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FIFTY YEARS OF HYBRIDIZATION: INTROGRESSION BETWEEN THE ARIZONA TOAD (*BUFO MICROSCAPHUS*) AND WOODHOUSE'S TOAD (*B. WOODHOUSII*) ALONG BEAVER DAM WASH IN UTAH

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***Abstract.*—Recent guidelines for conservation of species subject to hybridization strongly urge long-term studies to determine the extent and the direction of hybridization and introgression, especially in relation to human-induced habitat alterations. This study extends previous work on hybridization between the Arizona Toad (*Bufo microscaphus*) and Woodhouse's Toad (*B. woodhousii*) along Beaver Dam Wash by evaluating morphological and genetic status of four populations. Populations occur at sites from the high elevation headwaters to the hybrid zone at the confluence with the Virgin River in extreme southwestern Utah and extreme northwestern Arizona. Hybrid indices for individuals at the confluence shifted from predominantly *microscaphus*-like in 1949–1953 samples, to predominantly *woodhousii*-like in 1991-1992 samples, and back to *microscaphus*-like in 2001 samples. Nuclear and mitochondrial markers identified 49 individuals from the confluence site as "parental" types, "F1" hybrids, or reciprocal backcrossed hybrids. Although observed and expected frequencies of individuals in each of six cytonuclear categories were similar, numbers of hybrids with the *woodhousii* cytotype were significantly greater than those with the *microscaphus* cytotype. By contrast, hybrid indices of toads upstream (45–97 km) from the confluence were predominantly *microscaphus*-like in the 2001 samples, similar to earlier reports. Nonetheless, individuals with *woodhousii* mtDNA and *microscaphus* nuclear markers were found at sites 45.0 and 64.4 km, but not 96 km, upstream from the confluence of Beaver Dam Wash and the Virgin River. These results indicate that the confluence site is a hybrid swarm, and that introgression of *woodhousii* mtDNA into putatively "pure" *microscaphus* populations occurs for a considerable distance upstream along Beaver Dam Wash.**

Key Words.—Arizona; *Bufo microscaphus*; *Bufo woodhousii*; disturbance; hybridization; introgression; Nevada; Utah

INTRODUCTION

Natural hybridization often results from disruption of habitats leading to reproductive interactions between otherwise allopatric taxa (e.g., Vogel and Johnson 2008; Walters et al. 2008). If first generation hybrids are fertile, there is the potential for backcrossing to one or both parental species, with genes of one species entering the gene pool of the other (introgression). Extensive introgression can lead to the swamping of the gene pool of one species by the other (Rhymer and Simberloff 1996). Alternatively, strong selection against hybrids can result in narrow contact zones with a bi-modal distribution of phenotypes representing primarily parental forms, as recently documented for two gartersnakes (*Thamnophis butleri* and *T. radix*) in Wisconsin (Fitzpatrick et al. 2008).

Conservation biologists have long recognized that hybridization can complicate recovery efforts for threatened and endangered species (for recent examples, see Trigo et al. 2008; Gligor et al. 2009). Many guidelines for conservation of hybridizing species strongly urge studies to determine extent and direction of hybridization and introgression, and some knowledge of whether hybrid zones are naturally occurring or human influenced (Allendorf et al. 2001). Walters et al. (2008) found that introduction of Red Shiner (*Cyprinella lutrensis*) leads to hybridization with Blacktail Shiner (*C. venusta*), a closely related fish, and

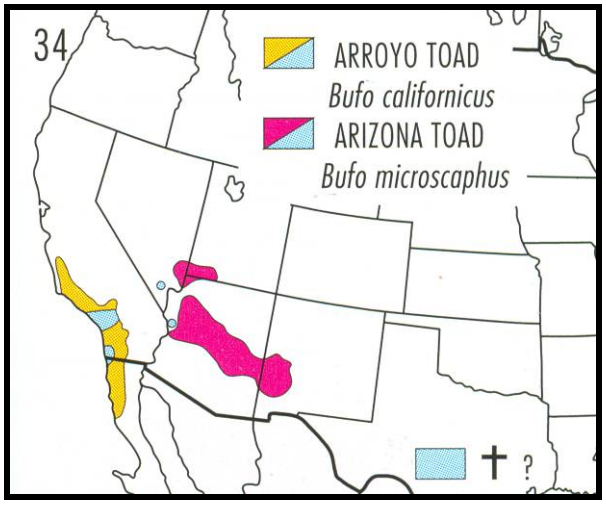

FIGURE 1. Distribution of *Bufo microscaphus* in the southwestern United States (from *A Field Guide to Western Reptiles and Amphibians*, 3rd edition, by Robert C. Stebbins. © 2003 by R.C. Stebbins. Used by permission of Houghton Mifflin Harcourt Publishing Co. All rights reserved). Pale blue indicates regions of significant loss.

