5	0.63	0.87	137.9	
	0.25	0.32	0.39	124.8	
White Sucker	No dilution	4.84			0.90
	0.75	3.64	2.99	82.4	
	0.5	2.42	2.99	123.8	
	0.25	1.21	1.34	110.4	
				Mean=128.2	Mean=0.90

Table 7. Cortisol assay validation by measuring the range and average of the coefficient of variation (CV) of observed cortisol concentrations among replicates from three species, one individual per species.

Species	*CV (Range)*	*Average CV*
Bluntnose Minnow	0.8 – 8.7%	4.9%
Smallmouth Bass	1.0 – 7.5%	4.0%
White Sucker	0.7 – 16.5%	6.4%
		Mean=5.1%

Table 8. Glucose assay validation by spike and recovery of glucose in whole fish homogenates from three fish species, one individual per species. All samples were run in triplicate.

Species	*Glucose spike level*	*Expected (µg /mL)*	*Observed (µg /mL)*	*Recovery %*
Bluntnose minnow	Low (25 µg /mL)	4386	4333	98.8
	Med (50 µg/mL)	5886	5881	99.9
	High (100 µg/mL)	8886	7802	87.8
Smallmouth Bass	Low (25 µg /mL)	6983	7180	102.8
	Med (50 µg/mL)	8482	8213	96.8
	High (100 µg/mL)	11480	7019	61.1
White Sucker	Low (25 µg /mL)	4958	5036	101.6
	Med (50 µg/mL)	6458	6250	96.8
	High (100 µg/mL)	9458	8228	87.0
				Mean=92.5

Table 9. Glucose assay validation by measuring recovery and linearity of dilution of glucose in whole fish homogenate from three species, one individual per species, diluted in water. All samples were run in triplicate.

Species	*Dilution factor*	*Expected (µg /mL)*	*Observed (µg /mL)*	*Recovery %*	*Linearity (R^2)*
Bluntnose minnow	No dilution	2491			1.00
	0.75	1869	1896	101.5	
	0.5	1246	1218	97.8	
	0.25	623	700	112.3	
Smallmouth Bass	No dilution	2899			0.97
	0.75	2174	2536	116.7	
	0.5	1449	1733	103.5	
	0.25	725	821	113.3	
White Sucker	No dilution	2512			1.00
	0.75	1884	1921	102.0	
	0.5	1256	1300	103.5	
	0.25	628	672	107.0	
				Mean=106.4	Mean=0.99

Table 10. Glucose assay validation by measuring the range and average of the coefficient of variation (CV) of observed glucose concentrations among replicates from three species, one individual per species.

Species	*Glucose CV (Range)*	*Average CV*
Bluntnose Minnow	0.1 - 11.0%	2.7%
Smallmouth Bass	0.1 - 7.8%	2.7%
White Sucker	0.5 - 9.4%	3.0%
		Mean=2.8%

Table 11. Triglyceride assay validation by spike and recovery of glycogen, the quantified product of triglyceride digestion, in whole fish homogenates from three fish species, one individual per species. All samples were run in triplicate.

Species	*Spike Level*	*Expected (mg/mL)*	*Observed (mg/mL)*	*Recovery %*
Smallmouth bass	Low (0.313 mg/mL)	2.49	2.44	98.0
	Med (0.625 mg/mL)	2.80	2.83	101.0
	High (1.25 mg/mL)	3.41	3.39	98.9
Spotfin shiner	Low (0.313 mg/mL)	1.03	1.01	97.6
	Med (0.625 mg/mL)	1.34	1.34	99.6
	High (1.25 mg/mL)	1.97	1.92	97.5
White sucker	Low (0.313 mg/mL)	2.35	2.36	100.6
	Med (0.625 mg/mL)	2.66	2.61	98.3
	High (1.25 mg/mL)	3.29	3.28	99.9
				Mean=99.0

Table 12. Triglyceride assay validation by measuring recovery and linearity of dilution of true triglyceride (measured as glycogen from triglyceride minus unincorporated glycogen) in whole fish homogenate from three species, one individual per species, diluted in water. All samples were run in triplicate.

Species	*Dilution Factor*	*Expected (mg/mL)*	*Observed (mg/mL)*	*Recovery %*	*Linearity (R^2)*
Bluntnose minnow	No dilution	0.52			
	0.75	0.39	0.37	95.3	0.99
	0.5	0.26	0.24	93.3	
	0.25	0.13	0.10	74.1	
Smallmouth Bass	No dilution	0.23			
	0.75	0.18	0.17	106.9	0.95
	0.5	0.15	0.11	132.2	
	0.25	0.06	0.06	101.8	
White Sucker	No dilution	0.50			
	0.75	0.42	0.37	112.7	0.97
	0.5	0.22	0.25	90.3	
	0.25	0.11	0.12	92.2	
				Mean=99.9	Mean=0.97

Table 13. Triglyceride assay validation by measuring the range and average of the coefficient of variation (CV) of observed true triglyceride concentrations among replicates from three species, one individual per species.

Species	*True Triglyceride CV (Range)*	*Average CV*
Bluntnose Minnow	2.5 – 17.0%	7.4%
Smallmouth Bass	4.8 – 18.8%	11.1%
White Sucker	2.9 – 8.6%	5.7%
		Mean=8.1%

Table 14. Variables measured for nine mayfly species during warm season thermal regimes (i.e., constant at 75°F, and variable between 68 and 82°F with a temperature change rate of 1.5°F/h, 2.0°F/h, 4.0°F/h, 8.0°F/h).

	Primary Stressors		Tertiary Stressors				
Macroinvertebrates	**Larval Drift**	**Metabolic rate**	**Egg Development**	**Growth**	**Development Time**	**Adult size/ condition**	**Fecundity**
Procloeon fragile		X	X	X	X	X	X
Procloeon viridoculare		X	X	X	X	X	X
Centroptilum minor	X	X	X	X	X	X	X
Cloeon cognatum	X	X		X	X	X	
Centroptilum triangulifer	X	X	X	X	X	X	X
Callibaetis fluctuans	X			X	X	X	
Acerpenna macdunnoughi			X	X	X	X	X
Leucrocuta hebe				X	X	X	X
Maccaffertium modestum				X	X	X	X

Table 15. Variables measured for five mayfly species during cold season thermal regimes (constant at 46°F, and variable between 39 and 53°F with a temperature change rate of 1.5°F/h, 2.0°F/h, 4.0°F/h, 8.0°F/h).

	Primary Stressors		Tertiary Stressors			
Macroinvertebrates	**Larval Drift**	**Metabolic rate**	**Growth**	**Development Time**	**Adult size/ condition**	**Fecundity**
Cloeon cognatum	X	X	X			
Centroptilum triangulifer	X	X	X			
Ameletus ludens	X	X	X	X	X	X
Ephemerella invaria		X				
Ephemerella subvaria	X		X	X	X	X

Table 16. A summary of the statistical analyses used for the macroinvertebrate studies.

Measure	Level of Replication	Replication Summary Type	Transformation	Analysis
Respiration	Jar/Tank	None	log10	ANCOVA w/ bug mass as covariate (in a regression format)
Drift	Trough	Total #of bugs (i.e. sum)	arcsine-square-root of pct data	ANOVA across and within time periods (i.e. lights on, temp increase, temp decrease, lights off)
Survivorship	Jar/Tank	Total # of bugs (i.e. sum)	arcsine-square-root of pct data	ANOVA
Growth (mass)	Jar/Tank	Mean within each Jar	not transformed	ANOVA
Growth (emergence)	Jar/Tank	Median # of days to emergence	not transformed	ANOVA
Hatch (days to)	Jar/Tank	Median # of days to hatch	not transformed	ANOVA
Hatch (as percent)	Jar/Tank	Total # of bugs (i.e. sum)	arcsine-square-root of pct data	ANOVA

Table 17. Respiration rate (mg/hr) regressed against individual weight (mg) with both variables $\log_{10}$-transformed. Regression intercept (β_0) and slope (β_1) along with associated R^2 and number of observations used are provided for each thermal regime. Significant results, based on an $\alpha = 0.05$, are indicated by the '*'. Separate regression analyses were run to compare each of the thermal regime results to that of 1 of 2 controls: the 0 °F/hr and 1.5 °F/hr thermal regimes.

	Control (0 °F/hr)			1.5 °F/hr			2 °F/hr			4 °F/hr			8 °F/hr		
Summer	β_0	β_1	R^2 (n)	β_0	β_1	R^2 (n)	β_0	β_1	R^2 (n)	β_0	β_1	R^2 (n)	β_0	β_1	R^2 (n)
C.minor	-0.14	-1.71	0.05 (10)	0.29	-0.84	0.16 (5)	---	---	---	0.50*	-0.62*	0.50 (13)*	0.26	-0.62	0.08 (11)
C. triangulifer	0.60	0.81	0.04 (11)	0.23*	-0.83	0.01 (13)	0.55 *‡	-0.77	0.25 (12)	0.18	-0.83	0.10 (13)	---	---	---
C. cognatum	0.30*	-0.21	0.01 (10)	0.31*	0.80	0.10 (15)	0.48*	0.00	0.00 (10)	0.37*	0.12	0.01 (13)	0.49*	-0.11	0.00 (15)
P. fragile	0.71*	-0.48*	0.32 (13)*	0.71*	-0.2	0.01 (13)	---	---	---	0.45*	-0.66 *	0.41 (13)*	0.63*	-0.66	0.18 (14)
P. viridoculare	0.73*	0.08	0.07 (6)	---	---	---	0.72*	-0.35	0.09 (6)	---	---	---	0.59 *†	-0.41 *†	0.92 (9)*
Winter															
A. ludens	0.64*	-0.57 *	0.16 (25)*	0.67*	-0.33	0.13 (15)	0.78	-0.29	0.03 (14)	0.72*	-0.74	0.33 (12)	0.40	-0.39	0.28 (8)
C. triangulifer	-2.84	-5.62	0.42 (5)	-19.9	-58.5	0.98 (3)	-0.25	1.00	0.04 (6)	---	---	---	-0.32	-1.26	0.05 (9)
C. cognatum	0.14	-0.17	0.01 (10)	-0.46	-0.77	0.12 (8)	-0.12	-0.28	0.26 (7)	-0.96	-1.63	0.18 (4)	-0.09	-0.18	0.08 (7)
E. invaria	-0.26	0.00	0.00 (10)	0.87 †	-1.36	0.13 (10)	-0.01	-0.21	0.04 (10)	---	---	---	0.05	-0.39	0.13 (6)

† Coefficients significantly different from the associated 0 °F/hr thermal regime control coefficient

‡ Coefficients significantly different from the 1.5 °F/hr thermal regime control

--- No regression results due to either strong positive relationships between respiration rate and individual weight or regressions based on too few observations (i.e., P. viridoculare, 1.5 °F/hr regime)

Table 18. Summary of stress responses (1°, 2°, 3°), growth and histopathology observations during the cool season. Y - indicates a significant effect of higher rate of temperature change, N- indicates no significant effect.

		1°-Cortisol		2°-Glucose		3°-Triglyceride		Growth rate	Histo
		Short term	Long term	Short term	Long term	Short term	Long term		
Predator	Smallmouth bass	N	N	N	Y	N	N	N	N
Benthic	White sucker	Y	N	N	N	N	N	Y/N[†]	N
Forage	Bluntnose minnow	N	N	N	Y	N	N	N	N
Forage	Spotfin shiner	N	N	N	N	N	N	N	N

[†] The growth rate of white suckers exposed to 8°F/h was not significantly different than the constant temperature control, but both of these treatments gained little or no weight while white suckers exposed to 1.5, 2 and 4°F/h gained significantly more weight.

Table 19. Summary of stress responses (1°, 2°, 3°), growth and histopathology observations during the warm season. Y - indicates a significant effect of higher rate of temperature change, N- indicates no significant effect.

		1°-Cortisol		*2°-Glucose*		*3°-Triglyceride*		*Growth rate*	*Histo*
		Short term	Long term	Short term	Long term	Short term	Long term		
Predator	Smallmouth bass	N	N	N	Y	N	Y	N	N
Predator	Walleye	N	N	Y	Y	N	N	N	N
Benthic	Channel Catfish	N	N	N	N	N	N	N	N
Benthic	White sucker	-	N	-	N	-	N	-	N
Forage	Rosyside Dace	N	N	N	Y	N	N	N	N
Forage	Spotfin shiner	N	N	N	N	N	N	N	N

Table 20. Summary of evidence for significant findings of thermal stress (SFTS) for the eight variables measured for mayflies during both warm and cold seasons (expressed as the number of SFTS of the number of species examined). Number of species examined ranged between 4 and 9, depending on the variable measured.

	Warm season	Cold season
Drift	1 of 5 (*P. fragile*, only 8°F)	1 of 4 (*C. cognatum,* only 8°F)
% Egg hatch	0 of 5	—
Egg development time	1 of 5 (*A. macdunnoughi*, only 8°F)	—
Larval survivorship	0 of 9	0 of 4
Larval development time	0 of 8	—
Larval growth	2 of 8 (*L. hebe*, only 8°F; *M. modestum*, 4°F for females)	0 of 4
Fecundity	1 of 6 (*L. hebe*, only 8°F)	0 of 4
Metabolism	0 of 5	0 of 4

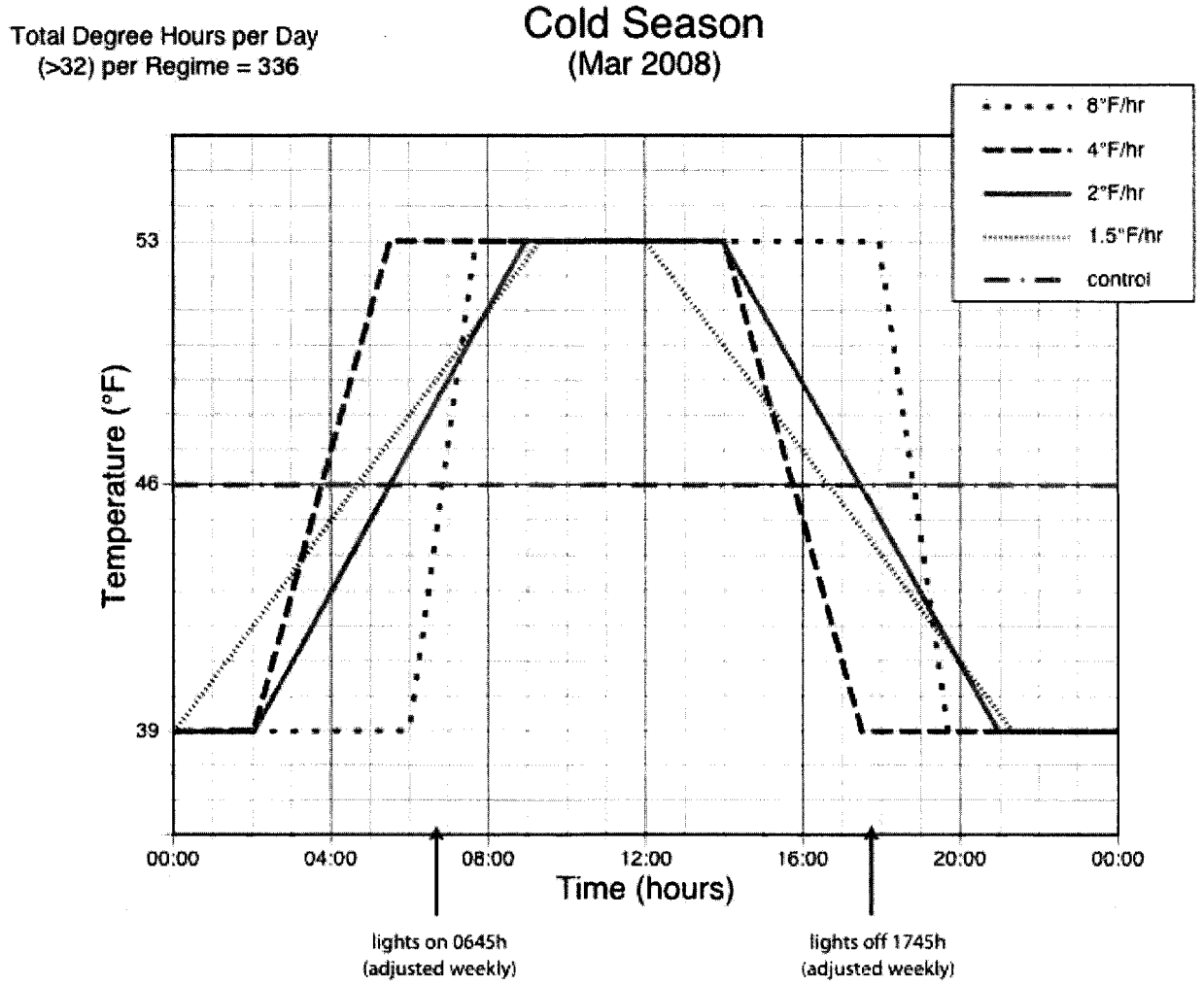

Figure 1. Actual cold season conditions (experiment began in Mar 2008) in the laboratory with five thermal regimes (constant at 46°F, and variable between 39 and 53°F with a temperature change rate of 1.5°F/h, 2.0°F/h, 4.0°F/h, 8.0°F/h), and a photoperiod of approximately 11:00h light and 13:00h dark. Treatments are staggered to insure that heating and cooling demands of the different treatments could be met.

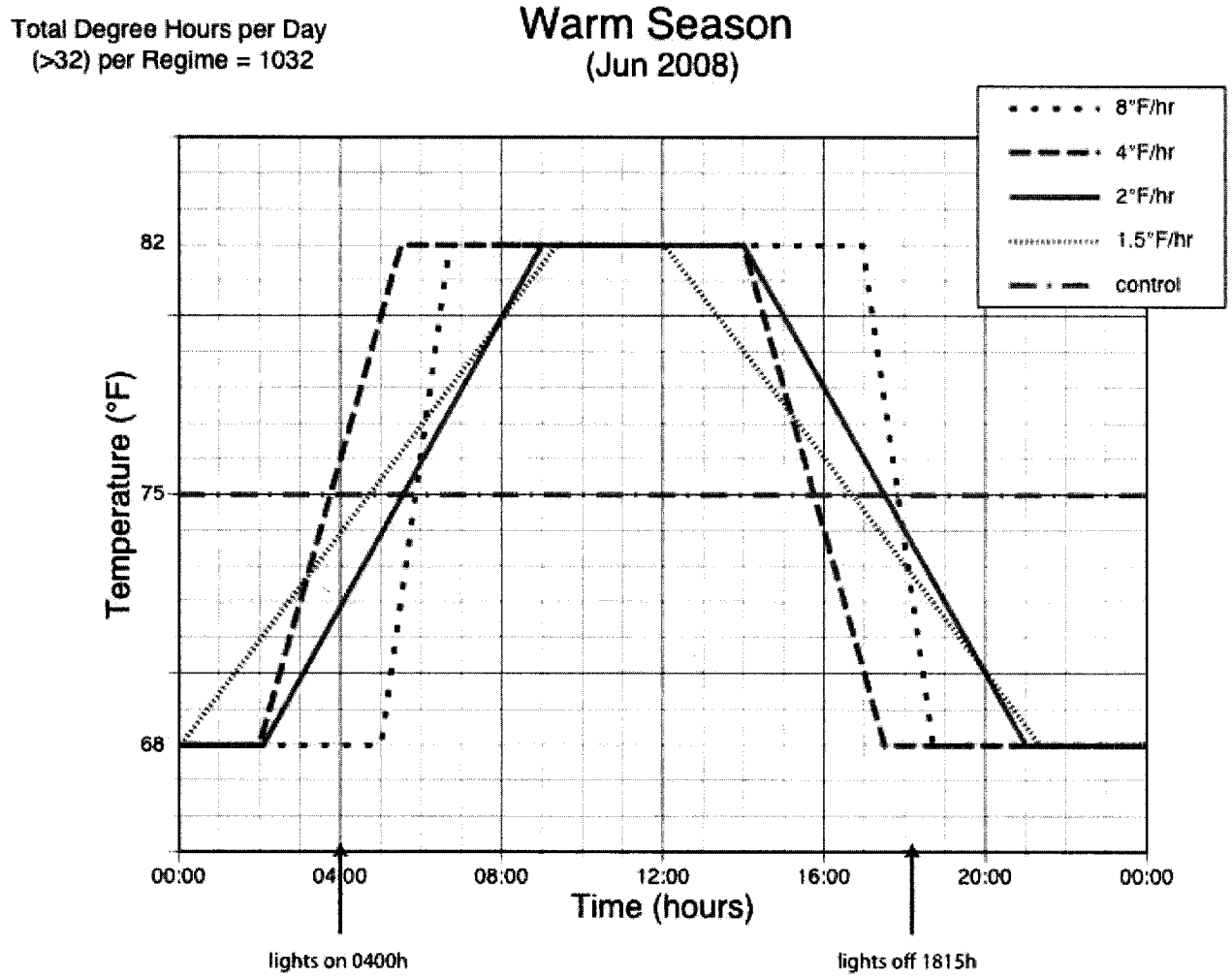

Figure 2. Actual showing warm season conditions (experiment began in Jun 2008) in the laboratory with five thermal regimes (constant at 75°F, and variable between 68 and 82°F with a temperature change rate of 1.5°F/h, 2.0°F/h, 4.0°F/h, 8.0°F/h), and a photoperiod of approximately 14:15h light and 9:45h dark. Treatments are staggered to insure that heating and cooling demands of the different treatments could be met.

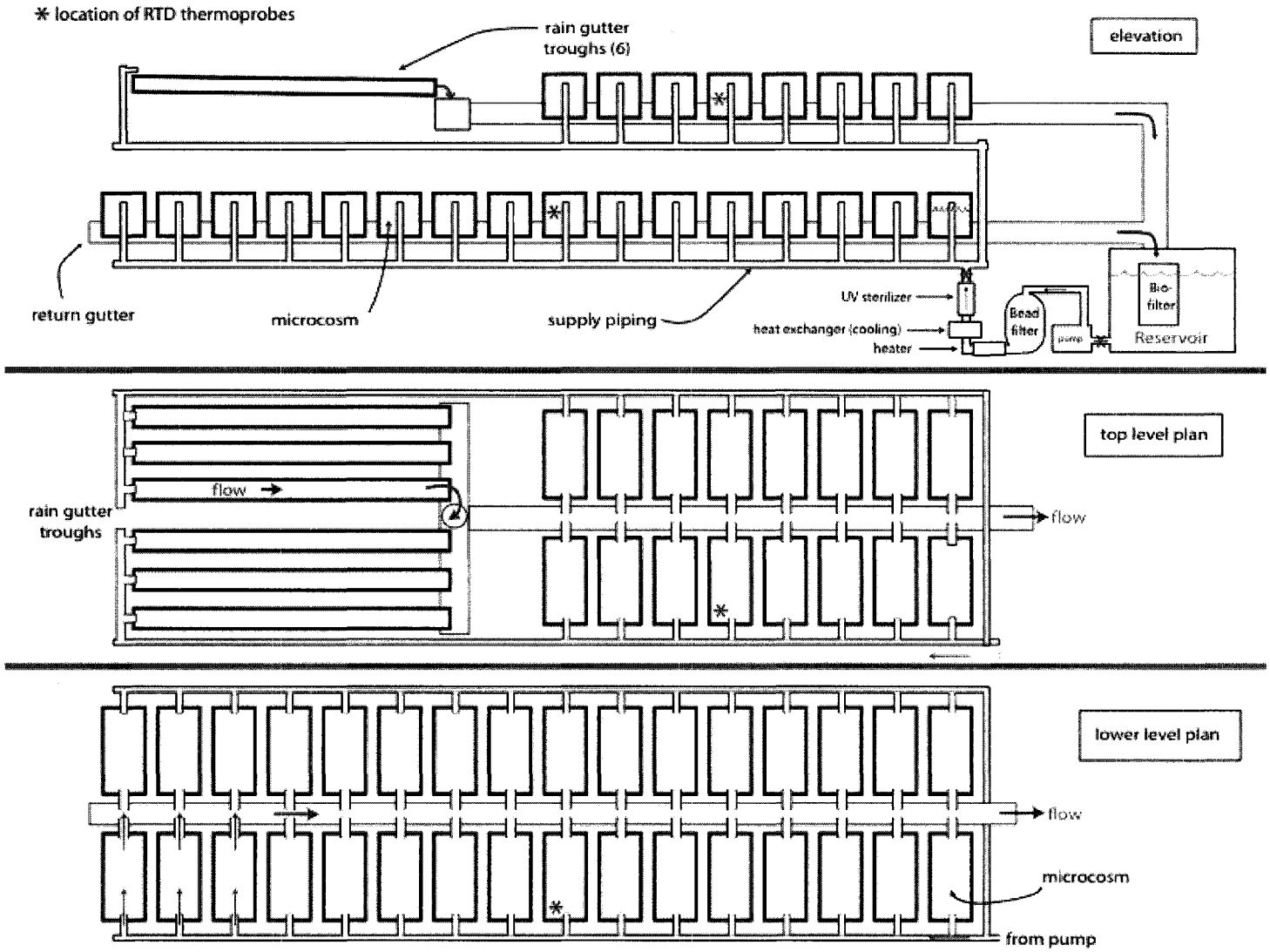

Figure 3. Side and top views of the rack system showing the connections between the water control and purification systems and the insulated trays used for rearing fish and insects and the rain gutter troughs for insect drift.

