Rehydration of Forensically Important Larval Diptera Specimens

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ABSTRACT Established procedures for collecting and preserving evidence are essential for all forensic disciplines to be accepted in court and by the forensic community at large. Entomological evidence, such as Diptera larvae, are primarily preserved in ethanol, which can evaporate over time, resulting in the dehydration of specimens. In this study, methods used for rehydrating specimens were compared. The changes in larval specimens with respect to larval length and weight for three forensically important blow fly (Diptera: Calliphoridae) species in North America were quantified. *Phormia regina* (Meigen), *Cochliomyia macellaria* (F.), and *Chrysomya rufifacies* (Macquart) third-instar larvae were collected from various decomposing animals and preserved with three preservation methods (80% ethanol, 70% isopropyl alcohol, and hot-water kill then 80% ethanol). Preservative solutions were allowed to evaporate. Rehydration was attempted with either of the following: 80% ethanol, commercial trisodium phosphate substitute solution, or 0.5% trisodium phosphate solution. All three methods partially restored weight and length of specimens recorded before preservation. Analysis of variance results indicated that effects of preservation, rehydration treatment, and collection animal were different in each species. The interaction between preservative method and rehydration treatment had a significant effect on both *P. regina* and *C. macellaria* larval length and weight. In addition, there was a significant interaction effect of collection animal on larval *C. macellaria* measurements. No significant effect was observed in *C. rufifacies* larval length or weight among the preservatives or treatments. These methods could be used to establish a standard operating procedure for dealing with dehydrated larval specimens in forensic investigations.

KEY WORDS *Phormia regina*, *Cochliomyia macellaria*, *Chrysomya rufifacies*, preservation, larval length and weight

Identifying and aging of Diptera larvae collected from decomposing human remains is an important component of death investigations (Catts and Goff 1992, Amendt et al. 2004, Byrd and Castner 2010). Specimens collected from human remains are typically placed in alcohol and stored until they can be sent to a forensic entomologist for identification and analysis. Despite efforts to keep them preserved, instances occur in which the preservative is lost and the larvae become dehydrated, resulting in stiff, rigid specimens that are difficult to identify. Dehydration of specimens can result in shrinkage and distortion of larval characters. If an identification of the specimen can be made, its body length, which is an important indicator of its age, cannot be accurately determined. Data have been published on using the dry weight of larvae to estimate larval age using prediction interval estimations, but the identity of larvae was known before drying, and at certain times during larval development the weights of different ages remain difficult to distinguish (Wells and LaMotte 1995). Thus, dried specimens become much less informative.

Curation of alcohol-preserved specimens is a challenge for any collections manager. One of the earliest published records of a method for rehydrating dried specimens is that of Van Cleave and Ross (1947). This method involves soaking the specimen in a 0.25–0.5% solution of commercial trisodium phosphate (TSP) and water. The qualitative descriptions given indicate that it was a successful method for reclaiming many different kinds of arthropod specimens. Marhue (1983) described three techniques to rehydrate specimens, but stated that TSP was a good technique to make crustacean specimens flexible again. Vogt (1991) attempted to quantify the change in rehydrated specimens by recording weight before and after a 10% acetic acid treatment, followed by soaking in several solutions, including TSP. Despite the fact that an analysis was attempted, the experimental design did not include an acetic acid-negative control. Thus, there was no endorsement of one technique over another, but rather just a documentation of the results. Commercial TSP was widely available in many different detergents in the United States (Hammond 1971) and abroad (e.g., Kroes 1980) to prepare walls for

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painting and was easily obtained commercially, but has largely been replaced with other chemicals that maintain the same mode of action, but are less harmful to water quality.

Initial preservation methods can have important implications for the fate of specimens in storage (Adams and Hall 2003). Recent efforts have focused on developing best practices for initial specimen preservation (Amendt et al. 2007, Byrd and Castner 2010). Many specimens are preserved by the recommended practice of hot-water killing (HWK), followed by placement into 80% ethanol (EtOH) (Lord and Burger 1983, Adams and Hall 2003, Amendt et al. 2007); however, the most commonly employed methods in the field are directly placing live specimens into 80% EtOH or 70–90% isopropyl alcohol (IPA; rubbing alcohol). The use of formalin as a preservative for insect specimens is strongly discouraged as it will degrade the exoskeleton (Huber 1998), render the specimens inflexible, and degrade extractable DNA in the specimens (Dillon et al. 1996). Practicality is often the driving factor when taking field samples, particularly in areas in which forensic entomology is a new tool in the investigation process.

With this practical approach in mind, the problem of rehydrating dried larval specimens and reclaiming their usefulness as forensic evidence was considered. The purpose of this study was to compare the ability of a commercially available TSP-substitute solution with an available source of TSP in reclaiming specimens using quantitative methods (length and weight) and the effect of the initial preservation conditions on this process. Because of the similar mode of action between TSP and TSP-substitute solution, we hypothesized that flexibility would be restored to specimens rehydrated with TSP or the TSP-substitute. We also hypothesized that specimens preserved and then rehydrated with these techniques would retain length and weight measurements recorded before initial preservation. To our knowledge, this represents the first application of rehydration methods to forensically important insect specimens and the first quantitative analysis of these methods.