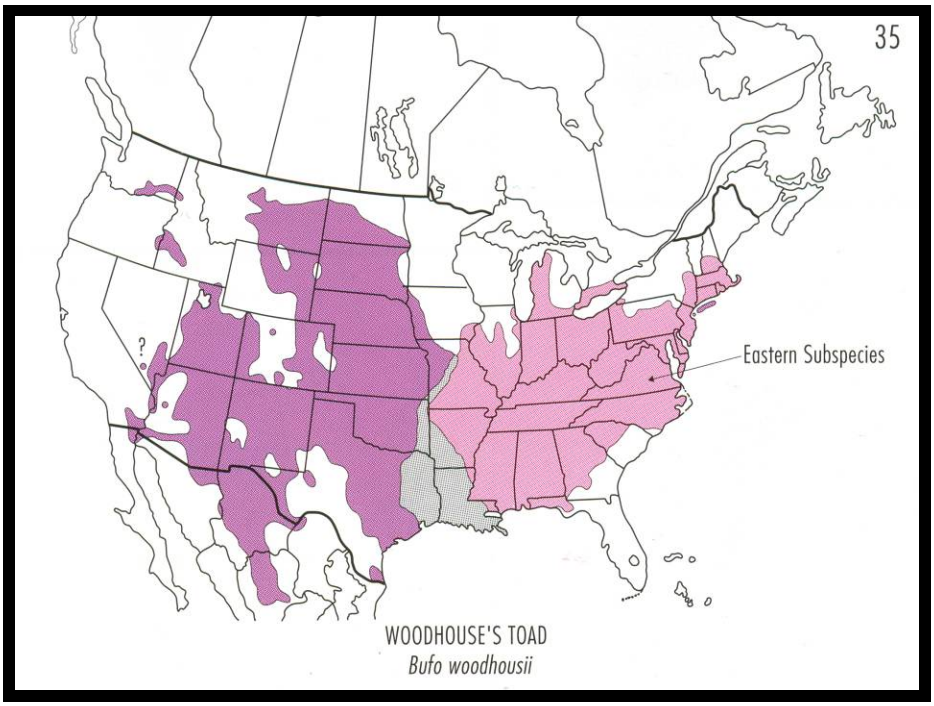

FIGURE 2. Distribution of *Bufo woodhousii* in the United States (from *A Field Guide to Western Reptiles and Amphibians*, 3rd edition, by Robert C. Stebbins. © 2003 by R.C. Stebbins. Used by permission of Houghton Mifflin Harcourt Publishing Co. All rights reserved).

that with increasing levels of disturbance in streams of the southeastern USA, Red Shiner replaces Blacktail Shiner. Long term studies are especially valuable in providing information on temporal and spatial stability of hybrids zones in relation to human induced habitat alterations.

The Arizona Toad, *Bufo microscaphus* (= *Anaxyrus microscaphus*) is distributed continuously within the Virgin River drainage of southern Utah and Nevada, and northwestern Arizona, and patchily across central Arizona to southwestern New Mexico, USA (Fig. 1; Price and Sullivan 1988; Gergus 1998). One of several species of toads in the region, *B. microscaphus* is perhaps the most sensitive to drought: it is typically found within a few meters of a stream, and generally breeds in these lotic habitats in early spring in Arizona and Utah (February-April). Woodhouse's Toad, *B. woodhousii* (*A. woodhousii*), is more widely distributed in the southwestern United States (Fig. 2), has a prolonged breeding period from early spring through summer (February-August), and generally breeds later (e.g., beginning in April) than *B. microscaphus* in the Virgin River area (Schwaner and Sullivan, unpubl. data). Both species are distinguished by a number of characteristics, and like many bufonids, hybridize when sympatric; *B. woodhousii* has expanded its range to co-occur with and even replace *B. microscaphus* in some disturbed habitats over the past 100 years (Sullivan 1993; Schwaner and Sullivan 2005).

