


Review

Biogeography of Selected Spring Endemics in Texas Interglacial-Drought Refugia with Unexpected Insights from a Spring-Dependent Nematode Parasite

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Abstract: The contemporary distribution of crenobiontic endemics in central Texas is enigmatic because only some springs are occupied by crenobionts despite other unoccupied springs having seemingly suitable habitats. In the absence of complete paleo-records, a cohesive and widely accepted explanation for this biogeography has eluded researchers for many years. We suggest that data on contemporary species with obligate coevolution, such as parasites with multiple obligate hosts in their life cycles, can help to fill intervening gaps in the paleo-record because the contemporary distribution of such a parasite indicates that its hosts cohabitated without interruption since the arrival of the parasite. To test this conjecture, we studied one such parasite endemic to a select few central Texas springs, *Huffmanella huffmanii*. By studying the distribution of the intermediate host, geologic and paleo-climatic records, performing lab experiments with live animals, and examining archived museum specimens of the definitive hosts from the 1950s, we were able to test multiple predictions about how the distribution of *H. huffmanii* became what it is today. Our results corroborate a narrative suggesting that several severe droughts since the Wisconsin glaciations are responsible for having sculpted the present-day distribution of central Texas crenobionts.

Keywords: cider refugium; crenobiology; biogeography; pleistocene; holocene; interglacial drought; relict; *Hyalella*; *Huffmanella*



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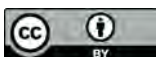
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1. Introduction

Unexpected Distributional Discontinuities

A transitional boundary zone that Huxley would later name “Wallace’s Line” was first described by Wallace as he explored the Malaysian Archipelago in the 19th Century [1]. Today Wallace’s Line is known to be a transition zone of floristic and faunistic distributions without an obvious and present cause separating the “radical distinctions of the Indo-Malayan and Austro-Malayan regions of the great Malay Archipelago” [2].

Since Wallace’s initial reports, scientists have been fascinated by such unexpected discontinuities in the distributions of taxa across the planet, at scales ranging from intercontinental to small springs that contain communities of clades unique to that region. Eventually, the fields of ecology, evolutionary biology, and biogeography began offering hypothetical causal mechanisms in attempts to develop synthesis narratives [3] that might help explain how some of these unexpected distributional discontinuities may have come about.

The unexpected discontinuities that brought our team into this study are, like Wallace’s Line, fuzzy and usually invisible boundaries; but unlike Wallace’s Line, our systems are on a much smaller geographic scale. Our discontinuities of interest are delineated by

the downstream end of spring-dominated conditions that are most easily detected by the downstream distributional limit of endemic crenobiontic clades [4]. The surface area of a typical spring run upstream from such defining boundaries, and to which the crenobionts are restricted, is usually only a few hectares, and sometimes much less.

We will attempt to establish a hypothetical narrative with theoretical cause/effect mechanisms supported by data and experiments to help explain how several of the crenobiontic endemics of central Texas might have become restricted to specific springs [5–10], and will narrow down the factors that still limit the distributions of these endemics to the small world upstream from the invisible boundaries that still restrain them. However, before we delve into this fascinating biogeography, we must first address the confusing array of ambiguous terms used throughout the literature in respect to endemism and refugial concepts (see Appendix A for our comprehensive review of the terminological morass and attempts to resolve ambiguities). After considerable deliberation and reflection on the terminological confusion, we have decided to (1) avoid the use of modifiers to the term “endemic” and will simply refer to clades as endemics *sensu* Darwin’s original glossary entry, “peculiar to a given locality;” (2) avoid use of the term “relict” all together, and (3) use the term “refugia” to refer only to biotopes that have protected biocenoses from extirpation for durations that are sufficiently long to be evolutionarily relevant.

2. Background Review and Proposed Model

In this section, we describe:

- the geographical setting and the geological and chronological scopes of our study,
- the biocenotic communities and their crenal biotopes that led us to develop the proposed model, and
- the functional aspects of our proposed refugial model, the logic underpinning it, and the new name we have proposed for this unique category of evolutionary refugia.

2.1. Our Study

Our study deals with contemporary biocenoses of endemic crenobiontic clades representing diverse higher taxa that are collectively localized in perennial springheads of central Texas. We hypothesize that these biocenoses have persisted, in one form or another, through multiple landscape-scale climate cycles wherein the surrounding terrain apparently varied from lush forest to extreme aridity and back again three or four times over the last several million years. While some biogeographers would probably be comfortable referring to the springheads in our study as “interglacial evolutionary refugia,” this expression alone is inherently ambiguous (see Appendix A) in that it does not distinguish between the functionally and historically different refugia shaped by fundamentally different combinations of forces and constraints that were applied during glacial/interglacial transitions.

2.2. The Setting

The focal setting for the study is the geographic province known as the Edwards Plateau in central Texas (Figure 1) but inferentially extends to the adjacent regions of the southwestern USA.

The refugial ecosystems of focus in our study are the perennial rheocrenic springs that emerge along the southern and eastern boundaries of the Edwards Plateau. All of the springs of interest are fed by numerous interconnected pools of the Edwards/Trinity Aquifer System [5–10].

Geographic Region of Interest

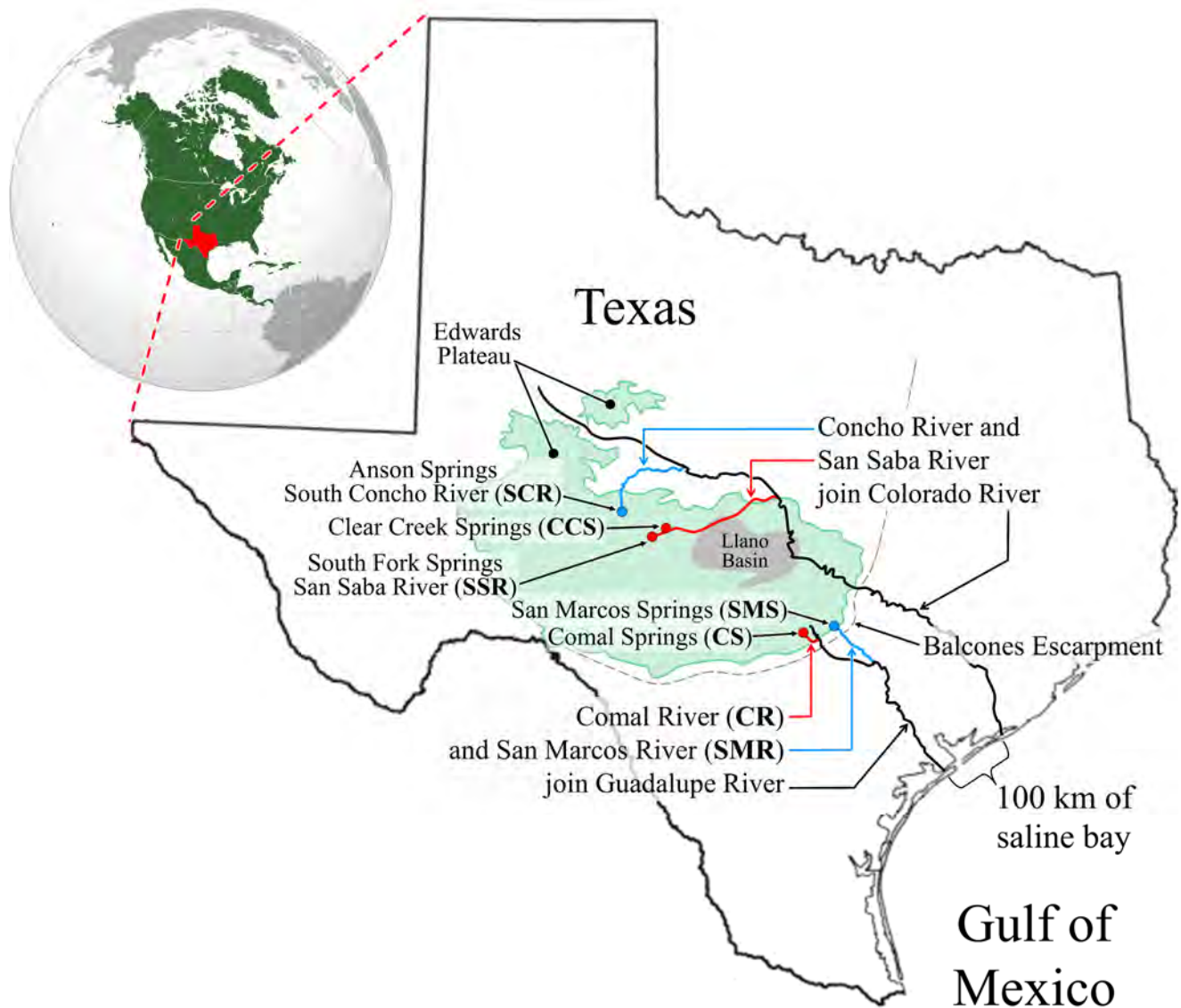


Figure 1. Map of Texas showing a rough outline of the Edwards Plateau with the springs of interest and their connecting streams. Map of NA from Wikimedia Commons.

2.3. Pre-Pleistocene Geological History of the Region

Much of western North America was formerly inundated by an inland sea throughout most of the Mesozoic until around the time of the K-Pg (Cretaceous-Paleogene) boundary [11]. Subsequent to the K-Pg boundary, the seas inundating Central Texas began to regress [12], partially due to a decrease in eustatic sea level, and partially due to regional uplifting associated with the Laramide Orogeny [13]. The Texas component of the uplifting is known as the Llano Uplift and is thought to have raised the strata of Central Texas several thousand meters [14,15]. This uplifting provided forces contributing to the Balcones faulting during the mid-Cenozoic [14–16]. The faulting resulted in a vertical displacement of strata by as much as 260 m [17]. The eroded karstic remnant of the footwall scarp now survives as the Balcones Escarpment, and the now eroded plateau formed by the footwall block is the Edwards Plateau.

Today, the only remaining marine deposits exposed in Central Texas are Cretaceous or early Cenozoic in age [18,19]. There is some evidence that, on several occasions subsequent to the Cretaceous, eustatic sea level rose high enough to have submerged the terrain east and south of the Edwards Plateau, perhaps as far inland as the Balcones Escarpment [20–24], but we could find no report of a stratigraphic record of marine transgressions over the Plateau since the Cretaceous.

One of the Cretaceous formations (Edwards Limestone) in the footwall block still exposed along the eastern and southern slopes of the Balcones Escarpment is riddled with caverns, the youngest of which are below the fault trace and support permanent subterranean streams of the Edwards Aquifer [14], with estimates of total water capacity ranging upwards to over 200 km³ [17]. At progressively higher strata of Edwards in the now vadose portion of the formation above the trace there are progressively older caverns which formerly carried aquifer water but now harbor terrestrial endemics, all this indicating a long Cenozoic history of coastward hydrological evolution in the Edwards Plateau [16]. It is in the perennial rheocrenic springs that have been issuing from this series of caverns for millions of years that we focus our attention.

2.3.1. The Wisconsin and Holocene Climates

To set the stage for our discussion, we must establish the presumptive history of a series of very wet and then very dry periods following in relatively quick succession in the region since about 22 kya. During most of the Cenozoic Era, the mean annual temperature of Earth apparently experienced a gradual cooling trend [21] and then dipped dramatically to near 0 °C during the four glacial maxima of the Pleistocene.

The climate of the SW USA during the latter stages of the Wisconsin Glaciation and the beginning of the Holocene is thought to have been much wetter than the present. Cooke et al. [25] provided convincing isotopic evidence that soil thickness over the Edwards Plateau during late Pleistocene averaged nearly 2 m thick, but starting at about 21 kya, soils eroded during the wet period at a rate of 11 cm/ky until about 5 kya. This rapid precipitation/erosion narrative is corroborated by various findings, including a fossil record indicating the presence, then decline, and then disappearance of burrowing mammals that require deep soils [26]. Within that erosional interval, there apparently was also a period of intense channelization of regional streams between about 15 and 11 kya [27–29] and another episode at 9.6–7.4 kya [29]. The soil erosion estimates of [25] suggest that most of the precipitation was probably in the winter, with droughts in the summers.

Following the transition between the wet Pleistocene and the present, a period of “exceptionally rapid warming” occurred [30,31] that is thought to have been accompanied by several periods of extensive and severe droughts [32]. Speleothem (cave formations consisting of minerals deposited from percolating water) studies from the southwestern U.S.A. indicate that stalactites grew rapidly immediately following the last glacial maximum, but then abruptly stopped dripping completely at about 10 kya and ceased to grow at all until about 2.7 kya when the dripping started again but at a slower rate [33]. This speleothem record, which is corroborated by parallel records of lakes in the western and southwestern U.S.A. becoming desiccated [34,35], is consistent with an extremely arid period 10 kya–2.7 kya. While the dates of onset and durations of the early Holocene wet period and Mid-Holocene droughts vary with locality, there is widespread consensus that the Pleistocene/Holocene transition was much wetter than present, and that the Mid-Holocene droughts were much drier than present, with the most extreme of the Holocene droughts primarily occurring between 6 and 2 kya [30,32,36–38]. The effective outcome of these droughts is that the volume of freshwater exposed at the surface in some parts of the western U.S.A. may have been reduced by 90% relative to contemporary surface water [39] and by 75% within the Edwards Plateau region [40,41].

Climatological Effects on Local Aquatic Communities

Given this condensed narrative of the Holocene climatic history in Texas, and that the highest concentrations of endemic aquatic biocenoses in the study area are found in the eucrenal reaches of rheocrenic spring runs, it seems reasonable to surmise that the Holocene droughts caused a dramatic reduction of available aquatic habitat exposed at the surface in Central Texas [40,41] which forced surviving aquatic taxa into isolated refugial patches of groundwater-sourced surface water, and some were even forced into the aquifer itself. We are obviously not the first to propose such a narrative for the Edwards Plateau, but intend to add several lines of clarifying information to the narrative that had not been reported before. In so doing, we will use a proxy in the form of a crenobiotic nematode parasite that has two obligate contemporary hosts in its life cycle, both of which are common and cosmopolitan in most Texas streams.

The fragmentation and rarefaction of surface waters during the mid-Holocene droughts are thought to have lasted for long durations relative to the generation time of most aquatic invertebrates. This would have halted the gene flow that was previously occurring between the populations of the permanently aquatic clades via fluvial conduits that were formally perennially interconnected. The genetic bottlenecks caused by reductions in local abundance, and the restriction of gene flow caused by fluvial fragmentation, are thought to have provided isolated natural laboratories for extreme adaptation to the stable conditions of groundwater-sourced surface water. Over time, genes that had, for probably since the Precambrian, provided complex systems of cellular damage control and repair necessary for ectotherms to survive in physicochemically variable waters were either lost or repurposed. Once these springs were later reconnected to fluvial surface waters, the crenobionts would be unable to re-disperse downstream into thermally ambient surface streams. Thus, unexpectedly abrupt distributional discontinuities are now common just downstream from some of the perennial spring sources flowing from the Edwards Aquifer. Another factor that enriches the drama playing out in these abrupt distributional discontinuities is that the returning fluvial relatives of the endemics taper in abundance upstream from the discontinuities because they cannot outcompete the endemics in their native biotope.