Materials and Methods

Collection of Specimens. Third-instar larvae were collected from decomposing animals used as teaching materials in the Texas A&M University forensic entomology course (College Station, TX). These animals included the following: one 75.5 kg feral hog, *Sus scrofa* (L.) (placed immediately in the field for colonization and never frozen), and three 4.4–9.9 kg domestic hogs, frozen after death and allowed to defrost in the field during exposure for colonization, which provided teaching scenarios for the spring and summer 2007 semesters. Additionally, a road-killed armadillo *Dasypus novemcinctus* L., two unprocessed domestic meat rabbits, *Oryctolagus cuniculus* (L.), and a processed defrosted domestic turkey, *Meleagris gallopavo* L., were collected from in the study. Third-instar larvae were collected from the remains from 8 March 2007 to 28 August 2007 (six collection dates). This wide range of dates was used to provide seasonal temperature variation and allow for the collection of three different blow fly (Diptera: Calliphoridae) species, including the following: *Phormia regina* (Meigen), *Cochliomyia macellaria* (F.), and *Chrysomya rufifacies* (Macquart). Larvae were preserved by three different methods, as follows: HWK in near boiling water (~100°C) for ≈30 s, followed by placement into 80% EtOH, placement of live larvae directly into 80% EtOH, and placement of live larvae into 70% IPA (Kroger, Cincinnati, OH).

Preserved larvae were stored until the last collection date (28 August 2007); thus, there were differences for storage period depending on the species. Collections of *P. regina*, which arrive early in the year in central Texas, were stored for the longest period of time at ≈25 wk (collected in March and April 2007); *C. macellaria* were stored for ≈16–20 wk (collected in April and June 2007); and *C. rufifacies* were stored for the shortest period of time before processing at ~1 wk (collected in August 2007).

Measurements. Once all collections were made, the larvae were processed by selecting the largest from the samples until a total of 90 larvae (30 per preservation technique) for each species was collected. This design was done in an attempt to simulate actual conditions in which the largest larvae are typically collected and used to estimate time of colonization of the remains. Each larva was moved into a separate labeled plastic 1.5-ml microcentrifuge tube (Fisher, Pittsburgh, PA) for storage (≈2 wk) until they were measured by placing them under a Meiji Techno model EMZ-8TR microscope (Meiji Techno America, Santa Clara, CA) fitted with an Infinity 1-3C digital camera (Lumenera, Ontario, Canada). An image of each larva was taken using the Infinity Analyze Version 4.4 software package (Lumenera), and its length was recorded to the nearest millimeter in the image, also using this software package. The *Caliper* tool was used in the Infinity Analyze program to accurately measure the length of the larva from mouthparts to anal protuberance by laying the larva laterally in a petri dish (Fig. 1A) and following the curvature of the preserved larva.

In addition to length measurements, each larva was also weighed using a method similar to Vogt (1991) in that they were blotted dry to remove all exterior liquid before weighing on an ER-182A (A&D, Tokyo, Japan) or an Adventurer Pro AV64 (Ohaus, Pine Brook, NJ) scale. Both scales were calibrated to the nearest 0.0001 g.

Dehydration. In an attempt to simulate the slow drying of a slightly opened container or a nonevaporation proof container, we used an ordinary thumbtack to punch a hole (~1 mm) in the top of each microcentrifuge tube and left to evaporate at room temperature (≈22–25°C). By the end of 6 wk, evaporation and dehydration of the larvae had not progressed to the point of completely drying the specimens (absence of all visible liquid); thus, the microcentrifuge tubes were placed in a sealed plastic box filled with ≈2.5-cm Drierite desiccant (W. A.