Based on morphological analyses, A.P. Blair (1955) first reported hybridization between *B. microscaphus* and *B. woodhousii* in the Virgin River drainage in southwestern Utah and northwestern Arizona. Using analysis of advertisement calls of male toads, W.F. Blair (1957) supported the morphological analyses of A.P. Blair (1955) in revealing intermediate hybrid call types in some locations. Building on the investigations of the 1950s, Sullivan (1995) found that the spatial pattern of hybridization documented by A.P. Blair (1955) was unchanged over 40 years, with no evidence of the influence of *B. woodhousii* on populations of *B. microscaphus* beyond the putative hybrid zones at the junctions of tributaries entering the main stem of the Virgin River (Fig. 3; see map in Sullivan 1995 for additional information). Sullivan (1995) suggested that a natural mechanism, late spring floods, which delayed the typically early breeding period of *B. microscaphus*, resulted in males and females being reproductively active during the breeding period of *B. woodhousii*. However, he also noted that hybrid zones were invariably associated with human disturbance of the breeding areas, usually by housing developments, impoundments associated with water sources for grazing cattle, or golf courses (Sullivan 1995). For central Arizona, Sullivan and Lamb (1988) hypothesized that hybrid zones in altered habitats were unstable, with *B. woodhousii* replacing *B. microscaphus* in at least one river system.

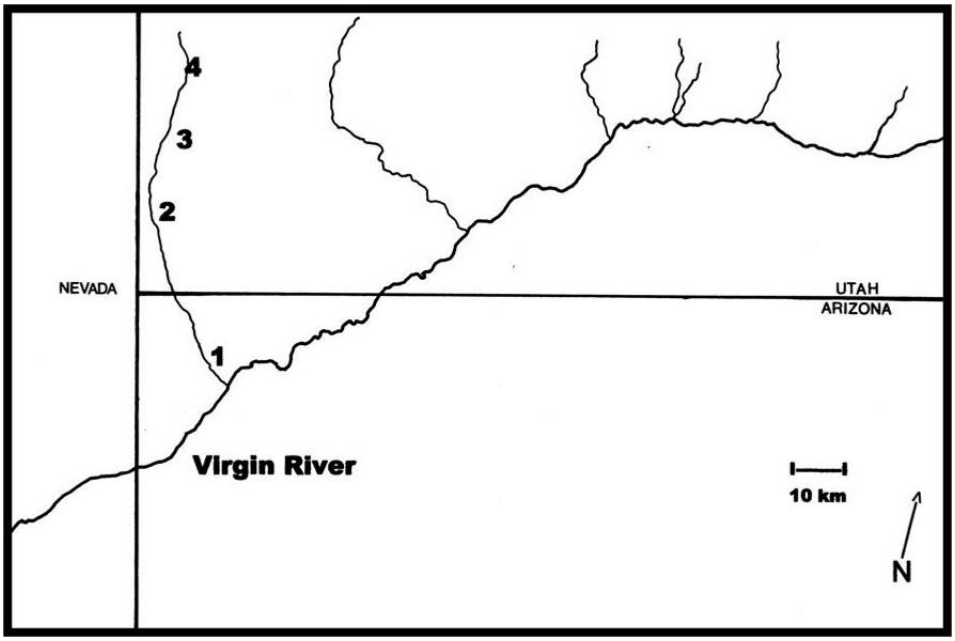

FIGURE 3. The Virgin River and its primary tributaries in southwestern Utah, northwestern Arizona, and southeastern Nevada, USA. The four study sites along Beaver Dam Wash are labeled: 1) Beaver Dam Wash and Virgin River Confluence; 2) Lytle Preserve; 3) Motaqua; and 4) Pine Park.

Historically, *B. microscaphus* has been a protected or "sensitive" species in Utah, Nevada, and Arizona. If hybridization and introgression occur primarily in waterways associated with human disturbance, and minimally (if at all) in undisturbed areas, an argument can be made to minimize at least development that encourages dispersal of *B. woodhousii* into habitat presently occupied only by *B. microscaphus*. *Bufo microscaphus* along the Virgin River are potentially threatened by human-induced disturbance in all regions by dams and lowering water levels from diversion of water for irrigation in addition to urbanization. Damming even small streams (e.g., to form "cattle tanks" for water retention) could cause further migration of the ecologically labile *B. woodhousii* into habitat of the relatively more specialized *B. microscaphus*, perhaps increasing the amount of hybridization. Consequently, characterizing the extent of hybridization and documenting introgression in natural populations is an important prerequisite for determining management strategies. Perhaps even more important is to identify the mating structure of hybrid populations and mechanisms promoting hybridization.