2.3.2. The Chronology of Our Model

The chronology of our study begins sometime after the last marine regression, which permanently exposed the Edwards Plateau of Central Texas and which probably occurred no earlier than the late Cretaceous, and no later than the mid-Pliocene [42]. Although there have been four or five interglacial warming periods during the Pleistocene that have probably contributed to the aquatic endemism of the Edwards Plateau, the main period of our investigative focus starts about 20 kya just before the transition from the Wisconsin glaciation to the interglacial warming of the Holocene.

2.4. The Model System

2.4.1. The Clades of Focus

There are numerous permanently aquatic clades in this landscape that form very local biocenotic communities of low-dispersion invertebrates having narrowly restricted and sharply defined distributions. Some of the clades are constrained to a single spring [12,43] despite many of the springs being permanently interconnected via underground conduits and perennial surface streams throughout recorded local history.

The mosaic of endemism observed across diverse higher taxa within this region is difficult to explain comprehensively—how could so many species, both related and unrelated, share such similar distributional patterns, and why are there so many abrupt distributional discontinuities associated with the Edwards Plateau? The most parsimonious explanation seems to be that these extant biocenoses share a common evolutionary history while co-habiting in their isolated spring biotopes, and that the highly restricted contemporary

distributions resulted from some series of events that acted simultaneously in sequence across the entire region.

The water of the Edwards Aquifer, which supports >100 stygobiontic clades [10], breaks through the surface at the bottom of the exposed Balcones Escarpment and feeds large perennial and thermally stable rheocrenic springs such as San Marcos Springs (SMS) and Comal Springs (CS; Figure 1) in Hays and Comal counties, TX, USA. The SMS supports diverse communities of aquatic organisms that include many endemic crenobiontic clades and is the headsprings of the San Marcos River (SMR).

One such clade is an innominate hyalellid amphipod we will call the SMS *Hyaella*; a mucronate (possessing dorsal spines) and crenobiontic amphipod restricted to the eucrenic waters of the first 3 km of the Upper SMR. This amphipod coexists with, and is reproductively isolated from [44] the cosmopolitan *Hyaella* cf. *azteca*. Both hyalellids also serve as functional intermediate hosts [45] in the life cycle of a locally endemic histoparasitic nematode of centrarchid fishes, *Huffmanella huffmanii* [46] (Nematoda: Trichosomoididae: Huffmanellinae). *H. huffmanii* is the only reported freshwater species of the subfamily and its distribution in the SMR is also restricted to the upper 3 km of the spring run, where prevalence of *H. huffmanii* is often 100% in several centrarchids species [47]. Recently, two other endemic freshwater populations of *Huffmanella* were reported from Texas headsprings some 230 km NW; one from Clear Creek Springs (CCS) on the San Saba River (SSR) [48] and the other from the headsprings of the South Concho River (SCR) [49]. No adults have been recovered from these new populations, but their eggs cannot be reliably distinguished from those of *H. huffmanii* in the SMR. Another mucronate and crenobiontic hyalellid amphipod clade distinct from the SMS *Hyaella* occurs at CCS (*H. texana* [50]). A fourth, but now extinct population of *Huffmanella* was discovered to have been infecting centrarchids in the nearby Comal Springs until the drought of 1956 [45]. Recently, adults and eggs that are morphometrically and genetically undisguisable from SMR *H. huffmanii* were discovered infecting freshwater poeciliids from ornamental fish ponds in Ruskin, Florida [51]. The 21 other nominate species and about 15 additional innominate but distinctive populations of *Huffmanella* have all been reported exclusively from marine or estuarine fishes, and *H. huffmanii* remains the only reported freshwater species. Interestingly, and perhaps pertinent to our focus, many of the other endemic invertebrates of the Edwards Aquifer and its springs appear to be marine-derived freshwater representatives of higher taxa that are mostly or exclusively marine [10,52].

The extremely narrow endemism of *H. huffmanii* and its SMS *Hyaella* host in freshwaters of Central Texas provides an opportunity for a novel line of proxy reasoning to enhance the biogeographic inferences that underpin the existing narrative regarding the region's geologic and climatic history.

2.4.2. Overview of Corroborating Experiments

We performed a series of stepwise, mostly experimental investigations using this nematode/amphipod/fish host–parasite system as a model to explore some of the mysteries of the biogeographic history of these springs in central Texas and their diverse biocenoses of narrowly endemic crenobionts. Firstly, we tested the possibility that the contemporary distributions of these crenobiontic clades could have been sculpted out of previously widespread distributions by the severe droughts of the Pleistocene interglacials. We focused on the most recent Holocene droughts, for which we have a relatively high resolution chronology, as an example. Secondly, we explored the possibility that these nonaerial aquatic invertebrates were forced by desertification into the stable physicochemistry of rheocrenic springs for tens or hundreds or thousands of generations resulting in genetic losses that are currently preventing those endemics from colonizing the now-reconnected surface streams. Thirdly, we investigated evidence that the evolution of the SMS *Hyaella* and its *Huffmanella* parasite were affected by the same large-scale environmental events.

2.5. Our Research Hypothesis

Our overarching hypothesis that we are investigating through a series of specific and testable hypotheses is that the *Hyaella/Huffmanella* host parasite system can shed light on the dynamical paleohistory of these functionally related biotopes populated richly with other endemic clades from diverse higher taxa, and that the descriptive name we have chosen to label the type of karstic rheocene refugia we are dealing with in Central Texas will be strengthened by skeptical challenges and serve as an example for uniquely labeling several other types of refugia that have traditionally been cast into the functionally meaningless categories of glacial or interglacial refugia.

2.5.1. “Crenal Interglacial-drought Evolutionary Refugia” (CIdER)

Our study focuses on several biocenoses of contemporary, narrowly endemic, crenobiontic clades of central Texas that are hypothesized to have once been widespread in the interconnected crenic and lotic freshwaters of the region. The refugia to which our endemics are currently restricted are karstic-spring microrefugia sourced by deep, expansive, largely interconnected subterranean reservoirs; thus, spring discharge is largely uncoupled from local precipitation/drought/temperature transients and many of the springs are perennial rheocrenes. We will start with brief descriptions of stages in the chronology of events that are hypothesized to have led to the endemism we find there today, and will apply a new term to help clarify the distinctions between this type of evolutionary refugium vs. other evolutionary refugia associated with glacial/interglacial cycles.

Following the general admonition of Ashcroft [53] (Appendix A.3.4), we will be using the new term and acronym “Crenal Interglacial-drought Evolutionary Refugia (CIdER).” While the name is longer than most category names, the modifiers capture the biotope and biocenosis, the climate, the dynamics, and the duration (long enough to be evolutionarily relevant). Although details in composition, timing and location will vary from site to site and region to region, the term will uniquely represent a cohesive type of refugium with a specific set of contemporary and historical factors that must be understood in order to fine-tune conservation efforts and potentially, to protect the many biotopes that are examples of this type of refugium and their delicate biocenoses. It also provides an example of how to address some of the issues revealed by workers who have published extensively about the terminological morass we must all wade through when reviewing the literature on the subject of biodiversity hot spots [4,53–59] (see Appendix A).

At this writing, we know of no other named refugial type or classification of endemics that encapsulates the salient and peculiar combination of features, driving forces, and evolutionary consequences just described above for the karstic springs of the Edwards Plateau. Yet, investigators dealing with glacial/interglacial refugia and related endemism often refer collectively to systems that have widely differing functional dynamics using a single ambiguous terminological scheme. For instance, endemics in terrestrial refugia will have arrived there having been driven by dramatically different forces with different consequences compared to aquatic endemics in crenal refugia in the same general region. The two communities may be experiencing the same climatic changes at the same time, but they cannot all be meaningfully referred to as simply “interglacial refugia.”

Note that since we are limiting our definition to groundwater-sourced aquatic refugia, the main factor defining a CIdER refugium is hydrological dynamics driven by advancing aridification followed by a return to precipitation sufficient to maintain perennial fluvial conduits, rather than long-term changes in average annual temperature, although temperature usually occurs as a covariate with precipitation.

If we could show that the SMS is indeed a CIdER refugium, then the occurrence of *Huffmanella* in the other springs of the Edwards Aquifer would suggest, by inference, that those springs also represent CIdER refugia that had earlier presented groundwater at the surface uninterrupted through the Holocene droughts, and perhaps much longer (potentially as far back as just after the last marine regression). Furthermore, if we could provide corroborative evidence that both the parasite and its obligatory amphipod host probably

coevolved and shared the same environment through the period of forced isolation, this finding would provide additional corroboration for the CIdER refugium hypothesis.

2.5.2. Hypothetical Stages in the Development of a CIdER Refugium

Stage 1: Abundant precipitation (Figure 2A). In order to explain how all of these once cosmopolitan clades from diverse higher taxa became so narrowly endemic in the same springs, we hypothesize that, at some time prior to the mid-Holocene Droughts, the spring runs of these rheocrenic springs were interconnected via surface-fed perennial streams.

Generalized Evolution of a CIdER Refugium

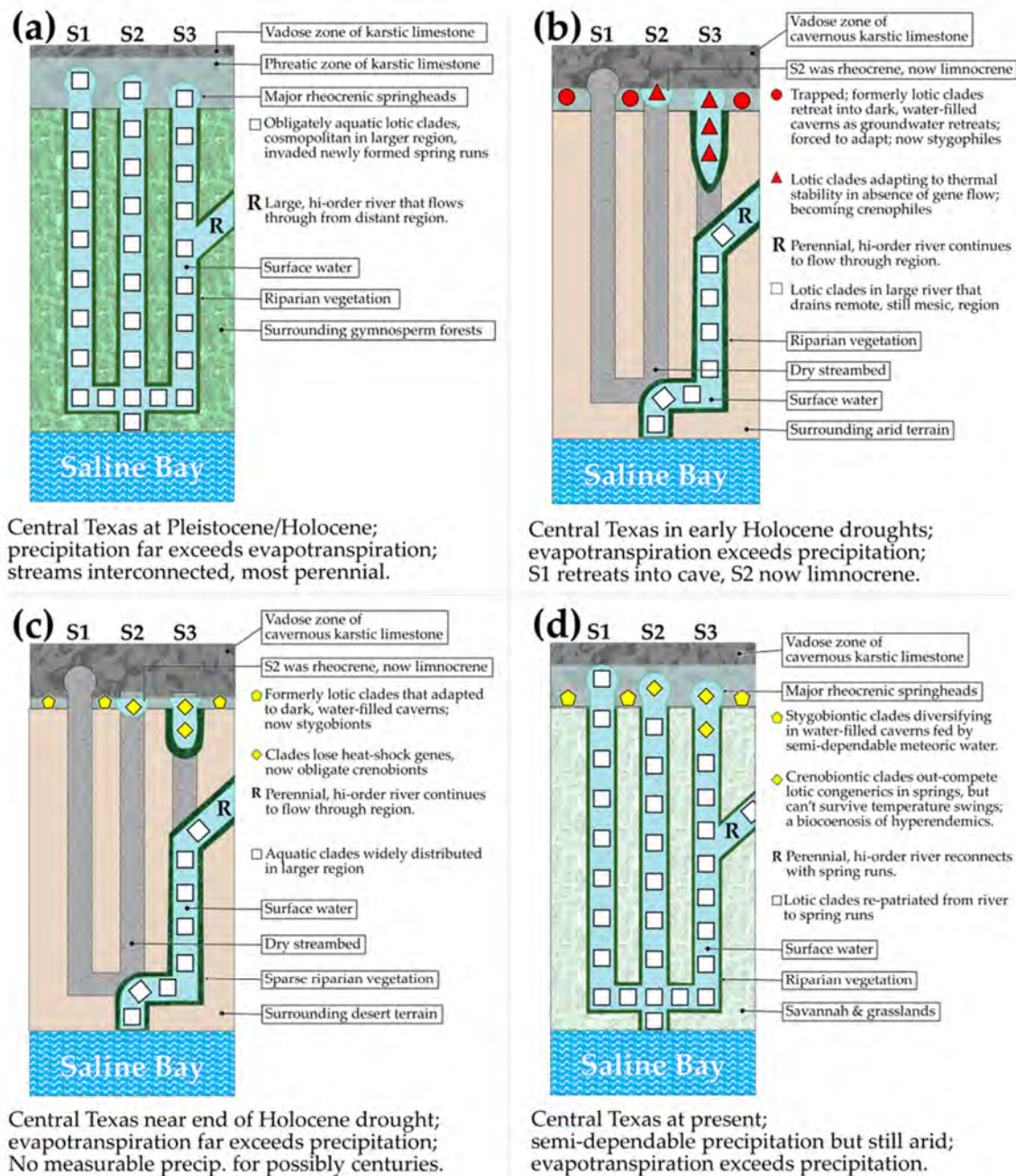


Figure 2. Schematic of the hypothesized sequence of events leading to the development of a CIdER refugium, using Central Texas as an example. S1–S3 represent three springs interconnected underground by conduits fed by the same aquifer pool, with S1 opening at the highest elevation and S3 at

the lowest elevation of the three. (a) Conditions during late Wisconsin Glaciation and early Holocene (ca 20–9 kya) with abundant precipitation well in excess of evapotranspiration, and continuous discharge from all springs flowing into progressively higher order streams and then to coastal bays. (b) Holocene droughts started ca. 9 kya and most aquatic biota were locally extirpated but some biota adapting: S1 withdrew underground with some vertebrates and invertebrates adapting to stygobiosis; S2 stopped flowing out but maintained groundwater-sourced limnocratic pool at surface; S3 is still flowing out into short spring run that disappears into air and ground with no connections to other surface water. (c) Near end of most severe of Holocene droughts ca. 4.5–2.7 kya with all springs still isolated and most surviving clades now stygobiontic or crenobiontic; (d) Abundant moisture returns and cosmopolitan fluviially adapted congeners are repatriated to interconnected spring runs and are mixing with the now-endemic crenobionts.

Stage 2: Aridification (Figure 2B). Reduced precipitation at the beginning of the mid-Holocene droughts (about 9 kya) eventually reduced discharge from the springs, interrupted surface connections with remaining streams, and isolated the aquatic biota of each spring into microrefugial crenal biotopes [60]. The bulk of the regional aquatic species were either extirpated, retreated into subterranean habitats where they transitioned into stygobionts, or retreated into major rivers that continued to drain remote regions. Hypothetical spring S1 (Figure 2B) stopped flowing and retreated mostly into a cave, S2 maintained a pool of water receiving sunlight at the surface as a limnocratic, and S3 continued to flow into a short unconnected spring run that disappeared due to evaporative and hyporheic losses and lack of phreatic input. The aquatic fauna of S2 and S3 became endemic remnants of clades once widespread in the region, but many of the clades had relatives that we will call sister populations that managed to survive the aridification in major rivers near the region.