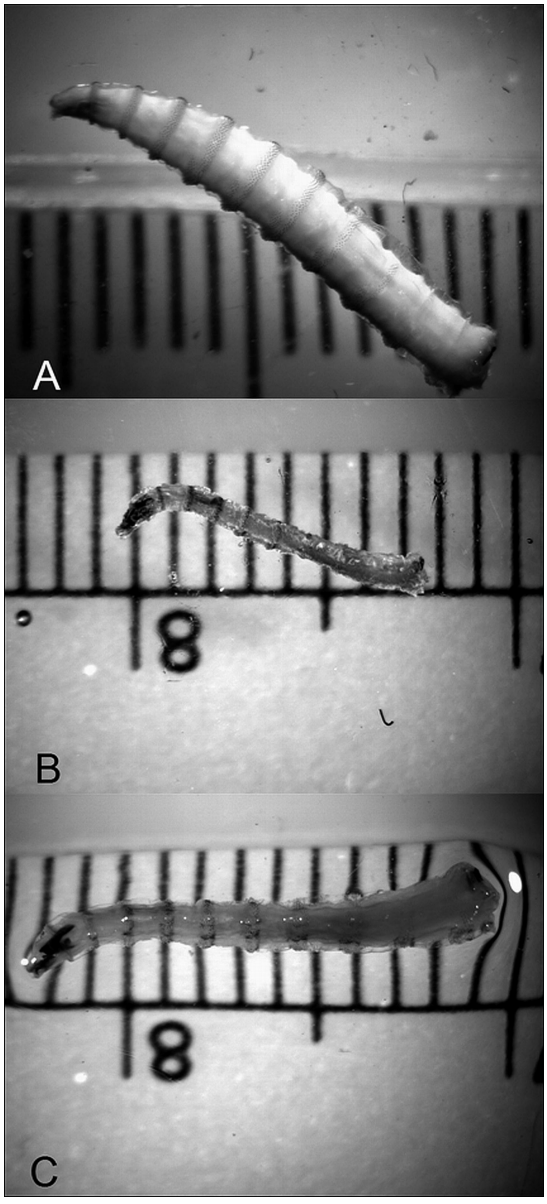

Fig. 1. Light microscope images of a single *P. regina* larva through preservation (A), dehydration (B), and rehydration (C) that was initially fixed by the HWK method and preserved in 80% EtOH, and then rehydrated in TSP-substitute solution. Flexibility of specimens was partially restored by use of detergent-based rehydration solutions.

Hammond Drierite, Xenia, OH) in an attempt to speed the drying process (13 d). Finally, the tubes were removed from the Drierite-filled boxes, and the lids were clipped from the microcentrifuge tubes to evaporate off the last fraction of liquid left in each tube (~2–3 d). Once all visible liquid was gone from each microcentrifuge tube, each larva was again imaged and measured for length and weight, as described above.

Rehydration. Ten larvae were randomly selected from each set of 30, in their respective preservative, and assigned to one of three different rehydration fluids, as follows: 5% TSP (Alfa Aesar, A. Johnson Matthey, Lancashire, United Kingdom) in deionized water, Klean-Strip TSP-substitute with 1–5% sodium silicate as an active ingredient (W. M. Barr and Co., Memphis, TN), or 80% EtOH. The larvae were transferred to a new microcentrifuge tube with 1 ml of rehydration fluid and allowed to sit overnight (≈15 h). After exposure to rehydration fluids, those specimens in the 5% TSP and TSP-substitute were transferred to another microcentrifuge tube with 1 ml of 80% EtOH and left for 3 d to allow for any possible changes in the larval length or weight as a result of gradual changes in resorption of EtOH to finalize. Larvae were again imaged and measured for length and weight, as previously described.

Statistical Analysis. All data analysis was conducted with SPSS 14.0.1 (SPSS 2005), with significance observed at the $\alpha \leq 0.05$ level.

Analysis of data from *C. macellaria* and *P. regina* was conducted with a split-plot analysis of variance (SP-ANOVA) design (SPSS 2005). In the split-plot model, blocks were defined as the animal that larvae from which they were collected and was considered a random factor. The preservation and rehydration methods were fixed factors in the model representing whole plots and subplots, respectively. The response variable of either length or width was transformed by calculating the proportion difference between initial and final measurements ([initial – final]/initial). Arcsine-square root (on the absolute value of length data) or the arcsine (on weight data) transformation was applied before ANOVA to normalize the data. Post hoc multiple comparison analyses were conducted with Bonferroni.

Data for *P. regina* length presented a problem for the SP-ANOVA because the number of larvae from certain treatments was low as a result of the randomization procedure used when selecting larvae for the study, which led to inadequate degrees of freedom for analysis of certain factors. Data for *P. regina* length were modified by collapsing larvae that were from animals that were most similar into the same groups and analyzing with a standard full factorial ANOVA. This method provided results similar to those obtained with a preliminary SP-ANOVA, but with adequate degrees of freedom.

C. rufifacies larvae were sampled from a single animal (domestic turkey), so a repeated measures ANOVA (RM-ANOVA) was employed. The data failed to meet the assumption of equality of variance (Mauchly's sphericity) for length or weight, so correction values were selected based on the criteria suggested by Girden (1992). Tamhane's T2 test for multiple comparisons was used to account for unequal variances and determine differences among between-subject groups.

Results

Fig. 1 illustrates the process for a single *P. regina* larva preserved by HWK-80% EtOH and rehydrated with TSP-substitute solution. In general, rehydration

Table 1. Average lengths (mm; ±SD) for larvae of three blow fly species (*Phormia regina*, *Cochliomyia macellaria*, or *Chrysomya rufifacies*) preserved with three different preservation methods, dried, and rehydrated with one of three different methods