Using the methods developed for assessing genetic evidence of hybridization and introgression between these two toads in central Arizona (Sullivan and Lamb 1988; Lamb et al. 2000), we examined in detail their interactions along Beaver Dam Wash, the best studied tributary of the Virgin River hybrid zone in southwestern Utah and northwestern Arizona. This study extends the pioneering work of Blair (1955), as well as that of Sullivan (1995), on hybridization between these anurans by investigating the genetic status of several of these populations evaluated over the past 50 years. We analyzed populations in Beaver Dam Wash, from the high elevation headwaters to the hybrid zone at the confluence with the Virgin River, for evidence of hybridization and introgression in morphology, allozymes, and mitochondrial DNA.

MATERIALS AND METHODS

Study site and specimen collection.—Beaver Dam Wash drains a watershed of extreme southwestern Utah and southeastern Nevada, before reaching its confluence with the Virgin River in extreme northwestern Arizona (Fig. 3). Toads were first collected in large numbers along this stream between 1949 and 1953 (A.P. Blair 1955; R.C. Stebbins, pers. comm.), and subsequently in 1966 (F.T. Awbrey, pers. comm.) and 1992 (Sullivan 1995). We scored these specimens and those collected during the present study for morphological characters indicative of hybridization using a hybrid index (HI; Sullivan 1986, 1995). In brief, we scored each toad for four qualitative characters (degree of ventral spotting, presence of a dorsal stripe, presence of cranial crests and presence of a pale bar between eyelids), and obtained a hybrid index score for each character of 0, 1, 2, or 3: 0 = typical *B. microscaphus*, 3 = typical *B. woodhousii*, and 1–2 are degrees of intermediacy between the two species (*B. microscaphus*-like = 1, *B. woodhousii*-like = 2; Sullivan 1995; see Fig. 4). Scores for these four

TABLE 1. Distinguishing characteristics of *Bufo microscaphus* and *Bufo woodhousii* (Sullivan and Lamb 1988; Sullivan 1995; Lamb et al. 2000).

Characteristics	*Bufo microscaphus*	*Bufo woodhousii*
Aat-1 locus	Alleles 100 and 120	Alleles 40 and 60
Ldh-1 locus	Allele 100	Allele 140
Ventral spotting	None	Extensive
Cranial crests	Absent	Present
Dorsal stripe	Absent	Present
Pale bar across eyelids	Present	Absent
Cyt*b* *Bsm*A I digest	Marker pattern	Marker pattern
Cyt*b* *Sfa*N I digest	Marker pattern	Marker pattern
16S *Tsp*509 I digest	Marker pattern	Marker pattern

characters were summed to produce a single (cumulative) hybrid index score (HI = 0–12) for each individual (Table 1). Because prior studies have documented some ontogenetic variation in these characters, we used only adult individuals (> 50 mm snout-vent length) for all morphological analyses.

To supplement original collections from 1949 through 1966, we collected toads by hand along Beaver Dam Wash primarily in the spring breeding period (February-July) during 1991–1993 and 2000–2001. We sampled four sites along Beaver Dam Wash in Arizona and Utah: the mouth of the tributary at Beaver Dam (site 1: southernmost, confluence with the Virgin River; 36º54'05.33"N, 113º55'54.06"W), Lytle Preserve (site 2: 45.0 km from the confluence with the Virgin River; 37º08'36.99"N, 114º01'22.01"W), midway between Lytle Preserve and Pine Park at Motoqua (site 3: 64.4 km from the confluence with the Virgin River; 37º18'32.23"N, 113º59'57.8"W), and Pine Park (site 4: 96.6 km from the confluence with the Virgin River; 37º31'21.65"N, 114º01'26.4"W). For the 1991–1993 samples, individual toads were scored for the morphological characters, euthanized by overdose of MS 222, and tissues harvested for genetic analysis (see below). We retained tissues for protein analysis in liquid nitrogen and for DNA analysis in a saturated salt-DMSO buffer (Amos and Hoelzel 1991). Prior to fixation in 10% formalin, and transfer to 70% ethanol for permanent storage, individuals were scored for a variety of morphological variables. Toads collected between 1949–1993 are now housed in the ASU Vertebrate Collection (BKS, A.P. Blair), Museum of Vertebrate Zoology (Stebbins) or the San Diego Natural History Museum Collection (Awbrey); a complete listing, including ascension numbers, is provided in Sullivan (1995). For the 2000–2001 samples, toads at site 1 were not euthanized; tissue for protein and DNA analysis came from two toe clips taken with sterile instruments, both placed in sterile Eppendorf tubes, snap frozen over dry ice in the field, and later stored at -75°C in an ultracold freezer. We recorded scores for morphological hybrid indices at the time of capture, and individuals were subsequently released at the capture site. Toads at sites 2–4 were euthanized and processed essentially as described for samples in 1991–1992; however, after euthanasia each toad was wrapped in foil and snap frozen on dry ice before storage and later processing for proteins, DNA, and morphology.