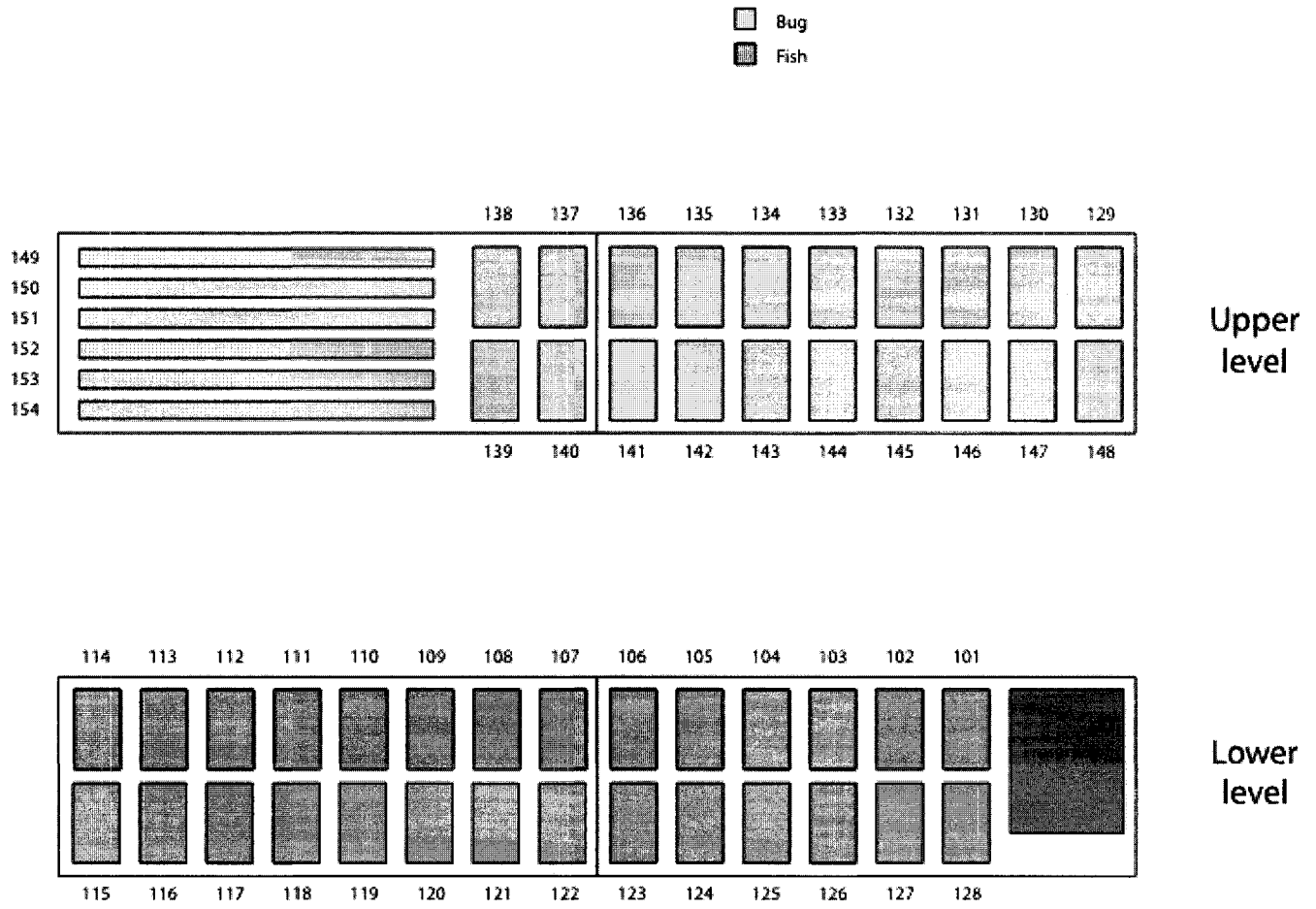

Figure 4. Top views of upper and lower levels of the rack system showing number system used to identify the insulated trays used for rearing fish and insects and the rain gutter troughs for insect drift.

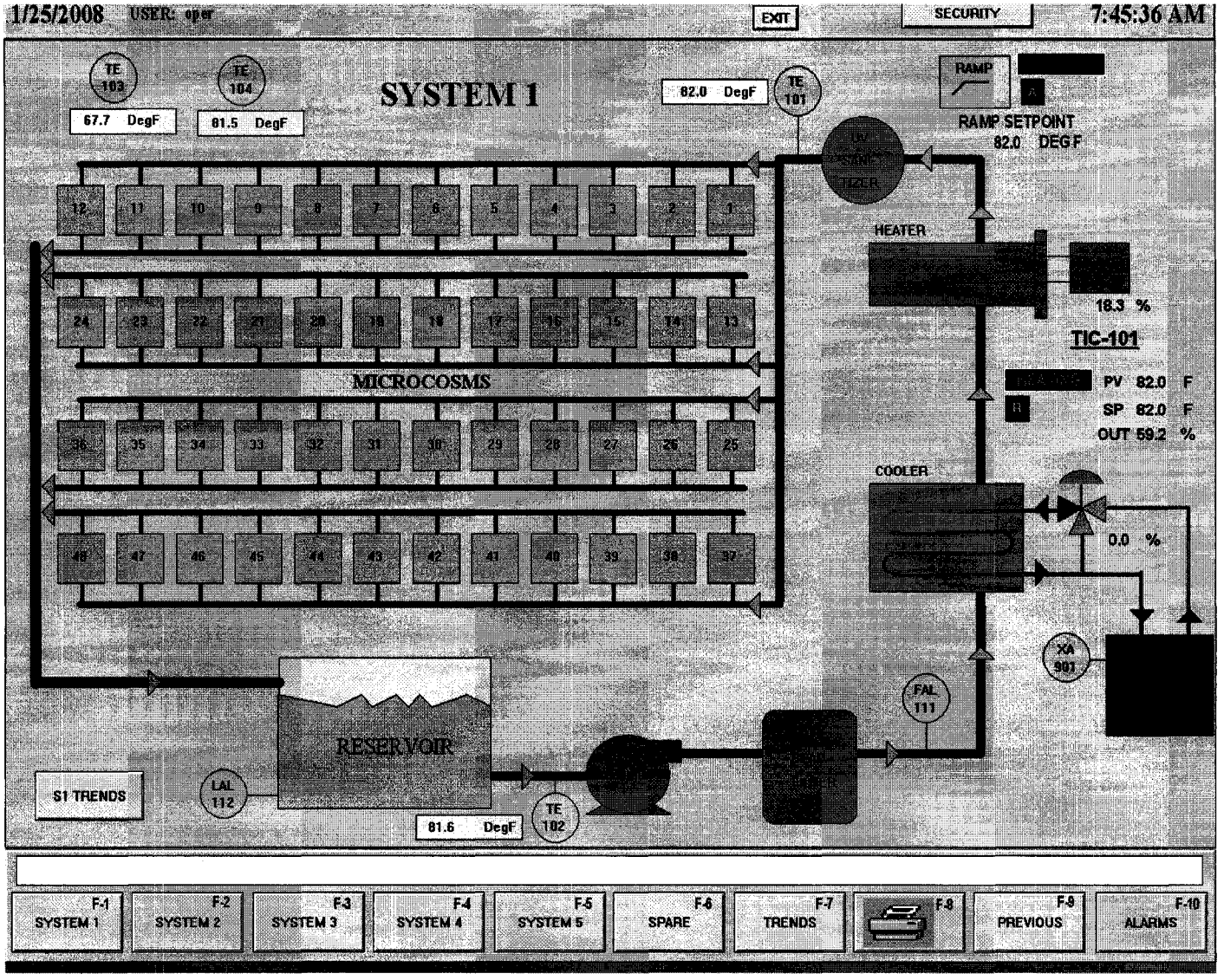

Figure 5. Captured screen image of System (rack) 1 controls.

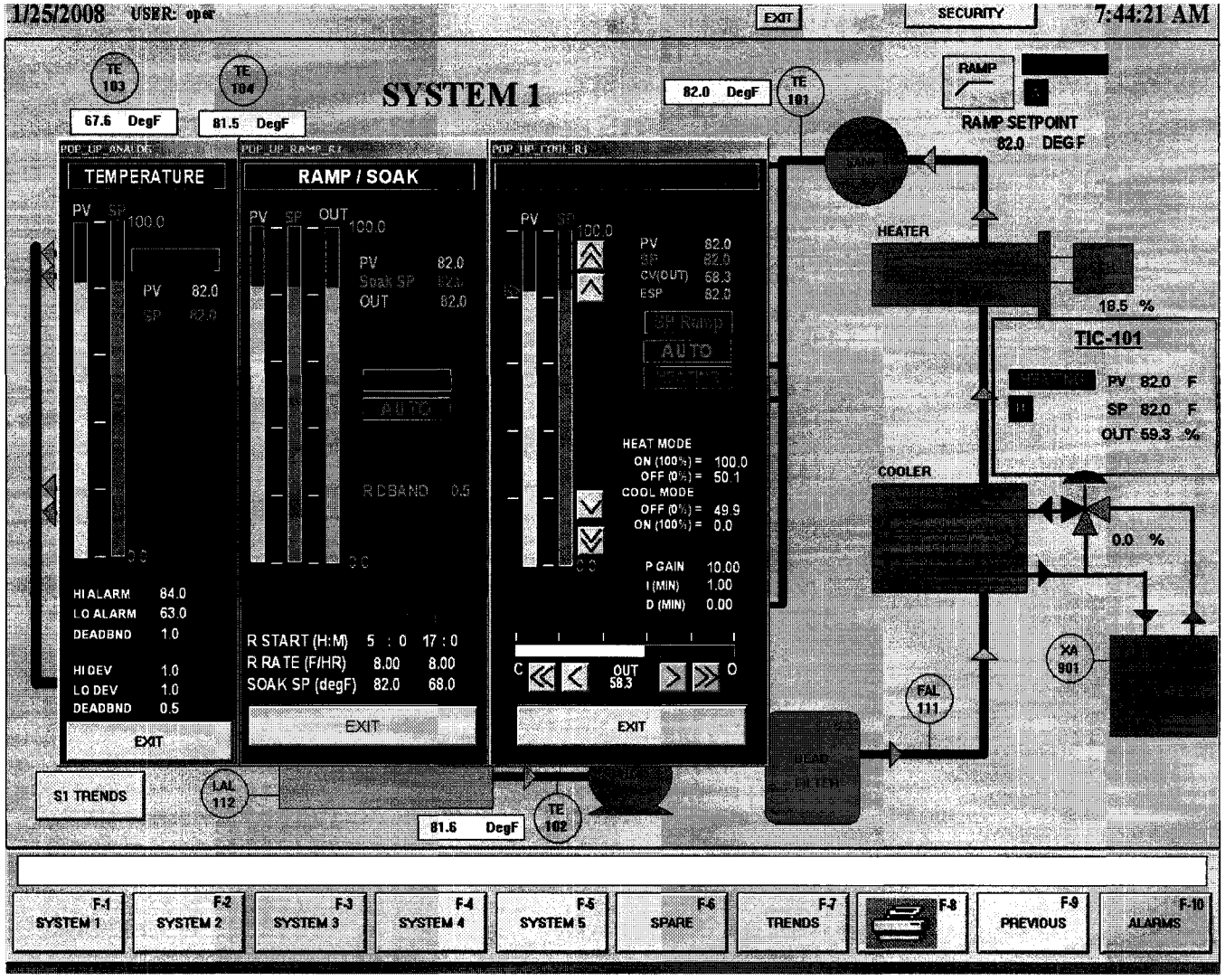

Figure 6. Captured screen image of pop-up window where details of temperature regime (maximum, minimum, rate of change, alarm levels) can be controlled for System (rack) 1

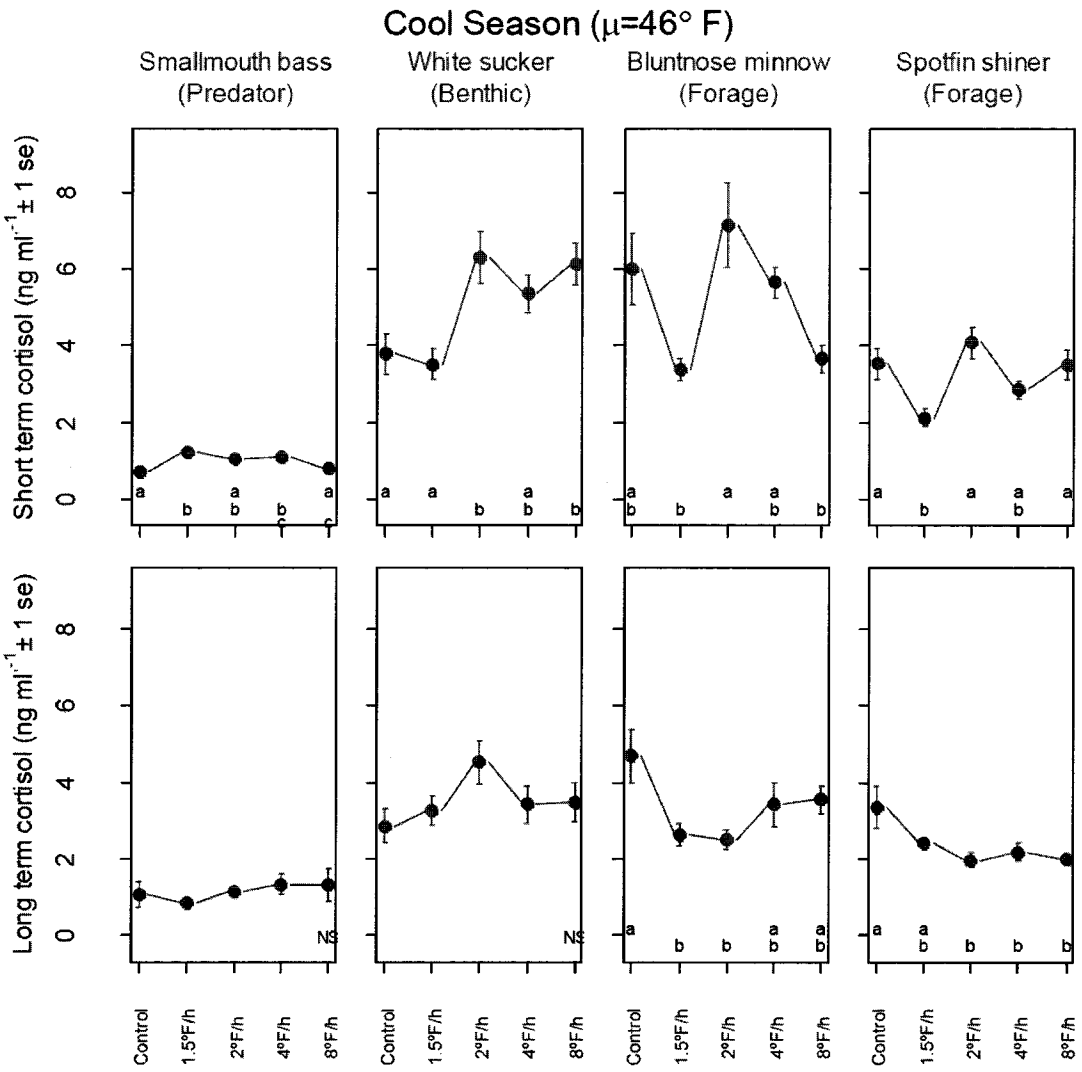

Figure 7. Short term and long term cortisol concentrations in four fish species experiencing constant mean temperature or diel temperature cycling during the cold season.

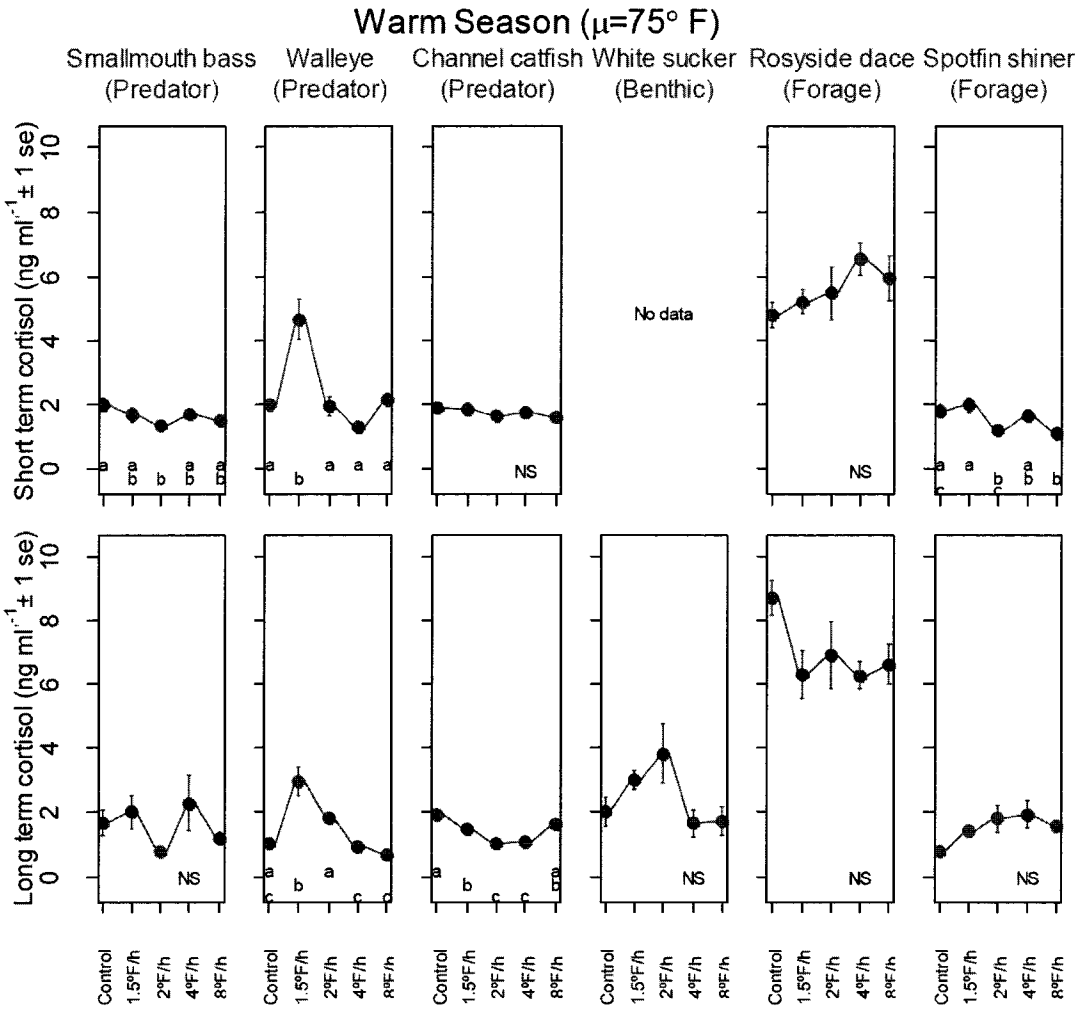

Figure 8. Short term and long term cortisol concentrations in six fish species experiencing constant mean temperature or diel temperature cycling during the warm season.

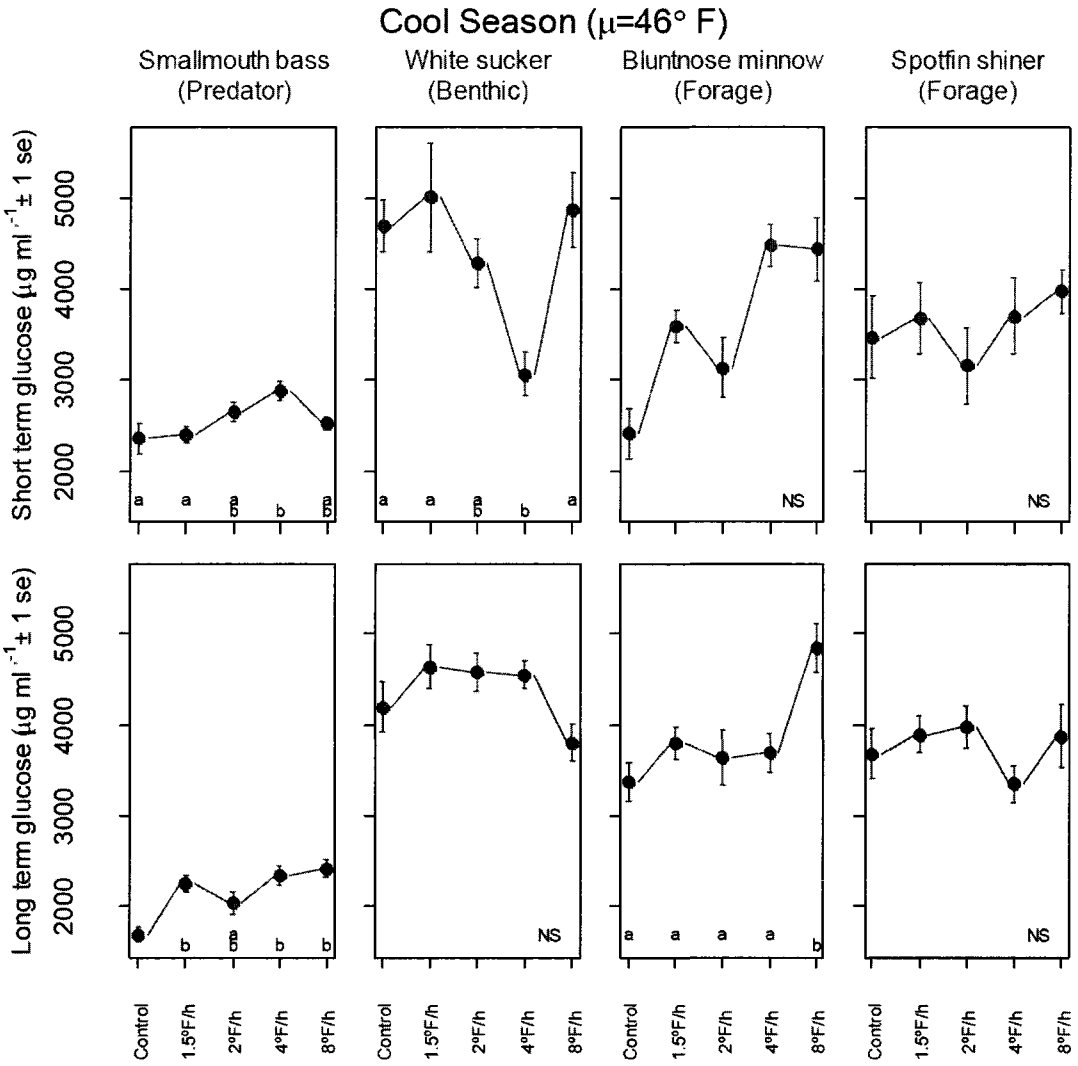

Figure 9. Short term and long term glucose concentrations in four fish species experiencing constant mean temperature or diel temperature cycling during the cool season.