Species	Preservation treatment[a]	Rehydration treatment[b]	Initial length (±SD)	Dried length (±SD)	Rehydrated length (±SD)	% difference
Phormia regina	IPA	EtOH	10.90 (2.09)	8.21 (1.66)	10.06 (1.51)	−6.74
	IPA	TSP	9.71 (2.29)	7.76 (1.69)	9.76 (1.93)	+2.22
	IPA	TSP-sub	10.21 (1.43)	8.21 (1.33)	9.43 (1.56)	−8.77
	EtOH	EtOH	10.92 (2.60)	8.12 (2.00)	9.90 (2.30)	−8.66
	EtOH	TSP	10.31 (3.02)	7.95 (2.23)	10.95 (2.16)	+8.88
	EtOH	TSP-sub	10.29 (1.80)	7.44 (0.98)	11.48 (1.98)	+12.71
	HWK-EtOH	EtOH	9.40 (3.76)	6.10 (2.61)	7.54 (3.07)	−19.24
	HWK-EtOH	TSP	10.55 (3.09)	6.51 (2.17)	8.92 (3.14)	−16.63
	HWK-EtOH	TSP-sub	10.73 (3.50)	6.69 (2.37)	10.58 (3.93)	−2.94
Cochliomyia macellaria	IPA	EtOH	10.92 (1.58)	7.57 (1.11)	9.54 (1.04)	−11.79
	IPA	TSP	10.37 (1.62)	7.63 (1.01)	10.11 (1.05)	−1.06
	IPA	TSP-sub	11.48 (1.04)	8.34 (1.10)	11.63 (1.04)	+1.34
	EtOH	EtOH	8.93 (1.75)	6.30 (1.41)	8.00 (1.50)	−8.92
	EtOH	TSP	9.39 (1.03)	6.98 (1.74)	9.18 (1.88)	−2.25
	EtOH	TSP-sub	9.33 (1.16)	6.28 (1.18)	9.86 (2.13)	+5.52
	HWK-EtOH	EtOH	11.00 (2.55)	7.06 (1.71)	8.88 (1.93)	−18.70
	HWK-EtOH	TSP	13.02 (1.51)	8.13 (1.13)	11.52 (1.63)	−10.40
	HWK-EtOH	TSP-sub	11.22 (2.58)	6.71 (1.26)	10.46 (2.01)	−5.70
Chrysomya rufifacies	IPA	EtOH	11.90 (0.95)	10.08 (2.37)	12.47 (2.41)	+4.19
	IPA	TSP	12.57 (1.09)	9.79 (2.12)	12.68 (2.07)	+0.99
	IPA	TSP-sub	11.59 (1.26)	9.78 (2.47)	12.56 (2.10)	+8.10
	EtOH	EtOH	12.82 (0.76)	9.49 (1.12)	11.62 (1.52)	−9.54
	EtOH	TSP	12.84 (0.62)	9.87 (0.83)	12.60 (0.86)	−1.92
	EtOH	TSP-sub	13.07 (0.66)	10.17 (0.85)	12.59 (0.91)	−3.71
	HWK-EtOH	EtOH	13.95 (1.17)	9.03 (1.05)	11.67 (1.65)	−16.45
	HWK-EtOH	TSP	14.13 (0.99)	9.24 (0.94)	12.73 (1.31)	−10.05
	HWK-EtOH	TSP-sub	13.66 (1.20)	9.03 (1.37)	13.61 (0.98)	+0.04

The average percentage of difference between initial and final length is also presented.
[a] IPA, 70% IPA; EtOH, 80% ethanol; HWK-EtOH, hot-water kill and 80% ethanol.
[b] EtOH, 80% ethanol; TSP-sub, TSP-substitute.

with the detergent-based solutions produced rehydrated larvae that were flexible and conducive to manipulation for visualization of identifying structures such that we failed to reject this hypothesis. The results for length and weight measurements before and after rehydration allowed for the rejection of the hypothesis that rehydration would restore these measurements to initial values observed after preservation and before dehydration. Table 1 displays the mean initial length, dried length obtained after dehydration, and the final rehydrated length for larvae treated with each preservation method and rehydration treatment along with the mean average percentage of difference between the initial and rehydrated lengths ([initial − final]/initial × 100), for each of the three species. Mean initial weight, dried weight after dehydration, and final rehydrated weight for each preservation method by rehydration treatment, as well as the mean percentage of difference are presented in Table 2 for all three species. The results for each individual species will be discussed in the following sections.

P. regina. Table 3 displays the results from factorial ANOVA for length and SP-ANOVA for weight data of *P. regina*.

Length. All *P. regina* larvae were collected from porcine carcasses that were available during the earliest collection dates. There was no significant effect of collection animal on the proportion difference between initial and final length. When considered alone, the length of the larvae was not significantly affected by preservation method or rehydration treatment; however, when the two were considered together, a significant interaction effect was observed (Table 3).

In general, rehydrated larvae of *P. regina* were within 20% of their initial preserved length. Specimens preserved in 80% EtOH and rehydrated in either of the detergent solutions had mean final lengths greater than the original preserved length. Larvae initially preserved in 80% EtOH and rehydrated with either the TSP or TSP-substitute had mean lengths slightly larger than the initial measured length (Table 1). Interestingly, specimens preserved with the HWK-80% EtOH method resulted in mean final lengths shorter than those of the original preserved length regardless of rehydration treatment (Table 1). However, the smallest observed difference was among those specimens initially preserved with the HWK-EtOH method and rehydrated in the TSP-substitute solution (Table 1).

Weight. The SP-ANOVA results revealed that the preservation and rehydration process appeared to have a greater effect on the weight of *P. regina* larvae than on their length (Table 3). The rehydration treatment had a significant effect on the proportion difference between initial and final larval weight (Table 3). Bonferroni multiple comparisons based on transformed data indicated that each of the rehydration treatments differed significantly. Neither collection animal nor preservation methods were significantly different, but their interaction was significant. This effect could be potentially explained by the relatedness of individuals from a particular collection animal both in terms of age and genetic relatedness.