Specimen preparation for allozyme and DNA protocols.—Sullivan and Lamb (1988) found two marker protein loci (*Aat*-1 and *Ldh*-1) by analyzing liver, kidney and serum samples. Methods for protein analysis followed Richardson et al. (1986). Total genomic DNA was isolated from each 1991–1993 sample using a Pharmacia RapidPrep DNA isolation kit; in 2000–2001 samples DNA was isolated by modifications of a standard phenol / proteinase K digestion, chloroform / isoamyl extraction method. The 1991–1993 samples were amplified for the mitochondrial cyt*b* gene using primers developed by Lamb et al. (2000), and a portion of the 16S rRNA gene using primers LGL 286 and LGL 381 developed by Bickham et al. (1996), in an Eppendorf Gradient Mastercylcer (licensed for PCR). For the 2000–2001 samples, only the 16S rRNA gene was amplified. Amplification reaction mixtures for both cyt*b* and 16S segments were 50µl volumes amplified for 32 cycles at 92C for 1 min, 55 C for 1 min, and 72C for 2 min, with appropriate negative controls to guard against contamination. Restriction fragments for digests of amplification products, using *Bsm*A I and *Sfa*N I for cyt*b* and *Tsp*509 I for 16S in a 12 µl volume (manufacturer's specification, New England Biolabs, Ipswich, Massachusetts, USA), were separated through 4% agarose minigels (3:1 Nusieve agarose; Fisher standard agarose), stained with ethidium bromide, and photographed under UV light with a Kodak EDAS290 system (Eastman Kodak, Rochester, New York, USA). Fragment lengths were confirmed by comparison to a 100 bp molecular weight standard (GibcoBRL). We used one marker allozyme (*Aat*-1; unfortunately, we were unable to resolve variation for *Ldh*-1) to identify hybrids and *Tsp*509I restriction fragment patterns of the amplified mitochondrial 16SrRNA gene to determine the parentage of all individuals (Table 1), following Lamb et al. (2000).

We used a Kruskal-Wallis (KW) test ($\alpha = 0.05$) to assess shifts in hybrid index scores at sites along the

FIGURE 4. Dorsal views of *Bufo microscaphus* (right) and *B. woodhousii* (left) showing presence of dorsal stripe and cranial crests in *B. woodhousii*, and their absence and the presence of a pale head bar in *B. microscaphus*. Photographed by Brian K. Sullivan.

wash. For analysis of the confluence hybrid site, we selected three temporally distinct samples that provided adequate sample sizes for comparison: 1949–1953 (N = 6), 1992 (N = 36) and 2001 (N = 56). Frequencies of individuals in six genotypic groups (MMw, MWw, WWw, MMm, MWm, WWm), as determined by cytonuclear markers, were tested for conformity to Hardy-Weinberg equilibrium (Asmussen et al. 1987).

RESULTS

Hybrid index scores for toads at the confluence of Beaver Dam Wash and the Virgin River (site 1) varied significantly over the five decades of study (KW: χ^2 = 33.03, $P < 0.001$, df = 2, N = 98; Fig. 5). Toads collected by Stebbins (1949-1953), although a small sample, were typical *B. microscaphus* (HI: X = 1.67, range = 1–3, N = 6), while those of Sullivan, 40 years later (1992) revealed a hybrid swarm (X = 8.72, range = 0–12, N = 36) with no hint of a bimodal distribution of phenotypes (in which primarily parentals but few hybrids are found). The sample collected for the present study (2001) was dominated by typical *B. microscaphus*, but still a hybrid swarm with individuals spanning the range of morphotypes typical of hybrids and both parental forms (X = 4.38, range = 0–12, N = 56). Hybrid index scores of individuals from sites 2–4, 45–96 km upstream of site 1 were almost exclusively typical *B. microscaphus*. A single individual from Lytle Preserve (site 2, 45 km from the confluence with the Virgin River) had characteristics approaching intermediacy (HI = 5).