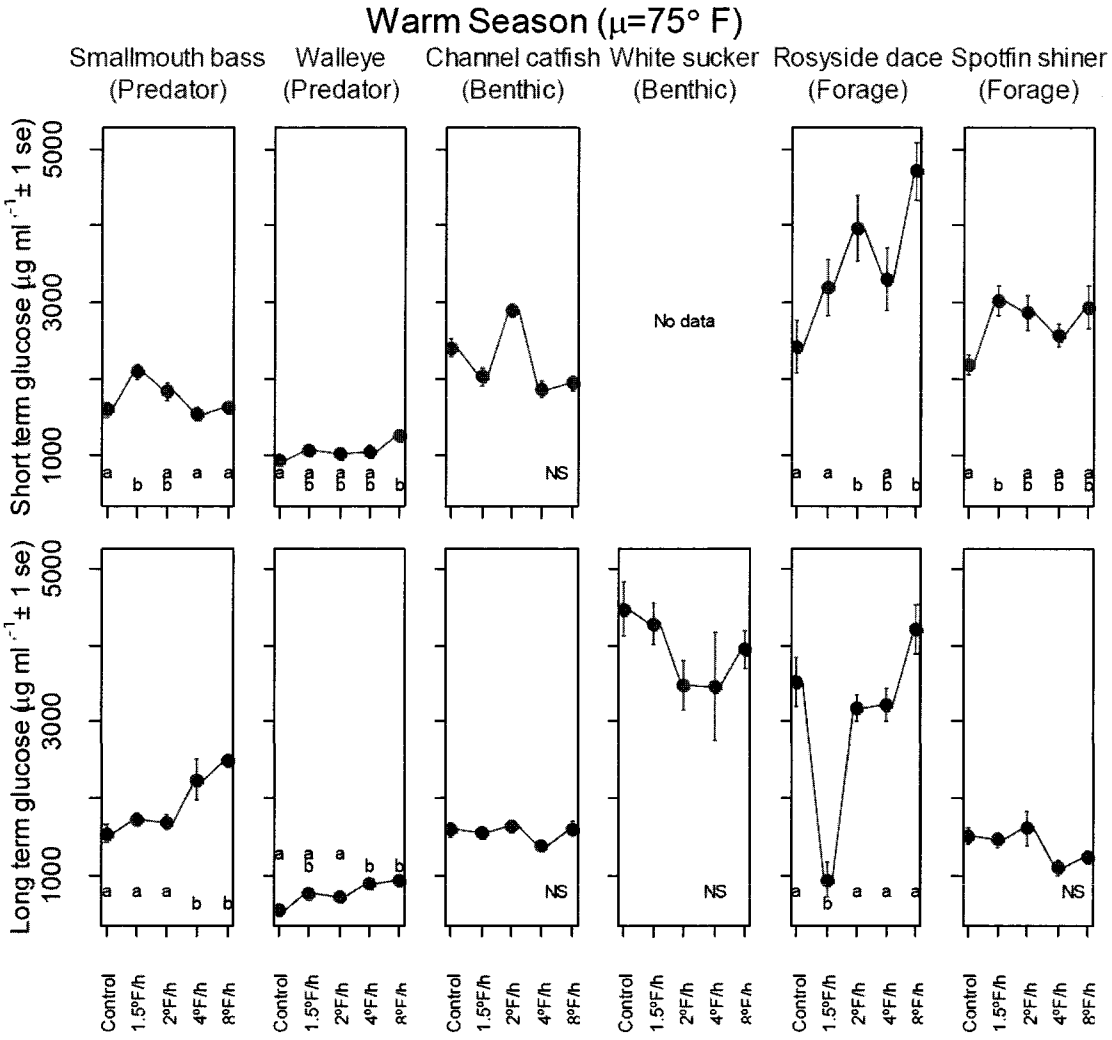

Figure 10. Short term and long term glucose concentrations in six fish species experiencing constant mean temperature or diel temperature cycling during the warm season.

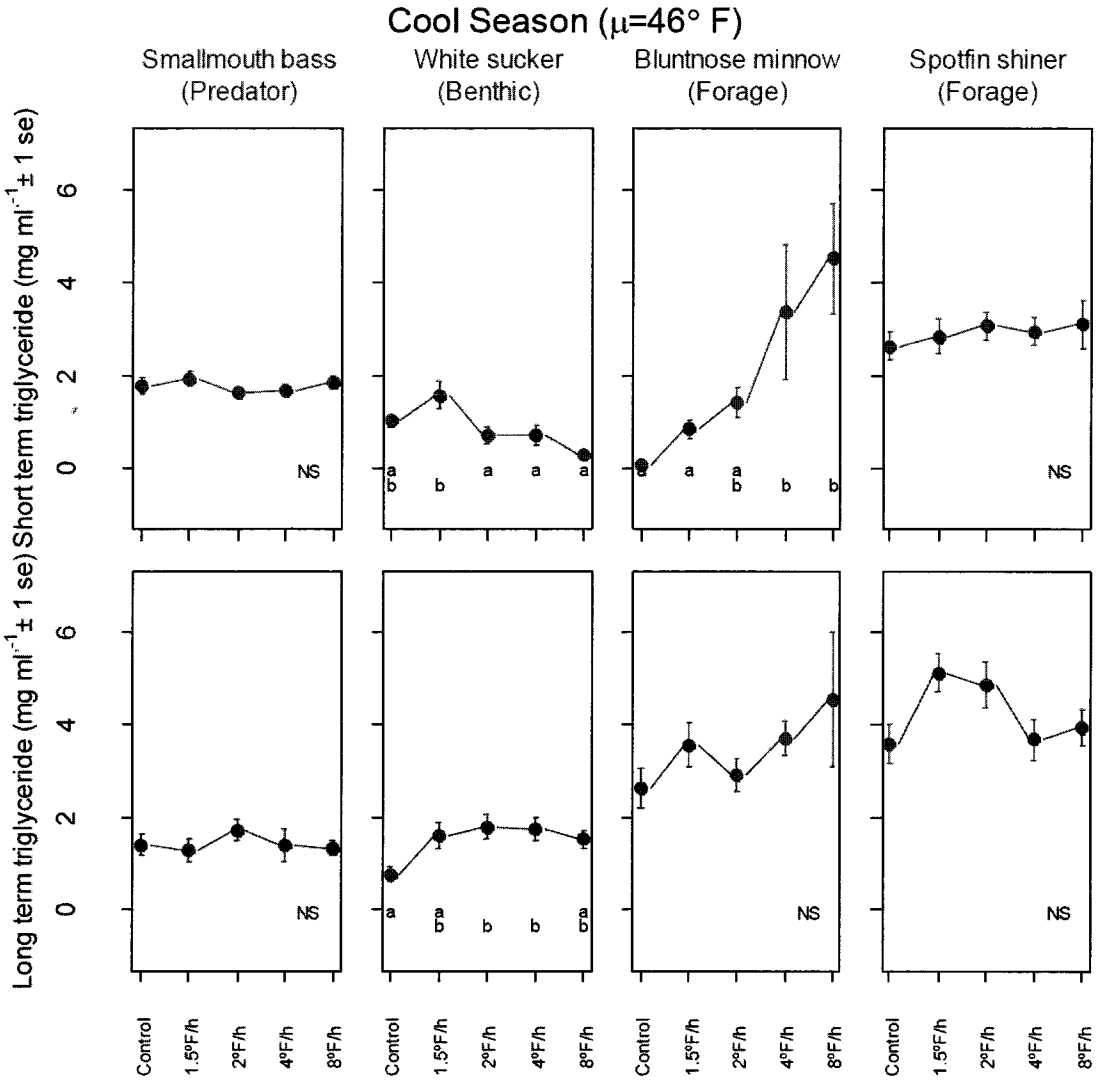

Figure 11. Short term and long term triglyceride concentrations in four fish species experiencing constant mean temperature or diel temperature cycling during the cool season.

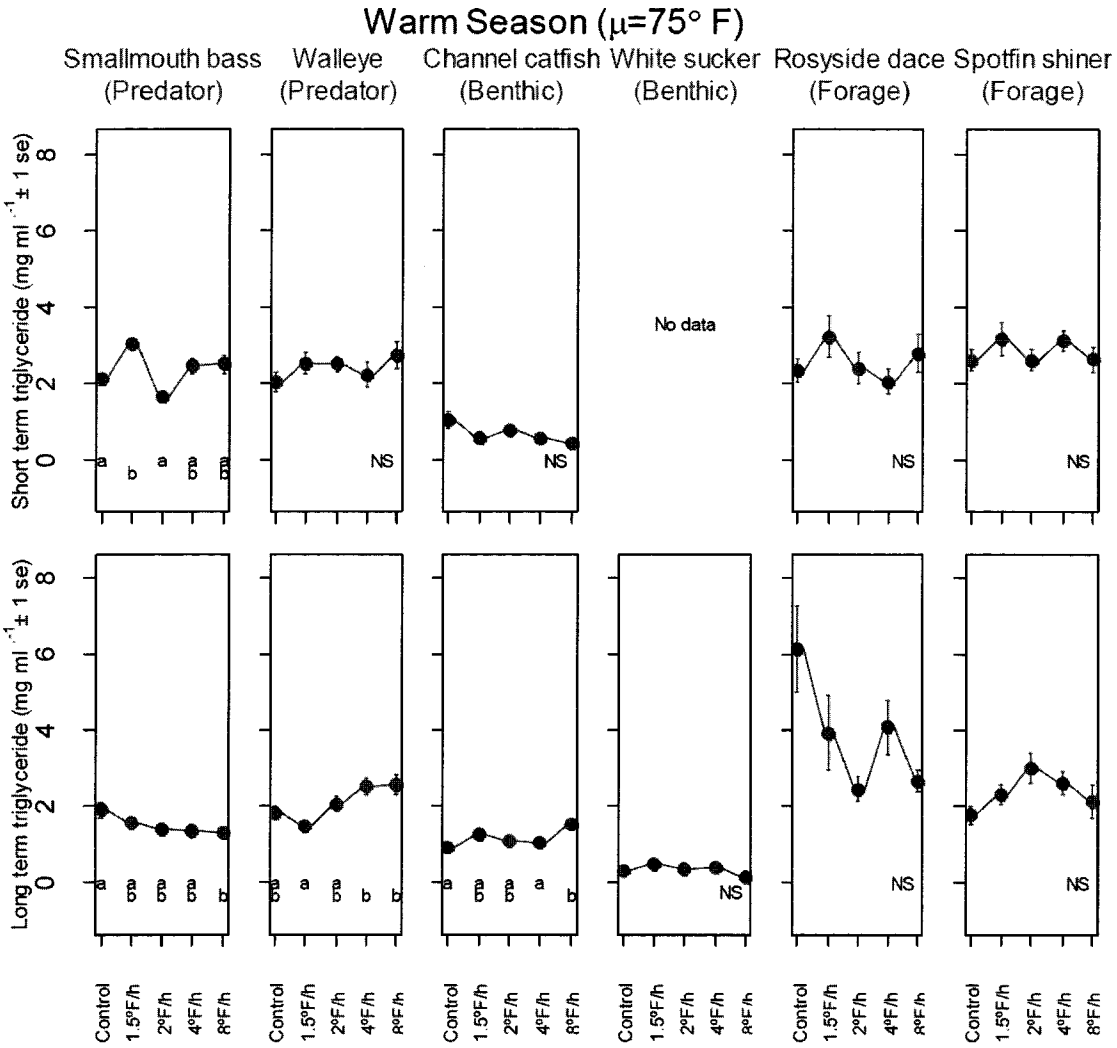

Figure 12. Short term and long term triglyceride concentrations in six fish species experiencing constant mean temperature or diel temperature cycling during the warm season.

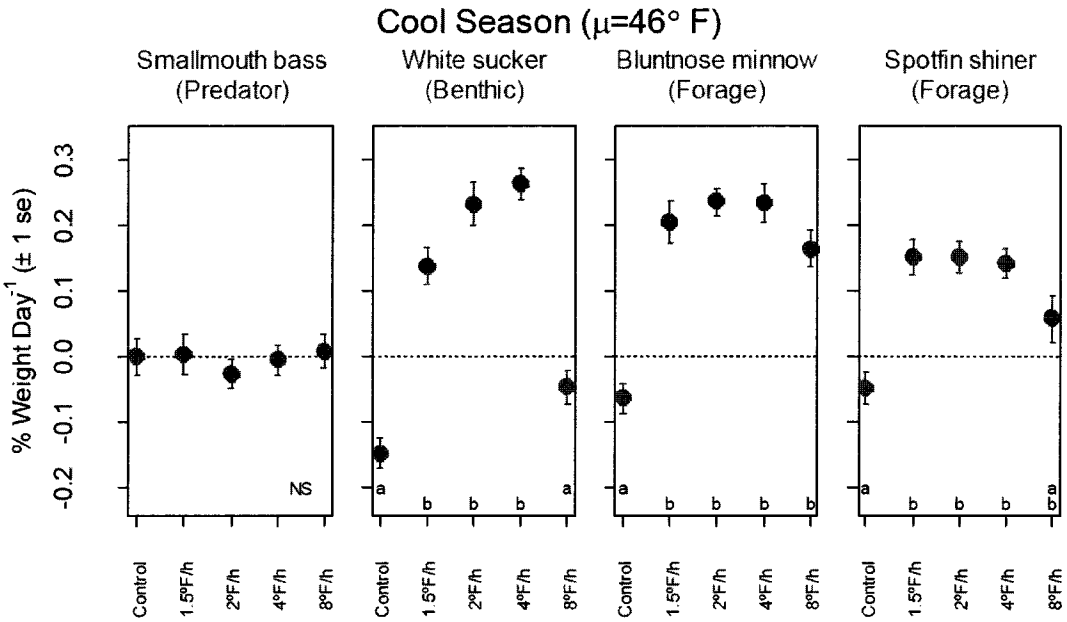

Figure 13. Growth rate over six weeks in four fish species experiencing constant mean temperature or diel temperature cycling during the cool season.

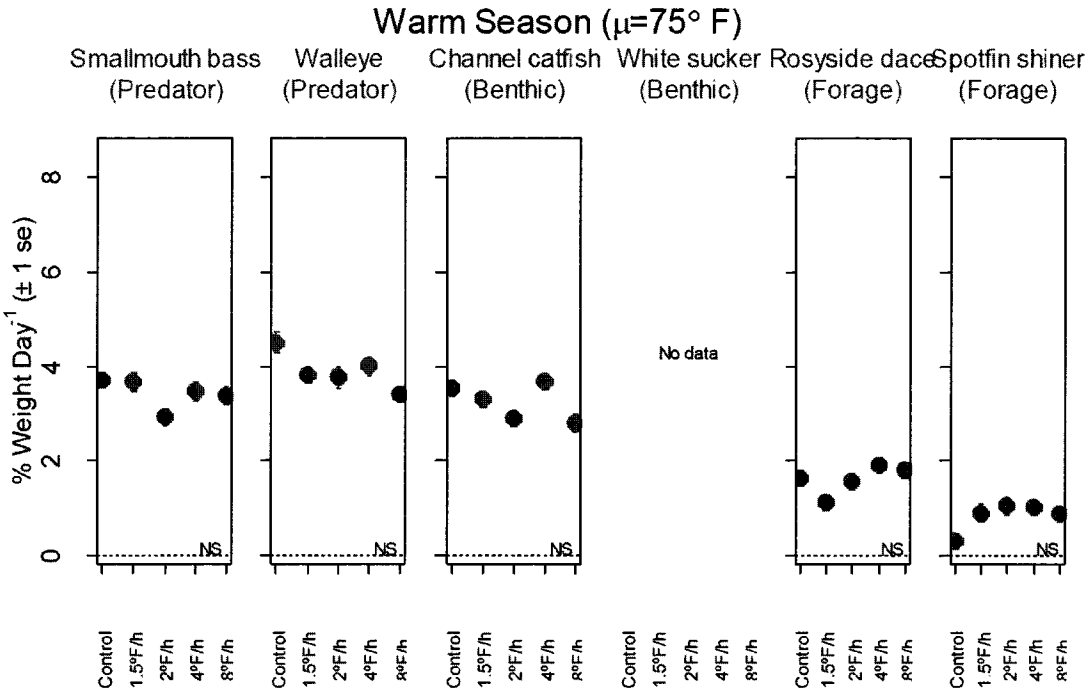

Figure 14. Growth rate over four weeks in five fish species experiencing constant mean temperature or diel temperature cycling during the warm season.

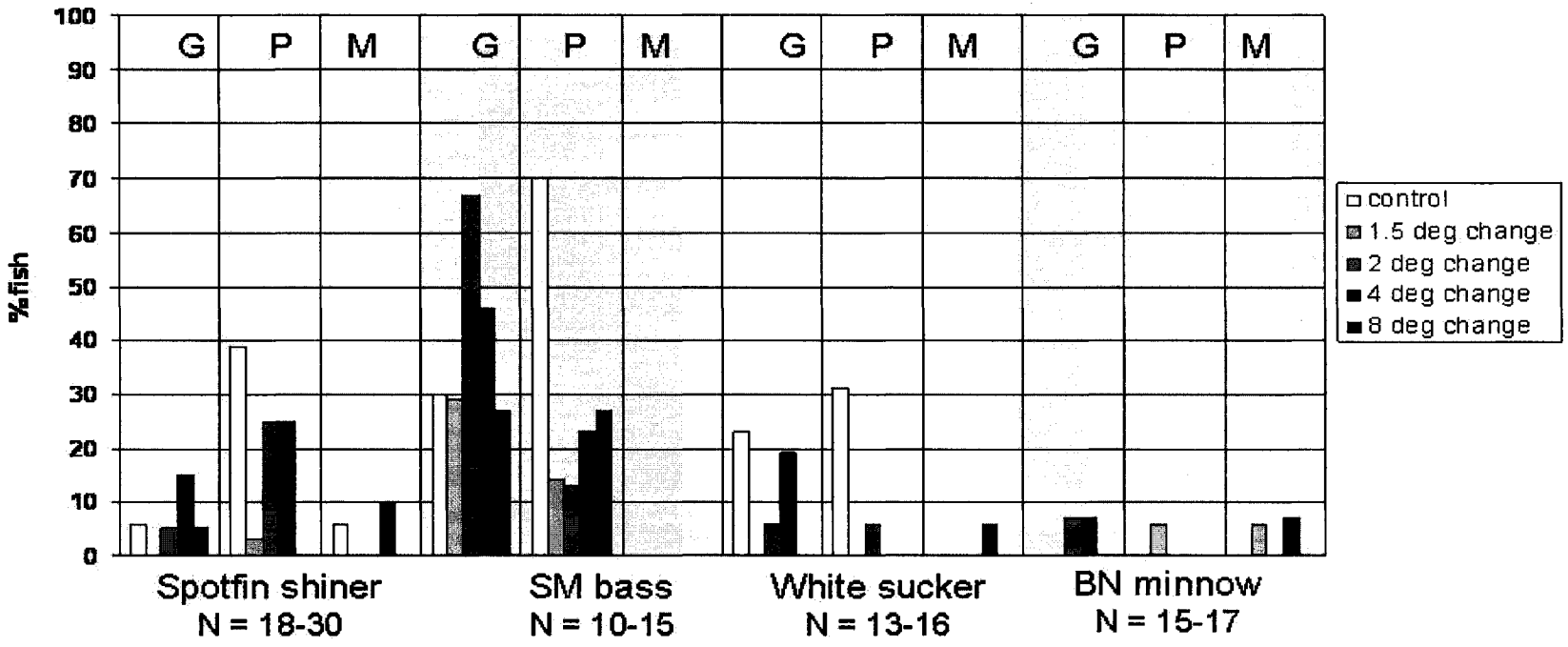

Figure 15. Histology observations during the cold season. G- granulomas observed in the viscera; P- parasites observed in the viscera, encysted or granulomatous; M- pigmented macrophage aggregates observed in the viscera.

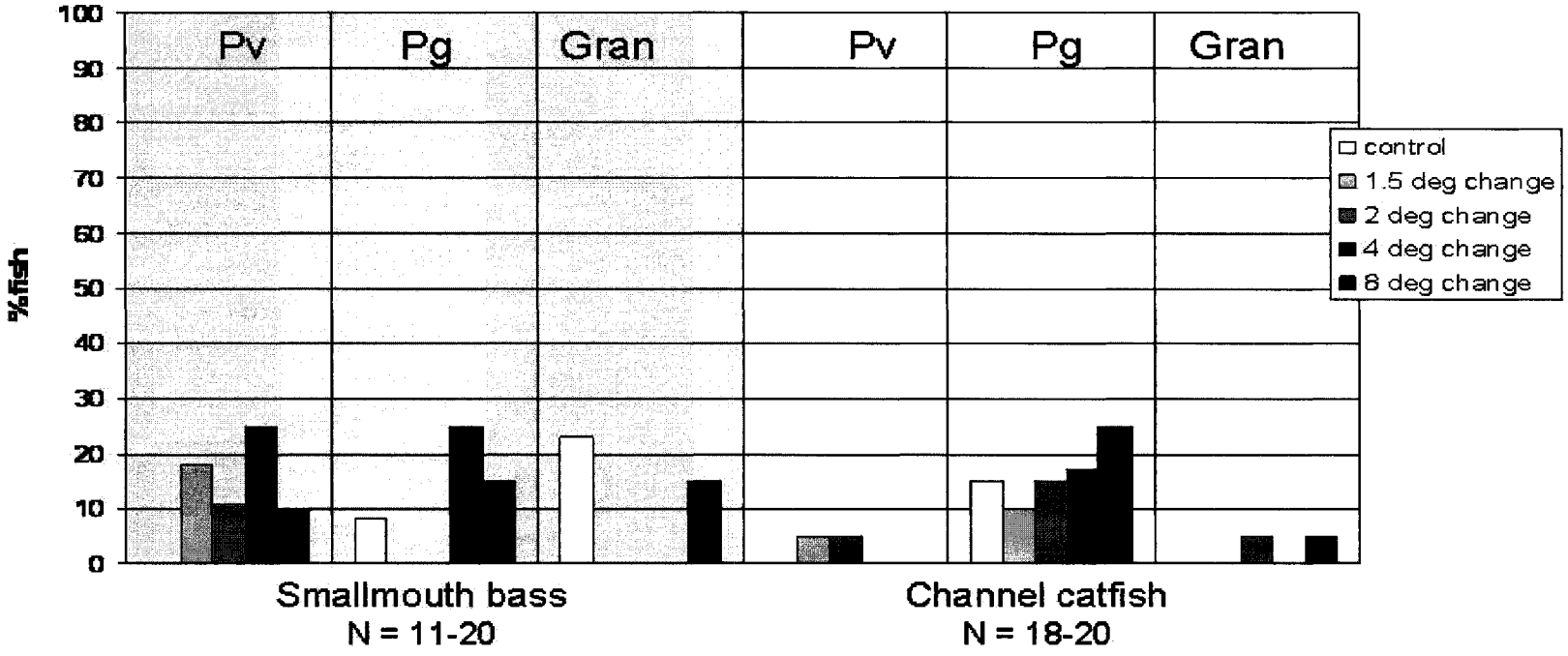

Figure 16. Histology observations during the warm season. Pv- parasites observed in the viscera, encysted or granulomatous; Pg – parasites observed in the gills; Gran - granulomas observed in the viscera.