Table 2. Average weights (g; ±SD) for larvae of three blow fly species (*Phormia regina*, *Cochliomyia macellaria*, or *Chrysomya rufifacies*) preserved with three different preservation methods, dried, and rehydrated with one of three different methods

Species	Preservation treatment[a]	Rehydration treatment[b]	Initial weight (±SD)	Dried weight (±SD)	Rehydrated weight (±SD)	% difference
Phormia regina	IPA	EtOH	0.0158 (0.0120)	0.0007 (0.0010)	0.0010 (0.0012)	−94.99
	IPA	TSP	0.0019 (0.0079)	0.0003 (0.0004)	0.0043 (0.0036)	−61.43
	IPA	TSP-sub	0.0102 (0.0080)	0.0004 (0.0005)	0.0030 (0.0038)	−77.77
	EtOH	EtOH	0.0188 (0.0092)	0.0012 (0.0008)	0.0021 (0.0015)	−89.18
	EtOH	TSP	0.0184 (0.0133)	0.0040 (0.0074)	0.0080 (0.0075)	−50.69
	EtOH	TSP-sub	0.0159 (0.0057)	0.0018 (0.0015)	0.0103 (0.0069)	−40.65
	HWK-EtOH	EtOH	0.0170 (0.0135)	0.0026 (0.0023)	0.0037 (0.0033)	−74.55
	HWK-EtOH	TSP	0.0202 (0.0143)	0.0035 (0.0022)	0.0070 (0.0049)	−64.39
	HWK-EtOH	TSP-sub	0.0195 (0.0128)	0.0029 (0.0021)	0.0121 (0.0096)	−38.52
Cochliomyia macellaria	IPA	EtOH	0.0176 (0.0060)	0.0018 (0.0013)	0.0032 (0.0013)	−79.94
	IPA	TSP	0.0137 (0.0042)	0.0010 (0.0008)	0.0044 (0.0024)	−64.46
	IPA	TSP-sub	0.0197 (0.0054)	0.0020 (0.0021)	0.0099 (0.0035)	−48.94
	EtOH	EtOH	0.0124 (0.0066)	0.0025 (0.0021)	0.0036 (0.0020)	−71.14
	EtOH	TSP	0.0124 (0.0028)	0.0030 (0.0025)	0.0055 (0.0027)	−54.54
	EtOH	TSP-sub	0.0134 (0.0050)	0.0027 (0.0021)	0.0097 (0.0048)	−26.56
	HWK-EtOH	EtOH	0.0207 (0.0104)	0.0039 (0.0028)	0.0061 (0.0053)	−70.22
	HWK-EtOH	TSP	0.0277 (0.0095)	0.0043 (0.0019)	0.0096 (0.0038)	−63.83
	HWK-EtOH	TSP-sub	0.0168 (0.0054)	0.0032 (0.0010)	0.0106 (0.0040)	−34.86
Chrysomya rufifacies	IPA	EtOH	0.0394 (0.0090)	0.0170 (0.0092)	0.0233 (0.0110)	−44.00
	IPA	TSP	0.0462 (0.0154)	0.0183 (0.0096)	0.0300 (0.0136)	−37.49
	IPA	TSP-sub	0.0358 (0.0119)	0.0139 (0.0090)	0.0213 (0.0099)	−42.44
	EtOH	EtOH	0.0394 (0.0089)	0.0155 (0.0063)	0.0206 (0.0087)	−49.60
	EtOH	TSP	0.0423 (0.0056)	0.0178 (0.0041)	0.0268 (0.0043)	−36.40
	EtOH	TSP-sub	0.0414 (0.0103)	0.0173 (0.0073)	0.0222 (0.0086)	−48.39
	HWK-EtOH	EtOH	0.0424 (0.0112)	0.0091 (0.0033)	0.0156 (0.0061)	−64.42
	HWK-EtOH	TSP	0.0471 (0.0110)	0.0105 (0.0031)	0.0214 (0.0061)	−55.14
	HWK-EtOH	TSP-sub	0.0389 (0.0122)	0.0081 (0.0032)	0.0251 (0.0062)	−31.40

The average percentage of difference between initial and final length is also presented.
[a] IPA, 70% IPA; EtOH, 80% ethanol; HWK-EtOH, hot-water kill and 80% ethanol.
[b] EtOH, 80% ethanol; TSP-sub, TSP-substitute.

From Table 2, it can be observed that rehydration was not effective in restoring initial weight. Those specimens rehydrated in TSP-substitute solution appeared to regain the most amount of weight after rehydration, but most of the weight was not recovered (Table 2). Those rehydrated with placement in 80% EtOH failed to recover the largest percentage of original values (Table 2).