Genetic markers revealed abundant evidence of hybridization and backcrossing at site 1, based on allozymes and mtDNA markers (Table 2). Observed and expected numbers of toads in each of the six cytonuclear genotypes were similar to Hardy-Weinberg expectations (contingency χ^2 = 3.60, df = 5, P = 0.61), although more numbers of MWm and fewer numbers of MWw hybrids were observed than expected (χ^2 = 5.73, df = 1, $P < 0.05$). Thus, genetic evidence indicates that the majority of hybrid matings at site 1 involved female *B. woodhousii* and male *B. microscaphus*, similar to the findings of Malmos et al. (2001) for central Arizona.

Although hybrid index and nuclear genetic markers indicated "pure" populations of *B. microscaphus* above site 1, we observed mtDNA indicative of *B. woodhousii* in three individuals in each of two samples of 20 individuals at each of two sites (sites 2 and 3; Fig. 3), approximately 45 km and 64 km, respectively, upstream of the confluence site. Hence, long distance introgression of mtDNA, but not the nuclear markers, was apparent.

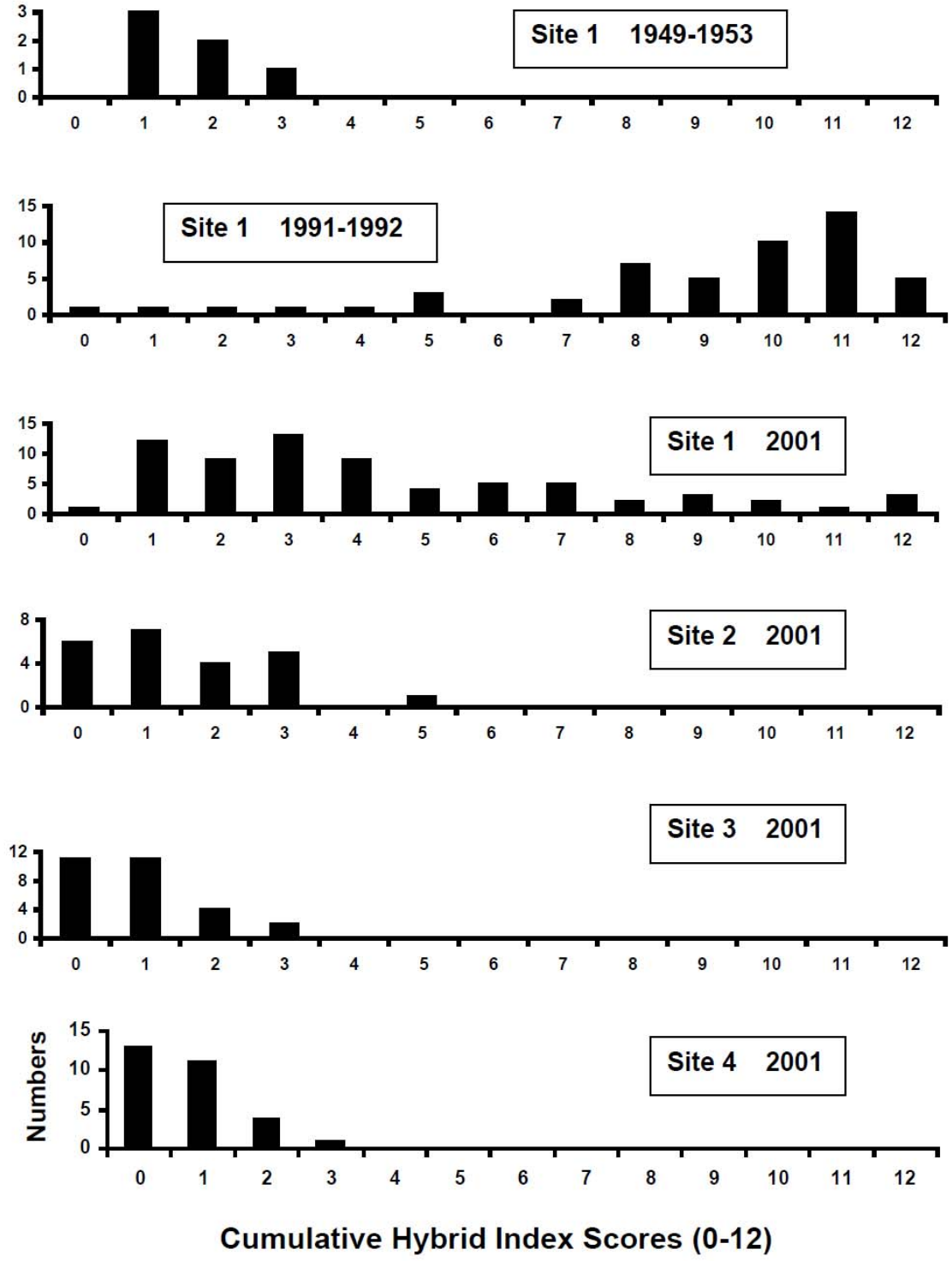

FIGURE 5. Cumulative morphological hybrid index scores (0 = *Bufo microscaphus* and 12 = *B. woodhousii*) for toads observed along Beaver Dam Wash from site 1 (upper three panels), and sites 2–4 (lower three panels). Vertical bars represent the numbers of toads observed for each index score category. Site 2 (Lytle Preserve), site 3 (Motaqua), and site 4 (Pine Park) are 45.0 km, 64.4 km, and 96.6 km, respectively, upstream from site 1.

TABLE 2. Observed/expected numbers of toads from site 1 in each of six cytonuclear genotypes for the cytoplasmic marker, 16s rRNA (m for *Bufo microscaphus* and w for *B. woodhousii*), and nuclear marker, aspartate aminotransferase (MM = *B. microscaphus*; MW = *B. microscaphus* x *B. woodhousii*; WW = *B. woodhousii*). Numbers in parentheses are frequencies of observed toads.

	Nuclear			
Cytoplasmic	MM	MW	WW	Totals
m	21 (0.43) / 17.8	2 (0.04) / 5.5	7 (0.14) / 6.7	30 (0.61)
w	8 (0.16) / 11.2	7 (0.14) / 3.5	4 (0.08) / 4.2	19 (0.39)
Totals	29 (0.59)	9 (0.18)	11 (0.22)	49 (1.00)

DISCUSSION

