Chapter 13: Defining PC/QC Standards for Mass-Rearing HWA Predators

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ABSTRACT

We have defined guidelines to help mass-rearing laboratories establish programs of process control (PC) and quality control (QC) in the production systems where *Sasajiscymnus tsugae* and *Laricobius nigrinus* are reared. We have included process and quality characteristics that span a range of technological difficulty and organizational levels. The tests for measuring these characteristics include biomass, morphology, biochemistry, and behavior. We emphasize the importance of using statistically-based QC and PC systems according to the protocols established by the industrial engineering community.

INTRODUCTION

Systems of process control (PC), quality assessment (QA) and quality control (QC) have been in use in industry for nearly 100 years, and they have been more recently applied to insect rearing over the past several decades. Using PC/QA/QC systems properly greatly adds to the value of the products in question and to the economy of their production and to consumer/user satisfaction. Chambers' (1977) excellent review of quality control explains the biological aspects of quality and how they can be applied in mass rearing systems.

MASS-REARING FRAMEWORK

Importantly, Chambers points out that numbers of insects produced in true mass-rearing systems over time must exceed 10,000 → 1,000,000 times the average fecundity for an individual female over the cycle of one generation. Along with the numerical constraints (quantities) that Chambers sets forth, he also treats the quality assessments that result in successful programs. These quality considerations have served as the basis of many mass-rearing systems and include biological features such as weight, mobility measurements, search capacity, fecundity, fertility, development rate, longevity, and Chambers also reviews other biological aspects such as pheromone production and response, sound production, and other features that reflect the health and vigor of insect to be used in large scale programs such as biological control or genetic pest management. We note that the rearing systems for HWA predators do not produce the numbers to qualify as mass-rearing by the standards of Chambers; however, the tenets of process control and quality control can still be applied profitably to these smaller-scale rearing programs.

Therefore, in this chapter, we focus on development of a practical system of process control and quality control. We distinguish these concepts as follows: 1) **process** is the series of events, procedures, and materials involved in the production of the product (in this case, the HWA predators); 2) the **quality** of the end-product (*S. tsugae* and *L. nigrinus*)

means the relative ability (or capability) of the product to do the job for which it is intended. In this chapter and in the PC/QC program that we are developing, we try to present a systematic analysis of the processes involved in mass-rearing these HWA predators to allow early detection of flaws in the process. We further try to define the standards that characterize the end product as capable of controlling HWA in hemlock forests. We also try to make the PC/QC system one that harmoniously fits the current efforts, minimizes the efforts of production teams, and adds to the value of the product without adding substantially to the cost. In accord with other PC/QC systems in industry, all these goals can be achieved by application of appropriate techniques that are based on careful study of the existing system of production. Several authors have treated quality control including reviews by Boller (1979), Boller and Chambers (1977), Chambers (1977), Calkins et al. (1996), and a comprehensive work that updates modern QC standards, Dyck et al (2005). Reviews of the genetics of mass-production of insects are provided by Bartlett (1984, 1985) and Mackauer (1976). Finally, an excellent introductory statement about the need for quality control and process control is provided by Bigler (1989). He expressed the treatment of quality control and process control in earlier applications to insects:

> Boller and Chambers (1977) divided the overall quality of fruit flies reared for sterile insect release programmes into major quality components, traits and measurable parameters. The question remains whether laboratory assessed traits or attributes have a predictive value for the performance of an insect in the field. Mackauer and Van Den Bosch (1973) and Messenger et al. (1976) concluded that it is hardly possible to identify attributes which will "precisely" characterize an effective biocontrol agent for a particular situation. The first problem is the clear definition of *what* attributes are to be measured.