Table 3. Full factorial ANOVA results for third-instar *Phormia regina* data for length and SP-ANOVA for weight

Factor[a]	*F* statistic	df	*P* value
Length			
Animal	0.254	1, 64	0.616
Preservative	0.805	2, 64	0.451
Treatment	2.598	2, 64	0.082
Preservative × animal	–	0	–
Preservative × treatment	2.751	4, 64	0.036*
Treatment × animal	0.424	2, 64	0.656
Preservative × treatment × animal	–	0	–
Weight			
Animal	0.078	3, 0.952	0.963
Preservative	0.714	2, 0.932	0.649
Preservative × animal	14.256	1, 63	<0.001*
Treatment	56.970	2, 63	<0.001*
Preservative × treatment	3.284	4, 63	0.017*

Length data were transformed using the arcsine (square root[p]) transformation, and weight data were transformed by applying the arcsine(p) transformation prior to analysis to meet the assumptions of equality of variance and normality. Exact *P* values are displayed. Bonferroni multiple comparisons were used to evaluate difference among groups with significance observed at the $\alpha < 0.05$ level. *, Indicates significant differences observed at the $\alpha < 0.05$ level.
[a] Animal refers to the animal carcass from which specimens were collected. Preservative refers to the initial preservation method of each specimen. Treatment refers to the rehydration treatment solution applied to the specimens.

C. macellaria. Table 4 displays the results from the SP-ANOVA for length and weight data.

Length. Analysis of length data showed that preservative was not a significant factor in the overall analysis. However, there was a significant interaction between preservative method and the animal carcass from which the larvae were collected (Table 4). Overall, lengths of larvae originally preserved in 80% EtOH were shorter than those from the other treatments (Table 1). Specimens collected from the rabbit carcass were shorter than those from the other two animals, and this may be because of differences in age as a result of stage of colonization and larval development. Bonferroni post hoc comparisons revealed differences among the lengths for larvae preserved with 70% IPA when compared with the other groups and are potentially attributable to the larger amount of length recovered after rehydration in the specimens rehydrated with either of the detergent solutions (Table 1).

Weight. A similar trend was observed in larval weight in which preservative method by itself did not have a significant impact on the proportion change in larval weight, but the interaction between preservative method and collection animal was significant (Table 4). Larvae collected from the armadillo had the

Table 4. SP-ANOVA results for third-instar *Cochliomyia macellaria* data for length and weight

Factor[a]	*F* statistic	df	*P* value
Length			
Animal	1.208	3, 5.072	0.396
Preservative	2.880	2, 3.883	0.171
Preservative × animal	3.471	4, 74	0.012*
Treatment	9.926	2, 74	<0.001*
Preservative × treatment	1.608	4, 74	0.181
Weight			
Animal	0.411	2, 4.044	0.688
Preservative	0.743	2, 3.914	0.533
Preservative × animal	4.850	4, 73	0.002*
Treatment	36.102	2, 73	<0.001*
Preservative × treatment	0.319	4, 73	0.865

Length data were transformed using the arcsine (square root[p]) transformation, and weight data were transformed by applying the arcsine(p) transformation prior to analysis to meet the assumptions of equality of variance and normality. Exact *P* values are displayed. Bonferroni multiple comparisons were used to evaluate difference among groups with significance observed at the $\alpha < 0.05$ level. *, Indicates significant differences observed at the $\alpha < 0.05$ level.

[a] Animal refers to the animal carcass from which specimens were collected. Preservative refers to the initial preservation method of each specimen. Treatment refers to the rehydration treatment solution applied to the specimens.

Table 5. Results for the RM-ANOVA of length and weight data of third-instar *Chrysomya rufifacies*

Factor[a]	*F* test	df	*P* value
Length			
Within subject			
Length	527.687	1.674	<0.001*
Length × treatment	5.239	3.348	0.001*
Length × preservative	26.300	3.348	<0.001*
Length × treatment × preservative	2.065	6.697	0.054
Between subject			
Preservative	0.853	2	0.430
Treatment	0.906	2	0.408
Preservative × treatment	0.225	4	0.924
Weight			
Within subject			
Weight	1,132.276	1.425	<0.001*
Weight × treatment	5.421	2.849	0.002*
Weight × preservative	15.206	2.849	<0.001*
Weight × preservative × treatment	3.742	5.699	0.002*
Between subject			
Preservative	1.209	2	0.304
Treatment	2.459	2	0.092
Preservative × treatment	0.444	4	0.776

Mauchly's sphericity test results indicated the data did not meet the assumption for equality of variance and were corrected as explained in the text. Length, $\tilde{E} = 0.837$; weight, $\acute{E} = 0.712$. Tamhane's T2 test for multiple comparisons was used to evaluate difference among groups with significance observed at the $\alpha < 0.05$ level. Exact *P* values are displayed. *, Indicates significant differences observed at the $\alpha < 0.05$ level.

[a] Animal refers to the animal carcass from which specimens were collected. Preservative refers to the initial preservation method of each specimen. Treatment refers to the rehydration treatment solution applied to the specimens.

largest range of weight of any of the other animals, and those larvae collected from the rabbit had the smallest range, suggesting that they were probably more similar in age and development.