Our results indicate that hybridization has been ongoing at the confluence of Beaver Dam Wash and the Virgin River for over 50 yrs with no obvious trend toward replacement of *B. microscaphus* with *B. woodhousii*, in spite of the long distance introgression we documented (based on cytoplasmic markers, to a distance upstream, of 64 km). Contrary to the replacement hypothesis of Sullivan (1986), hybrid index scores for toads (mean = 4.4 in 2001–2002) at the confluence site were closer to those of typical *B. microscaphus* (0–3) than *B. woodhousii* (9–12) at the close of our study (2001–2002).

Malmos et al. (2001) provided evidence for directional hybridization in the more recently established hybrid zones between these toads in central Arizona. He argued that the more abundant male *B. microscaphus* allowed female *B. woodhousii*, unable to find a conspecific, a potential mate relative to the less abundant *B. woodhousii* (all hybrid individuals they examined were F1 progeny of female *B. woodhousii*). The data of Malmos et al. (2001), in addition to those of Sullivan (1986) and Sullivan and Lamb (1988), are consistent with the replacement hypothesis in which *B. woodhousii* initially colonizes and then replaces *B. microscaphus* in disturbed habitats. These observations stand in contrast to those of older zones, such as the other sites along the Virgin River that appear to have remained stable for many years on the basis of morphological characters (Sullivan 1995). Nonetheless, our results reveal introgression (of *B. woodhousii* mtDNA) upstream from the primary region of hybridization, potentially resulting from the same mechanism identified by Malmos et al. (2001): mis-matings between local male *B. microscaphus* and female *B. woodhousii*.

The apparent persistence of a hybrid swarm at the confluence of Beaver Dam Wash with the Virgin River in Arizona (Table 2), in an area heavily disturbed by the creation and recent expansion of a golf course, is consistent with the hypothesis that disturbance plays a role in hybridization between *B. microscaphus* and *B. woodhousii*. As noted by Walters et al. (2008), disturbance may play a direct role in hybridization in the fish they studied in which disturbance-related turbidity is thought to increase the opportunity for mis-matings. For the two bufonids we surveyed, it is unclear if habitat change, such as that associated with the construction of impoundments, impacts aquatic larval stages, terrestrial juveniles or adults, or all of these life history stages, much less why the transition from lotic to lentic aquatic habitats appears to favor *B. woodhousii*.