Stage 3: Continued isolation (Figure 2C): Several thousand generations later toward the end of the mid-Holocene droughts, the spring-bound endemics had lost many alleles formerly used to produce enzymes adapted to diel and seasonal temperature swings [61], and for repairing damage to molecular infrastructure and metabolic systems following exposure to temperature extremes [62]. Such alleles could be lost from the refugial population without fitness penalties due to the direct connections to deep aquifer conduits restraining the thermal variability of the springs to within ± 1 to 2 °C. Indeed, Somero [62] showed that congeneric species of intertidal organisms replace each other along a subtidal/intertidal gradient, suggesting that the fitness costs of evolving competitive advantage at a given temperature are actually less than the costs of maintaining the metabolic wherewithal to operate competitively at a range of temperatures. At the same time, the refugial endemics had become much more efficient at exploiting resources from the physicochemically stable refugium. Meanwhile, the sister populations of the crenobiontic clades had adapted to surface water in the remote regions to which their population had retreated as the drought progressed.

Stage 4: Abundant precipitation returns (Figure 2D). Upon the return of precipitation beginning at probably around 4.5 kya, reconnection of the isolated refugia to perennial surface-fed streams began to allow contact between the long-isolated endemic clades and the now-repatriated, but still stream-adapted, congeners from remote regions where surface-fed streams had continued to flow. By this time, genetic divergence between the crenobiontic population and the displaced sister population may have progressed to the degree that even if the sister population is subsequently repatriated into sympatry with their crenobiontic congeners, reproductive incompatibilities may have accumulated to the extent that successful backcrosses between the two populations are now unlikely or impossible. However, if they are reproductive compatible, it is likely that the crenobiontic congener will soon be introgressed out of existence by the much richer allelic gene pool of the repatriated sister group.

At Stage 4 above, our research team steps onto the stage and makes several observations listed below that ultimately engender an array of questions.

1. Members of the spring-endemic population are now crenobionts because they are unable to disperse into the connected ambient stream.
2. The cosmopolitan congener (repatriated sister group) does not introgressively swamp the endemic population because of apparent reproductive incompatibilities.
3. The widespread congener is apparently unable to displace the endemic population from the spring by out-competing it.
4. Both populations remain in graduated sympatry [63] in the eucrenal and/or the spring-influenced upper reach of the spring run.
5. Our team is unable to determine by mere inspection whether the observed endemism of the crenobionts had originated through sympatric or allopatric processes.
6. Our team is also unable to determine by inspection what factors are limiting the downstream dispersal of the crenobionts.
7. Thus, if an endemic species occurs in a patch of habitat that is far from other such patches, that alone is insufficient evidence that the endemism developed in allopatry; likewise, when an endemic species occurs sympatrically with a closely related congener, that observation is not necessarily evidence that the endemism had developed in sympatry. Therefore, while speculation might be useful for developing testable hypotheses, the development of a definitive and supportable narrative that parsimoniously explains the cause of contemporary endemism should be based on multiple lines of reasoning that are corroborated by pertinent data and, where possible, experimental findings.

2.6. The Need for Rigor

We are not the first to have proposed that the springs of the Edwards Plateau were once perennially isolated refugia. While conjecture and speculation abound regarding potential mechanisms that forced the hypothesized range contractions and caused the endemism among the aquatic fauna of the Edwards Plateau, there have also been some rigorous attempts to test such hypotheses.

Adams et al. [64] provided an interesting account of the presumed evolutionary history of saline and freshwater gammarid amphipods in previously isolated springs of the Chihuahuan Desert which borders the Edwards Plateau on the west and has experienced many of the same major climatic and geologic events. Krejca [65] used evolutionary patterns of stygobionts to aid in interpreting the hydrogeologic history of the central Texas karst aquifers and adjacent aquifers of northern Mexico. White et al. [16] studied a troglomorphic spider clade from vadose caverns of the Edwards karst and established links among phylogeography, geomorphology, and hydrogeology. They also developed a robustly supportable interpretation of the Pleistocene evolutionary history of the clade indicating that the spiders had apparently been epigeal during successive glacial maxima and then retreated into caves that had been left at progressively lower elevations in the vadose zone during the three interglacial warming periods of the Pleistocene as the aquifer evolved coastward [66]. Craig et al. [8] studied distributions of fishes noted for their preference for springs of the Edwards Plateau and compared these with distributions of related river fishes in connecting streams. They reported findings consistent with one or more episodes of lengthy isolation during the Pleistocene interglacials, each followed by reconnection.

While host/parasite systems are rarely used to test proposed causes of paleogeographic endemism, there have been some productive attempts. Mejía-Madrid et al. [67] investigated the phylogeography of *Rhabdochona lichtenfelsi*, an intestinal nematode parasite specific to endemic goodeid fishes in Mexico, and provided new information regarding the biogeographical history of fragmentation and recent evolution of the Mesa Central drainages. Martínez-Aquino et al. [68] used molecular dating of cladogenetic events of the intestinal trematode *Margotrema* spp. and found good correspondence with inferred phylogeographic history of their hosts (goodeine fishes of the western slope of central Mexico). Lumme et al. [69] investigated the historical zoogeography of a monogenetic trematode of a small fish in Europe, and discovered findings consistent with several sequential

episodes of isolation bottlenecks and re-expansions in numerous allopatric refugia during the Pleistocene.

Our main goal in this study was to investigate the Crenal Interglacial-drought Evolutionary Refugium (CIdER) Hypothesis as a possible mechanism to explain the extreme endemism of the diverse freshwater clades that are now restricted to tiny distributions of a few hectares in the Edwards Plateau and surrounding regions. A secondary goal was to explain how these same organisms might have continued to remain constrained entirely to the spring-influenced portion of their ancient habitats, despite the return of interconnectivity thousands of years ago between the springhead habitats and surface-fed streams. Therefore, our report will utilize the present biogeography of an extant endemic host/parasite system to make inference into ancient abiotic factors possibly contributing to the original isolation of these populations. In particular, a host/parasite system consisting of a narrowly endemic nematode parasite with two obligately aquatic but ubiquitous hosts (centrarchid fishes and hyalellid amphipods) was used as a proxy for palaeoecological inference. This three-species host/parasite system serves as a more useful proxy than would three free-living crenobiotic endemics, because the occurrence of this narrowly endemic parasite in a CIdER refugium today indicates that not just the parasite, but also both of its obligate host species (or their ancestral clades), were continuously present in the habitat from the hypothesized contraction of the parasite's formerly broad range to its current very limited range of about 20 hectares of spring-influenced surface water. By using this parasite as the test species, we hope to improve understanding of the likely distributional history and co-evolution of not only the parasite, but also the codivergence of its crenobiotic amphipod intermediate host, and by broader inference, some of the other endemics of the region.

Our study involves findings from many different types of investigations, from purely descriptive to rigorously experimental. In order to facilitate assimilation and application of the findings, we will accumulate all the issues directly associated with each finding, including purpose, methods, results, and discussion together in narrative form in the section related to each finding.

3. Methods, Results and Discussion

We have organized this section into a question/investigations/answers format to facilitate assimilation of data consistent with our research hypothesis.

3.1. Q1: Is There Evidence That SMS Has Continuously Maintained Groundwater at the Surface since the Last Glacial Maximum?

A major component of the CIdER refugium hypothesis is that the SMS is a permanent spring that has maintained a supply of flowing groundwater at the surface continuously and without interruption through the Holocene and probably much earlier. It has not stopped flowing in recorded history, but the CIdER refugium hypothesis requires that it has not stopped presenting at least a springbrook at the surface for at least the last 14 ky. Interestingly, support for the SMS being a CIdER refugium was obtained indirectly from neighboring Comal Springs (CS).

3.1.1. An isolated *Huffmanella* Clade Was Extirpated from CS by a Historic 4.5-Month Cessation of Spring Discharge

Comal Springs (Figure 1), the headsprings of the Comal River, has the highest average discharge of any naturally occurring freshwater spring in the southwestern USA [70], is located <30 air km southwest of the SMS (Figure 1), derives its flow from the same pool of the Edwards Aquifer, and is confluent with the same river as the San Marcos River. Despite the close proximity of the Comal and San Marcos springs and the remarkable similarity between their aquatic communities and physicochemistry [71], *Huffmanella* had never been detected in centrarchids from the CR, despite many attempts (Table 1). Additionally, both hosts of *Huffmanella huffmanii* are known to occur abundantly and continuously throughout the streams connecting the two springs. Thus, the absence of *Huffmanella* in CS has

remained an intriguing enigma since the discovery of *Huffmanella* in the SMS in 1978, and represented a challenge to the CIDER hypothesis in its early stages of development.

One evening while discussing frustration over our failure to find *Huffmanella* in the centrarchids of the Comal Springs, one of us (MLDW) recalled that the CS had ceased flowing (due to over-pumpage) for >4 months during the drought of record in 1956 [70]. This drought is known to have caused the local extirpation [72] of the fountain darter, *Etheostoma fonticola* [73], which was formerly endemic to both the CS and the San Marcos Springs, and MLDW conjectured that it might possibly have also caused the extirpation of an ancient clade of *Huffmanella* there as well. During this drought, the SMS, which issues from the same aquifer, also experienced reduced discharge, but because the elevation of its spring openings is about 14 m lower than that of CS, the SMS had flowed continuously throughout the drought with a minimum discharge of $\geq 1.3 \text{ m}^3 \text{ s}^{-1}$ [70], and thus spared the SMS populations of the fountain darter and *Huffmanella* from extirpation there. If we could find evidence consistent with our conjecture that *Huffmanella* actually had existed in the CS prior to the cessation of flow in 1955, this would be consistent with CS having continuously presented groundwater at the surface throughout the Holocene until over pumpage during the drought of the 50's caused the spring to stop flowing for the very first time (perhaps since its formation) and brought about the extirpation of not only the fountain darter, but perhaps also an ancient clade of *Huffmanella* and probably other unreported crenobiontics. It would also mean that the first and *only* time the CS went dry was in 1955, and that it otherwise had not stopped presenting groundwater at the surface since the Wisconsin glaciation, and probably much earlier.

Table 1. Texas springs searched for the presence of mucronate *Hyaella* spp. or *Huffmanella* spp. prior to this study.

Spring Name	References	Coords. (Deg W, Deg N)	Crenobiontic <i>Huffmanella</i> spp.	Crenobiontic <i>Hyaella</i> spp.
Barton Springs	[47]	30.263759, -97.770876	N	?
Blue Springs	[48]	29.893691, -100.994661	N	N
Caroline Spring	[48]	30.469016, -101.803561	N	N
Clear Creek Springs	[48]	30.907044, -99.960929	Y	Y
Comal Springs (CS)	[45] (fish from [5])	29.714441, -98.135296	Y ¹	N ²
Comal Springs (CS)	[47,48,74–76]	29.714441, -98.135296	N ³	N
Fessenden Springs	[47,48]	30.166927, -99.342635	N	N
Finnegan Springs	[48]	29.901371, -100.999576	N	N
Hueco Springs	[47]	29.759169, -98.140878	N	?
Las Moras Springs	[48]	29.309747, -100.420961	N	N
San Felipe Springs	[48]	29.373565, -100.885139	N	N
San Marcos Springs (SMS)	[48,75–77]	29.893931, -97.930088	Y	Y
San Saba Springs	[48]	30.825901, -100.119022	Y	Y
South Concho Springs	[49]	31.135639, -100.493499	Y	N

¹ Fish were collected in 1951 and deposited in museum; examined in 2015. ² Arrangements only allowed us to examine swim bladders. ³ Local *Huffmanella* clade apparently went extinct when flow stopped during drought of 1956–7.

To test the hypothesis, we searched museum records for centrarchid collections from anywhere in the Comal River prior to 1956 and found a collection by the Texas ichthyologist Clark Hubbs [5] in the Ichthyology Collection at the University of Texas Natural History Museum. These fish had been collected in December of 1951, well before the drought had become serious enough to stop surface flow. We acquired access to seven centrarchids in the collection. Preserved fish were temporarily removed from jars and examined on site. We excised the retia mirabilia from the swimbladders of these fish (jar catalog # TNHC2331, TNHC2332, TNHC2335, TNHC2338, and TNHC2353) which were returned to their jars less the rete. The retia were transported to our lab and examined for evidence of infection with *Huffmanella* and we found that 3 of 3 *Lepomis miniatus*, 1 of 2 *L. microlophus*, and 0 of 2 *L. macrochirus* were positive for eggs that were morphometrically indistinguishable from eggs of *H. huffmani*. Interestingly, these host-specific prevalences mimicked prevalences

we had been seeing in the SMS for decades. So, an extinct clade of a crenobiontic endemic parasite of fish from the headsprings of the Comal River had thus been discovered.

To also confidently confirm that *Huffmanella* is, indeed, still currently absent from CS, we collected 60 more centrarchids of various species from Landa Lake (which impounds Comal Springs) in 2014 and inspected them for the presence of *Huffmanella*. The effort was repeated in 2015 for a total of 120 centrarchids. We also extensively searched the literature from the years following the drought of 1956 for any suggestion that *Huffmanella* (or any nematode of centrarchid swimbladders) may have been reported from Comal Springs. The literature search and all 120 fish and were negative, corroborating that *Huffmanella* had likely been extirpated from the CS sometime between 1951 and 1978, and also suggesting that the eggs of *H. huffmani*, which had been shown to survive for years at 22 °C in sealed vials with no detectable decrease in infectivity [45], are unable to survive desiccation lasting less than 5 months in 1955–1956.

The discovery of an extinct clade of *Huffmanella* in Comal Springs seemed to be exciting circumstantial corroboration of the CIdER refugium hypothesis. However, if we could experimentally demonstrate that *Huffmanella* eggs are, indeed, sensitive to desiccation, the corroboration would be much stronger. Therefore, we sought to determine how long the eggs could survive a drying experience similar to what one would expect eggs to experience as they are slowly exposed subaerially while the wetted perimeter of the spring retreats back into the spring openings during an extended drought.

We pooled eggs from the shredded swimbladders of several freshly wild-caught centrarchids from the SMS, stirred them into a container of artesian water, and pipetted a 100 µL aliquot of the egg suspension into each of two 100 mL beakers filled with the same artesian water. One beaker was randomly assigned to the desiccation treatment and the other established as a continuously hydrated control. Both beakers were maintained uncovered in the same conditioned room at about 22 °C and exposed to evaporation, differing only in that the control beaker was periodically topped off with deionized water to maintain original volume. At the end of the first week, the treatment beaker had become completely desiccated. We continued to incubate both beakers at 22 °C until the end of the second week (the treatment beaker remained dry while water lost from the control beaker was replenished daily). We then rehydrated the treatment beaker with aquifer water and incubated both beakers until the end of the third week, providing deionized water to both as needed.

Twelve adult lab-reared amphipods (*Hyaella* cf. *azteca*) of about the same size were randomly divided into two groups of six. One group was added to the beaker of control eggs and the other six to the treatment beaker and incubated for 5 d at 22 °C (amphipods exposed to eggs become infective to fish 5 d after exposure to eggs [45]). Each experimental amphipod was then carefully examined for *Huffmanella* larvae under a dissecting scope. All amphipods exposed to non-desiccated control eggs were infected with multiple active *Huffmanella* larvae (100% prevalence), while none (0% prevalence) of the amphipods exposed to desiccated eggs were observed to contain any *Huffmanella* larvae either dead or alive (Table 2).