Rehydration treatment was found to be highly significant (Table 4), and Bonferroni comparisons indicated that all the treatments differed from each other. Those larvae rehydrated with the TSP-substitute solution had mean final weights 51.7% of their original weight regardless of treatment, whereas those rehydrated with the TSP and those rehydrated in EtOH gained an average of 43.4 and 27.9% of their original weight through rehydration, respectively. As in the results for *P. regina*, those specimens initially preserved by the HWK-EtOH method gained a higher percentage of their initial weight after rehydration regardless of method (Table 1).

***C. rufifacies*.** Results for the RM-ANOVA of length and width data are displayed in Table 5. The RM-ANOVA design provides within-subject comparisons that were expected to be different and uninformative because they reflect the change in the larval length and weight through the process of drying and rehydration. The between-subject analysis is the most informative part of the RM-ANOVA result for *C. rufifacies* and will be discussed in the following sections.

Length. There was no significant difference among preservative methods or rehydration treatments (Table 5). From Table 1, it can be observed that those specimens initially preserved in 70% IPA had mean lengths longer than originally preserved values regardless of rehydration treatment, but this was not determined to be statistically significant (Table 5). Those specimens initially preserved in 80% EtOH or with the HWK-EtOH method were shorter on average than those preserved with 70% IPA regardless of rehydration treatment. The single exception to this trend was found among those larvae initially preserved with the HWK-EtOH method and rehydrated in TSP-substitute, which were very close to original length values (Table 1).

Weight. There was also no significant difference observed in the between-subject analysis of larval weight (Table 5). No rehydration treatments restored larval weight to the predehydration value, and the trend was similar across preservation and rehydration methods (Table 2). The trend was similar to that observed with the larvae of the other blow fly species in that those specimens rehydrated with one of the detergent solutions recovered a larger percentage of original weight than those rehydrated by placement in 80% EtOH (Table 2).

Discussion

The rehydration of dried larval Diptera specimens is a feasible solution to reclaiming their usefulness as forensic evidence. This study showed that using 80% EtOH, TSP, or TSP-substitute solutions to rehydrate dried specimens restored larval length and weight to varying levels. These results suggest the amount of restoration depends on the initial preservation method, fly species, and potentially on the length of time that specimens were preserved. Larval length is often used as an estimator of larval age and then

extrapolated out to the time a body or carcass was colonized (Catts and Goff 1992). For specimens in this study, partial original larval length was restored regardless of initial preservation method or rehydration treatment. However, larval weight was not restored by any of the rehydration treatments to levels close to predehydration values.

The interaction between initial preservation method and rehydration treatment has the potential to complicate length to age estimates. Both *P. regina* and *C. macellaria* larvae rehydrated with the TSP-substitute were on average <1% different from their original length, whereas those rehydrated in the TSP solution had final rehydrated mean lengths on average 3.2% shorter than the original values (Table 1). However, for *P. regina* and *C. macellaria* larvae initially preserved by HWK and 80% EtOH and then rehydrated in TSP-substitute solution, rehydrated mean lengths were on average 4.3% shorter than the initial mean values, whereas those rehydrated with the same method, but preserved by placement directly into 80% EtOH, had average mean rehydrated lengths of 9.1% longer than initial values (Table 1), thus suggesting that the permeability of the larval cuticle was affected by the initial preservation. The length of *P. regina* larvae preserved in 80% EtOH was similarly affected by rehydration in TSP solution. The cause of this phenomenon was not examined in this study, but the mode of action for both of the detergent solutions is to break down the lipid bilayer of the cell wall and allow for osmotic flow of liquid back into the cells (Vogt 1991). Third-instar larvae of *C. rufifacies* initially preserved in 70% IPA and rehydrated in TSP-substitute solution also had a mean rehydrated length 8.1% longer than initial values. Taken together, these results suggest that initial preservation methods may affect the permeability of the cuticle, and consideration of the initial preservation method in the selection of a rehydration treatment may help to reduce error in length to age estimates made from rehydrated larvae.

Dried specimens may have value in determining larval age; however, there remain stages during larval development in which resolution is poor and the data relating larval weight to age are known only for *C. macellaria* from a single locality (Wells and LaMotte 1995). In addition, the species identity of the larval specimen has to be determined; thus, the specimens would require some level of rehydration before age determination by dry weight if the specimens arrive to the forensic entomologist in an already dehydrated state. Although storage of dry specimens in the long-term might be an appealing solution because it would eliminate the need for curation of liquids and might preserve extractable DNA better than preservative solutions (Dillon et al. 1996), further development of this approach would require obtaining development data on dry larval weight for every species in which only length data have to date been obtained.

The importance of initial preservation methods on the accuracy of larval age estimation has been noted by previous authors (Tantawi and Greenberg 1993, Adams and Hall 2003), especially when relating published development curves to forensic specimens. The initial preservation method may also be important prior knowledge to have when choosing a method to rehydrate a dried specimen. Rehydration may be better accomplished with one of the detergent solutions for particular species after a lengthy storage time. Although not quantified during this study, the general observation was that greater specimen flexibility was obtained through rehydration with either of the detergent solutions, which suggests that they may be more useful for rehydrating specimens for identification purposes. There was no statistical evidence to promote one rehydration technique over the others; however, the use of a detergent solution may provide a more flexible specimen that is closer in length to the original preserved specimen.