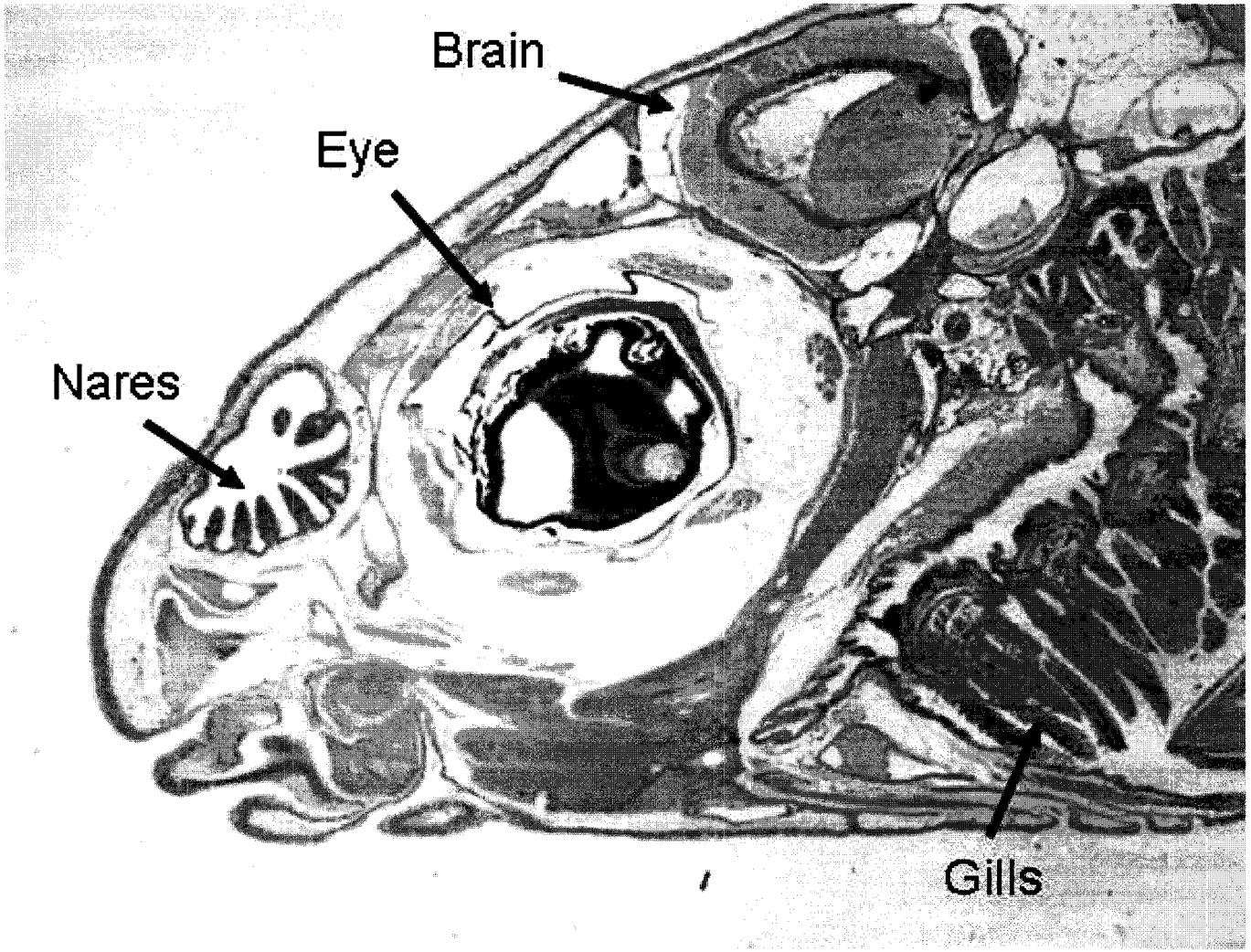

Figure 17. Normal tissues from the head of a bluntnose minnow, control treatment.

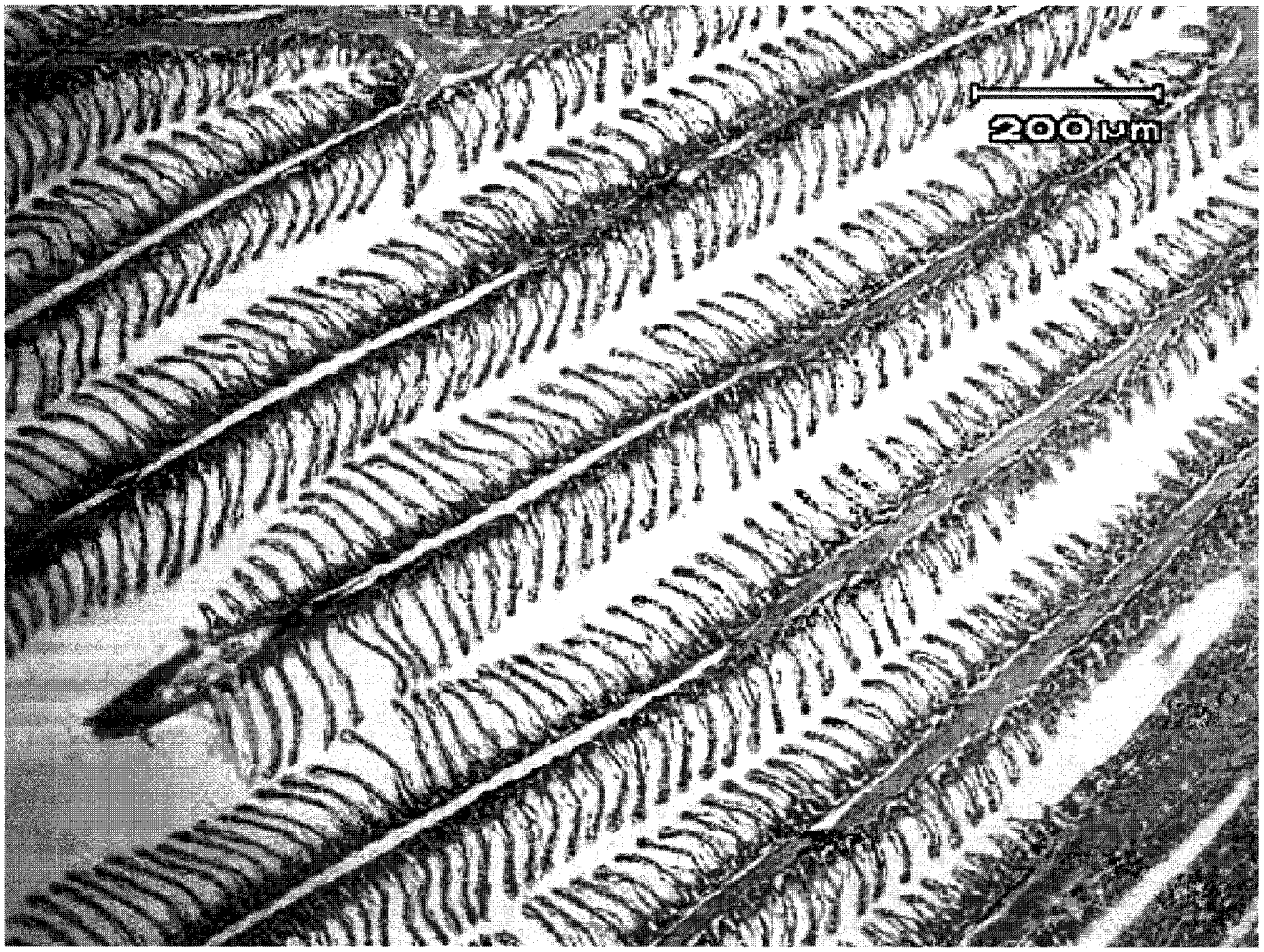

Figure 18. Normal gills from a white sucker, control treatment.

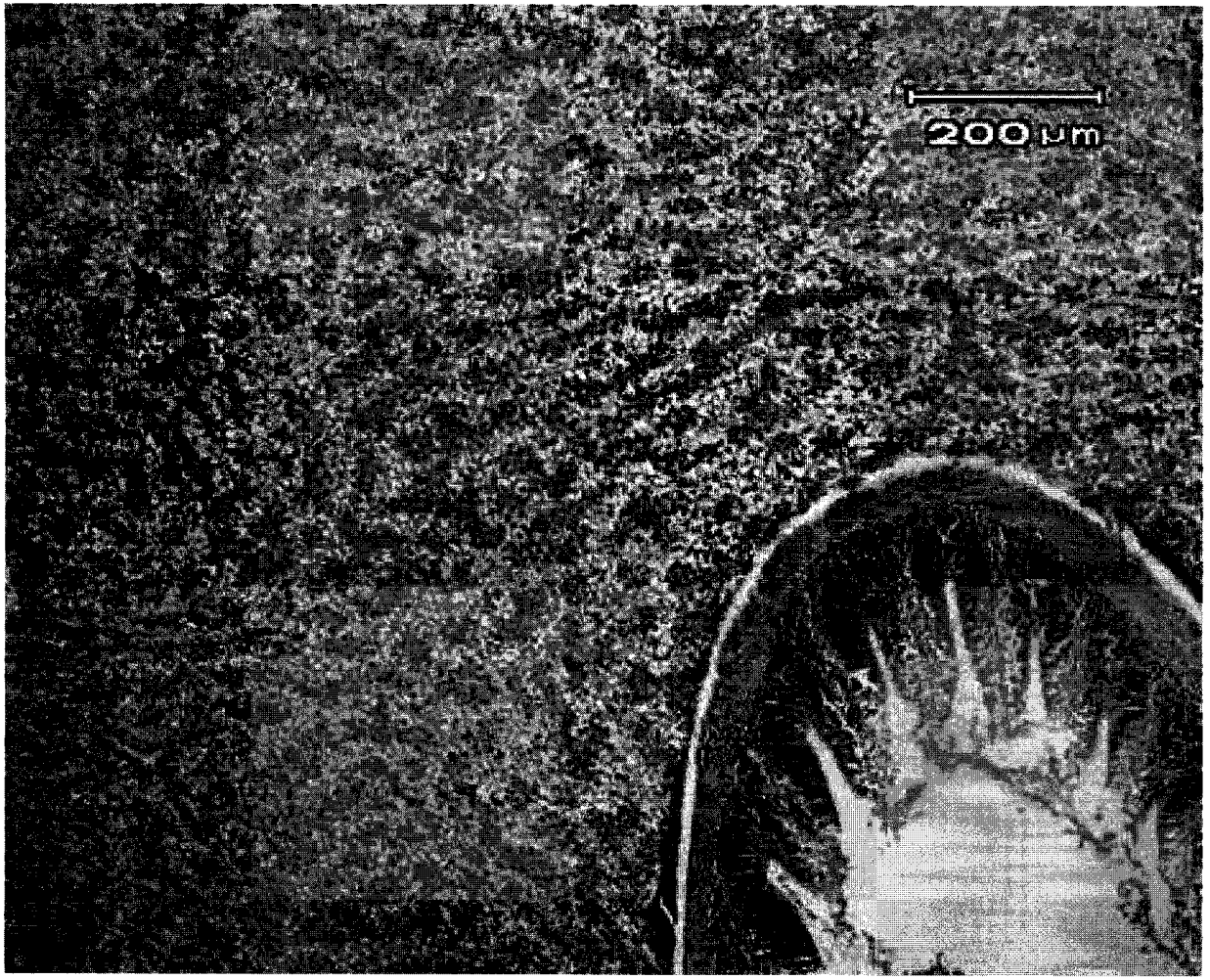

Figure 19. Moderately vacuolated liver from a white sucker, control treatment. Most fish had normal tissues such as liver, intestinal tract, etc. This liver is moderately vacuolated due to lipid and glycogen storage (common finding in fish, usually diet-related).

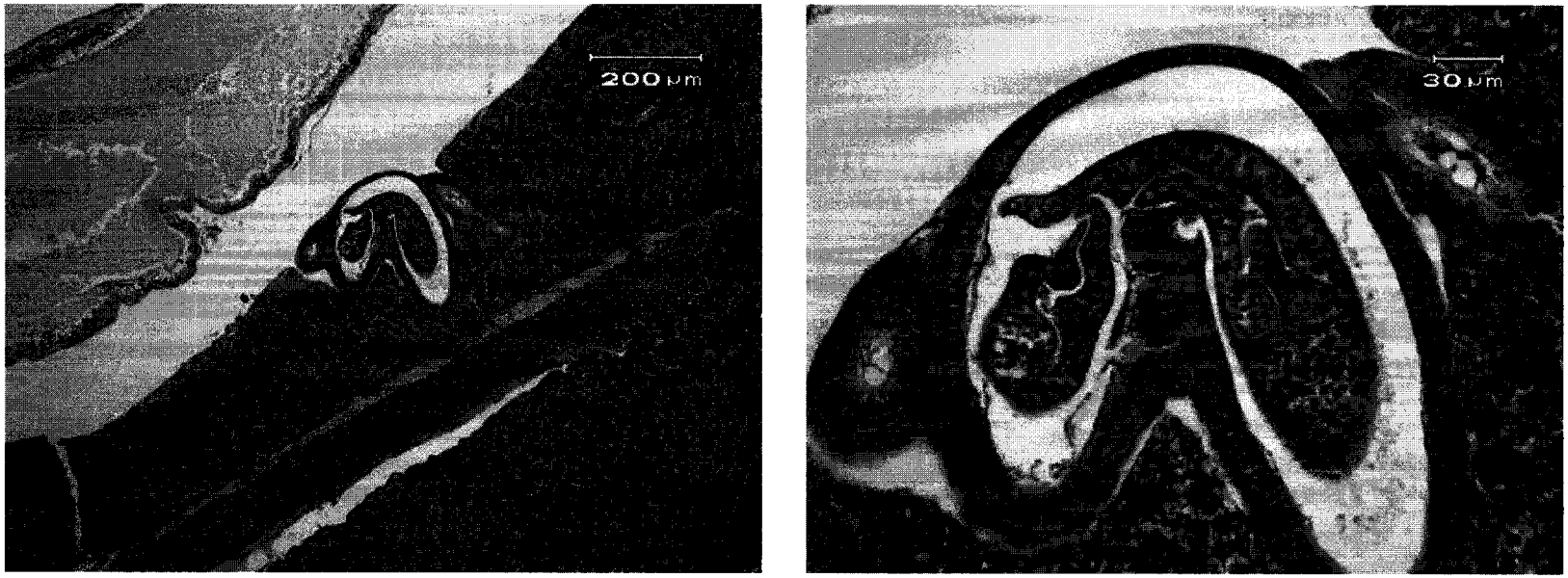

Figure 20. Myxozoan parasite (sporoplasm) in a bile duct of the liver, shown enlarged on the right, of a spotfin shiner from the 4°F/h treatment, cold season. These are well encysted, host-adapted parasites with minimal inflammatory reaction.

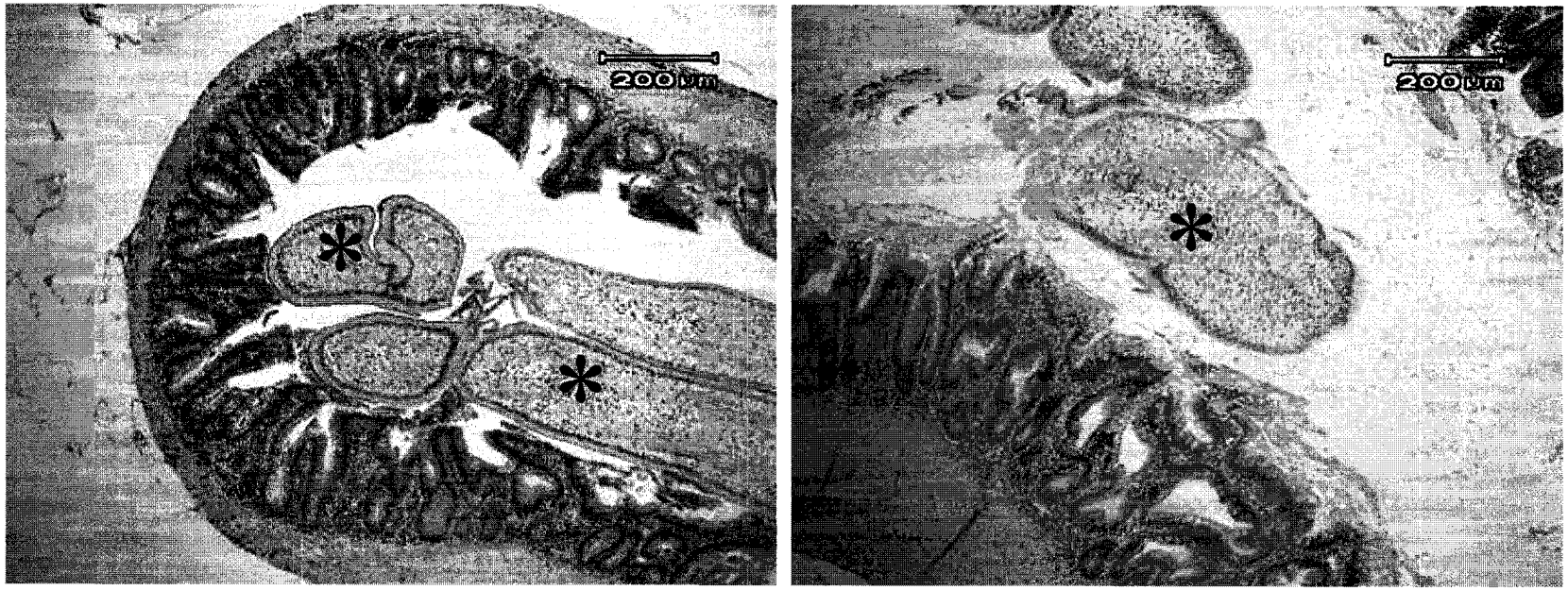

Figure 21. Smallmouth bass intestinal sections showing flukes (*) in control (left) and 4°F/h (right) warm season treatments.

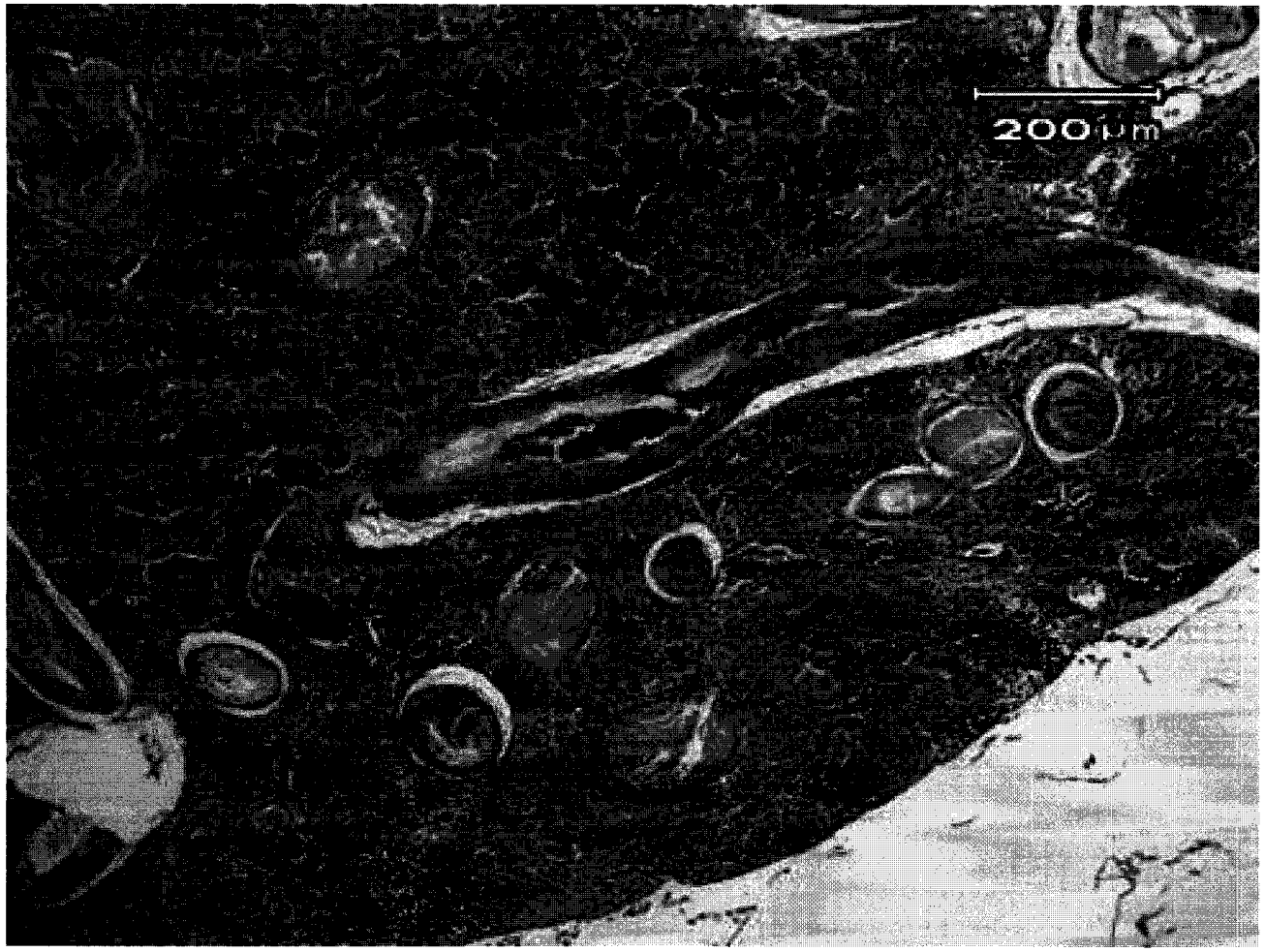

Figure 22. Granulomas in the spleen of a smallmouth bass from the cold season 4°F/h treatment. This is a rare fish with multiple granulomas within the same organ. The granulomas are inflammatory lesions producing the circular areas and are apparently due to parasite migration. One granuloma in this photo is even shaped like a worm (black arrow).

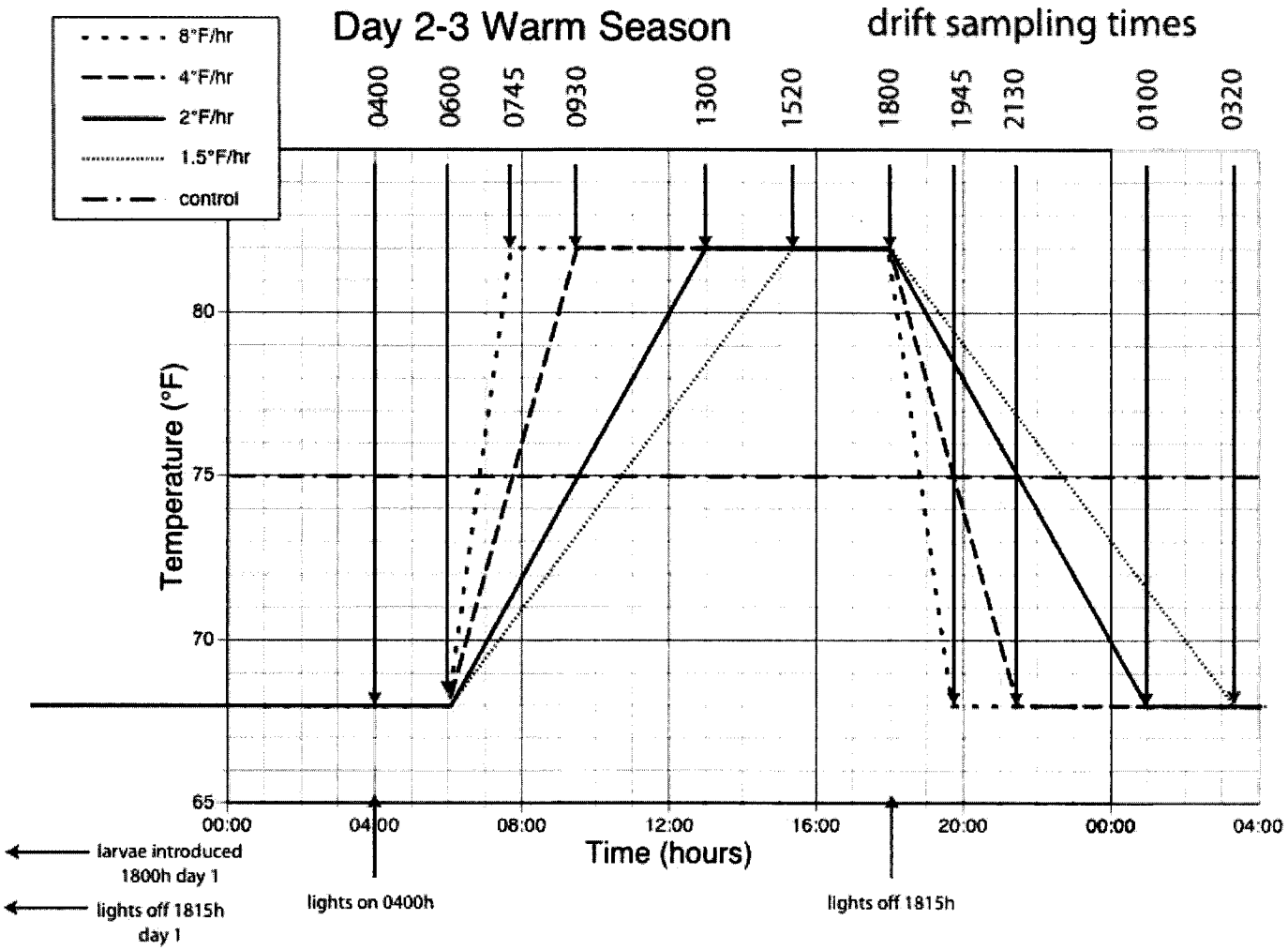

Figure 23. Actual showing warm season conditions in the laboratory drift studies with five thermal regimes (constant at 75°F, and variable between 68 and 82°F with a temperature change rate of 1.5°F/h, 2.0°F/h, 4.0°F/h, 8.0°F/h), and a photoperiod of approximately 14:15h light and 9:45h dark. Treatments are staggered to insure that heating and cooling of the different treatments begin at the same times. The 11 drift sampling times are indicated by vertical arrows originating from the top of the figure.