Table 2. Effects of desiccation on the ability of *Huffmanella huffmani* eggs to infect amphipods (*Hyaella* cf. *azteca*).

Experimental Group	Days 1–7: Incubation	Days 8–14: Treatment	Days 15–21: Incubation	Day 22: Challenge	Day 27: Evaluation
Desiccated	Evaporating w/o replenishment	Dry all week	Rehydrated and replenished	Six lab-reared amphipods added	All 6 amphipods negative for larvae
Control	Evaporating with replenishment	Continued replenishment	Continued replenishment	Six lab-reared amphipods added	Many live larvae in all 6 amphipods

This finding is consistent with our expectation that the *Huffmanella* population that was present in CS prior to 1952 had been extirpated from CS when it stopped flowing for several months during the drought of record in 1956, and that the CS and SMS had otherwise never ceased to maintain groundwater at the surface since before the Last Glacial Maximum, even during the much more severe droughts of the Holocene.

The continued discharge from CS during the subsequent drought of 2008–2014, despite a near doubling of regional population between 1960 and 2000, means that the cessation of flow in the CS in 1956 was caused by over pumpage, rather than by the drought itself. The difference was the effectiveness of the staged pumpage restrictions mandated by Texas Senate Bill 1477 to control withdrawals from the aquifer during droughts [78] as they become more severe. Had this legislation been implemented early in the 1950's, it would probably have also protected the Comal population of fountain darters that were extirpated, the now extinct *Huffmanella* sp., a hypothesized crenobiontic *Hyaella* clade similar to the SMS *Hyaella*, and probably other unknown crenobiontic endemics unique to the Comal River system.

3.1.2. More Unexpected Contributions from Texas Wild Rice (*Zizania texana*)

Another corroborating observation in support of the permanency of flow in the SMR spring run through the Holocene (relative to other springs of the Edwards Plateau) can be inferred from Texas wild rice (*Zizania texana* Hitchcock [79]), an endangered plant currently restricted to the upper 4 km of the SMR and never reported from the larger CS less than 30 km away.

Z. texana is thought to have been derived from a widespread clade of *Zizania* that had flourished during the Wisconsin glaciation when the southwestern USA was much wetter, but then retreated eastward during mid-Holocene aridity, with the isolated SMR population containing the only extant representatives [80].

We conjecture that another remnant population of *Z. texana* might have survived in the CR into perhaps the mid Holocene but was then extirpated from there by a drought that had stopped the flow of crenal water through the consequently stagnant shallows and marshes surrounding the spring openings where the plant had previously flourished when flowing. However, the fountain darter and the *Huffmanella/Hyaella*/centrarchid system had both survived these same conditions in crenal pools over the openings of CS. This conjecture is consistent with information obtained from two scientists having research experience with the plant. Hardy [81] (pers. comm.) said that, in experiments pertaining to the growth of potted *Z. texana* in varying velocities of spring water, the plants flourished in flows at characteristic spring-run velocities, but when cohorts were transferred to flow-through ponds fed with the same water but with no measurable velocity at the pot, the rice began yellowing within 1 week and then died. Heard [82] (pers. com.) also gave an account of a healthy stand of *Z. texana* observed in shallow flowing water in the SMR (at 29.888626, -97.934122). The root bed of the stand was later exposed to air during a low-flow event in 2018 which killed all the exposed plants within about 2 weeks. Additionally, Horne et al. [80] reported that both the roots and seeds of *Z. texana* are very sensitive to desiccation. Thus, it is possible that Holocene droughts had extirpated the remaining stands of *Zizania texana* from all springs lacking shallows permanently flooded with flowing crenal water for as little as 2 weeks, but had still allowed *H. huffmanii* and the fountain darter to survive in groundwater-sourced pools in both CS and SMS, with the latter being the only spring maintaining a flowing springbrook continuously through the Holocene with enough velocity to support *Z. texana*.

3.2. Q2: Can the Occurrence of Endemic *Hyaellids* Similar to the SMS *Hyaella* in Other Springs Reveal Other CIdER Refugia Harboring *Huffmanella*?

A literature search for other large, mucronate, narrowly endemic *Hyaella* similar to the SMS *Hyaella* revealed one other species in Texas (*H. texana* [50]) at Clear Creek Springs (CCS; also known as Wilkinson Springs; Table 1, Figure 1). Subsequently, Clear Creek

Springs (CCS), the type locality for the crenobiontic *Hyaella texana*, was found to be positive for *Huffmanella*, with species-specific infection patterns similar to the San Marcos River. This finding is consistent with our predictions that crenobiontic *Hyaella* in the Edwards Plateau are associated with CIDER refugia and that the endemic *Hyaella* coevolved in isolation with a local population of *Huffmanella* parasites, which is also endemic to that spring.

The CCS spring run (Clear Creek) is a short tributary (<5 km) of the San Saba River (SSR) and the headsprings of the SSR (30.825991, -100.119046) are only 23 km west and upstream of the confluence of Clear Creek with the SSR (Figure 1). Thus, centrarchids from the SSR headsprings were also checked for the presence of *Huffmanella* and found to be positive. Consistent with the hypothesis that springs bearing *Huffmanella* are former CIDER refugia, a crenobiontic population of mucronate *Hyaella* was found there as well. However, these crenobiontic amphipods are morphologically similar to, and were found to be reproductively compatible with, *H. texana* from CCS, while also being reproductively incompatible with the SMS *Hyaella* sp. from the San Marcos Springs [44].

Interestingly, the water level at Clear Creek Springs is about 25 m lower in elevation than any of the spring openings we have seen in the SSR headsprings, and so the mucronate crenobiontic *Hyaella* in the SSR headsprings is probably derived directly from *H. texana*, and the CCS probably becomes the permanent headsprings of the SSR during extended severe droughts such as those of the Holocene. Meanwhile, there are numerous submerged springs in the SSR between to two sites and plenty of opportunities for the Clear Creek *Hyaella* to exchange alleles with the headsprings clade. Thus, we consider the crenobiontic *Hyaella* from the SSR headsprings to simply represent an extension of the known range of *Hyaella texana*, and henceforth we will use that taxon to refer to the crenobiontic *Hyaella* from the SSR headsprings.

The South Concho River (SCR) headsprings (31.135647, -100.493471; also known as Anson Springs; Figure 1) just 50 air km NW of the CCS was also sampled and found to be positive for *Huffmanella* in the swimbladders of local centrarchids. However, after several thorough searches, all amphipods collected in the SCR appeared to be morphologically consistent with the widespread *Hyaella* cf. *azteca*, and we were unable to find a mucronate *Hyaella* similar to *Hyaella texana* or the SMS *Hyaella*. This observation makes the SCR and Comal Springs the only known sites for *Huffmanella* in the freshwaters of Texas where we have not also found a morphologically distinct crenobiontic *Hyaella* amphipod restricted to the same biotope.

However, since *Huffmanella huffmanii* is obligately dependent on *Hyaella* for its intermediate host, it is almost certain that there was a hyalellid amphipod in the SCR that carried *Huffmanella* through the Holocene period, but that this species had not developed reproductive incompatibility with the *Hyaella* cf. *azteca* before the latter was repatriated back into the spring. In this case, the crenobiontic form would have been introgressively swamped into extinction by the returning *H. cf. azteca*, which now serves as intermediate host for the *Huffmanella* population in the SCR.

3.3. Q3: Are the Downstream Distributional Limits of Crenobionts in the SMR Determined by Change in Some Aspect of Spring Physicochemistry?

3.3.1. Overview of Contextual Data

Studies of spring endemics from all over the world have reported gradual declines in abundance of the endemics as distance downstream in the spring run from the springhead and the crenobiontic *Hyaella/Huffmanella* host–parasite system in the SMR is no exception.

While Cox et al. [77] had previously shown that *Huffmanella* prevalence drops off precipitously at around 3 km downstream, we sought to verify that this downstream depression was still operating at about the same distance downstream, to quantify the decline in abundance, and to assess changes in various physicochemical parameters as possible causes of the decline.

We collected, by angling with artificial lures, a total of 59 fish belonging to five centrarchid species from five sampling sites along the upper 5 km of the SMR spring run. Captured fish were maintained in buckets with at least 10 L of aerated artesian water at 22 °C until euthanized according to IACUC-approved protocols the same day.

Retia mirabilia were excised from the swimbladder of euthanized fish and examined with a dissecting microscope for evidence of *Huffmanella* eggs. Since Cox et al. [77] had determined that an infected fish might contain upwards of millions of the tiny eggs of *H. huffmanii*, we used an egg-density rating protocol adapted from Worsham et al. [45] to serve as a proxy for the traditional parasite intensity as a measure of parasite load. The procedure per fish was to examine five non-overlapping fields of the rete that contained some of the densest deposits of eggs in the rete and rate each field according to the subjective ordinal scale in Table 3. The arithmetic mean of the five field ratings was then used to represent the load of that fish as its mean egg-density rating. We then averaged the egg-density ratings for all infected fish from a collection site to represent the mean egg-density rating (MnEggDensRat) for that site. Note that Worsham et al. [45] had used the term “intensity” rating, but we later determined that “egg-density” rating is probably more appropriate [49,83] since we were not actually counting eggs. Additionally, while egg number would accurately represent the history of *Huffmanella* infection of a fish, it would not necessarily represent the number of worms present at necropsy (intensity), which is usually zero.

Table 3. Protocol and criteria used to determine egg-density ratings for *Huffmanella* infection levels in centrarchid swimbladders.

Egg-Density Rating	Rating Criteria for Each Field
5	Transmitted light entirely blocked by multiple layers of eggs
4	More than 50% of field blocked by eggs
3	Between 25% and 50% of field blocked by eggs
2	Up to 24% of field blocked by eggs
1	Only a few spots of eggs observed in field
0	No eggs detected in field

Prevalence at a site was determined by dividing the number of fish whose swim bladder contained at least one egg by the total number of fish examined for infection at that site.

There was a decline in both prevalence and mean egg-density ratings when values were plotted on a map showing collection stations (Figure 3).

The prevalence of *Huffmanella huffmanii* infection in centrarchids wild-caught from various stations at or near the headsprings of the San Marcos River was consistently near 100% (Figure 3), but declined at sampled stations increasingly farther downstream from the headsprings and diminished to zero just beyond the 4.3 km station. The mean egg-density ratings for *Huffmanella*-infected fish at those stations tracked almost exactly in the same trend as the prevalence, with both suddenly dropping precipitously at about 3 km (Figure 4A). Indeed, over the distance downstream where *Huffmanella* declined to absent, the reach between 2.7 and 4.3 km revealed the sharpest declines relative to maximum values, with an 81% decrease in MnEggDensRat and a 59% decrease in prevalence (Figure 4a).

Since we suspect the SMS *Hyaella*, also crenobiontic, of being the ancestral intermediate host that carried *Huffmanella huffmanii* through the Holocene droughts, we also expected the SMS *Hyaella* to show a decline in abundance relative to the widespread *H. cf. azteca* downstream. We sampled habitats in eight near-shore low-flow stations varying from about 0.5–2.8 km downstream from the headsprings. Consistency among habitats sampled was maintained by finding beds of *Ceratophyllum* sp. and gently lifting single sprigs from the river at each station and swirling them around in a small bucket of locally obtained amphipod-free river water. This procedure was repeated at each station until about 50–75 amphipods had been captured. We temporally stored the amphipods from each

station in separate labelled jars for transportation back to the lab. We estimated relative abundance by sorting the amphipods from each station into the two pans representing the species of *Hyaella* that occur in the river (SMS *Hyaella* and *H. cf. azteca*), calculated the percent of total hyalellids that we had identified as SMS *Hyaella*, and then plotted the percentages against river kilometer at each collection site.

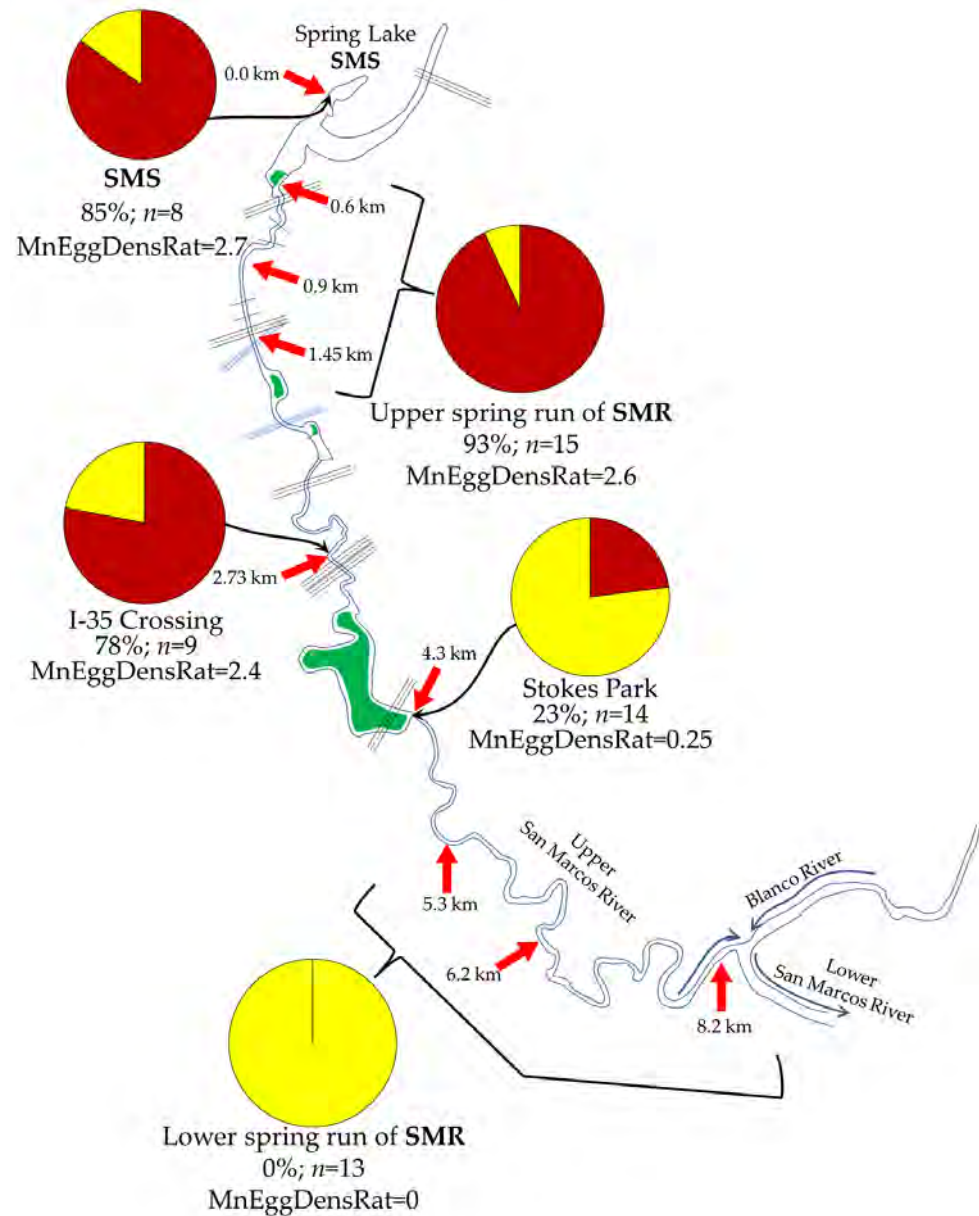


Figure 3. Map of San Marcos River, Hays County, TX, showing decline in *Huffmaniella* abundance at progressively more downstream stations as measured by prevalence and mean egg-density ratings in wild-caught fish (red pie slices = %fish positive; yellow slices = %fish negative for *H. huffmani*). Distances are approximate thalweg kilometers down-stream from headsprings (29.894038, -97.930154) based on a path traced in Google Earth Pro (map data ©12/21/2018 Google). *n* refers to number of fish sampled from each location.