Other studies have suggested that fixation by HWK fully extends the larval length and provides a more accurate description of the larva's length in life (Tantawi and Greenberg 1993, Adams and Hall 2003). We did not observe a significant difference among our preservation treatments. This could be for several reasons, including the inherent variation in our study design. Larval collections were made from field-deployed carcasses subjected to natural colonization and climatic conditions, unlike other studies that were laboratory based. The greater amount of variation present in the field would make it difficult to find evidence of small differences in age based on larval length, but may more accurately describe the field situation encountered in an actual investigation.

Another factor that may have contributed to the lack of significant differences in larval length among preservation methods may have been the length of time that the specimens were stored. An inadvertent factor that may have contributed to the study was that each species was stored for different lengths of time before processing. Adams and Hall (2003) found that storage caused changes in larval length with mean length increasing shortly after fixation and preservation and then declining slowly over time (0.6% shorter after 28 d). *P. regina* larvae and 36% of the larvae of *C. macellaria* larvae were stored for as long as 4 mo before processing, and this factor was not accounted for during the current study. It is reasonable to suggest that storage period may affect larval length and the ability to rehydrate a dried specimen. However, results in this study demonstrated that larvae of both species were rehydrated with some restoration of length.

In this study, the technique for the rehydration of dried museum specimens using 5% TSP solution was successfully applied to larval dipteran specimens, which are the most useful specimens in the current application of forensic entomology. The now widely available TSP-substitute solution appears to be a viable alternative to the old TSP detergents for rehydrating dried specimens. Rehydrating larvae can restore flexibility and allow for identification of these larvae, especially with the use of a detergent solution. Larval weight after rehydration is not a reliable indicator of larval age and is not restored by the rehydration process. Future studies that use standardized laboratory-

reared insects may reduce the overall variation in the current experimental design and give more predicative ability to the observations made in this study. Also, experiments using different ages of larval specimens would provide more information on how this factor may affect rehydration of specimens. Different life stages may also react differently to dehydration and rehydration and would add to the data set and expand the technique for future development as a standard operating procedure. Optimal data for use during forensic estimates will probably always be obtained from specimens freshly preserved using the HWK-80% EtOH technique (Adams and Hall 2003), but for those instances when specimens have become dried out, the techniques described in this study may become a useful tool in the reclamation of these specimens as forensic evidence and provide the framework for a standard operating procedure for dealing with dehydrated larval insect evidence.

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References Cited

Adams, Z.J.O., and M.J.R. Hall. 2003. Methods used for killing and preservation of blowfly larvae, and their effect on post-mortem larval length. Forensic Sci. Int. 138: 50–61.

Amendt, J., R. Krettek, and R. Zehner. 2004. Forensic entomology. Naturwissen 91: 51–65.

Amendt, J., C. P. Campbasso, E. Gaudry, C. Reiter, H. N. LeBlanc, and M.J.R. Hall. 2007. Best practice in forensic entomology: standards and guidelines. Int. J. Legal Med. 121: 90–104.

Byrd, J. H., and J. L. Castner (eds.). 2010. Forensic Entomology: the Utility of Arthropods in Forensic Investigations. CRC, Boca Raton, FL.

Catts, E. P., and M. L. Goff. 1992. Forensic entomology in criminal investigations. Annu. Rev. Entomol. 37: 253–272.

Dillon, N., A. D. Austin, and E. Bartowsky. 1996. Comparison of preservation techniques for DNA extraction from hymenopterous insects. Insect Mol. Biol. 5: 21–24.

Girden, E. R. 1992. ANOVA: Repeated Measures. Sage Publications, Thousand Oaks, CA.

Hammond, A. L. 1971. Phosphate replacements: problems with the washday miracle. Science 172: 361–363.

Huber, J. T. 1998. The importance of voucher specimens, with practical guidelines for preserving specimens of the major invertebrate phyla for identification. J. Nat. Hist. 32: 367–385.

Kroes, H. W. 1980. Replacement of phosphates in detergents. Aq. Ecol. 14: 90–93.

Lord, W. D., and J. F. Burger. 1983. Collection and preservation of forensically important entomological materials. J. For. Sci. 28: 936–944.

Marhue, L. 1983. Techniques to restore dried-up invertebrate specimens. Syllogeus 44: 175–177.

SPSS. 2005. SPSS for Windows, release 14.0.1 computer program, version 14.0. SPSS, Chicago, IL.

Tantawi, T. I., and B. Greenberg. 1993. The effect of killing and preservative solutions on estimates of maggot age in forensic cases. J. For. Sci. 38: 702–707.

Van Cleave, H. J., and J. A. Ross. 1947. A method for reclaiming dried zoological specimens. Science 105: 318.

Vogt, K. D. 1991. Reconstituting dehydrated museum specimens. Curator 34: 125–131.

Wells, J. D., and L. R. LaMotte. 1995. Estimating maggot age from weight using inverse prediction. J. For. Sci. 40: 585–590.

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