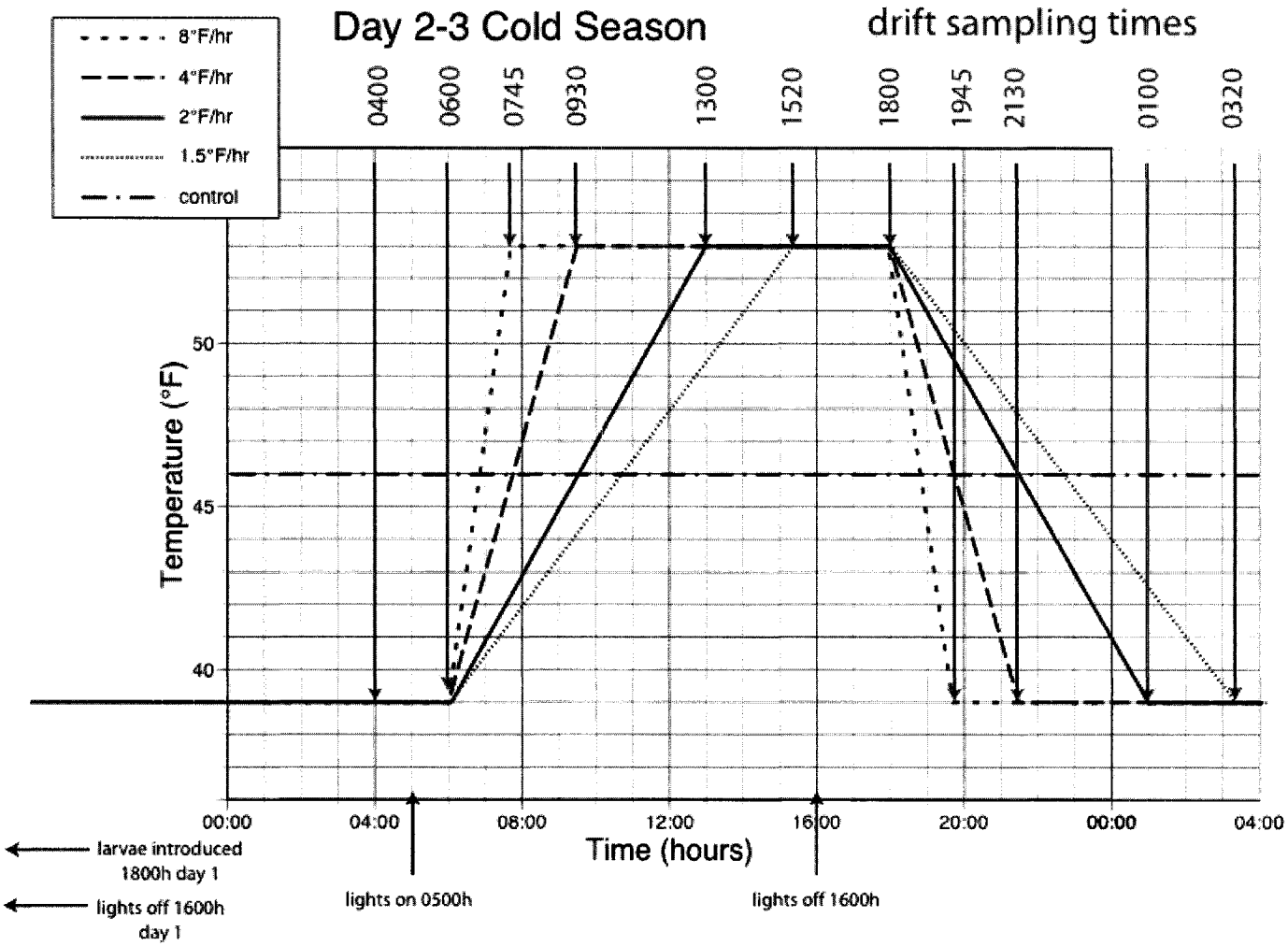

Figure 24. Actual showing cold season conditions in the laboratory drift studies with five thermal regimes (constant at 46°F, and variable between 39 and 53°F with a temperature change rate of 1.5°F/h, 2.0°F/h, 4.0°F/h, 8.0°F/h), and a photoperiod of approximately 11:00h light and 13:00h dark. Treatments are staggered to insure that heating and cooling of the different treatments begin at the same times. The 11 drift sampling times are indicated by vertical arrows originating from the top of the figure.

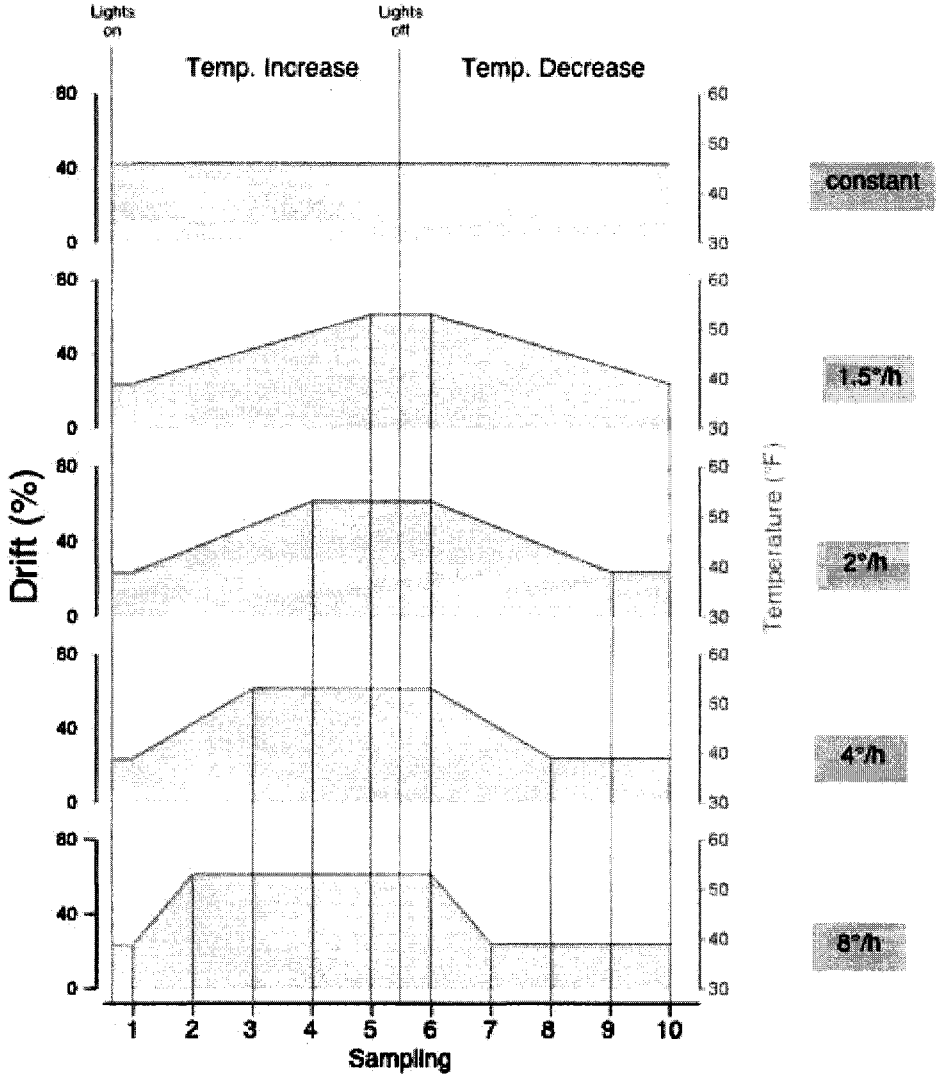

Figure 25. Illustration of drift sampling periods used in Post Hoc Tukey tests to assess changes in drift in one or more of the fluctuating thermal regimes (1.5°F/h, 2.0°F/h, 4.0°F/h, 8.0°F/h).

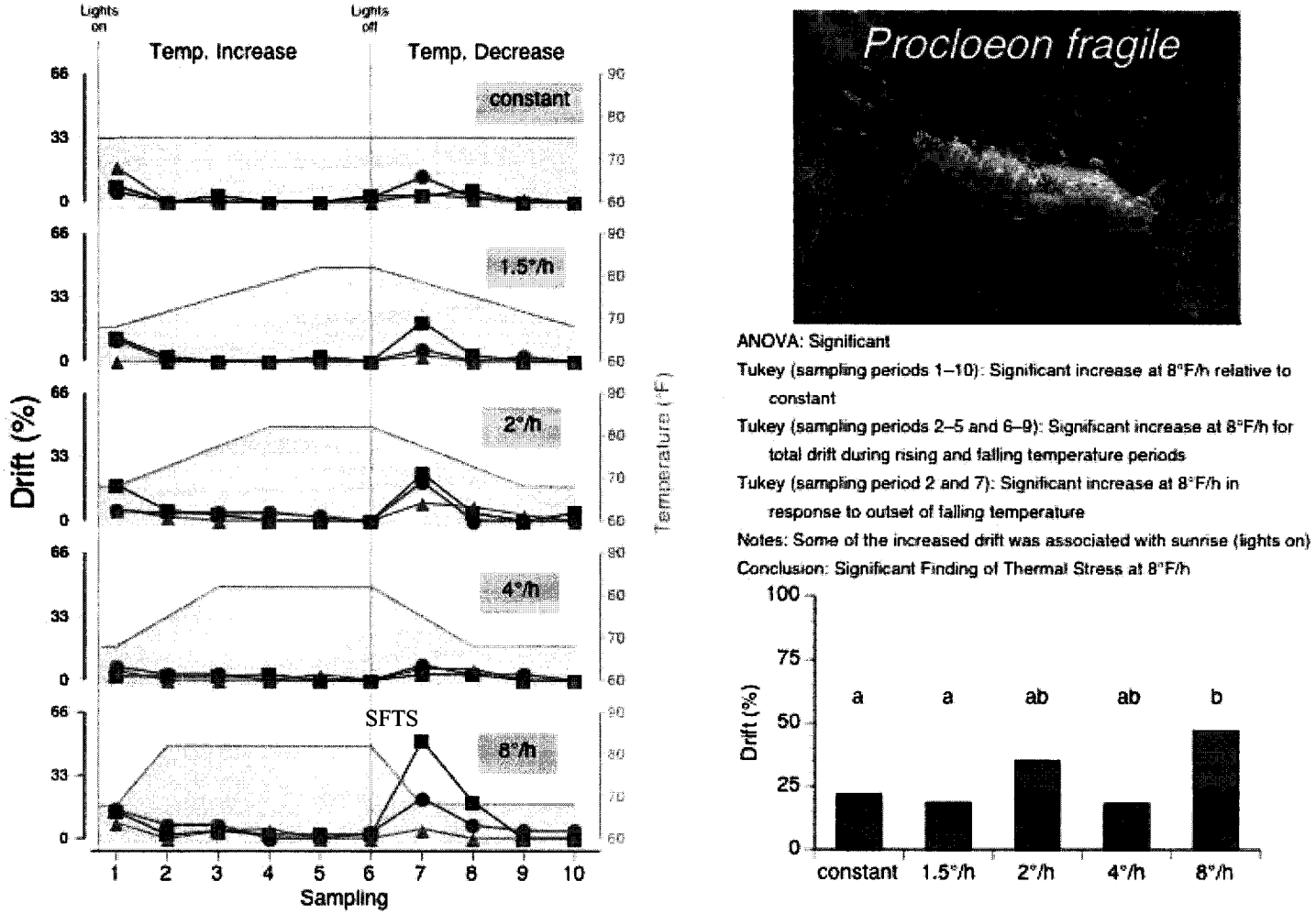

Figure 26. Drift patterns during summer thermal regimes for the mayfly *Procloeon fragile*, presented for three replicate drift troughs during each sample period (superimposed over the different thermal regimes), and summed across the 10 sample periods.

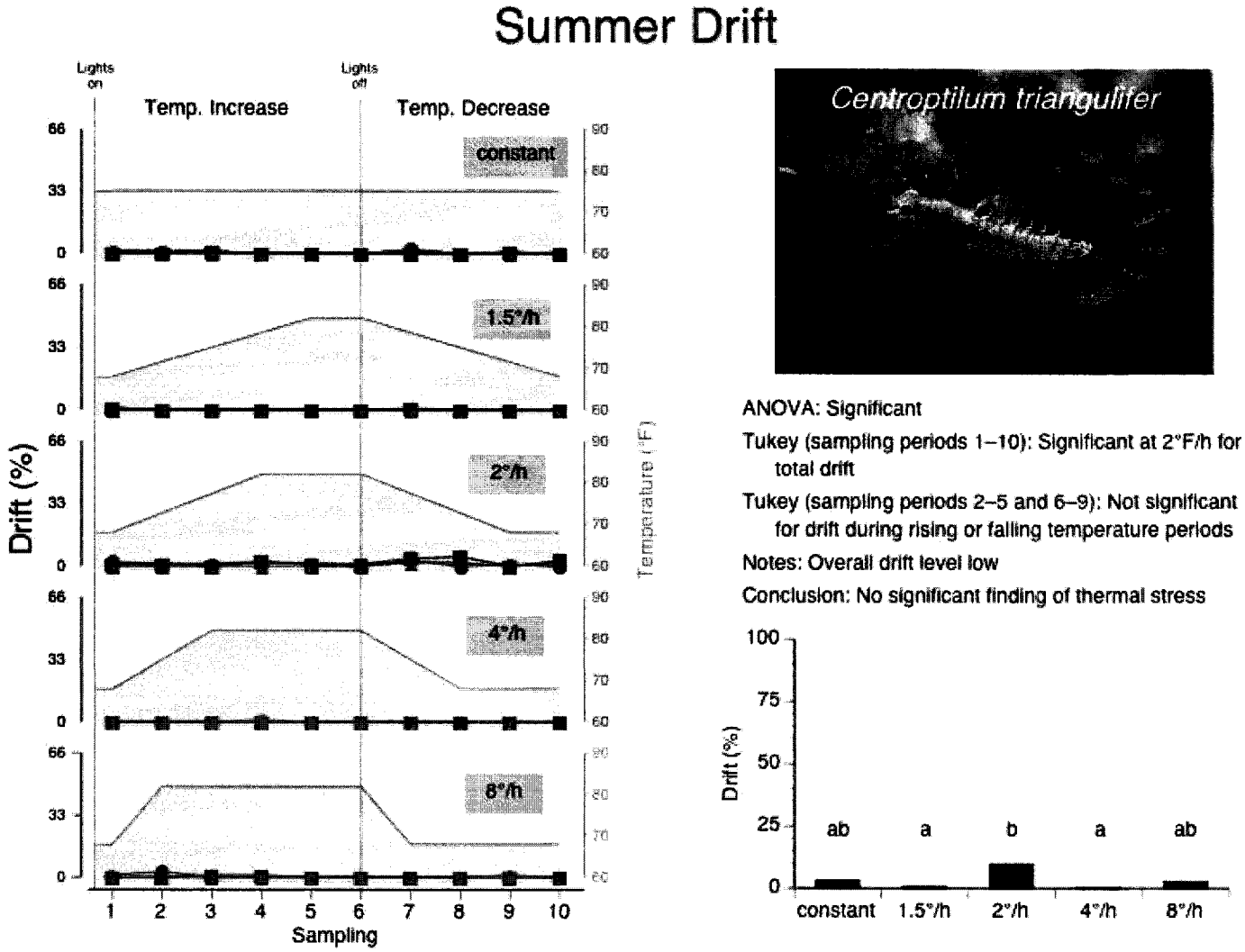

Figure 27. Drift patterns during summer thermal regimes for the mayfly *Centroptilum triangulifer*, presented for three replicate drift troughs during each sample period (superimposed over the different thermal regimes), and summed across the 10 sample periods.

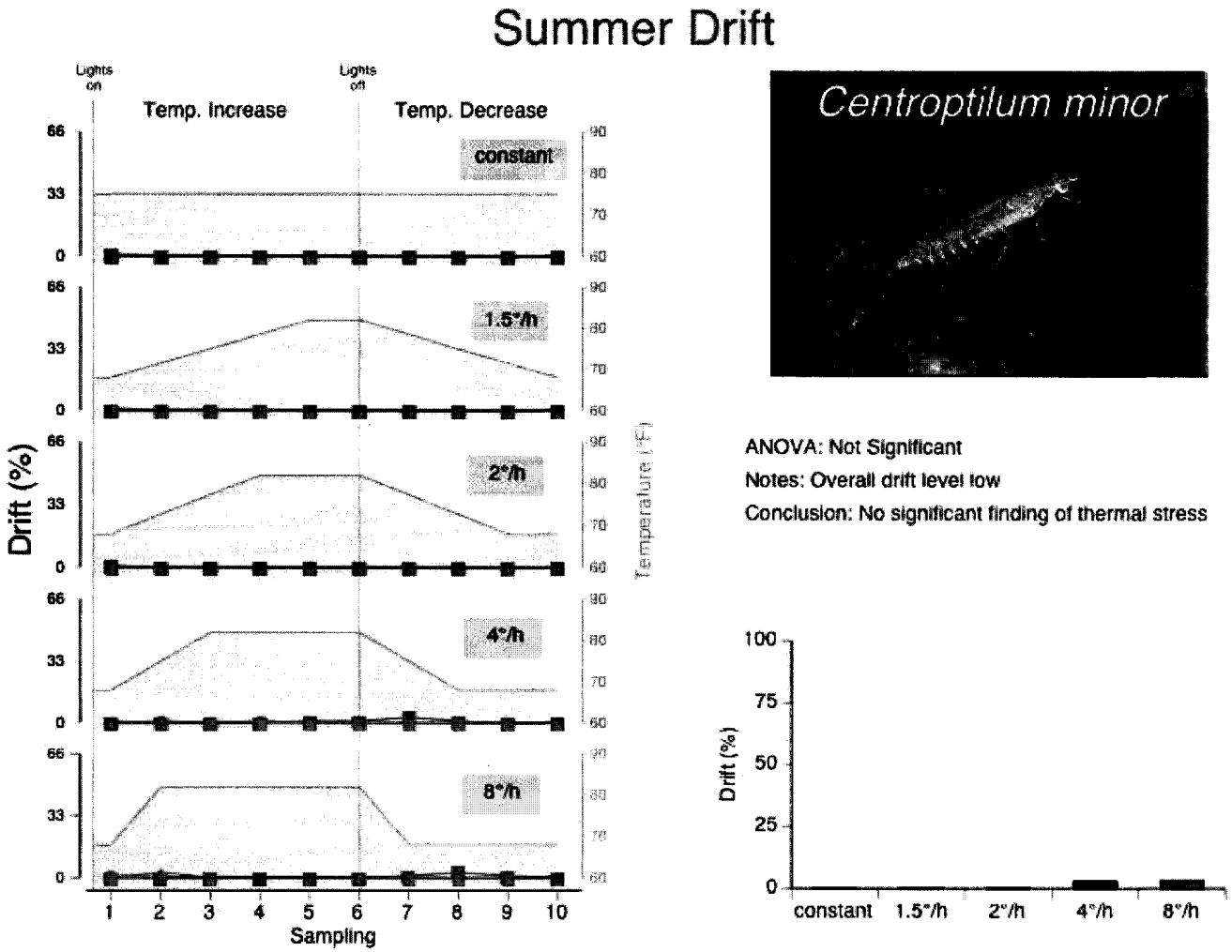

Figure 28. Drift patterns during summer thermal regimes for the mayfly *Centroptilum minor*, presented for three replicate drift troughs during each sample period (superimposed over the different thermal regimes), and summed across the 10 sample periods.

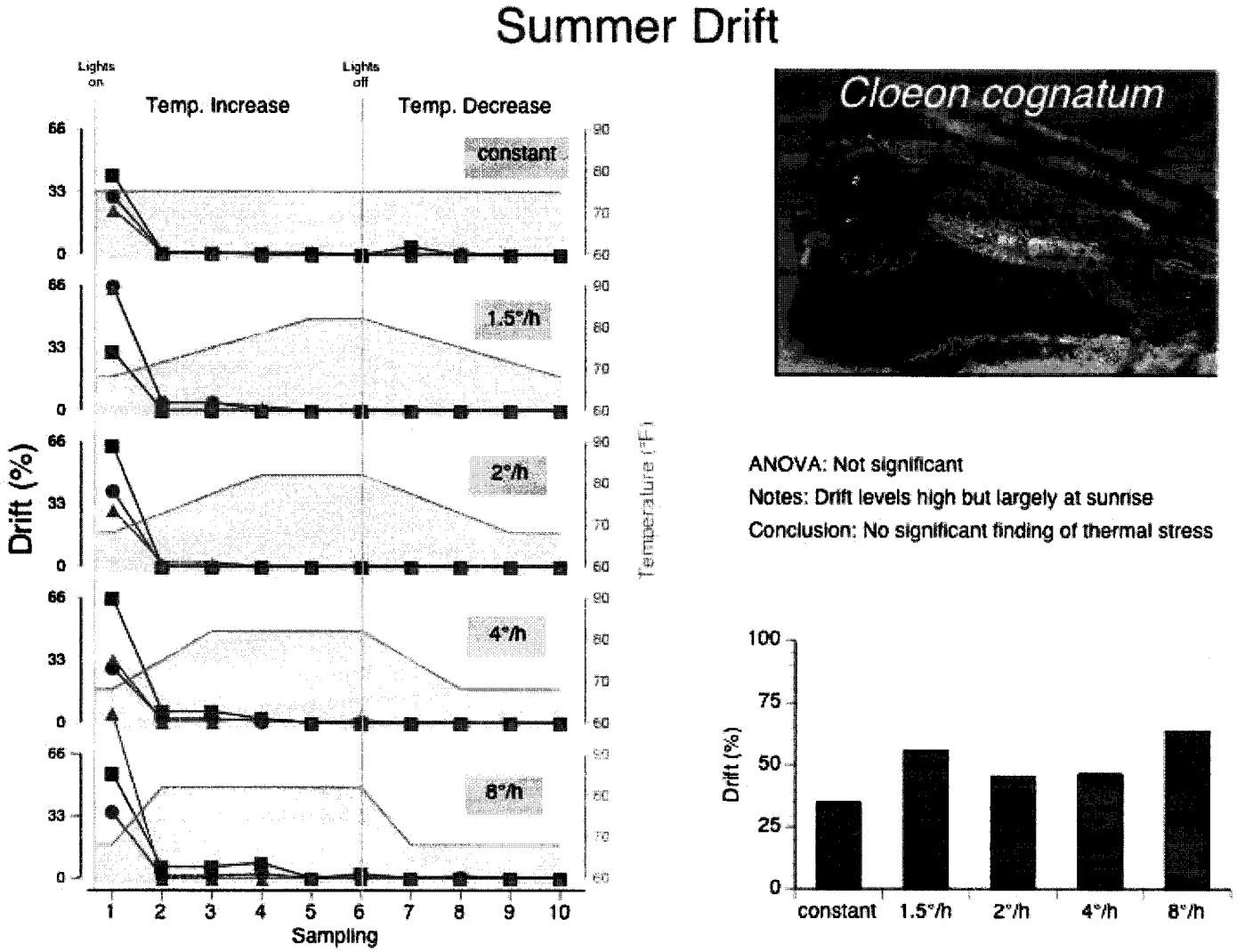

Figure 29. Drift patterns during summer thermal regimes for the mayfly *Cloeon cognatum*, presented for three replicate drift troughs during each sample period (superimposed over the different thermal regimes), and summed across the 10 sample periods.