Downstream Correlation of Parasite Abundance and Amphipod Mix with Temperature Variation in San Marcos River

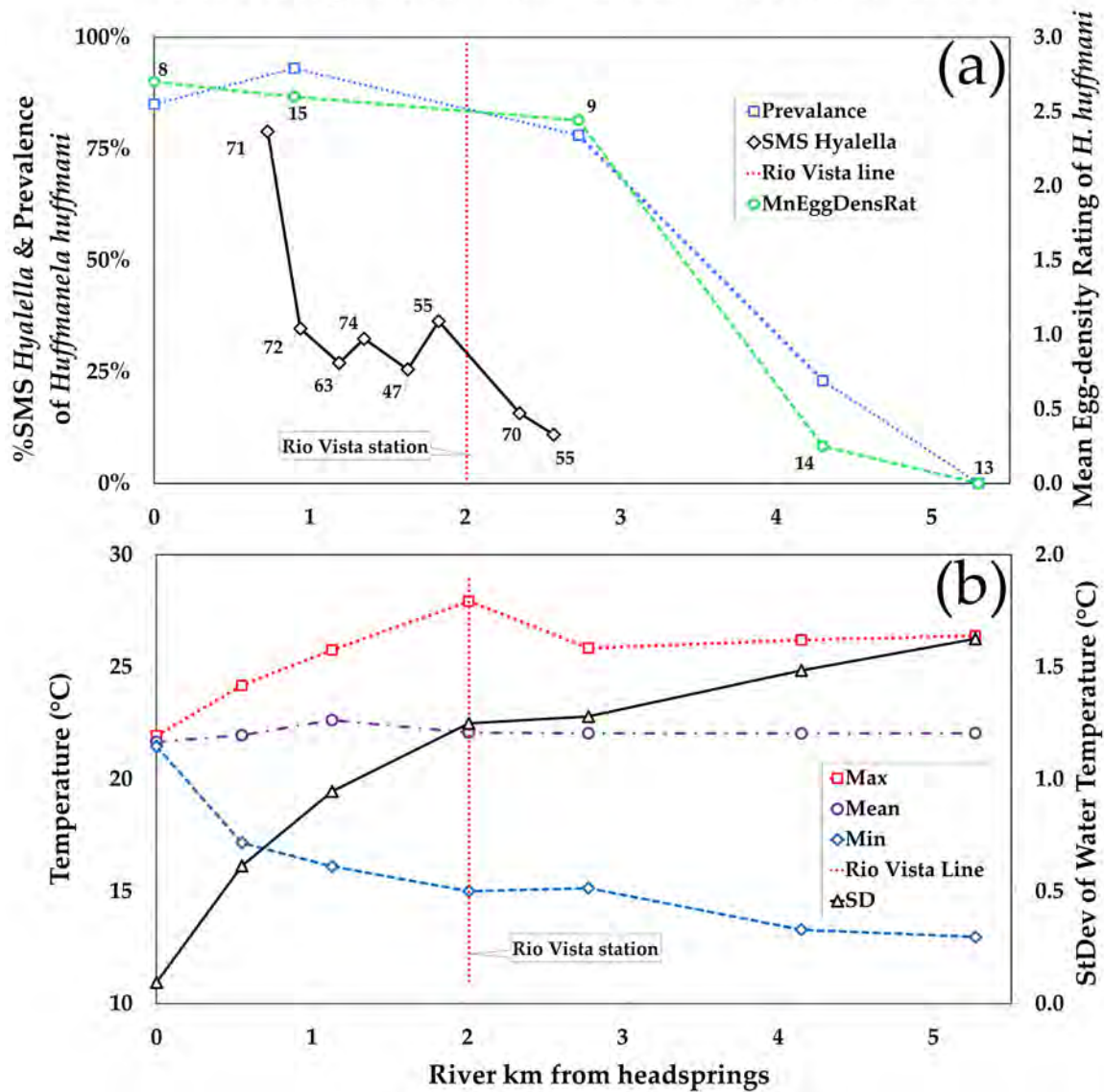


Figure 4. (a) Variation in prevalence and mean egg-density ratings for *Huffmanella huffmanii* in wild-caught centrarchids, and %relative abundance of amphipods sampled that were SMS *Hyalella*—all plotted against river kilometers down-stream from the headsprings of the San Marcos River; data labels are sample sizes. (b) Variation in mean temperature and standard deviation of temperature at various stations downstream from headsprings along with 14 y minima and maxima. All temperature data were recorded at 4 h intervals during years 2000–2014, which included years of severe drought followed by recovery.

Note in Figure 4a that the proportion of the amphipods that were identified as the SMS *Hyalella* at various stations downstream showed an even more pronounced decline downstream than did the prevalence of *H. huffmanii*. This does not necessarily mean that the SMS *Hyalella* is more sensitive to physicochemical instability than *H. huffmanii*, because the farther downstream distribution of *Huffmanella*-infected fish could also be explained by the relatively greater mobility and a much longer longitudinal home range than individual SMS *Hyalella*. A freshly infected fish host will be prepatent for nearly a year after its

initial infection [45], and during that prepatent period, its infection cannot be detected by the gross inspection method we used. During the prepatent period, an infected fish would probably wander up and down the stream some distance from the site of initial infection before the fish (if caught) would be classified as infected. Nonetheless, the downstream decline in *H. huffmanii* parasitism combined with the downstream decline in the relative abundance of the SMS *Hyaella*, the intermediate host thought to have carried the parasite's life cycle through the Holocene droughts, adds circumstantial corroboration to our hypothesis that *Huffmanella huffmanii* and the SMS *Hyaella* are coevolved endemics of the same CIDER refugium.

A collateral conclusion that can be drawn from these findings is that the centrarchid fishes in the study must have a surprisingly small home range over a period of several years. Indeed, given that (1) there were several flood events before and during the sampling period, (2) any prior *Huffmanella* infection remains grossly obvious in a fish for the rest of its life, and (3) the minimum time between a fish becoming infected and developing obvious signs of infection is 1 y, it then follows that any individual infected fish in the study had not moved up or down the stream more than a hundred meters or so in their entire life. Thus, *Huffmanella* could be used as permanent tags to indicate that a fish had at least once in its life been exposed to at least one *Huffmanella*-infected amphipod. Furthermore, since the eggs of many species of *Huffmanella* in a fish host have been shown by many authors to go through a predictable series of easily categorized changes as they age year after year, one could also determine approximately how long the fish had spent in a reach where it was exposed to infected amphipods.

One other noteworthy finding relative to the CIDER hypothesis is the uptick in the otherwise downstream decline in relative abundance of the SMS *Hyaella* at about river kilometer 1.8 (Figure 4a). At first we thought it was just sampling variability, but we noted that the same site where the uptick occurred was the *only* site sampled where we also collected any amphipods in the genus *Crangonyx*, which is hyporheic, but does not survive for long in surface water. *Crangonyx* amphipods are often collected near spring openings in Spring Lake, the impoundment over the SMS, but note that none were collected among the 452 amphipods collected at the other seven stations along the spring run, and yet 4 *Crangonyx* were collected among the 59 amphipods collected at this one station. Thus, the site at 1.8 km is probably receiving a local influx of cool hyporheic water from a seep from the rarely flowing Purgatory Creek that occasionally ejects *Crangonyx* amphipods, and that is corroborated by a leveling of the otherwise downstream-increasing slope in the standard deviation of temperature for a short reach starting at about km 2.0 (where the thalweg of Purgatory Creek joins the SMR). Indeed, the temperature spread over 14 y (Max-Min) upstream of the site is 12.9, 10.7 at the first station downstream from the site, and back up to 12.9 at the second site farther downstream. It is also very unlikely that *Crangonyx* would escape predation for more than a day after emerging into surface water, so there must be substantial hyporheic discharge from the Purgatory Creek channel to allow four to have been collected there in one grab sample. The uptick in SMS *Hyaella* there adds additional credibility to our conjecture that the general downstream decline in relative abundance of SMS *Hyaella* (Figure 4a) is due to some declining physicochemical attribute of spring water; but the question remains, "which attribute(s)?"

3.3.2. Downstream Increase in Temperature Variance Is Inversely (But Tightly) Correlated with *Huffmanella huffmanii* Abundance Measures in Wild-Caught SMR Fish

We reviewed previous studies of patterns of downstream change in various physicochemical parameters in the SMR and found almost all of them were stable at the springhead but showed progressively increased diel, seasonal and precipitation-related variability with distance downstream [84–86]. However, water temperature, among all these parameters, seemed to exhibit the highest degree of contrast between low variability at the springhead relative to variability downstream, and therefore seems the most likely candidate to be the limiting factor preventing the crenobionts from surviving more than a few

kilometers downstream. Changes in magnitudes of seasonal and diel variations in water temperature at various stations in the SMR over 14 y are demonstrated in Figure 5.

Diel and Seasonal Variation in Water Temperature of San Marcos River Every 4 h

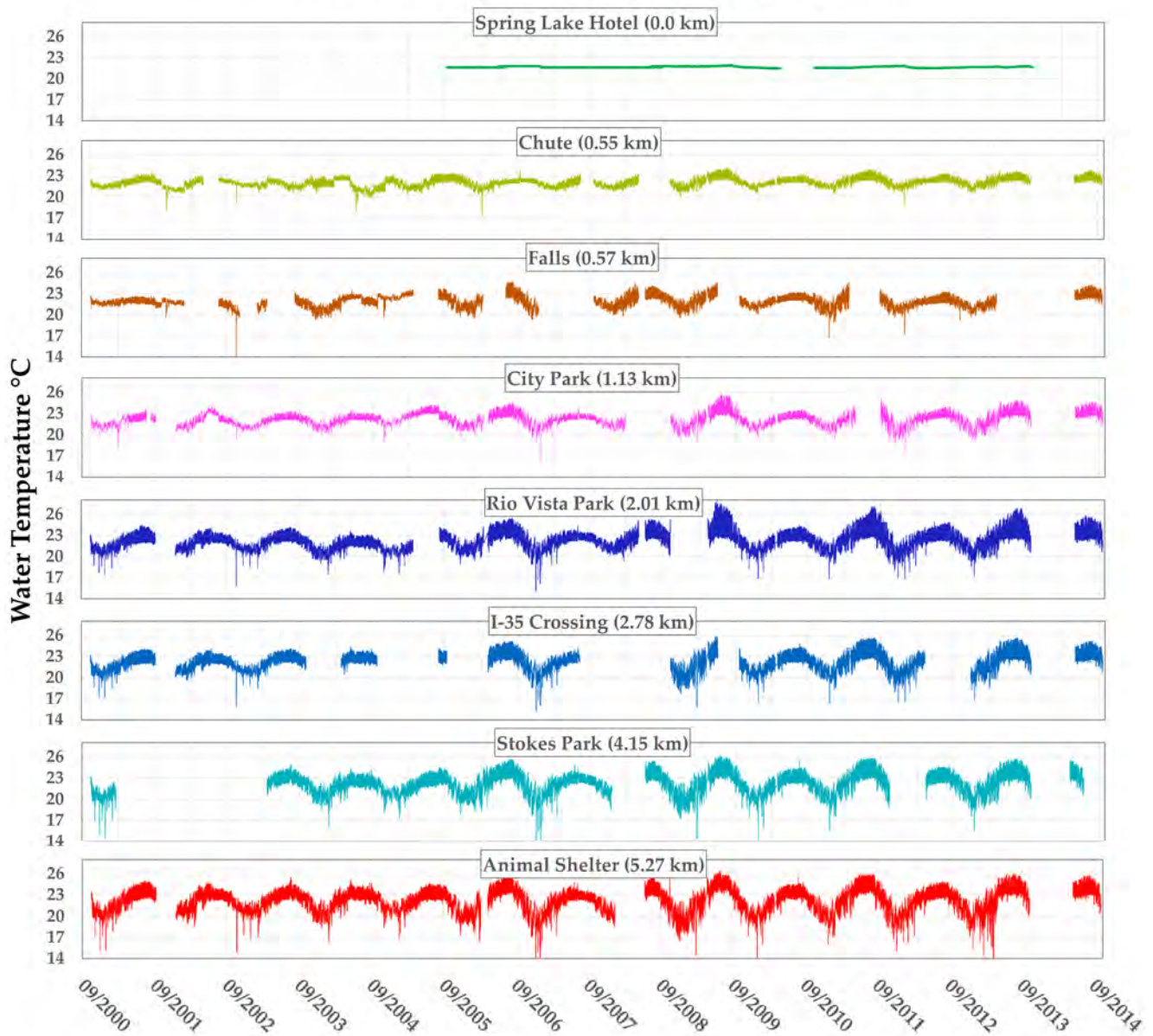


Figure 5. Seasonal and diel variability of water temperature over 14 years recorded at various stations downstream from headspring (graciously shared by BIO-WEST, Inc.; gaps are memory-full errors, losses due to spates, or vandalism; transients are due to cold fronts and winter spates from flashy tributaries).

The discharge of the eucrenal zone of San Marcos Springs is currently almost entirely from groundwater; therefore, the resulting diel and seasonal variability of water temperature near the headsprings is biologically trivial (Figure 5); indeed, out of all the 15,252 temperature records at the spring head between 2006 and 2013, the difference between very highest temperature and very lowest temperature was $21.94 - 21.46 = 0.48$ °C. It seems reasonable to assume that variation over a typical year during the geologic time frame under consideration would be similarly trivial, and that the invertebrate clades evolving

in this headspring over many thousands of generations would not have been made sensitive to such minute temperature swings by their loss of thermal-shock recovery systems through genetic drift. One complication to this portrayal of springhead tranquility is that the current locality of the main springs supplying the SMS discharge can only be traced back by 14 ky of sediments, and prior to that, the spring head may have been at a site about 1.2 km NE (29.902652, -97.922809) on the other side of Sink Creek. However, the entire Sink Creek valley across the springs from the escarpment to about 0.5 km southeast was scoured to bedrock until deposition was re-started about 13 kya [87], so the record is uncertain as to when the SMS discharge first started at the current location.