PRODUCTION PROCESS FOR *SASAJISCYMNUS TSUGAE*

An analysis of the rearing process must be performed, starting with a listing of all the elements of the rearing system. In the case of *S. tsugae*, the components of the process are as follows: 1) collection of insects to start colonization → 2) holding P generation adults in containers → 3) feeding them adelgid prey presented as infestations on hemlock → 4) supplementing natural diet with honey, Wheast, or other supplements → 5) adding water as a spray or in some other manner → 6) allowing oviposition and either collecting eggs or allowing them to remain in adult cages → 7) harvesting F_1 generation to start new cage → 8) continuing process by repeating steps 1)-8) to produce subsequent generations (F_2, F_3…F_n). At some point in the process P → F_n a harvesting step is added where some stage (usually adults) are removed from the colony and prepared for release. As Chambers (1977) points out, the harvesting/preparation/release step is very important and can be the point of great losses in quality and failure of the system. However, the scope of this chapter is confined to the production steps.

PRODUCTION OF *LARICOBIUS NIGRINUS*

The steps in production of *L. nigrinus* are similar to those involved in *S. tsugae* production, except that the former species includes a step that involves a complex life stage where *L. nigrinus* larvae enter the soil to pupate, aestivate for several months, then emerge as adults in the fall.

In Figure 1, we see the five major factors that can contribute to the loss of quality in a production program for an HWA predator: 1) microbial factors, 2) containers, 3) soil factors, 4) diet quality, and 5) environment. For example, if the production process allows microbial contaminants or pathogens to enter the target insects, these microbial components can either kill or sicken the incipient products (Cohen 2003). One microbe that has gained considerable attention in HWA predators is a species of protozoan known as a microsporidia. Although viruses, bacteria, and fungi have received lesser attention in HWA predators than microsporidia, they can be equally destructive.

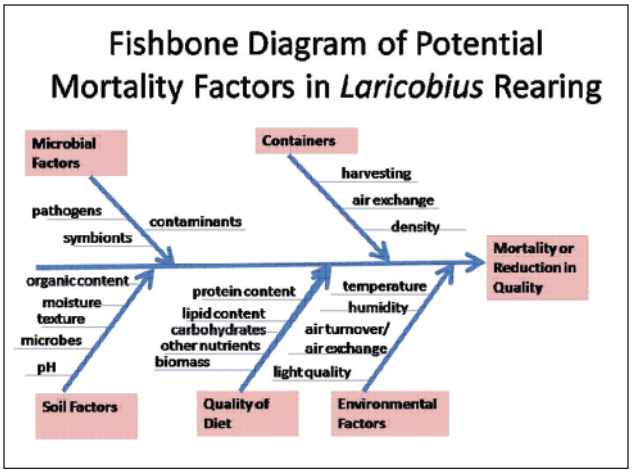

Figure 1. A fishbone diagram that covers five major facets of rearing of *Laricobius nigrinus*.

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Figure 2. A hypothetical Pareto plot that "dissects" or demonstrates the various potential causes of mortality (failure) in the soil phase of rearing *L. nigrinus*. The possible causes of failure or mortality were derived from discussions with workers in *L. nigrinus* rearing labs and from the literature such as Johnson et al. (2007).

Although containers seem to be a simple component, their design can lead to many problems in rearing HWA predators (and other insects as well). Frequently, in rearing situations, improvements in cages can influence gas exchange, heat exchange, moisture retention, and many other factors that can make the difference between a highly successful, economically-sound program and a fail.

For soil-dwelling insects, including *L. nigrinus*, soil features, including texture, moisture content, pH, microbial profile, etc. can be of huge significance in survival and health of the insects (Johnson et al. 2007), and this includes the predators that are products of our rearing systems.

In Figure 2, we extended the hypothesis that over-watering could be a substantial cause of mortality by causing the larvae to drown. Conversely, desiccation could also be a source of mortality, but in our measurements of rearing soils provided by the PABIL laboratory, all soil samples tested had a water activity of close to 1.00 (equivalent to 100% relative humidity). The sphagnum moss in the artificial soil is known to hold moisture to help keep soil air spaces humid. We also had input from rearing labs that they felt that larval nutrition had a strong impact on survival of larvae in the soil.

In the soil choice pupation experiment, soil sizes represented by coarse and fine hemlock soil, approximated coarse sand and larger particles vs. fine sand, silt, and clay-sized particles, sifted out from soil collected under a planting of urban hemlock trees. Results indicated that when *L. nigrinus* had a choice of different soil textures to pupate in, all pupae were located in coarse hemlock soil, and none in either the standard soil mixture used in laboratory rearing of this species or fine soil (Fig. 3). In all cases of successful pupation, new adults emerged from pupal chambers in the fall.