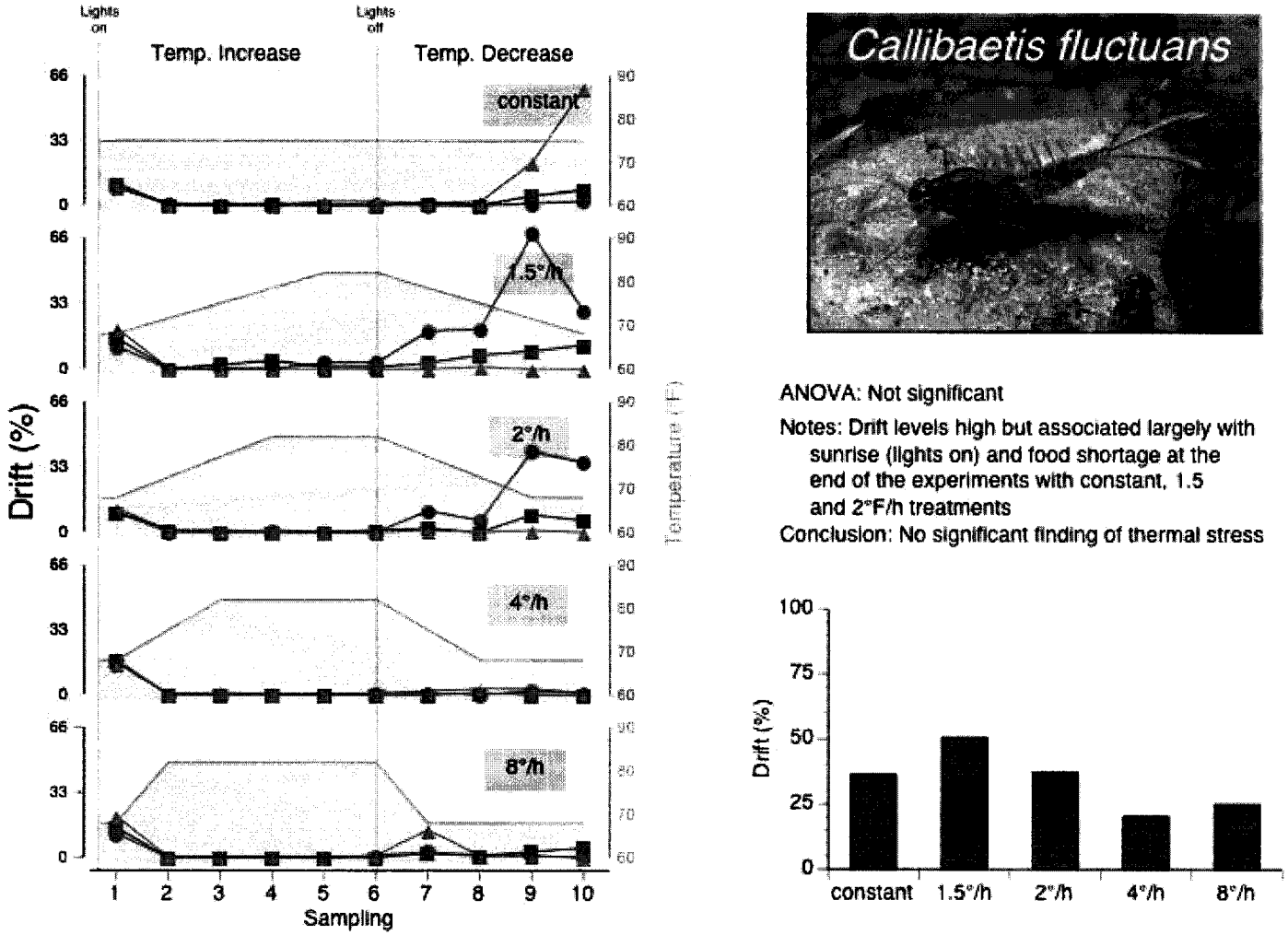

Figure 30. Drift patterns during summer thermal regimes for the mayfly *Callibaetis fluctuans*, presented for three replicate drift troughs during each sample period (superimposed over the different thermal regimes), and summed across the 10 sample periods.

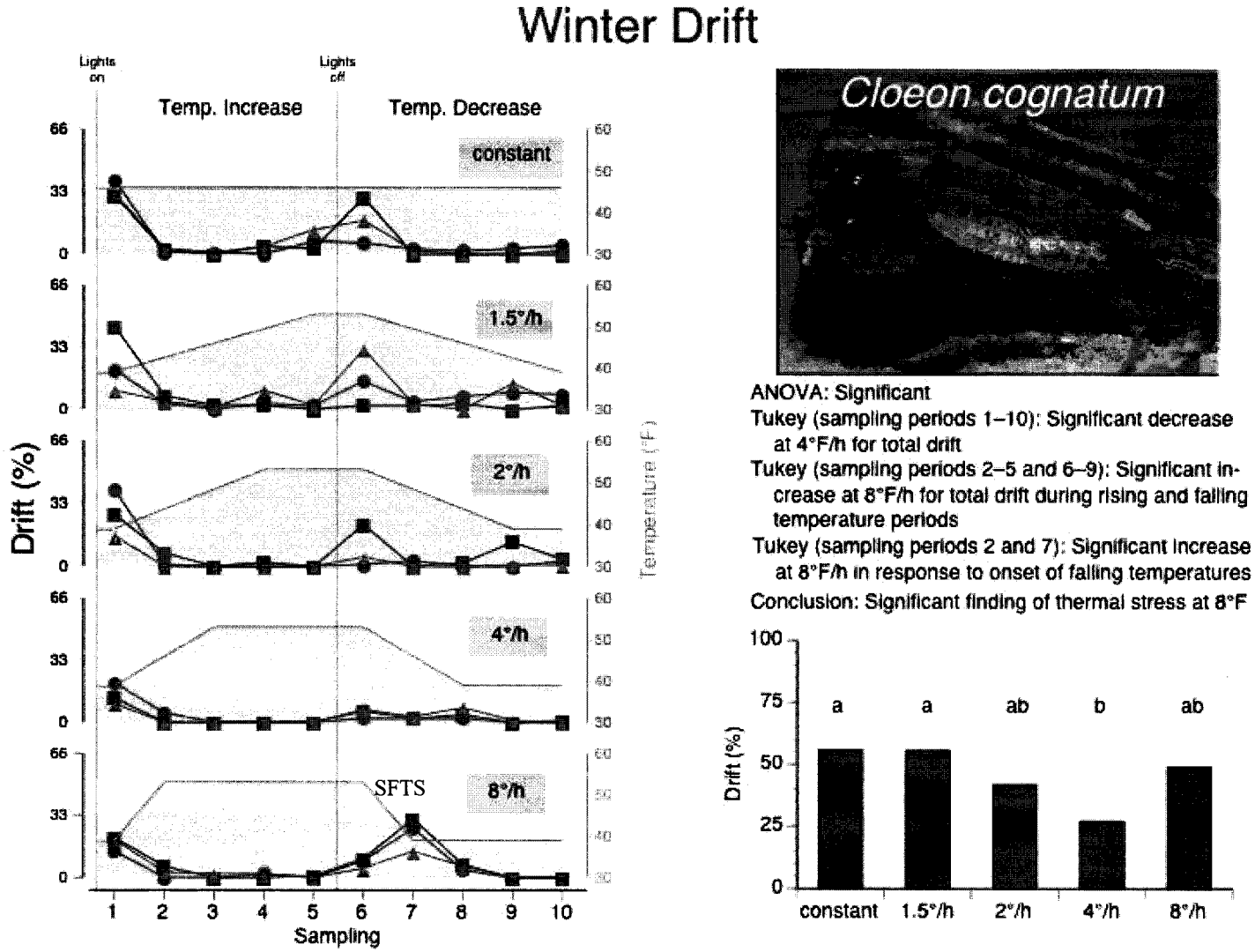

Figure 31. Drift patterns during winter thermal regimes for the mayfly *Cloeon cognatum*, presented for three replicate drift troughs during each sample period (superimposed over the different thermal regimes), and summed across the 10 sample periods.

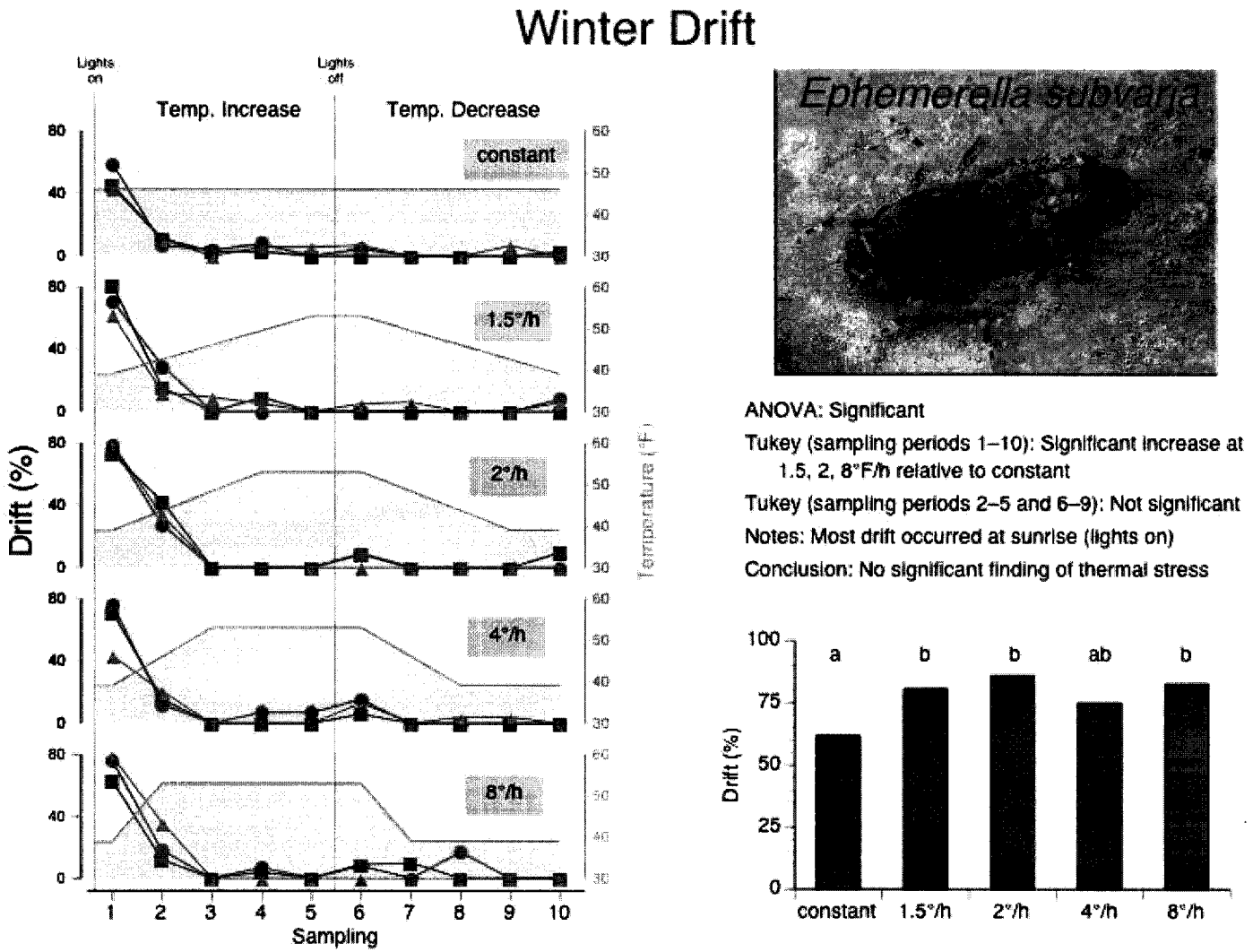

Figure 32. Drift patterns during winter thermal regimes for the mayfly *Ephemerella subvaria*, presented for three replicate drift troughs during each sample period (superimposed over the different thermal regimes), and summed across the 10 sample periods.

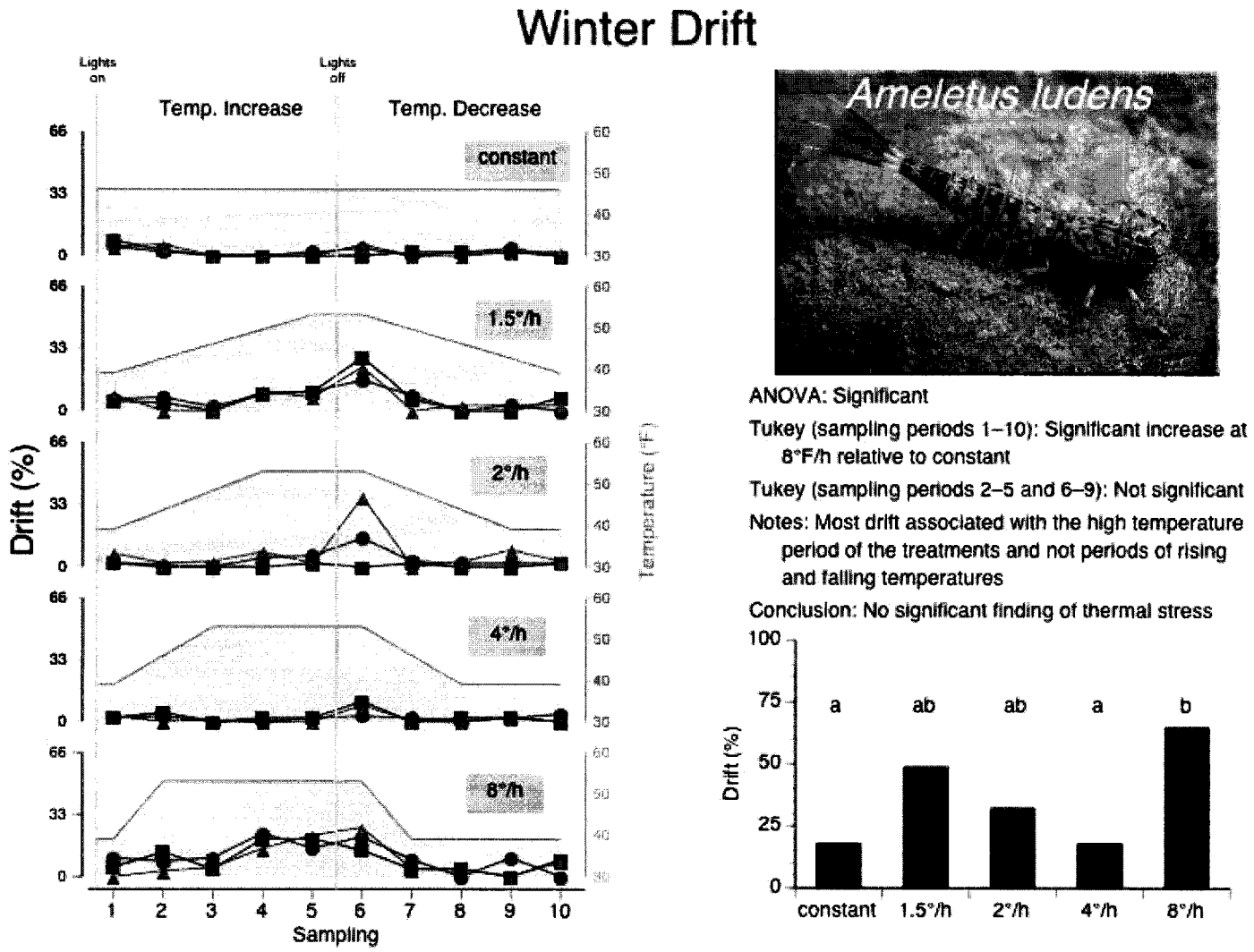

Figure 33. Drift patterns during winter thermal regimes for the mayfly *Ameletus ludens*, presented for three replicate drift troughs during each sample period (superimposed over the different thermal regimes), and summed across the 10 sample periods.

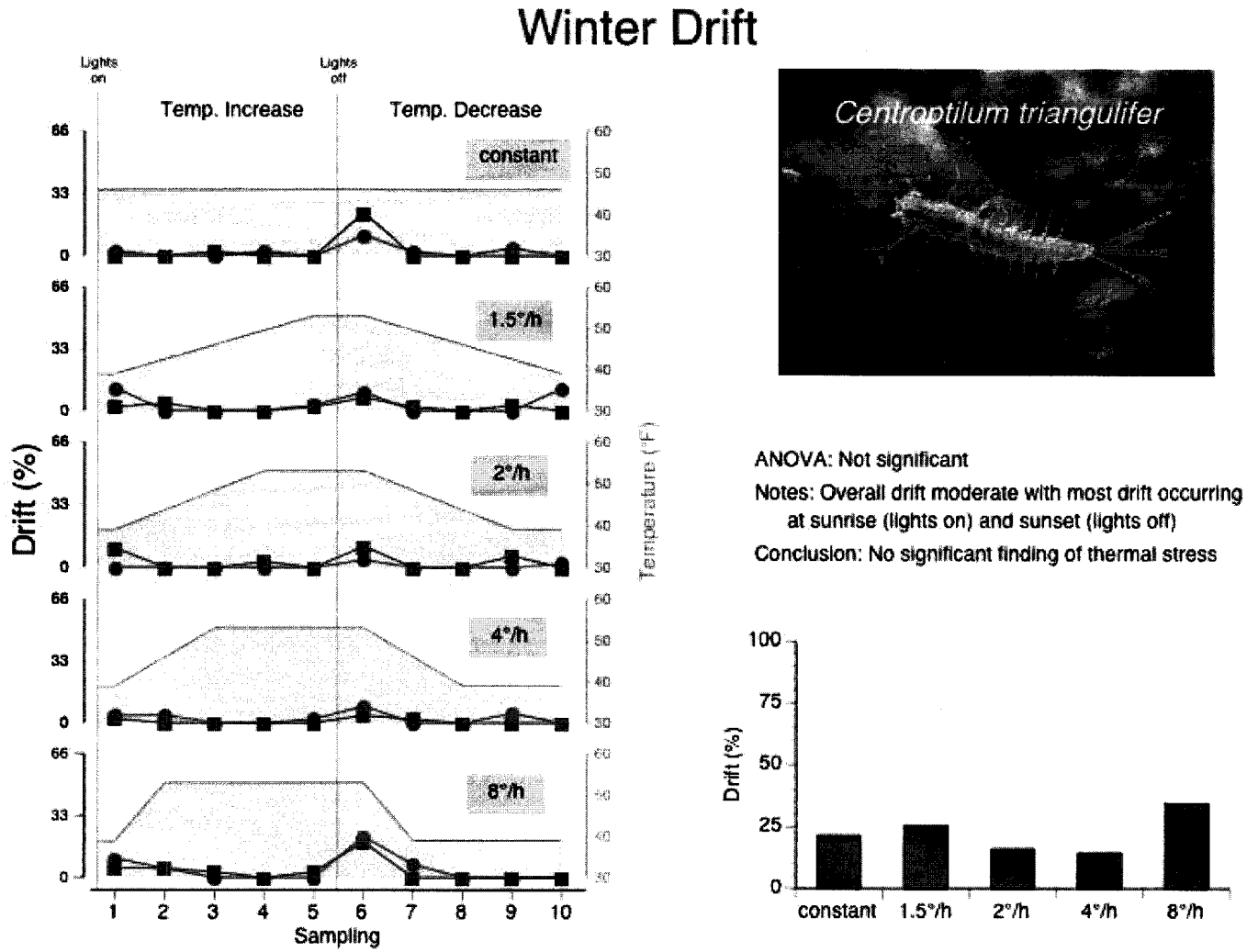

Figure 34. Drift patterns during winter thermal regimes for the mayfly *Centroptilum triangulifer*, presented for three replicate drift troughs during each sample period (superimposed over the different thermal regimes), and summed across the 10 sample periods.

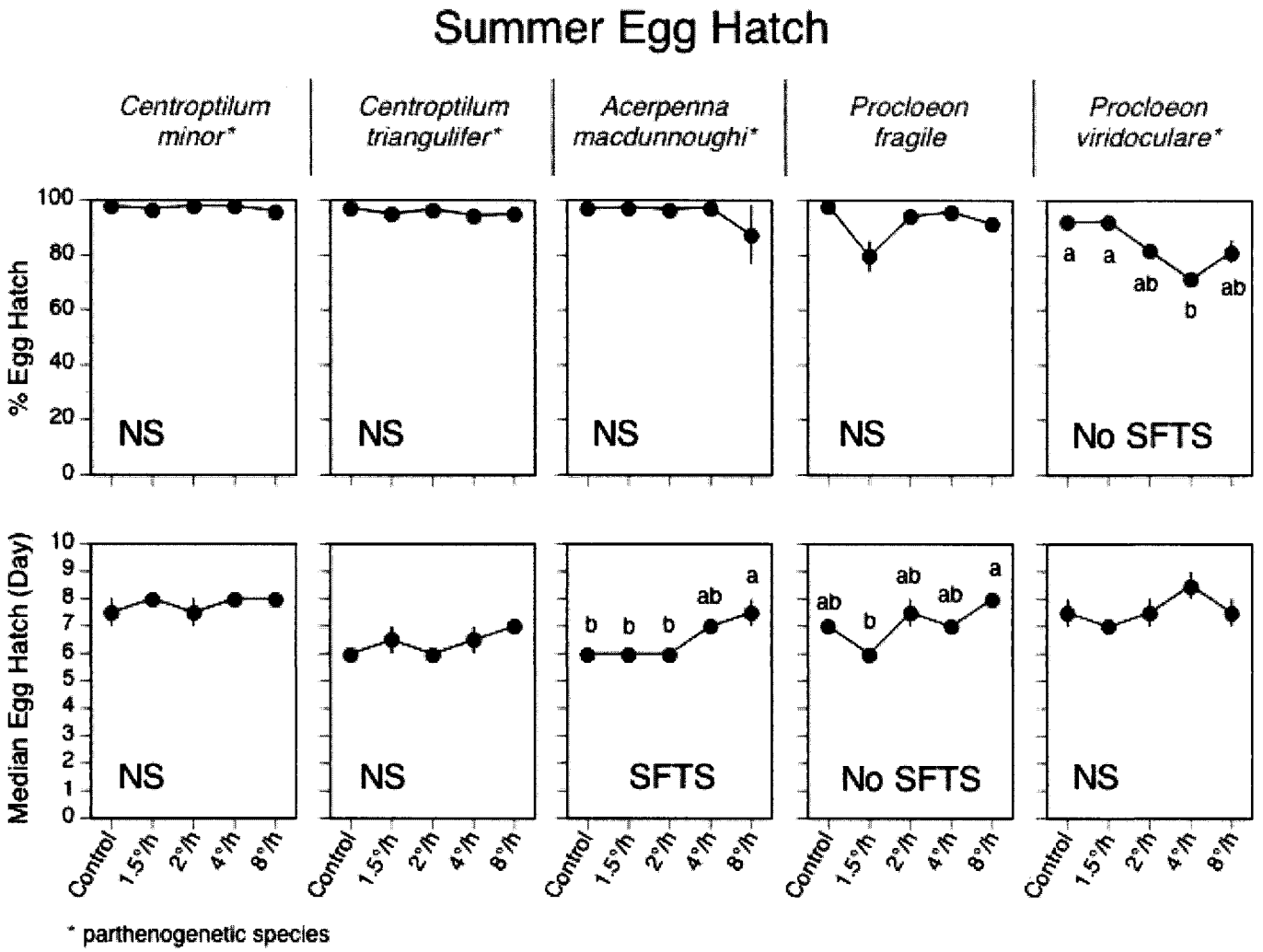

Figure 35. Egg hatching success (as a percentage of total oviposited and number of days between oviposition and hatching) for six mayfly species during warm season thermal regimes (i.e., constant at 75°F, and variable between 68 and 82°F with a temperature change rate of 1.5°F/h, 2.0°F/h, 4.0°F/h, 8.0°F/h).