While the water at the headsprings of SMS was found to be physicochemically stable year-round, the diel and seasonal variability increased, as expected, downstream. Interestingly, despite the local weather inducing major temperature swings and transients in downstream water, the annual means of water temperature at these stations varied only slightly from 22 °C at progressively more downstream stations, with the maximum difference between any two 14 y station means being 1 °C. However, the variance of the temperatures recorded at the station 2 km downstream during the same time interval (1.5595 C²) was 175 times larger than the variance at the headsprings (0.0089 C²) even though the annual means at the two stations differed by only 0.4 °C.

Downstream trends in prevalence and the mean egg-density ratings of *H. huffmanii* at (Figure 4a) are almost exact inverses of the trend in temperature variation (as standard deviation) at nearby stations (Figure 4b). Note in Figure 5 that the diel variation in temperature (thickness of the trace) increases consistently with downstream distance until at about 2 km where it more-or-less stops increasing. The amplitude of seasonal variation also more or less stabilizes at near 2 km. Then, notice in Figure 4a that both prevalence and egg density also drop precipitously just downstream from that location.

This observation suggests that increasing amplitude of variations in water temperature (or something else covarying closely with it) at progressively more downstream stations is reducing the likelihood that centrarchids captured at those downstream stations will be infected.

We statistically compared the mean egg-density ratings and prevalences from five sampling sites at varying distances downstream (from Figure 3) to the combined seasonal and diel variation in temperature at seven nearby monitoring stations. Variation in temperature was expressed as the standard deviation of temperatures in degrees Celsius. Spearman's Rho correlation procedure [88] was used to determine the likelihood that the apparent relationship between the prevalence of *Huffmanella* and the standard deviation of temperature could be explained by random variation, and the correlation was very highly significant with $p(r_s = |-1|) \rightarrow 0$.

3.3.3. Downstream Depression of *Huffmanella* Abundance Probably Caused by Progressively Increasing Thermal Instability: Experimental Corroboration

Even though we detected significant correlations between declining downstream *H. huffmanii* abundance measures and increasing downstream variation in temperature, such a correlation would not provide direct evidence of a cause-effect temperature-sensitivity mechanism, since there are many other water-quality factors in the SMR that also change predictably within the first few kilometers of a spring run [84,86]. Variation in water temperature can only be concluded to be a factor contributing to the endemism of *H. huffmanii* if we can experimentally demonstrate some sort of sensitivity to naturally occurring off-mean water temperatures similar to those that occur near where the abundance of *Huffmanella* infections begins to decline (3–4 km; Figure 4a). Thus, we designed an experiment that might demonstrate temperature sensitivity in a parasitologically meaningful way.

We examined temperature data at a station just upstream from where the abundance of the parasite declined sharply and determined that the minimum and maximum water temperatures there that were sustained for at least 4 h at any time in the 14 y period of mon-

itoring were approximately 15 °C and 28 °C. The monitoring period included the second most severe drought of record followed by return average temperature.

We designed an elaborate experiment to evaluate our hypothesis that the distribution of *H. huffmanii* is primarily limited by temperature extremes. The experiment was designed to assess varying subsets of the temperature transients observed at the chosen site for varying durations to determine, by titration, the temperatures at which the eggs (1) began to be affected and (2) at which they were all killed. We predicted that temperature swings to one extreme or another for several hours will depress the ability of *H. huffmanii* eggs to infect the intermediate host, *Hyaella* cf. *azteca*.

When we were in the final stages of implementing the experiment, our university announced an impending lockout of all labs due to COVID-19, which severely compromised how much of the original design we could complete in the two weeks remaining before the lockout. However, we decided to go forth with a simplified experiment, so that there might be some level of experimental findings pertaining to our hypothesized temperature sensitivity, albeit with threats to validity brought on by the compromises. The design of the simplified experiment is diagrammed in Figure 6.

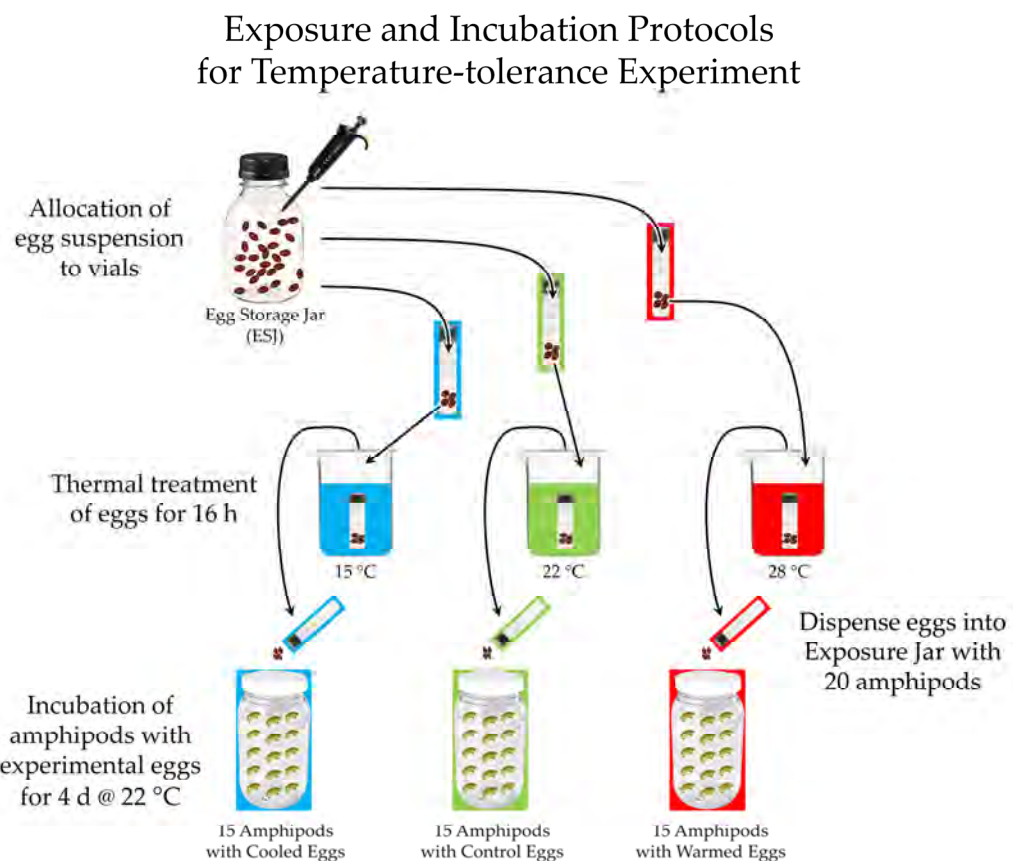


Figure 6. Design of experiment to determine if temperature extremes recorded at the site where *Huffmanella huffmanii* prevalence declined at the greatest rate can help explain the limited downstream distribution of the parasite in the San Marcos River.

The source of *Huffmanella* eggs for the experiment was a batch that had been pooled from several freshly caught SMR centrarchids and that had been validated for infectivity to amphipods. We gently stirred the eggs to suspend them and pipetted about equal aliquots (based on titrations by [45]) into each of three vials. We randomly assigned each vial into one of three experimental baths (15, 22, and 28 °C).

Then, we added to each bath a HOBO U-2 v2 thermal data logger programmed to record bath temperature every 60 s. At about 14:15 on 18 March 2020, we began ramping down the temperature of the 15-C bath over a period of 3.7 h to a mean treatment temper-

ature of 14.6 (14.5–15.4) °C and held for 16 h before ramping the temperature back up to 22 °C. During the same period, we ramped up the temperature of the 18-C bath over a period of 2.5 h to a mean treatment temperature of 27.6 (27.4–28.0) °C, which was held for 16 h before ramping back down to 22 °C. Meanwhile, the 22 °C bath was held at a mean of 21.8 (21.7–21.9) °C (close to mean annual river temperature) for the same length of time. The temperature ramps were not smooth (because of the impending lockout, we resorted to controlling temperature in the cold-treatment bath by adding ice chips to the outer bath and tracking cooling progress real-time with a mercury thermometer) and the overall rates of change in temperature ramps between start and end of a ramp was between 1.8 and 2.4 °C/h (Figure 7).

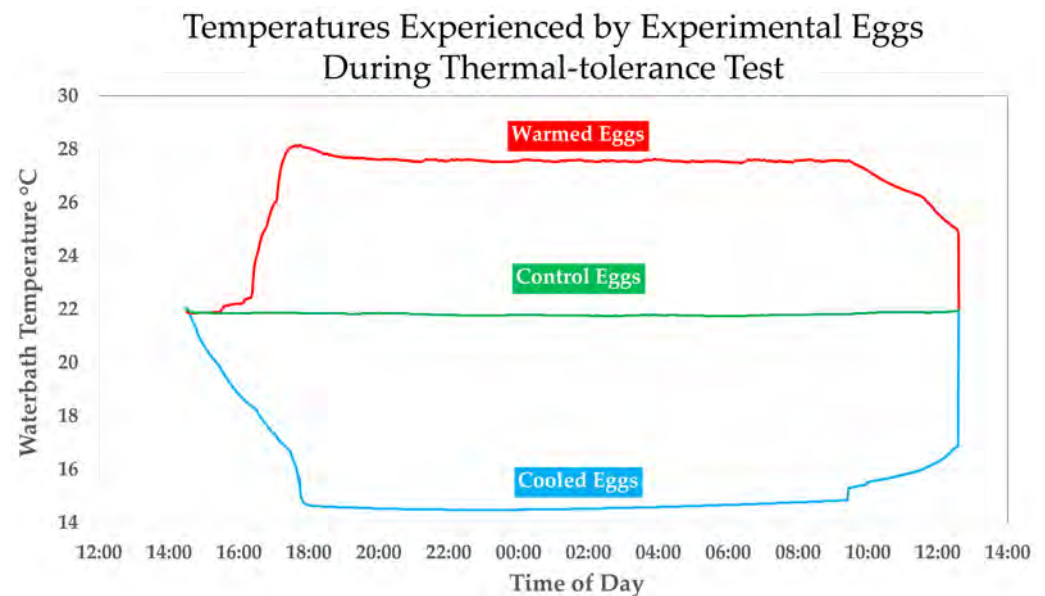


Figure 7. Record of the water temperatures experienced by the three experimental groups of *Huffmanella* eggs prior to feeding eggs to experimental amphipods.

The ramp rate we employed is 10 to 20 times faster than the fastest rate we observed in naturally occurring thermal transients at the site and in experiments involving animals adapted to environments within ambient temperature swings; such ramp rates would be a severe threat to validity because they would swamp the heat-shock response system. However, many stenothermal animals have lost an inducible heat-shock response [89], and extended ramp times (more gradual slope) would probably not have resulted in reduced effects; indeed, other investigators have reported that extending the ramp time to targeted hold temperature (slowing the rate of change) while maintaining the same exposure duration at the targeted temperature often increases the number of stenothermal subjects responding negatively to the targeted temperature because the extended ramps also extend the exposure of the subjects to off-mean temperature (see [90,91]). In other words, if we had ramped temperatures more slowly, we would likely have seen stronger effects of reduced infectivity, even at less extreme targeted temperatures.

Prior to the end of the thermal treatment of the egg vials, we placed 15 lab-reared amphipods (*Hyalella* cf. *azteca*) into each of four 1-L jars, three of which we had labelled to match the three experimental groups, and the fourth was to serve as a Death-rate Control (in case exposed amphipods started dying before they were ready for dissection, we could determine if the deaths were caused by over-dosing the treated amphipods). Immediately after the thermal treatment of experimental eggs concluded, we transferred the eggs from the vials in each experimental group to their corresponding amphipod exposure jars. After the amphipods had been exposed to the eggs for 4 d, we rinsed the amphipods and incubated them for an additional 24 h to allow any eggs consumed on the fourth day of exposure to migrate to the hemocoel prior to dissection. We then coded the amphipod

source containers, and had previously trained workers dissect all the amphipods live over a period of 6 h, with workers dissecting an amphipod from each of the four containers before repeating. Immediately after counting worm larvae in the experimental amphipods, we were locked out of the lab for several months.

The number of larval worms recovered from each amphipod was then used as a data point for that group and the three groups of data were analyzed with a one-way ANOVA with Tukey's HSD test for multiple-comparisons. Our expectation was that the amphipods in either (or both) of the off-mean-temperature groups would show significantly diminished larval counts relative to those in the control group (22 °C).

Both treatment groups (15 °C and 28 °C) showed a reduced mean infectivity of eggs relative to that of the 22-C control group (Figure 8). A one-way ANOVA of the three groups was highly significant [$p(F_{0.05(1),2,39} \geq 9.50) < 0.001$]. A Tukey's HSD test for multiple comparisons was then applied, and infectivity of eggs for both the 15-C and 28-C treatment groups were significantly lower than that of the 22-C control group [$p(q_{0.05,3,39} = 3.44) < 0.01$] for both groups. However, the difference in infectivity effects of the 15 °C and 28 °C groups did not differ significantly from each other, indicating that both summer and winter temperature extremes may be operating together to restrict downstream colonization of the spring run. None of the amphipods in the Death-rate Control jar died before the experimental amphipods were dissected, suggesting that all amphipods that died in the exposed groups died of infection-related causes.

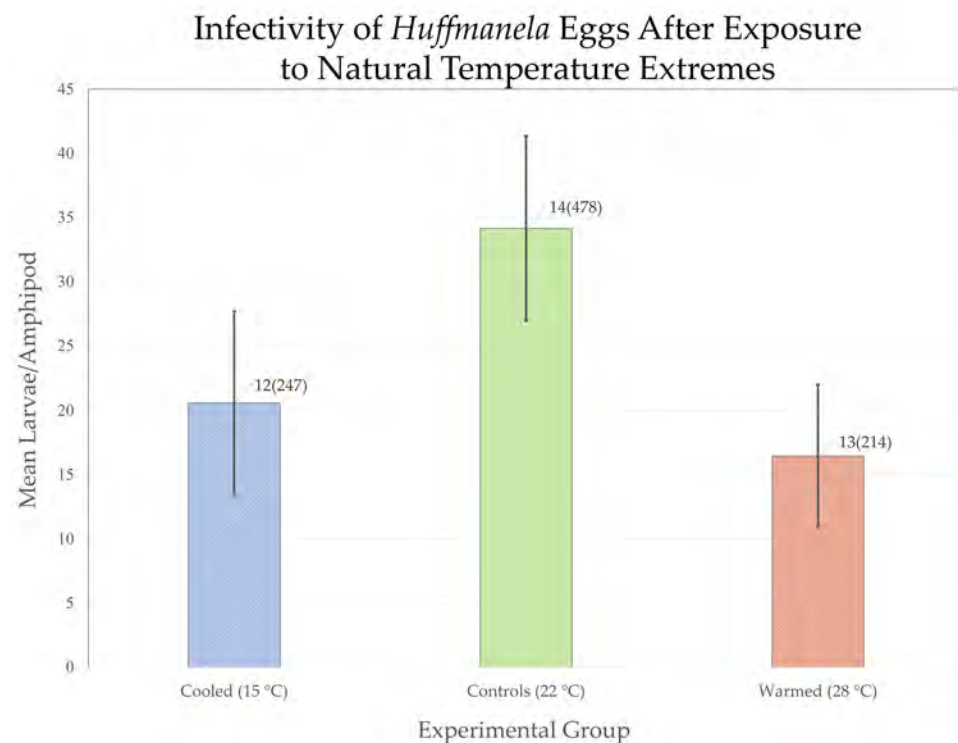


Figure 8. Effects of natural temperature extremes (15 °C and 28 °C) for 16 h on the ability of thermally treated *Huffmanella* eggs to establish infections in exposed amphipods relative to controls (22 °C); value labels are: # surviving amphipods (recovered larvae); error bars are 95% confidence intervals.