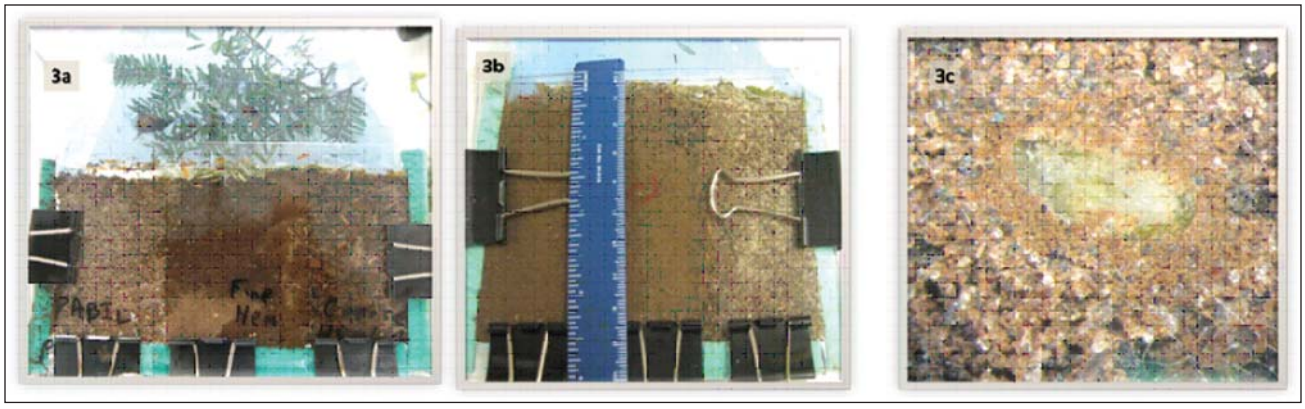

Figure 3. Plexiglass soil sandwiches used to test choices of *Laricobius nigrinus* mature larval responses to soil moisture, soil texture, organic content, pH, or other soil parameters. The dimensions of the sandwiches are about 9 cm x 10 cm and about 1 cm in width. (a) The soils used in these chambers are, from left to right, standard sand and milled sphagnum mix used in rearing *L. nigrinus*, fine and coarse hemlock soils sifted to particle sizes of ≤ 0.60 mm and > 0.60 mm, respectively; (b) depth at which a pupal chamber of L. nigrinus (circled in red) was detected in coarse hemlock soil; (c) close-up of a newly formed pupa of L. nigrinus in pupal chamber in coarse hemlock soil (photos by C. Cheah).

As we see in Figure 4, the collection and presentation of data allows us to treat quality in an objective and quantifiable way. The control chart in Figure 4 shows the weights measured in female *S. tsugae* produced over a 45 week period, where collections of 100 beetles were weighed, and average weights calculated. It is evident from this chart that during two periods over the whole rearing interval were notable for weights dipping below the lower control limit (LCL). These declines in weight during Week 24 and Week 38 must be considered indications that the process was out of control and that the products were of inferior and unacceptable quality during these periods.

It is important to note that the biomass or weights of *S. tsugae* have not definitively been shown to be indicators of quality, i.e. to be related to the desirable characteristics of voracity, large search capacity, longevity, and high fecundity, but we are assuming that the correlations exist. Therefore, the underlying hypothesis of this portion of our study is that a certain, minimal mean body weight is correlated with the biological characteristics stated above.

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Figure 4. Range charts based on data from *Sasajiscymnus tsugae* program at the North Carolina Department of Agriculture's Biological Control Laboratory.

This leads to the question of "upstream" issues having potential effects on production. Clearly, in light of the high cost of the HWA predators, it would be advantageous to catch the problems that cause defects before they are manifested in the final product. This is especially important in predators such as *S. tsugae* and *L. nigrinus*, which have very long life cycles with a large input of materials (hemlocks infested with HWA, cages, soil) and labor. The entire rearing process from adults in one generation to adults in the next generation takes months (nearly a year, in the case of *L. nigrinus*). Figure 5 and the ensuing discussion explain the distinctions between product control and production control.