Summer Larval Survivorship

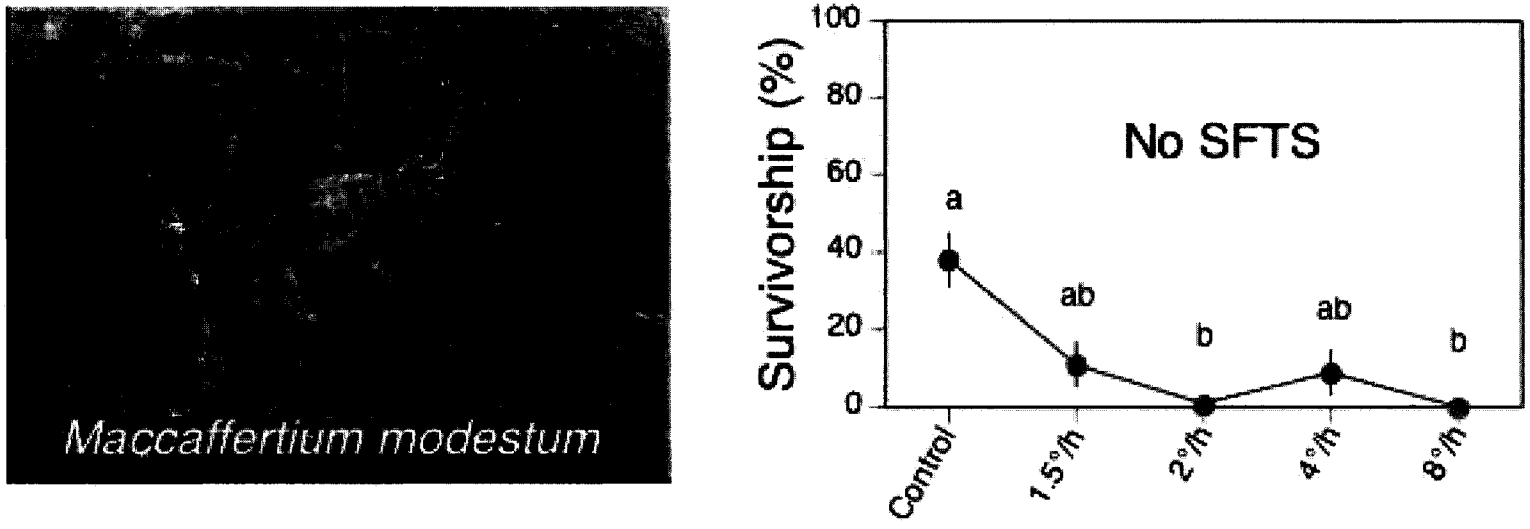

Figure 36. Larval survivorship (as a percentage) for the mayfly *Maccaffertium modestum* during warm season thermal regimes (i.e., constant at 75°F, and variable between 68 and 82°F with a temperature change rate of 1.5°F/h, 2.0°F/h, 4.0°F/h, 8.0°F/h).

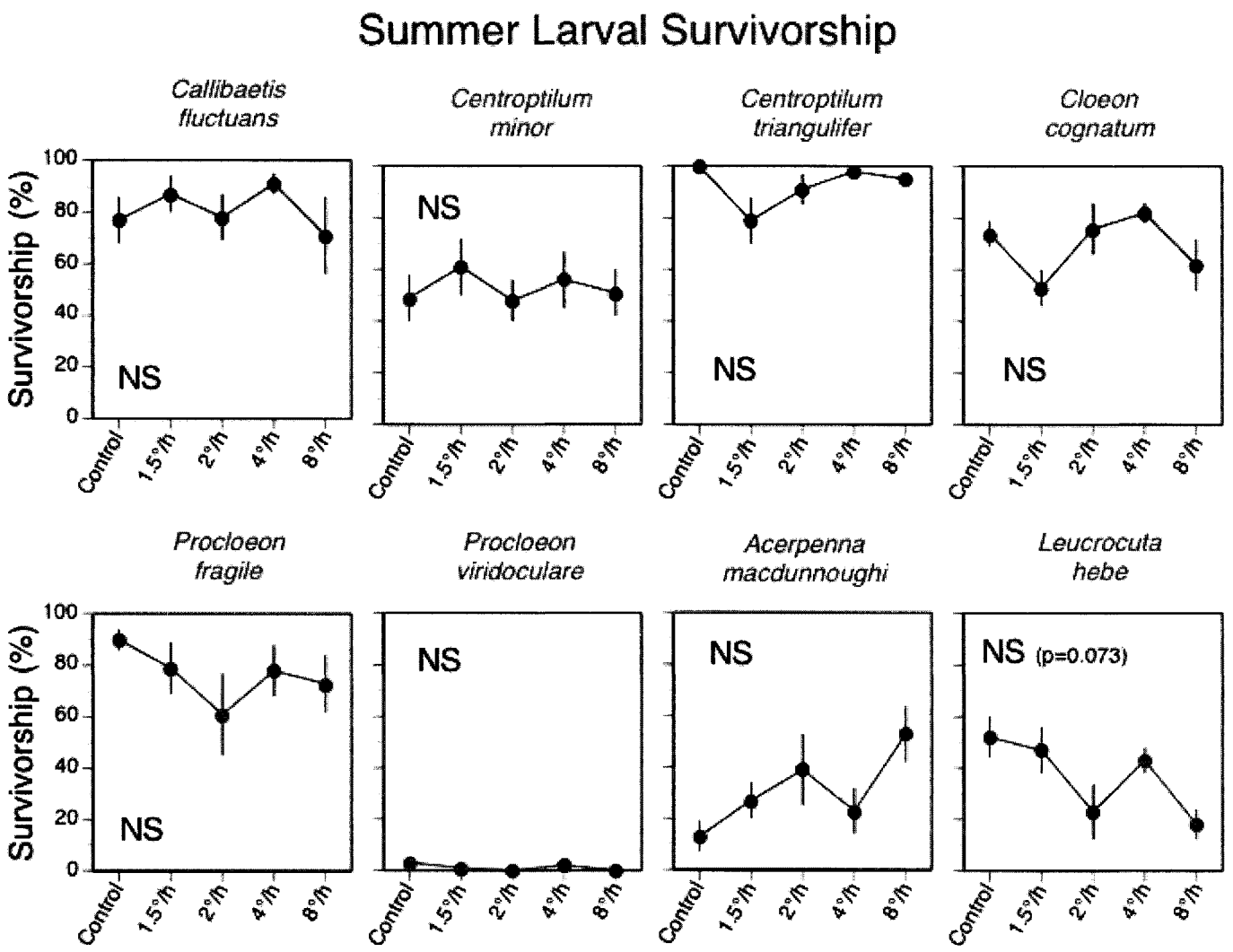

Figure 37. Larval survivorship (as a percentage) for eight mayfly species during warm season thermal regimes (i.e., constant at 75°F, and variable between 68 and 82°F with a temperature change rate of 1.5°F/h, 2.0°F/h, 4.0°F/h, 8.0°F/h).

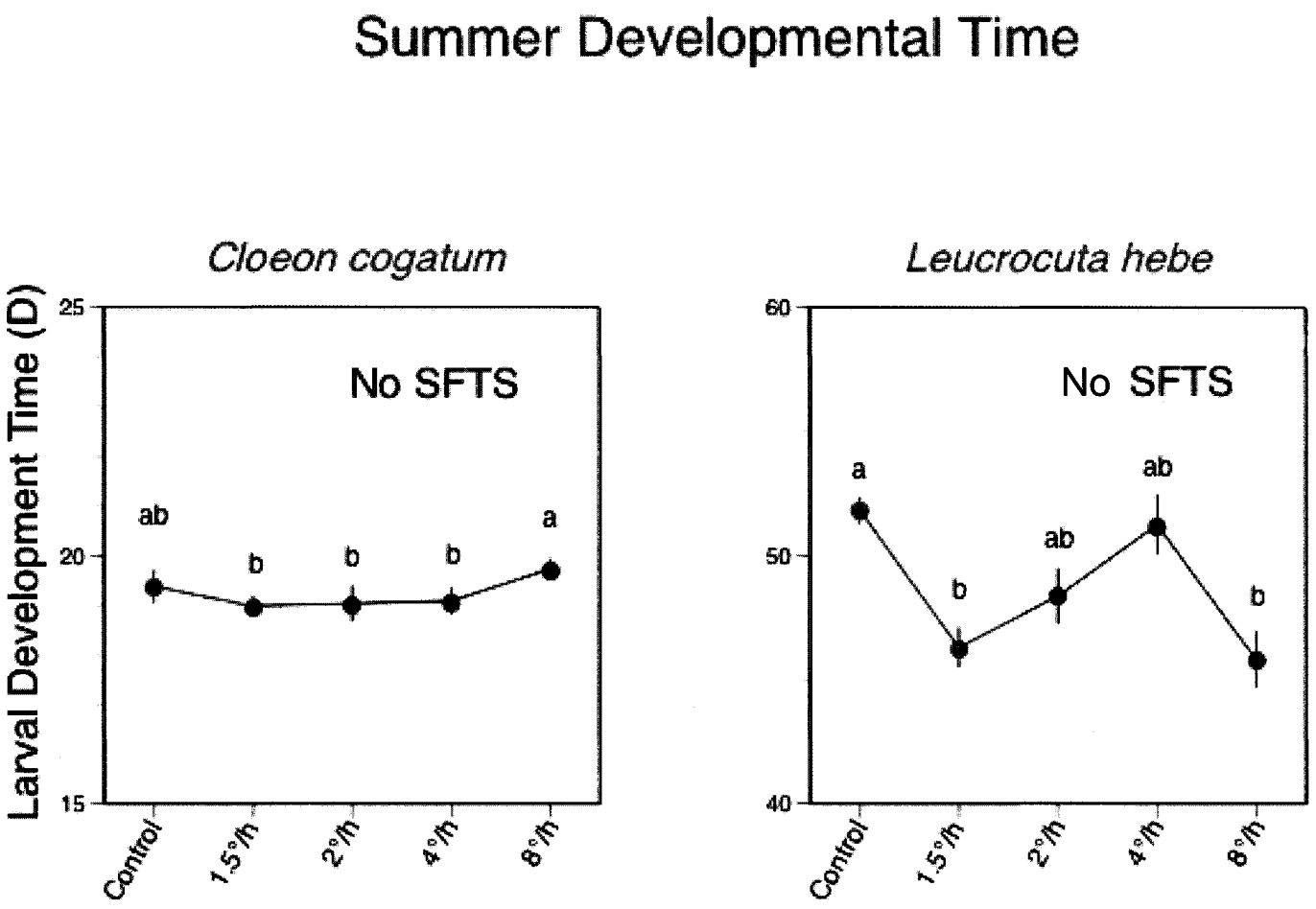

Figure 38. Larval development time (D) for two mayfly species during warm season thermal regimes (i.e., constant at 75°F, and variable between 68 and 82°F with a temperature change rate of 1.5°F/h, 2.0°F/h, 4.0°F/h, 8.0°F/h).

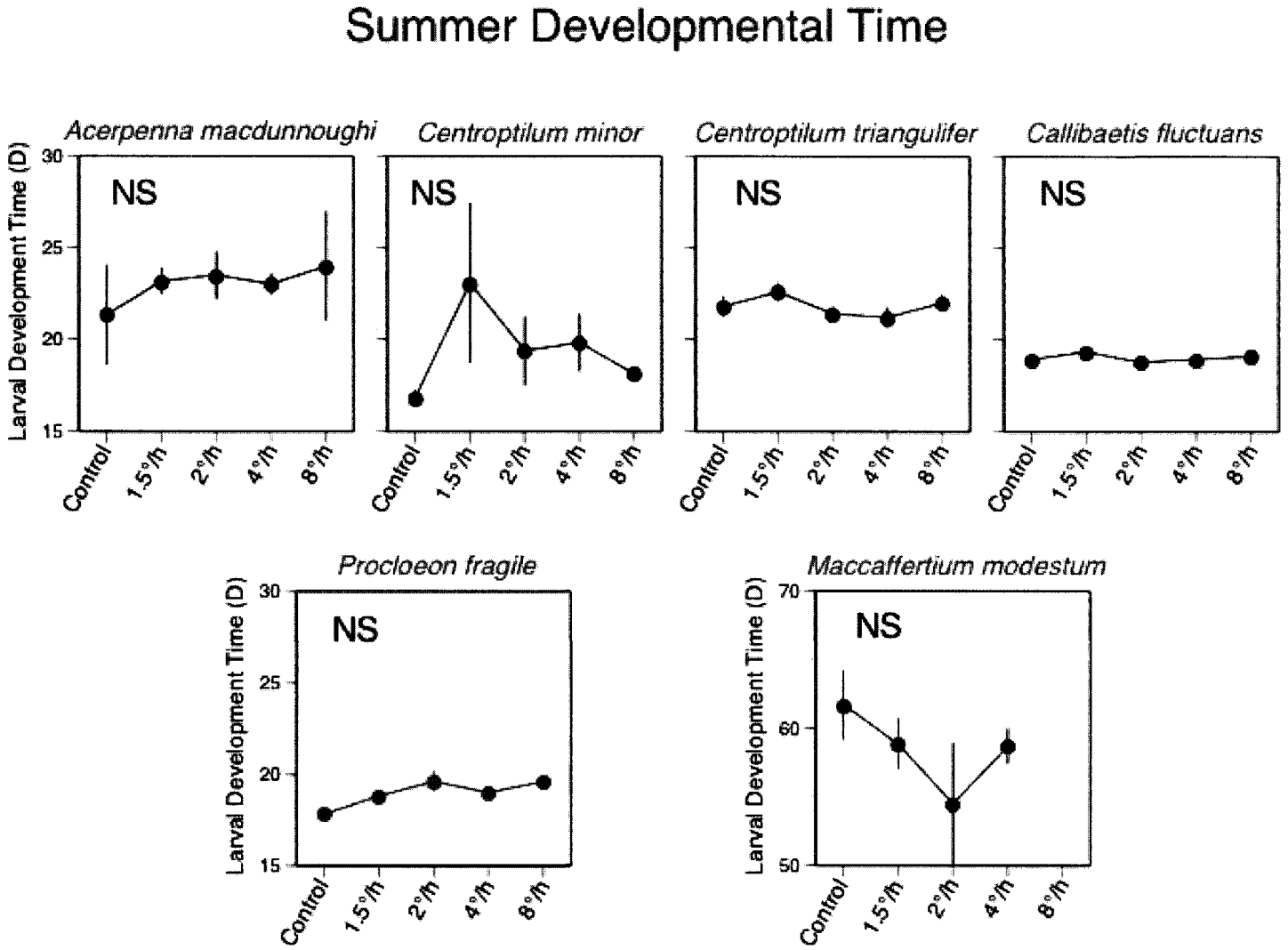

Figure 39. Larval development time (D) for six mayfly species during warm season thermal regimes (i.e., constant at 75°F, and variable between 68 and 82°F with a temperature change rate of 1.5°F/h, 2.0°F/h, 4.0°F/h, 8.0°F/h).

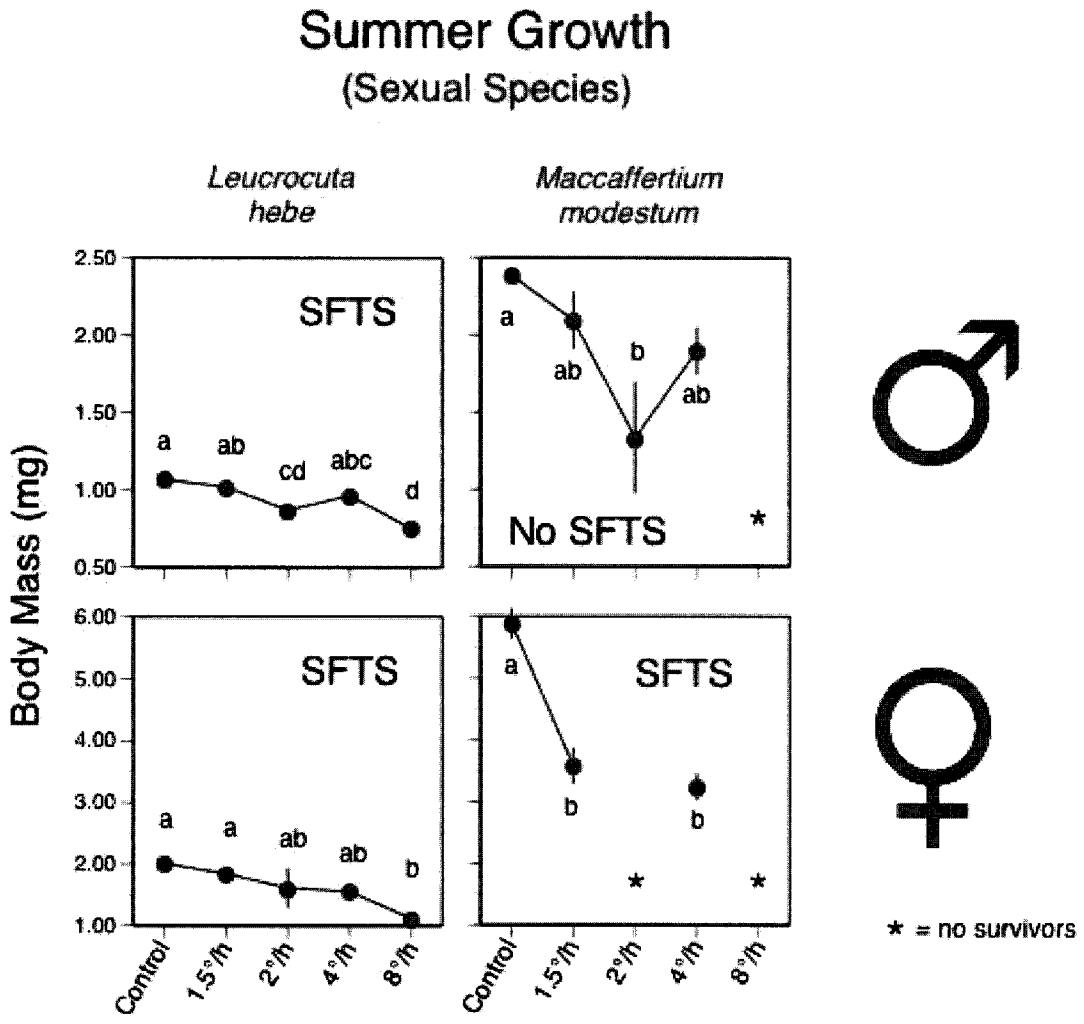

Figure 40. Growth expressed as adult size (mg dry mass) for two mayfly species (male and female) during warm season thermal regimes (i.e., constant at 75°F, and variable between 68 and 82°F with a temperature change rate of 1.5°F/h, 2.0°F/h, 4.0°F/h, 8.0°F/h).

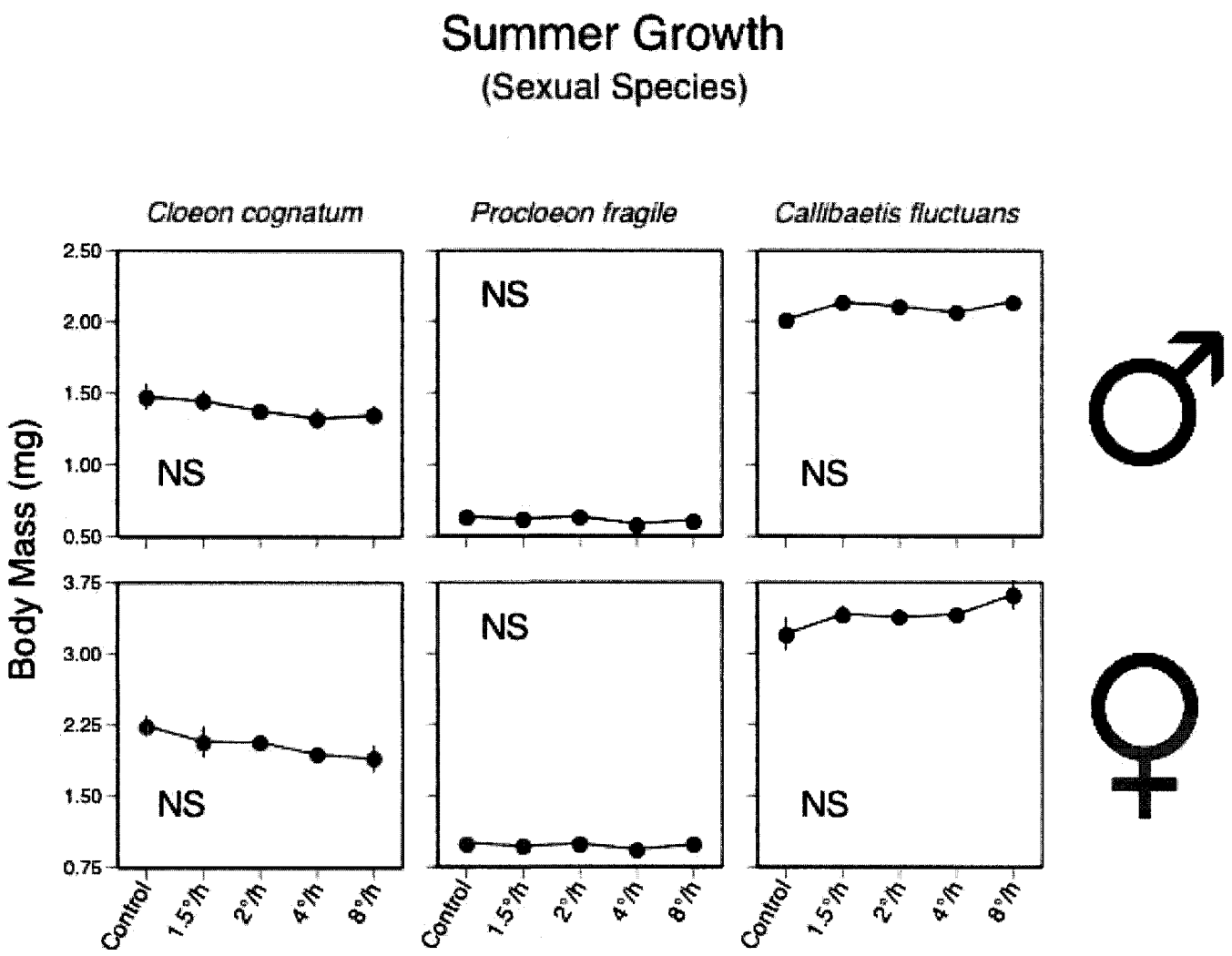

Figure 41. Growth expressed as adult size (mg dry mass) for three mayfly species (male and female) during warm season thermal regimes (i.e., constant at 75°F, and variable between 68 and 82°F with a temperature change rate of 1.5°F/h, 2.0°F/h, 4.0°F/h, 8.0°F/h).

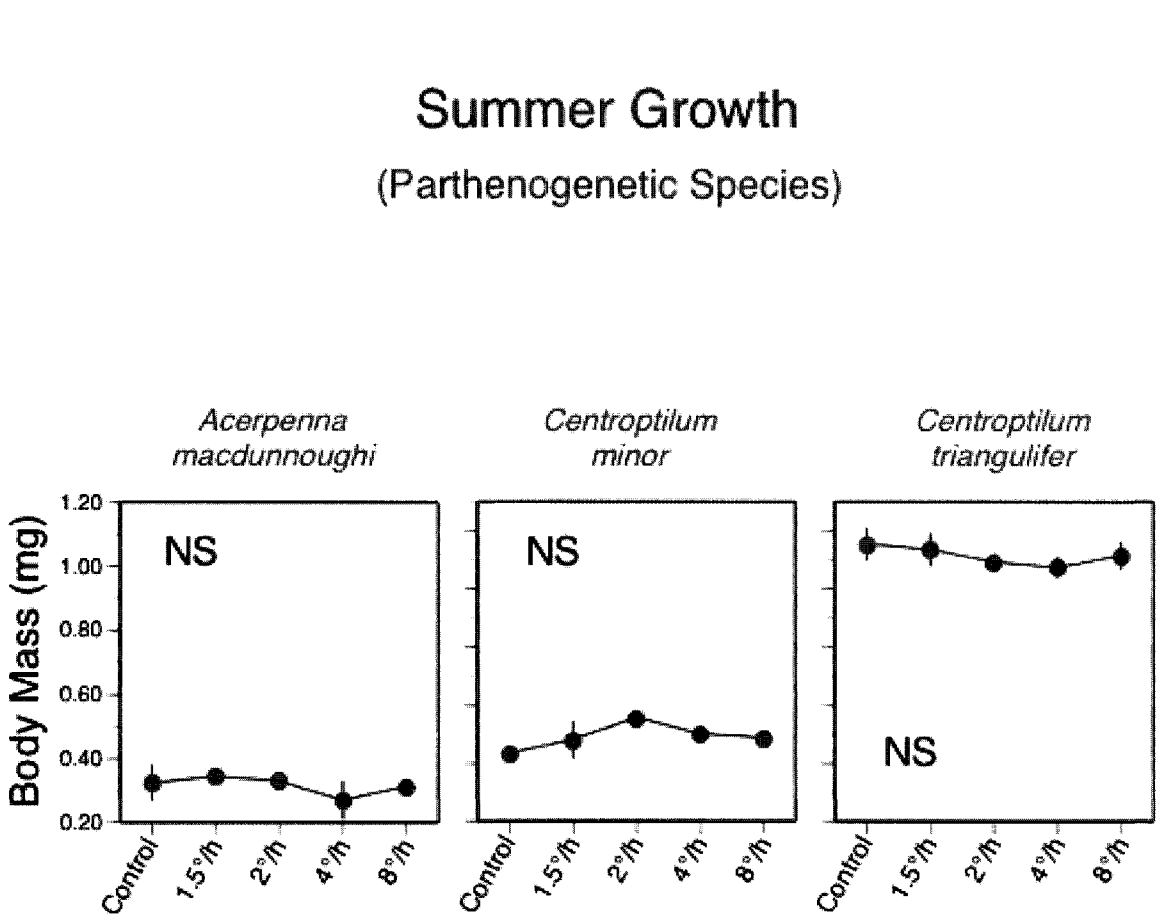

Figure 42. Growth expressed as adult size (mg dry mass) for three mayfly species (female only) during warm season thermal regimes (i.e., constant at 75°F, and variable between 68 and 82°F with a temperature change rate of 1.5°F/h, 2.0°F/h, 4.0°F/h, 8.0°F/h).