3.4. Q4: Are There Corroborating Data Consistent with the Two Cenobiontic Host/Parasite Systems Having Been Separated for Thousands of Amphipod Generations?

3.4.1. River Flow Patterns

The South Concho and the San Saba Rivers both flow east for about 150–250 river kilometers where they are confluent with the much larger Colorado River, which drains the eastern and northern margins of the Edwards Plateau (Figure 1). In contrast, the Comal and San Marcos rivers are confluent with the Guadalupe River, which drains much of the southern edge of the Plateau. Therefore, the two *Huffmanella* populations in CCS and the

SCR are not nearly as isolated from each other as they both are from the populations on the south side of the Edwards Plateau (SMS *H. huffmaniana* and the now extinct CS *Huffmaniana*). Likewise for *Hyaella texana* of the CCS vs. the SMS *Hyaella*. Indeed, ever since probably well before the Pleistocene, the San Saba and South Concho river populations have been separated from the two southern populations (San Marcos and Comal rivers) by what currently amounts to over 1000 river kilometers and 100 km of saltwater bays, and by 230 air km with no known intervening *Huffmaniana* or crenobiontic *Hyaella* populations (Figure 1).

3.4.2. COI Genetics of Crenobiontic Hyaellids

It is also worth noting that the CCS *Hyaella texana* is an ancient clade according to COI phylogeny, and much older than the more recent SMS *Hyaella* [44]. The *Hyaella* cf. *azteca* of the SMR is phylogenetically intermediate between the two crenobiontic forms.

Regarding the absence of a crenobiontic *Hyaella* in Comal Springs, it would be interesting to return to the museum collection of [5] and check out those centrarchids in which the now extinct Comal Springs clade of *Huffmaniana* was discovered, and look to see if their stomachs contain evidence of a mucronate, crenobiontic *Hyaella* that went extinct along with its *Huffmaniana* parasite during the drought of 1956.

3.4.3. Reproductive Isolation of the Crenobiontic Hyaellids

Assuming that the crenobiontic *Hyaella* population in the Clear Creek Springs and its *Huffmaniana* parasite have been genetically isolated for thousands of amphipod generations from their respective counterparts in the San Marcos Springs, one would also assume that some prezygotic or postzygotic reproductive isolation to have occurred. It turns out that [44] had already demonstrated that size-matched adults of these two crenobiontic amphipod species (*Hyaella texana* from CCS and the SMS *Hyaella*) occasionally entered amplexus in symmetrical cross experiments, but never produced first generation offspring (F₁) after 8 weeks together (neither $\sigma_{\text{SMS}} \times \text{CCS}$ nor $\sigma_{\text{CCS}} \times \text{SMS}$). Likewise with symmetrical crosses of either crenobiont with the *Hyaella* cf. *azteca* from the San Marcos River. However, conspecifics of all three clades readily mated and produced F₁ in as little as 2 weeks under identical conditions.

3.5. Q5: Are There Corroborating Data Consistent with the Two Crenobiontic Hyaellids Having Coevolved with Their Local Huffmaniana Parasites Prior to the Repatriation of Hyaella cf. azteca to Both Springs?

There are two aspects of this question that we were able to test: (i) an experimental comparison of the two crenobiontic *Hyaella/Huffmaniana* host/parasite systems with each other, and (ii) a retrospective comparison of one crenobiontic *Hyaella/Huffmaniana* system with its sympatric repatriated *Hyaella/Huffmaniana* system in the same spring.

3.5.1. The Two Crenobiontic Host/Parasite Systems: Exposing Both Crenobiontic Hyaellids to Heavy Doses of Local vs. Exotic Huffmaniana Eggs

While it would be useful to determine if adult *Huffmaniana* worms of the SMS and CCS clades could cross successfully, such an experiment would require either isolating rare adult worms of separate sexes of both species in rete tissue culture, or learning how to reliably sex viable eggs without harming them, and would take years to complete, if even feasible. However, if the two *Huffmaniana* clades in CCS and SMS have diverged genetically (as have their amphipod hosts; Section 3.4.3), then it would seem that an attempt to cross-infect the CCS hyaellid with *Huffmaniana* eggs harvested from the SMS centrarchids (and the reciprocal cross infection), the cross-infections should exhibit detectable differences in infectivity or some other manifestation of host reaction compared to infections of each crenobiontic hyaellid with eggs from its home spring. Worsham et al. [45] had already demonstrated that eggs from the CCS and SMS *Huffmaniana* clades would infect the crenobiontic amphipod from either spring, but what we needed to show was some kind of differences in the host/parasite dynamics that would be consistent with the two

host/parasite systems having evolved separately over thousands of generations in separate CIdER refugia.

We hypothesized that in order for such host parasites systems to survive through many boom-and-bust cycles in such a tiny, isolated biotope, the host amphipod clade in each system must have evolved some way of managing unusually high challenges with *Huffmanella* eggs. For instance, if a disturbance kills a substantial number of infected fish hosts in a short period, the amphipods would soon be challenged with ingestion of potentially orders of magnitude more *Huffmanella* eggs than they normally had to deal with, unless they had developed an immunological defense against such occasional excesses of eggs. We anticipated that the amphipod clade that had coevolved in a spring with the local *Huffmanella* clade would be more resistant to being swamped by eggs of that *Huffmanella* clade than from an equal dose of eggs from an exotic *Huffmanella* clade.

With these factors in mind, we designed an experiment to determine if the crenobiontic hyalellids (CCS & SMS) would show detectable resistance to a near lethal challenge with eggs of the local *Huffmanella* with which they presumably coevolved, but would be relatively immunologically naïve to an approximately equal challenge with eggs from the exotic *Huffmanella* clade from the other spring. Fortunately, in the life-cycle work of [45], an approximate lethal level of *Huffmanella huffmanii* eggs in the SMS *Hyaella* had already been titrated, and we decided to use that approximate abundance of eggs in the experiment.

A 2 × 2 experiment was set up consisting of four identical containers of amphipods: two containers with 20 each lab-reared crenobiontic CCS *Hyaella*, and the other two containers with 20 each lab-reared crenobiontic SMS *Hyaella*. Two containers of *Huffmanella* eggs were prepared: one harvested from fish naturally infected with the CCS *Huffmanella* clade and one harvested from fish naturally infected with the SMS *Huffmanella* clade. The CCS eggs were suspended by stirring and an aliquot of the suspension then split about equally, with half delivered to a container with 20 CCS amphipods and the other half to a container with 20 SMS amphipods. The procedure was then repeated with the other two containers of amphipods but with the SMS eggs (Table 4). It was not critical that the dose of CCS *Huffmanella* eggs be equal to the dose of SMS *Huffmanella* eggs, as long as both doses killed some amphipods and allowed others to survive. However, the dose of one egg source must be very close to the other dose from the same source, and great care was taken to split each source equally between the two recipient amphipod containers.

Table 4. Setup of differential mortality experiment to test for evidence of long-term genetic isolation and coevolution of the SMS and CCS *Hyaella*/*Huffmanella* host/parasite systems. Values represent number of *Hyaella* amphipods from the side-indicated spring being challenged with a heavy dose of *Huffmanella* eggs from the top-indicated spring.

		<i>Huffmanella</i> Egg Clade	
		CCS	SMS
Crenobiontic <i>Hyaella</i> clade	CCS	20	20
	SMS	20	20

The four beakers of exposed amphipods were monitored over a 2-week exposure period to determine the mortality rates of amphipods in the four cells of the experiment. Significantly higher mortality rates in the two cross infections (CCS/SMS and SMS/CCS) would be interpreted as consistent with our assertion that the two host/parasite systems had diverged genetically over many generations of isolation in CIdER refugia.

The mortality rates of the two crenobiontic amphipods, when over-exposed to *Huffmanella* eggs from both springs, were almost exactly opposite (Figure 9). Significantly more native *Hyaella* from each spring survived exposure to eggs of local *Huffmanella* than to about equal doses of eggs of exotic *Huffmanella*. A chi-square contingency test with Yates correction for continuity (Table 5) returned a very highly significant result. These results show

that both crenobiontic *Hyaella* clades are capable of withstanding massive exposure to eggs of the *Huffmanella* species with which they presumably coevolved, but not as well with eggs of the *Huffmanella* species to which they are naïve.

Cross-infection Experiment (CCS × SMS)

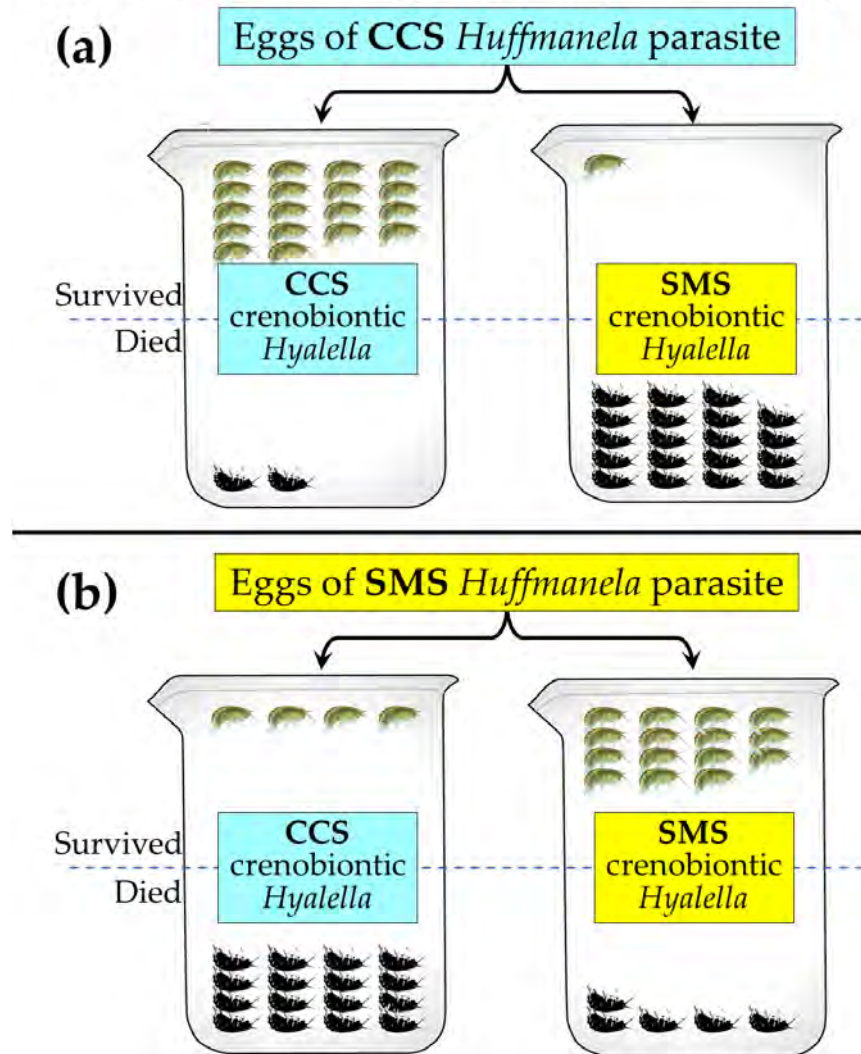


Figure 9. Results of a 2 × 2 cross-infection experiment to determine differential mortality rates of the two crenobiontic *Hyaella* clades when over exposed to eggs of *Huffmanella* clades from the local vs. the exotic springs. Panel (a) is exposure of *Hyaella* to CCS *Huffmanella* while panel (b) is exposure to SMS *Huffmanella*.

Table 5. Chi-square contingency analysis of the data from the differential survivability experiment depicted in Figure 9.

		# Died (of 20) <i>Huffmanella</i> Egg Source		Null Expectation <i>Huffmanella</i> Egg Source		Test Statistics	
<i>Hyaella</i> Source	CCS	CCS	SMS	CCS	SMS	$\Phi =$ χ^2 (Yates) = df = $p <$	-0.75 18.24 1 0.0001
	SMS	18	4	11	11		
	CCS	1	15	8	8		
	SMS	19	19	38			

3.5.2. Comparison of One Crenobiontic Amphipod/*Huffmanella* System with the Local Repatriated Amphipod/*Huffmanella* System: Differences in Chronic Reactions to the Parasite

This test was not a dedicated experiment but consisted of reviewing the records from many unpublished exposures of amphipods to *Huffmanella* eggs during the [45] life cycle experiments. Most of that data were derived from exposures of either of the two San Marcos Springs amphipods (SMS *Hyaella* or *Hyaella* cf. *azteca*) to eggs of *H. huffmanii* from the SMS, and we focus our analysis thereon.

The review revealed that anytime individual SMS *Hyaella* had been exposed to eggs from local SMS *Huffmanella*, the amphipods rather quickly, starting at about 2 w post-exposure, had immobilized, or killed many of the *Huffmanella* larvae from the experimental exposure to local eggs. In contrast, when individuals from the other local amphipod clade (*Hyaella* cf. *azteca*) had been exposed to the same local eggs of SMS *Huffmanella*, the larvae resulting from the exposures had survived for over 6 w in the *Hyaella* cf. *azteca*. A related finding from the review was that when either of the mucronate crenobiontic hyalellids from either the CCS or SMS had survived experimental exposure to *Huffmanella* eggs from their local springs, subsequent observations of the surviving amphipods had revealed that the larval *Huffmanella* from local eggs had become calcified and yellowed after 2–3 w. In contrast, not a single *Hyaella* cf. *azteca* that had been exposed to *Huffmanella* eggs was observed with yellowed larvae, even >6 w post-exposure. These findings from the review (summarized in Table 6) are consistent with our hypothesis that the SMS *Hyaella* and its *Huffmanella* parasite had coevolved in isolation for probably thousands of amphipod generations in the absence of *Hyaella* cf. *azteca*, the latter having recolonized the San Marcos Springs since the Holocene droughts.

Table 6. Differential reaction of the two SMS amphipod species to infection with eggs of local *Huffmanella*.