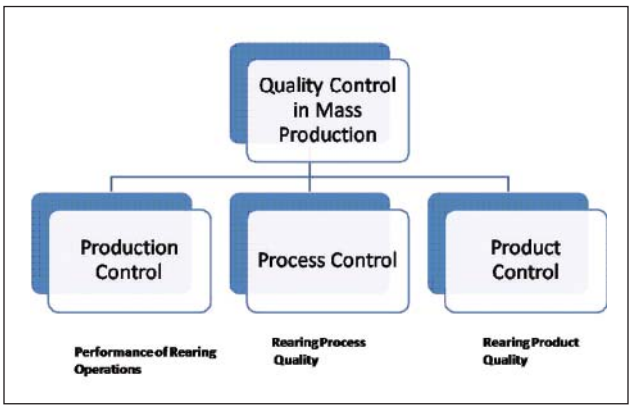

Figure 5. The components of a quality control system for mass-reared natural enemies, adopted from Leppla and Fischer (1989) and from discussions by Penn et al. (1998).

Strictly speaking, when we discuss product quality, we are focusing on the characteristics of the end-product and how they meet the needs for which the product was intended. In our case, this product must have the characteristics that lead to the control of HWA to reduce pest populations below a biological threshold, which translates into preventing HWA from killing hemlock trees. In the discussions by Leppla and Fischer (1989) and Penn et al. (1998), there is a separation of "process" and "production" control where the process is analyzed by measurement of the materials, including the biological materials such as the immature stages of the insects being produced, are examined and evaluated. We suggest that the diagram could be simplified for convenience, with Production Control and Process Control fused into one category. In our model, overall quality control would be divided into Process Control (where all elements of the production are potential elements of scrutiny) and Quality Control (where the final product is measured in a context of standards developed to assure product capability to perform as expected).

RECOMMENDED PROCESS CONTROL AND QUALITY CONTROL SYSTEM FOR HWA PREDATORS

This leads to the specifics of the **process control** measures in rearing *Laricobius* and *Sasajiscymnus*.

The areas that we have identified for Process Control are 1) environment, 2) diet materials, 3) microbial factors, 4) soil factors, 5) container factors, and 6) genetics. We note that genetics would be important to assess, but there has been little background established for assessing genetic factors. Therefore, we summarize our recommendations for the further development of measurement of the five components mentioned here and illustrated in Figure 1.

1) **Environmental Factors** can be evaluated by the use of a temperature, humidity, and light measurement data logger (or similar device). We have found that Hobo-type data loggers are small enough to fit into rearing containers, and they can be programmed to report temperature, humidity, and light intensity over a one-week period. The accumulated data can be downloaded to a computer so that deviations from standard conditions can be detected by examination of the graphs that the data loggers and their software support. This technique is discussed in detail by Cohen (2003). It is important to mention that for under $500, a laboratory can be equipped with four data loggers and the software required to read the loggers. We

further suggest that the nearly continuous output of the data loggers gives insectary workers a much more comprehensive sense of deviations in the rearing system. Other kinds of sensing systems can be used to detect other parameters such as CO_2 or O_2 concentrations.

2) **Diet Factors:** There is common agreement that predators' diets are among the most important determinants of quality. Yet, there is no clear definition of what is meant by high or low quality diets (Cohen, personal observation). Basing the following discussion on interviews with rearing personnel, judgments of prey quality are based on a) the condition of the tree, b) the number of woolly masses present, c) the size of the woolly masses, and d) the numbers of eggs present in egg masses. We have tried to elaborate on these factors to include biochemical/biomass factors including: a) weight of excised woolly masses, b) protein content, c) lipid content, d) carbohydrate content, and e) antioxidant content of woolly masses. We developed or modified tests for measuring 1 to 10 HWA for each test. We found that the presence of HWA "wool" complicated the analysis of the nutritional factors, especially lipid analysis.