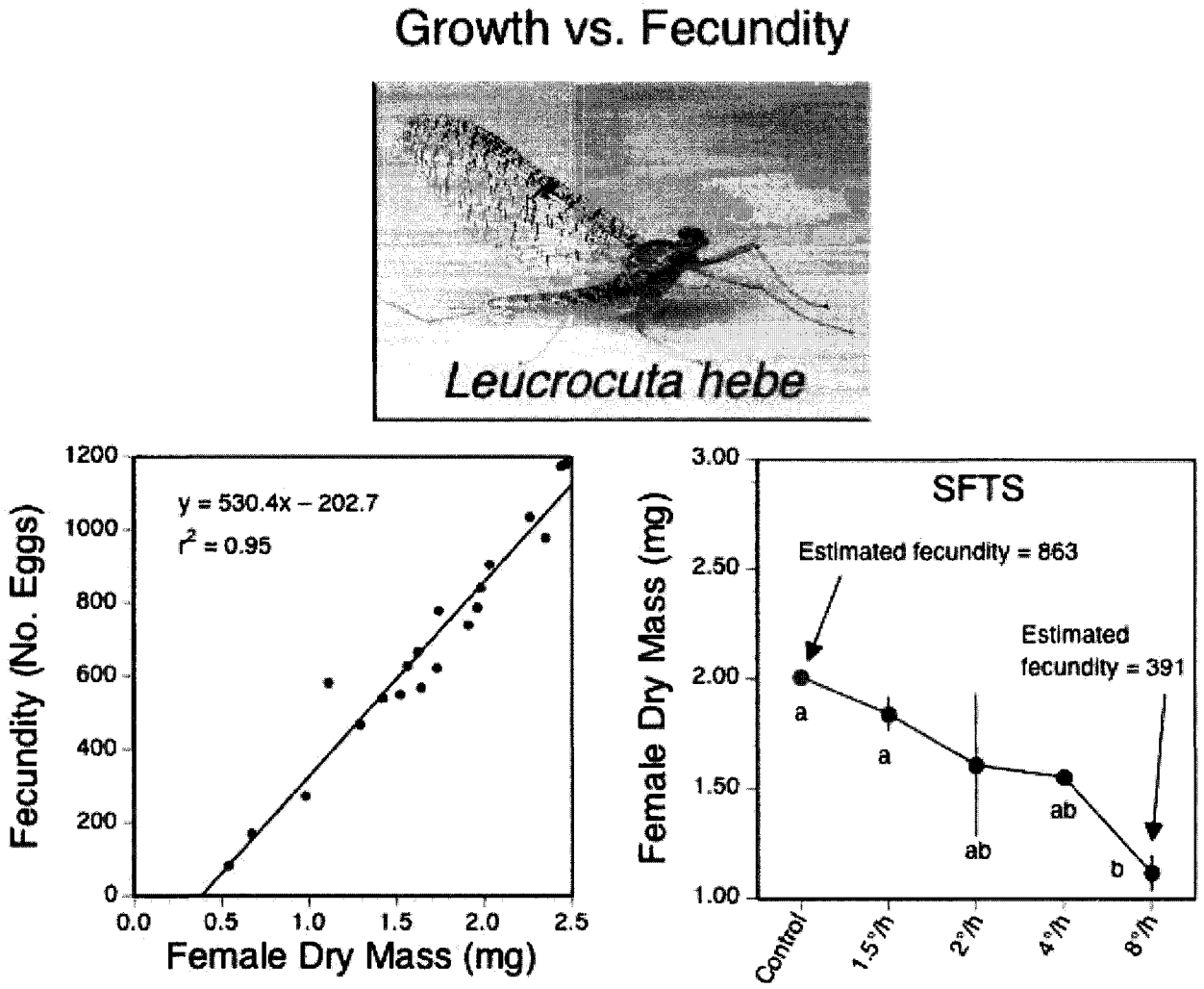

Figure 43. Growth expressed as adult size (mg dry mass) and fecundity (number of eggs per female) for the mayfly *Leucrocuta hebe* during warm season thermal regimes (i.e., constant at 75°F, and variable between 68 and 82°F with a temperature change rate of 1.5°F/h, 2.0°F/h, 4.0°F/h, 8.0°F/h). Relationship between female body size (as mg dry mass) and fecundity used to convert differences in body size into differences in fecundity.

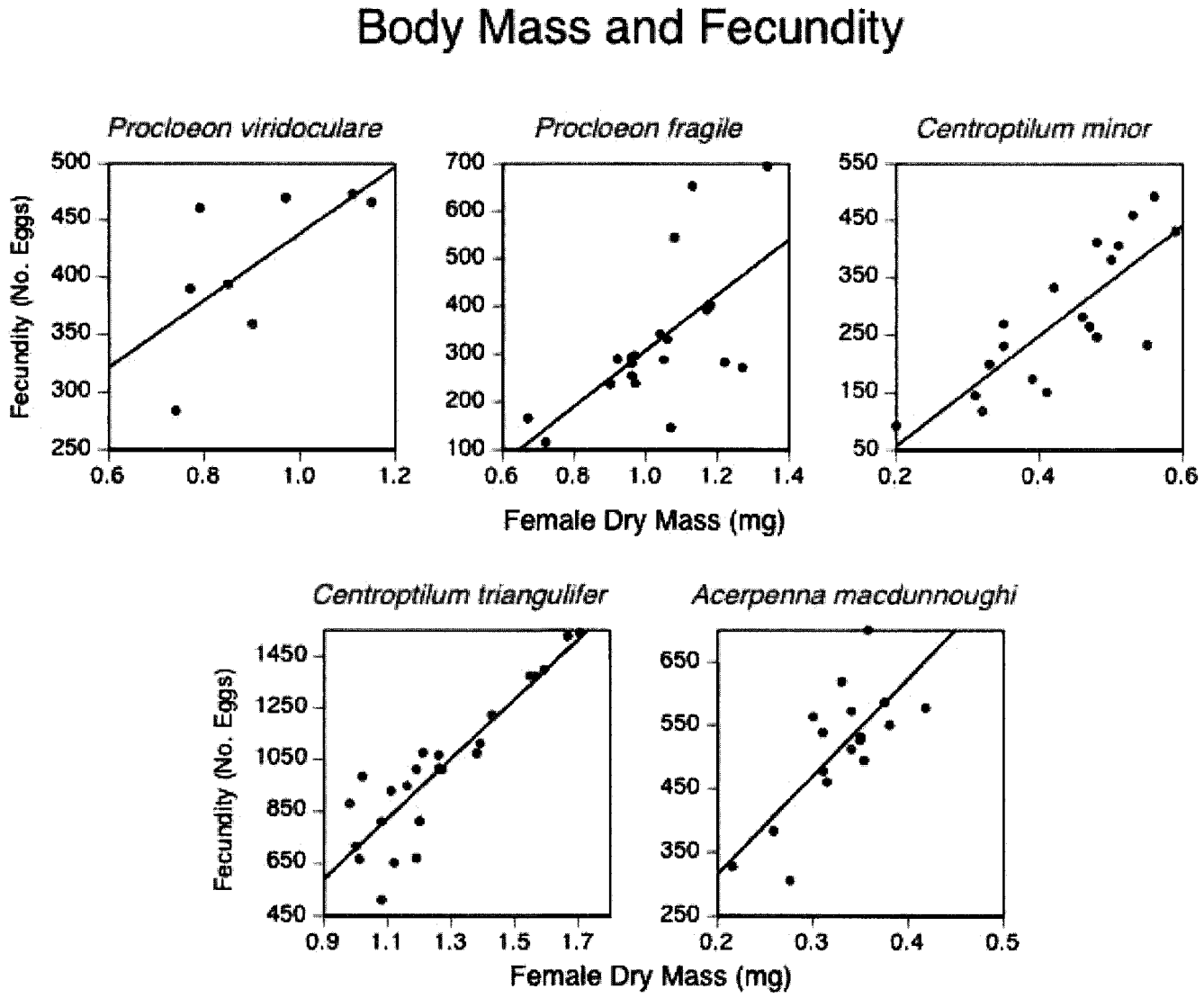

Figure 44. Relationship between female body size (as mg dry mass) and fecundity for five mayfly species.

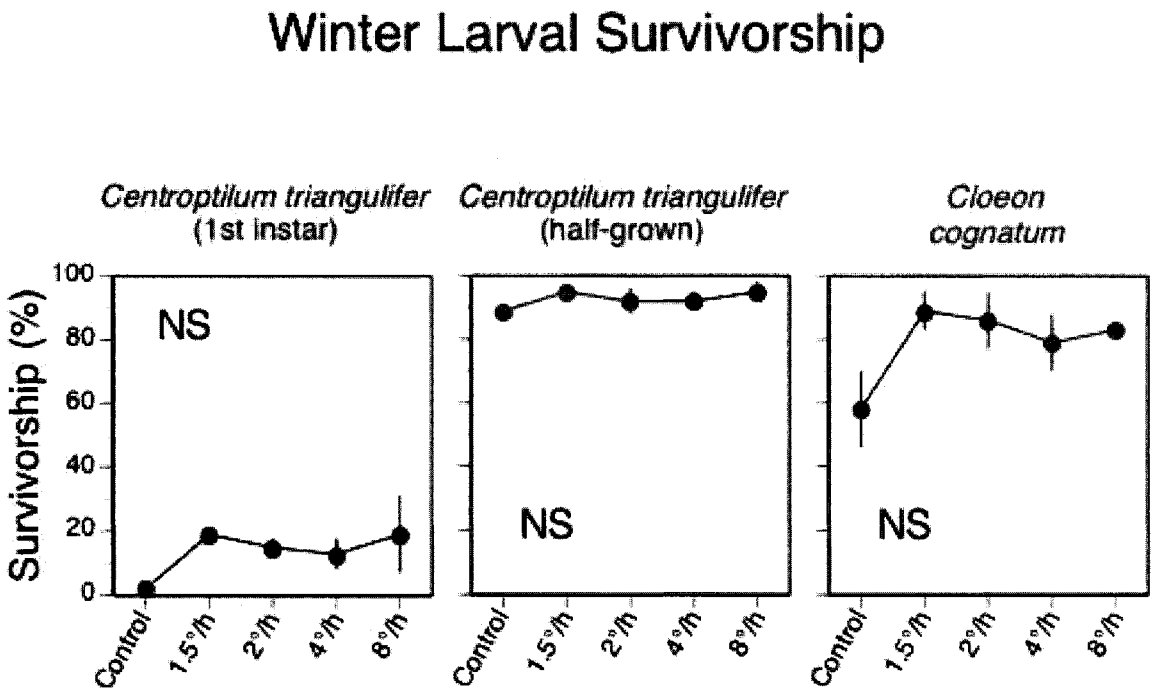

Figure 45. Larval survivorship (as a percentage) for two mayfly species during cold season thermal regimes (constant at 46°F, and variable between 39 and 53°F with a temperature change rate of 1.5°F/h, 2.0°F/h, 4.0°F/h, 8.0°F/h).

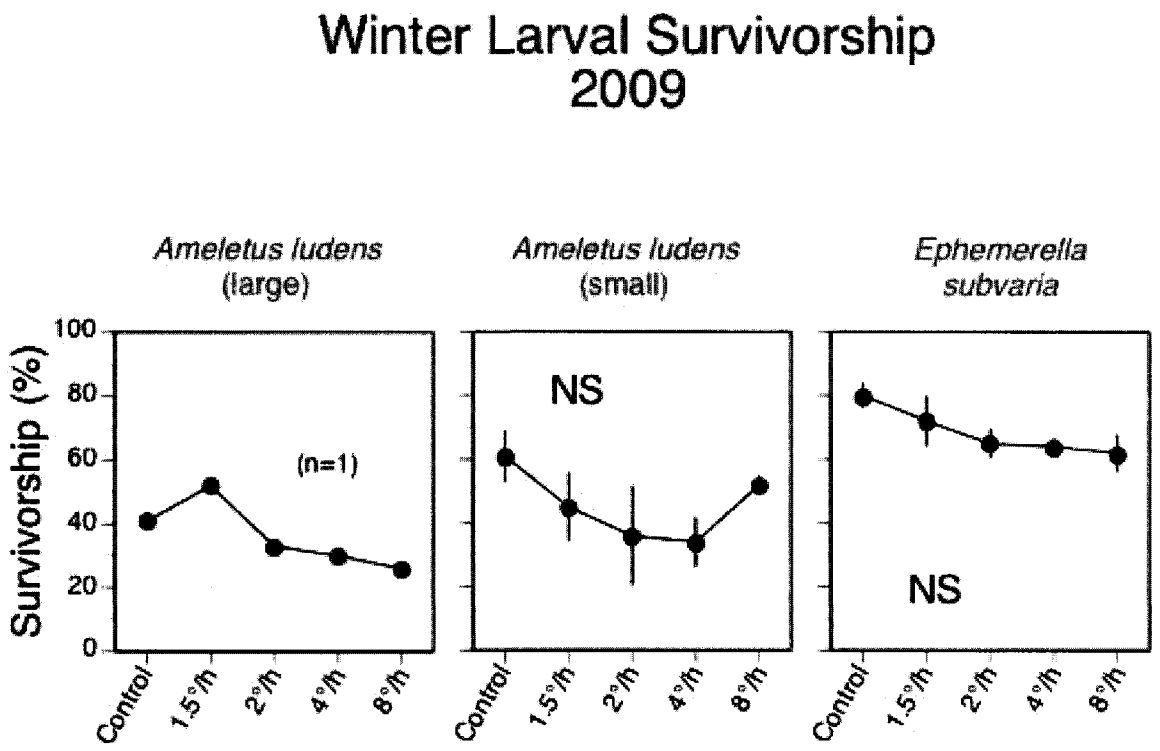

Figure 46. Larval survivorship (as a percentage) for two mayfly species during cold season thermal regimes (constant at 46°F, and variable between 39 and 53°F with a temperature change rate of 1.5°F/h, 2.0°F/h, 4.0°F/h, 8.0°F/h).

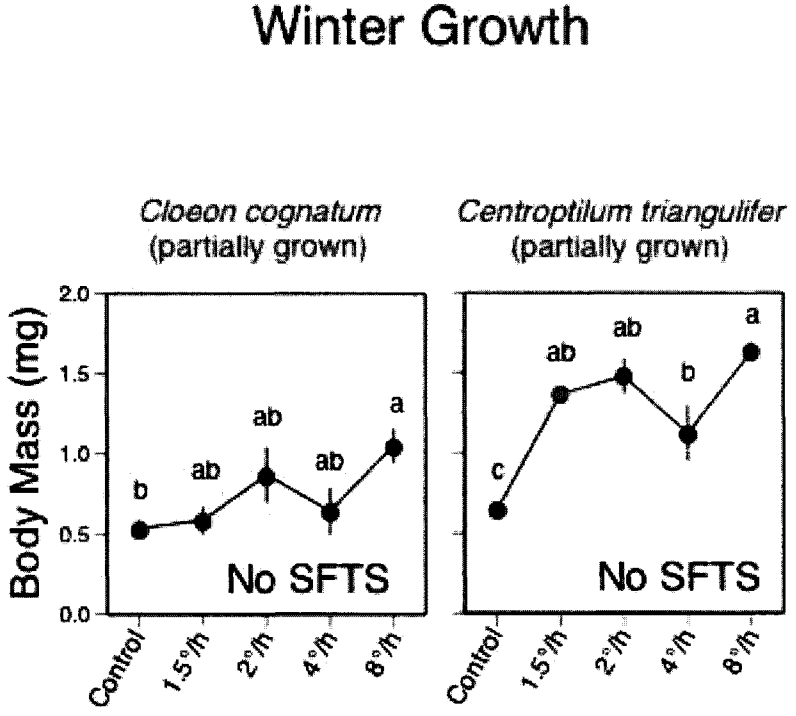

Figure 47. Growth expressed as final larval size (mg dry mass) for two mayfly species during cold season thermal regimes (constant at 46°F, and variable between 39 and 53°F with a temperature change rate of 1.5°F/h, 2.0°F/h, 4.0°F/h, 8.0°F/h).

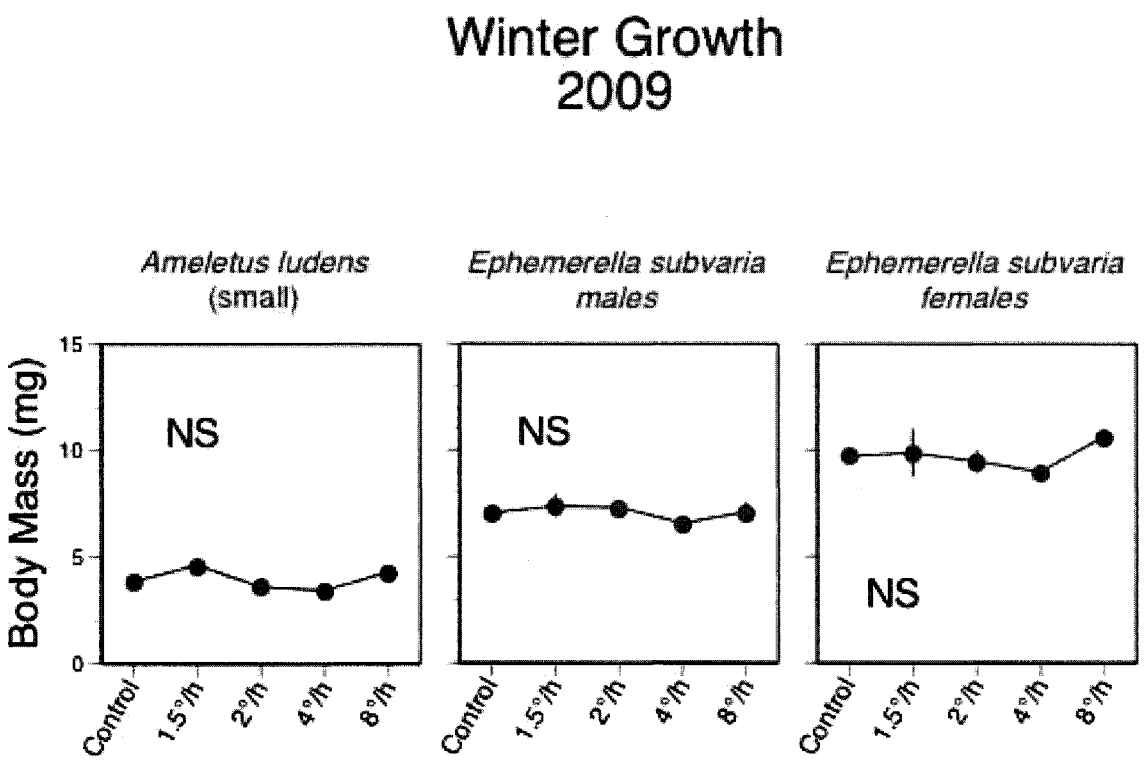

Figure 48. Growth expressed as final body size (mg dry mass) for two mayfly species during cold season thermal regimes (constant at 46°F, and variable between 39 and 53°F with a temperature change rate of 1.5°F/h, 2.0°F/h, 4.0°F/h, 8.0°F/h).

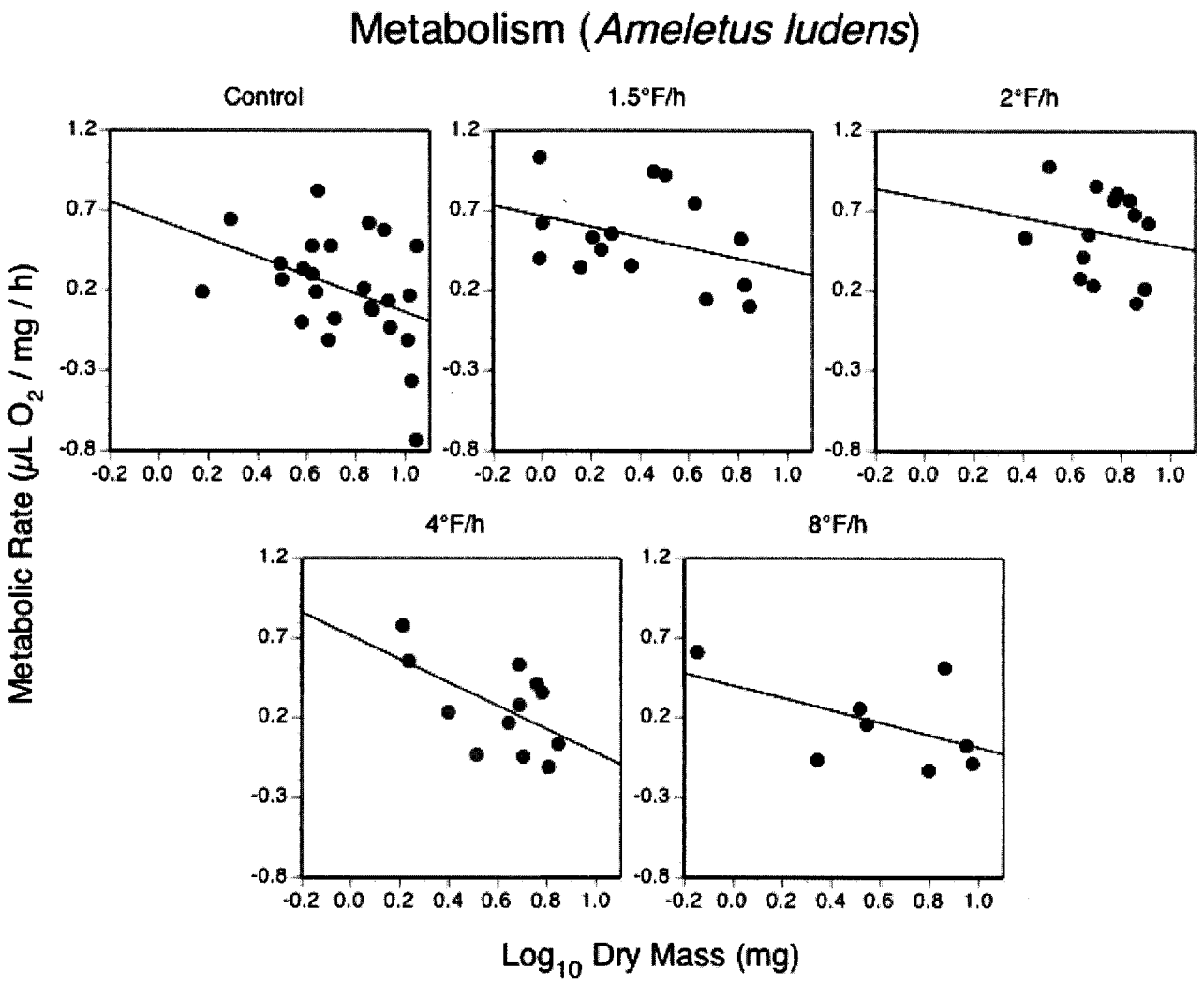

Figure 49. Metabolic rate at 46°F for different sizes of *Ameletus ludens* larvae reared in cold season thermal regimes (constant at 46°F, and variable between 39 and 53°F with a temperature change rate of 1.5°F/h, 2.0°F/h, 4.0°F/h, 8.0°F/h).