With respect to introgression, we found three of 20 individuals with nuclear markers for *B. microscaphus* and cytoplasmic markers for *B. woodhousii* at each of two locations, 45 km and 64 km upstream from the primary hybridization site. As noted by Trigo et al. (2008), such long distance introgression can compromise conservation of endangered taxa (e.g., when selecting populations for captive breeding), and can only be revealed by thorough sampling of even distant populations from hybrid zones using genetic methods. Thus, in spite of hybrid index scores revealing a recent shift (morphologically) toward pure *B. microscaphus* at the confluence of Beaver Dam Wash and the Virgin River, upstream from the confluence, introgression has occurred, as indicated by mtDNA but not nuclear genetic markers. One explanation for the trend might be that *B. microscaphus* males are far more abundant at site 1 than *B. woodhousii* males or females (Schwaner, pers. obs.) and their ubiquity might dilute the effects of current or past hybridization between the two species. By contrast, Gligor et al. (2009) suggested that asymmetrical introgression of nuclear DNA due to male dispersal explains the discordance between mtDNA and other markers in mouse lemurs (*Microscebus* spp.) in an ecotone in Madagascar. We have no data on dispersal in either of the toads in our study, and no *a priori* reason to expect female biased dispersal; it would seem the absence of selection would best explain the long distance introgression of mtDNA in our system. However, another consideration is that we had only one informative nuclear gene marker, *Aat*-1, to assess hybrid variability. Additional markers, perhaps microsatellites, could reveal more extensive hybridization and further elucidate the interactions between these toads in southwestern Utah.

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Division of Wildlife Resources, Arizona Game and Fish Department, and IACUC of Arizona State University.

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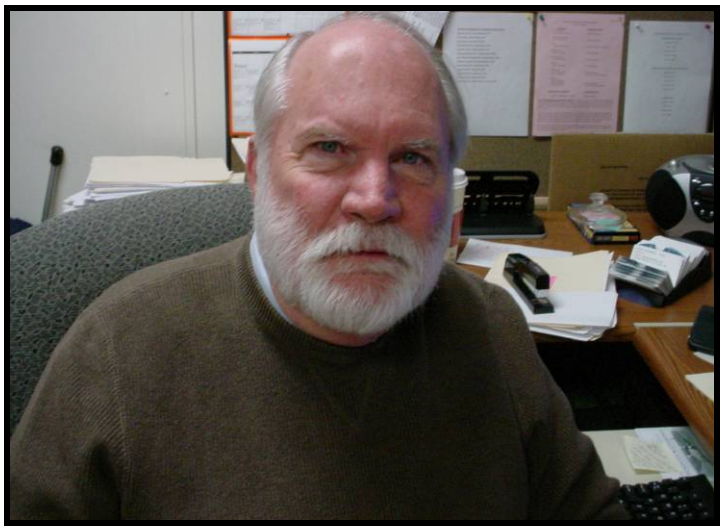
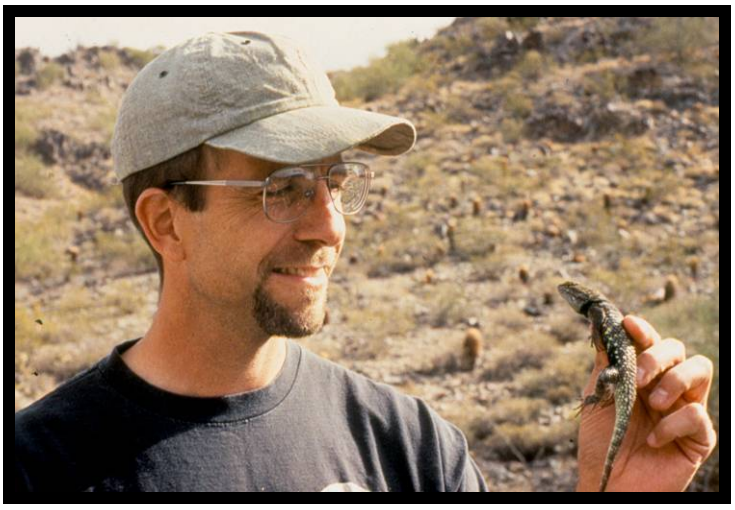


TERRY D. SCHWANER is Professor of Biology and Dean of the College of Sciences at The University of Findlay. His research interests are focused on population biology, ecology, genetics, and evolution of amphibians and reptiles. He has authored or co-authored 90 scholarly articles. (Photographed by Anonymous)



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