   Protein: To determine protein content, we refined a dye-binding test according to modifications of the method of Heller and Sherbon (1976), (see Udy website). This method involves the homogenization of HWA tissue in a solution of Acid Orange 12 Dye, then centrifuging and measuring with a spectrophotometer at 480 nm. When the disappearance of color is compared with a standard curve established with authentic proteins of known concentrations, the protein content of individual HWA woolly masses can be determined.

   Lipids: The total lipid concentration of egg masses is determined by the vanillin method, which is a colorimetric procedure performed similarly to the protein test, using a spectrophotometer. As with the protein determination, authentic standards are used to establish a standard curve. The method is explained by van Handel (1988) as is the following analysis of carbohydrate concentrations. In the vanillin method, the materials to be tested, such as insects, are homogenized in concentrated sulfuric acid and phosphoric acid, then reacted with the vanillin reagent.

   Carbohydrates: Both soluble (free) sugars and glycogen can be determined by using the anthrone test with samples of HWA and comparison with standard curves as described by van Handel (1988). Like the vanillin/lipid test, the anthrone test is performed with sulfuric acid, which breaks down (hydrolyzes) the organic components, including all kinds of carbohydrates, which then react with the anthrone molecules to form a colored product whose optical density can be read colorimetrically and compared with known carbohydrate standards.

   Free-Radical Scavengers: We have determined that the most simple and comprehensive test of free radical scavengers (anti-oxidants) in diet materials and in the insect products is the colorimetric DPPH method described by Cohen (2003) and Cohen and Crittenden (2004).

3) **Microbial Factors:** Although there is a potential that any of several taxa of pathogens may adversely affect the HWA predators in our production systems, Dr. Lee Solter (personal communication) has stated that the most common and serious microbial threats to HWA predator-quality are the protozoan pathogens known as microsporidia (Phylum: Microspora, by the classification of Undeen and Vavra 1997). In light of Solter's findings, we strongly recommend that HWA production facilities include a search for pathogens. A trained technician can perform tests of predators by making wet-mounts or Giemsa or Gram stains of dry mounts (Undeen and Vavra 1997). With these stains and a phase-

contrast microscope, microsporidia-infected individuals can be efficiently recognized. We must add, however, that the number of specimens that must be examined expressed as a percentage of the population that is to be released has yet to be determined.

4) **Soil Factors:** Predators belonging to the genus *Laricobius* spend more than half of their life-cycle (4-5 months) in the soil as pre-pupal larvae, pupae, and newly-eclosed adults, which must dig their way out of the soil to seek populations of HWA prey and mates. We found that *L. nigrinus* larvae burrow about 4-5 cm into the soil (Figure 3b). Once larvae reach the appropriate depth, they pupate and remain in their pupal cocoons (Figure 3c) until they are ready to emerge as adults. In Figure 1, we list several soil-related factors that we hypothesize as related to biological fitness and survival of *Laricobius*.

5) **Container Factors:** The environmental factors can be major forces in determining the quality or loss of quality in any insect, but researchers who study HWA predators have discovered that these insects are especially attuned to temperatures and light/dark cycles that signal seasons and potential prey availability. Given the importance of environmental conditions, we recommend a rigorous attention to light, temperature and humidity conditions in insectaries and especially within cages. The technology of the cages, including sites of foliage placement, degree of crowding of foliage and beetles, and mechanisms for harvesting can greatly affect numbers of predators being produced and also the quality of these predators. When we consider the architecture of a hemlock tree in nature, it becomes clear that the arrangement of shorn branches in cages can become a maze, rather than a natural series of corridors for beetles to discover their prey. As far as predator density is concerned, the derodontids and the coccinellids in the HWA predator programs are not strongly cannibalistic as are some predators, but they clearly can become competitors when resources such as high quality prey are scarce or inadequate.

6) **Genetic Factors:** Too often, declines in production and/or quality of insects is attributed to genetic truncation or deterioration, but too seldom has the genetic deterioration hypothesis been confirmed as being causative (Bartlett 1985). In fact, Cohen (2003) has summarized a number of failings in diet or environment, or simply personnel errors that were documented to cause declines in quality or production numbers. Hopefully, with molecular methods having become readily available, insect rearing programs will be able to develop a clearer understanding of causes and effects in genetic truncation or genetic shifts that are clearly inherent in mass-rearing.