	SMS <i>H. huffmanii</i> Larvae in Presumed-Naïve <i>Hyaella</i> cf. <i>azteca</i>	SMS <i>H. huffmanii</i> Larvae in Presumed Co-Evolved SMS <i>Hyaella</i> sp.
Larval viability	>6 w	~2 w
Evidence of rejection	none	Immobility → calcification

Another observation related to this test is the change in relative proportions of the two sympatric *Hyaella* clades at progressively more downstream stations from the SMS springhead (Figure 4a). This progressive downstream replacement of the crenobiontic amphipod, which had presumably survived the Holocene droughts in the spring, by the widespread amphipod, which had presumably reinvaded the springs after the droughts, is exactly what one would expect, and such patterns have been observed with other clades that were presumably brought back into contact after the Holocene droughts [8]. In such cases, the crenobionts would be so perfectly specialized for competition in the crenobiontic biocenosis of the springhead that the repatriated congener could not eliminate them there, but the crenobiont would lose this competitive advantage as it encountered more and more species downstream that have evolved to be competitive under more widely varying conditions and also cyclical variations in community structure on a seasonal basis.

4. Conclusions

4.1. Paleoecological Inference

How aquatic taxa evolve to become crenobiontic has yet to be directly tested but we hypothesize that, because Edwards Aquifer-fed springs are well known to experience little to no daily and seasonal variation in physicochemical conditions, the evolution of crenobionts is the result of extended periods of evolution in extremely stable spring refugia. Paleoclimatological evidence suggests that two Holocene thermal maxima occurred prior to the present warming trend and these thermal maxima coincided with severe droughts.

The finding that the drought of the present warming trend extirpated spring endemic fountain darters and *Huffmanella* from Comal Springs in the 1950s [45,72] clearly demonstrates that drought conditions have the capacity to sculpt the distribution of crenobiontic taxa. It should be noted that during the drought of the 1950s Comal Springs ceased flowing in fall of 1955 and early winter of 1956 but still had small patches of standing surface water that probably tracked ambient temperatures. Thermal tolerance studies suggest that ambient temperature swings are not survivable by *Huffmanella* eggs (Figure 8) which reinforces the notion that drought and thermal variability have sculpted the distribution of crenobionts; this finding is reinforced by the correlation between the contemporary biogeography of *Huffmanella* and temperature in the San Marcos River. The finding of a second endemic *Huffmanella* population in centrarchids from a spring it shares with the only other known crenobiontic *Hyaella* in Texas is consistent with our conjecture that the CIdER refugium hypothesis is a reasonable explanation for the present distribution of many endemic crenobiont clades in Central Texas; this finding is reinforced by evidence of coevolution between co-occurring *Huffmanella* and *Hyaella*. With this in mind, this study suggests that parasites with at least two distinct obligate hosts are far more useful for palaeoecological inference than most free-living taxa because the contemporary distribution of such a parasite indicates that the environmental and ecological conditions necessary to support all host species, as well as meeting the environmental and ecological requirements of the parasite itself, were met without interruption since the arrival of the parasite to that geographic region. The coupled coevolution of parasites and their obligate hosts provides rich insight into palaeoecological conditions; especially aquatic parasites with multiple hosts required to complete their life cycle. To our knowledge, this is only the third study to use biogeographic data on parasites with multiple hosts to inform palaeoecological reconstructions [67,68] and we suggest applying this strategy can prove insightful in other model systems.

4.2. A Proposed New Subcategory for Evolutionary Refugia

The term “Crenal Interglacial-drought Evolutionary Refugia” (CIdER) is proposed as a label under which research data pertinent to this special category of refugia can be kept separate from data pertaining to terrestrial glacial and interglacial evolutionary refugia, the latter two being driven by completely different forces, even while developing in the same region at the same time. Extant CIdER are characterized by the following generalized historical narrative:

- The starting point is a diverse community of obligately aquatic plants and mostly invertebrate animals that are widely distributed among interconnected streams during a period of high regional precipitation associated with extensive glaciation just poleward of the region.
- The community becomes challenged by region-wide gradually reduced precipitation as the glacial margins retreat poleward.
- Evapotranspiration begins to exceed precipitation and many streams and springs that were formerly perennial become progressively more intermittent, and the distribution of the obligate aquatic community becomes progressively more fragmented.
- Many smaller springs stop flowing and lotic habitats become restricted to isolated flows fed by a few perennially rheocrenic springs, and perennial high-order rivers draining remote regions.
- The largest rheocrenic springs have spring runs extending from the springhead but disappear into ground and air, never connecting to other surface waters; others shrink back to limnocrenes in which the water remains thermally constant a meter or so deep where there is movement of groundwater.
- This condition is maintained for thousands of generations of the obligate aquatic invertebrates, which are now members of an isolated biocenosis continually bathed in a thermally constant environment with a stable bottom undisturbed by spates.
- The surviving clades eventually lose (to drift or reassignment) genetic loci for heat shock proteins that once allowed the organisms to adapt to varying temperatures, but

also become extremely efficient competitors under these rigidly constrained physico-chemical conditions.

- The drought breaks, and dependable precipitation in excess of evapotranspiration returns to the region.
- Spring-streams lengthen, eventually forming permanent connections with surface-fed streams, but crenobionts that survived through the drought in the springs can no longer survive through the temperature swings of progressively more ambient waters downstream.
- Sister clades that are relatives of the crenobionts, but which had retreated to other regions with dependable precipitation during the drought, now return and intermingle with the crenobionts.
- If the crenobionts are now reproductively isolated from the repatriated sister clades, a longitudinal density gradient is established, with the repatriated sister clades diminishing in density toward the spring head, and the crenobiontic clades diminishing downstream with physicochemical instability.
- The obligate aquatic community at the springhead now consists of a highly diverse mixture of surviving, but still endemic crenobionts and repatriated sister clades.

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Appendix A. Review of Terminology Related to Refugia, Relicts, and Endemics

This section addresses a confusing array of ambiguous terms that we had no intention of dealing with, but which will continue to unnecessarily encumber the communication and integration of related findings unless some attempt is made to clear the terminological fog.

Appendix A.1. Terms Related to Endemics

Much of the recent attention to the concept of endemism has been from the perspective of how best to focus limited mitigation resources for maximum conservation impact. Conservationists have developed algorithms that can purportedly identify sensitive geo-

graphic hotspots of endemism that are contributing substantially to global diversity and then triage them for prioritization in preparation for anticipated rapid climate change. The CANAPE method for the Categorical Analysis of Neo- and Paleo-endemism is claimed by Mishler et al. [92] to allow, “... for the first time, a clear, quantitative distinction between centres of neo- and paleo-endemism ...” Given the urgency of biodiversity conservation concerns and the need to prioritize regions of endemism for the establishment of reserves, this appears to be a reasonable way to go about parsing the growing body of ecological and taxonomic data available, and then prioritizing resource allocation. However, some authorities have concluded that the protocol for classifying regions by these terms (neo-vs. paleo-endemism) introduces investigator bias into the decision-making process [56] because the investigator must impose artificial boundaries on the limits of time and space under consideration. Another complication is that the clades we are studying would transition from one category to the other over the time interval we are considering for our narrative. Fattorini [93] provides a detailed history of the endemism concept and some of the problems associated with the varied uses of paleo- and neoendemism while Myers et al. [56] proposed a detailed classification of different applications of endemism. However, we finally chose to avoid the use of these modifiers and will simply refer to our clades as endemics sensu Darwin’s glossary entry [94], “peculiar to a given locality,” with the qualifier “peculiar” distinguishing our use of the term from the way it is sometimes used to imply “indigenous to” or “native to.” Thus, we will avoid the prefixes “Neo” and “Paleo-.” Furthermore, we will only characterize an ecosystem as being the habitat of an endemic if it is the smallest geographic area to which at least two endemic clades are restricted, following criteria established by Platnick et al. [95] and Harold et al. [57].

Appendix A.2. Terms Related to Relicts

Some of the endemic clades in our study have been loosely referred to as relicts; but, unfortunately, the jaded application history, and the almost sensational intrigue sometimes associated with the term “relict,” have reduced the utility of the term to that of a colloquialism. Grandcolas et al. [96] attempted to break out the functionally different historical applications of the term and apply unique names to them, but did not provide for a term that would apply to a geographically restricted population of “left-behind” survivors of a formerly widespread but climatically displaced and still extant sister group which is later repatriated; a situation we appear to be dealing with in our study (along with many other researchers dealing with glacial “relicts”). Since almost any definition of any kind of relict will technically require that the relict population represents the sole survivors of a formerly widespread (but now extinct) source population, we have avoided using the term “relict” for the endemic clades we are studying.

Appendix A.3. Terms Related to “Refugia”

Appendix A.3.1. Misapplications of “Refug-”

Frequencies of words derived from ‘refuge’ and ‘refugium’ have seen dramatic increases in life-science literature in recent years. Keppel et al. [97] searched for literature containing references to keywords ‘refugia,’ ‘refugium,’ and ‘refuge’ in the title, abstract, or keywords and published between 1991 and 2010. They noted that the number of papers using the terms derived from “refuge” increased by about 5-fold in that time interval, but papers using latinized terms derived from “refugium” increased by about 13-fold. Unfortunately, these terms have been increasingly applied indiscriminately to a variety of scenarios having presumptive origins that are functionally unrelated [53,54,98,99]. Indeed, we know of cases where the term “refugia” has been conflated with many other terms, including refuges, remnants, and reserves [97]; captive or artificial cultures maintained by humans [100]; an anthelmintic treatment strategy wherein a portion of stock in a pasture had been treated with anthelmintics and then all stock rotated out of that pasture (which becomes “the refugia” pasture) [101]; diurnal retreats for nocturnal species (and v.v.); and is sometimes even used as the plural form of refuge, i.e., using “refugia” where one would

expect to see “refuges” [102]. Clearly, a literature search for findings pertaining to a specific functional type of refugium will inevitably return hits bloated with ambiguity.

Appendix A.3.2. Problems with “Evolutionary Refugia”

The modifier “evolutionary” has been applied to refugia since 1970 and perhaps before. Davis et al. [60] attempted to refine the application of the term in arid regions as, “permanent, groundwater-dependent habitats (subterranean aquifers and springs) supporting vicariant relicts and short-range endemics.” However, they unnecessarily (in our opinion) included the term “vicariant” in the definition, thus technically excluding its application to climate-change endemics, and ignored the applications of the same term to terrestrial habitats. Davis et al. [60] also defined the minimum tenure of the refugia in chronological units (millennia) rather than potential number of life-cycles completed during the tenure, despite the fact that a given chronological time span will impose profoundly different effects on clades that typically produce four generations per year vs. those that require a minimum of 4 years per generation. Despite these reservations, we consider the modifier “evolutionary” to be useful (less the constraints referenced above) in that it implies an isolation experience sufficient to have an evolutionarily persistent effect on the clade(s) of interest.

Appendix A.3.3. “Refugia” with “Glacial” Modifiers

The term refugia has often been used with Pleistocene modifiers in the past several decades to refer specifically to “glacial refugia,” in which the distribution of a formerly widespread temperate clade is subsequently compressed, by the colder temperatures of an approaching glaciation, into geographically much more limited patches or margins where conditions are milder, and from which the species ultimately disperses to widespread occurrence again following glacial retreat [54]. Later, the inverse term “interglacial refugia” was used to refer to limited areas into which the distribution of a once widespread boreal species that thrives in low temperature conditions (e.g., *Dryas integrifolia*) is subsequently compressed into north-facing alpine patches or retreating glacial margins by increasingly warmer interglacial conditions, only to return to abundance during the next glacial advance [103]. However, both refugia types are driven by changing thermal patterns, and the terms, as they have been defined, should not be used to refer to glacial or interglacial refugia driven by regional-scale variation in other factors such as meteoric moisture, even though the factors usually covary with temperature. Indeed, conservation plans designed for a refugium formed by changing thermal regimes require dramatically different approaches compared to management plans for refugia formed by changing precipitation patterns. Thus, the term “interglacial refugium” alone does not provide sufficient specificity to distinguish between scenarios representing dramatically different functional models of refugia.

Appendix A.3.4. Recent Attempts to Rein in Refugial Misuses

Recent papers have attempted, with marginal success, to rein in how these terms are used by defining boundaries for them (e.g., “What do we mean by ‘refugia?’” [54]. Ashcroft [53], in an extensive review of the application and misapplication of terms derived from refugium, indicated that they are “used without distinguishing between macrorefugia and microrefugia, ex situ refugia and in situ refugia, glacial and interglacial refugia or refugia based on habitat stability and refugia based on climatic stability.” This is a sad state of affairs for a discipline determined to sort out, clarify, and prioritize the issues related to diversity conservation. Ashcroft’s [53] recommendation to rectify the situation was, “More care needs to be taken to properly define the context when referring to refugia . . . so that the validity of methods and the conservation significance of refugia can be assessed.”

Appendix A.3.5. The Need New Refugial Categories

There are functionally different subcategories of refugia that have been identified by researchers but which are not adequately distinguished by any of the frequently used terms. For instance, glacial refugia and interglacial refugia are both cyclical in nature, with a few major and many minor cycles involving the mass displacement of entire communities, often leaving behind remnants in small climatological alcoves. This is followed by the return of the main sister population which then either swamps the left-behind endemics, or sympatrically contends with them, (these outcomes varying from one refugium or cycle to another). Yet, there is no specific term that we can find to represent the cyclically precipitation-restricted endemics in our study and their kilo-generational crenal (freshwater spring) refugia. Indeed, most related terms we can find refer to refuges that either originated from one-time dispersal driven processes or as one-time vicariance-driven processes. Furthermore, these terms have been hijacked by workers studying the cyclical glacial/interglacial processes in which cyclical trends in either precipitation or temperature (or both) are the primary displacing stimuli. Some investigators have attempted to mitigate this problem by declaring that climate change is a form of vicariance, but that just muddies the literary water, because landform vicariance by tectonic disjunction is usually on a very long time scale and cannot be merged with climate-induced cyclical displacement/return scenarios that involve very different implications for population dynamics, perturbation resilience, and habitat management plans.

Appendix A.4. Consequences of Terminological Ambiguity and Recommended Solutions

Continued ambiguity in the use of these short-term safe-haven and long-term refugial terminologies complicates literature searches and confounds attempts to advance understanding of the evolutionary pressures experienced by occupants of functionally distinct types of refugia. This, in turn, complicates ongoing attempts to prioritize application of limited resources set aside for conservation of biodiversity and can result in application of ineffective or even counter-productive management strategies.

We consider terminological ambiguity to be a substantial cause of the confusion that has historically confounded attempts to develop effective management strategies for protecting the biodiversity of widely varying types of refugia, especially those associated with delicate spring ecosystems. This is a functional, rather than merely a semantic ivory-tower problem, because properly established and well-thought-out categorical terms can serve as virtual repositories into which pertinent descriptive and cause-effect information can be accumulated and efficiently retrieved with less confusion and conflation.

Future work in the arena of evolutionary refugia should be required to either (1) use an established and unambiguous compound term with a representative abbreviation and use explicit language to describe the reasons why the term applies to the situation, or else (2) develop a new compound term with a recommended abbreviation and describe in explicit language how subsequent workers can know when they are dealing with a case that comes under the new refugial type, and also describe how the refugial type being named differs from established unambiguous refugial terms in use.

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