THE FINAL PRODUCT: QUALITY CONTROL OF PREDATORS THAT ARE TO BE RELEASED.

The tests of quality that we recommend for both species are 1) weights of adult beetles, 2) sex ratios, 3) internal morphology/development, 4) protein content, 5) carbohydrate content, and 6) voracity.

1) Weights of individual adults can be determined with a balance sensitive enough to read to 0.01 mg (10 µg), or if a less sensitive balance must be used (such as analytical balances that read to 0.10 mg (100 µg), collections of either 10 individuals or 100 can be weighed in pools. Weights can be evaluated for their fit to process control charts (Figure 4).
2) Sex Ratio: For S. tsugae, sex ratios can be determined externally, using live beetles. For *L. nigrinus*, sexing would have to be done by examining internal morphology. Normal sex ratios of both species are approximately 1:1 (males: females).
3) Internal morphology/development: These tests must be performed with dissected insects, and we have determined that a sampling of 6-10 insects is adequate to reflect the condition of the population as a whole (Fig. 6).

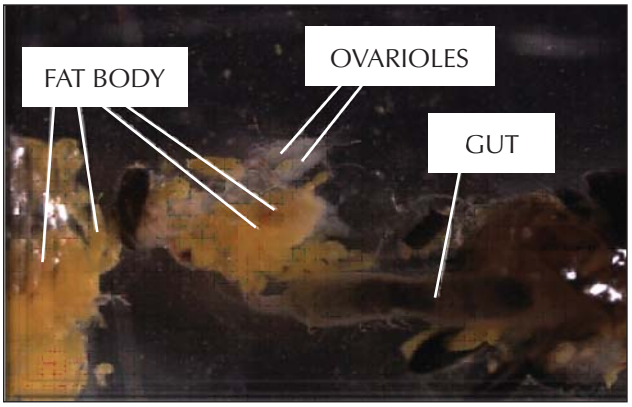

Figure 6. The internal structures of a female *Sasajiscymnus tsugae*, showing the poorly developed ovaries and fat body. It is evident from this image that the insect had recently fed, but the internal organs are not developed to a point where the insect could soon reproduce. On a scale of 0-10, with 10 being fully developed and ready to lay eggs, this insect would be rated as a 2-3.

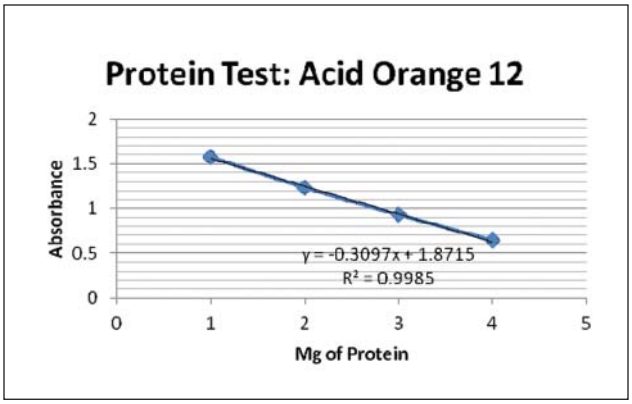

Figure 7. A graph of absorbance at 482 nm vs. protein concentration (1-4 mg).

4) Protein content: We recommend use of the dye-binding test known as the Acid Orange Test, which is outlined above under "Process Control." In Figure 7, we present a standard curve for authentic proteins measured as a comparison with the proteins from either diet materials (HWA) or predators.

5) Carbohydrate content: We suggest the anthrone test, which is same type of analysis used for HWA (above in section on Process Control).

6) Voracity: A voracity or feeding vigor test of a sub-sample of beetles that are to be released is important. Each beetle should be given a twig with 30 HWA adults with eggs confined in a 9 cm diameter Petri dish at optimal temperatures and light cycles for each species. After 72h, the number of prey consumed should be measured by visual observation of disturbed woolly masses and/or consumed adelgid stages. The numbers consumed should be compared with a control chart to determine whether or not the voracity is comparable to the established mean.

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