Does position along the watershed affect hybridization dynamics between the native *Orconectes sanbornii* and invasive *O. rusticus*?

Elisa C. Henderson

Oberlin College Department of Biology

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Abstract

Introduction of nonnative species into new ranges can have broad implications for local biodiversity. The rusty crayfish (*Orconectes rusticus*), has been recorded to have expanded outside of its endemic range of Southwest Ohio and Kentucky, displacing a variety of native crayfish. *Orconectes rusticus'* range expansion is likely due to human use as live bait, resulting in its introduction into the native range of the Sanborn's crayfish (*O. sanbornii*). We are investigating the morphological and genetic impacts of invasion and possible hybridization in two invaded watersheds in north-central Ohio. Crayfish were collected from multiple sites along two invaded rivers. In both rivers, the ratio of invasive to native individuals varies with position in the watershed, with a higher proportion of invaders downstream than upstream. Here, we ask whether the genetic composition of populations sampled along the watershed agrees with the morphological pattern. Here, we present morphological data on species distribution along sympatric rivers. A closer look at the distribution of genetic diversity along the length of the watershed may provide insight into the consequences of the invasion for both invader and native.

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Introduction

Species invasions can have broad ecological and evolutionary implications for biodiversity within the invaded region (Allendorf & Lundquist 2003, Vitousek *et al.* 1997.) Introduced species may have the ability to outcompete native species through greater ability to acquire resources, high genetic variation conducive to rapid evolution, or the lack of stresses that may be alleviated in the non-endemic habitat (Vitousek *et al.* 1997, Lee *et al.* 2002). Crayfish constitute a high amount of biodiversity in freshwater streams, with over 390 species native to North America alone (Hobbs 1989). Many of these species inhabit disjoint, restricted ranges, so may be prone to respond negatively to the presence of invasive species (Lodge *et al.* 1998, Hill

& Lodge 1999). *Orconectes rusticus* (the Rusty crayfish) has a high propensity for successful invasion, having large body size, large chelae relative to body size, and high growth rate (Butler & Stein 1985). Additionally, Sargent and Lodge (2014) found evidence that invasive Rusty crayfish have evolved faster growth rates and greater survival than the same species in its native range. These characteristics have allowed *O. rusticus* to expand from its native range of Indiana, western Ohio, and northern Kentucky to displace other crayfish species located north, east, and west of the Rusty's endemic range (Butler & Stein 1985). The Rusty crayfish has also been found to hybridize upon invasion with some native congeners, such as *O. propinquus* (Perry *et al.* 2001), and *O. sanbornii* (Zuber *et al.* 2012).

Factors such as displacement, reduction in prey, hybridization, and landscape effects can change the dynamics and impacts of crayfish invasions (Vitousek *et al.* 2997, Lee *et al.* 2002). Especially interesting are the evolutionary impacts of crayfish invasions that involve hybridization between a native and an invasive species. When hybridization and invasion occur together, additional considerations must be made to understand the process. Hybridization can have variable effects on an invasion depending on the amount of hybridization that is occurring and the fitness of the hybrid offspring (Ellstrand & Schierenbeck 2000, Perry *et al*. 2001). Our categorization of hybrid individuals will change our perception of the invasion. On the one hand, the genetics of the native species may be at least partially maintained within the hybrids in the face of invasion, and thus the native species in some respect is able to resist the effects of invasion. On the other hand, hybridization to a great enough extent yields a cohort of hybrid individuals that are distinct from the native parental species. In this way, the invasive species is able to wipe out the resident population without exhibiting total displacement or out-competition. The notion that hybrids are separate entities from either parent species can potentially cause

extensive conservation management problems in the case of endangered species (Wayne & Shaffer 2016).

Landscape heterogeneity can also cause the dynamics of an invasion to change, especially when hybridization between the resident and the introduced species is possible. Combining information on landscape characteristics, such as flow or connectivity of a river, with population genetics is a field of study known as landscape genetics. This relatively new approach can be especially informative in invasion situations for predicting colonization, migration, and introgression processes that result from the introduction of a non-native species (Paulson & Martin 2014). Here, we address an invasion between the native Sanborn's crayfish (*Orconectes sanbornii*) and the invasive Rusty crayfish (*O. rusticus*) in north-central Ohio watersheds. In this system, the flow of the rivers presents a heterogeneous environment, along which we may find different dynamics of the Rusty invasion. We compare morphological and genetic characteristics of sympatric populations (along rivers that have been invaded by the Rusty crayfish) with characteristics in allopatric populations that only contain one of the two species in its native range. We studied two sympatric rivers, the Huron River and the Kokosing River, and we compared downstream populations with upstream populations within each of these rivers to identify patterns of the invasion that result from landscape heterogeneity. Hybridization has been found previously in the Huron River (Zuber *et al.* 2012), so we were also interested in whether this has a strong effect on the invasion dynamics along these two rivers. Based on previous unpublished data (Roles Lab), we expected to find a higher abundance of Rusty crayfish in downstream populations and a higher abundance of Sanborn's crayfish in upstream populations, when identifying by morphospecies. We also expected that genetic data would agree with morphological findings, but may uncover that genetic characteristics of sympatric populations

are somewhat intermediate between what we would expect for an allopatric *O. sanbornii* populations and an allopatric *O. rusticus* population.

Materials and Methods

Sample Collection

Crayfish were collected in summer 2016 from five sympatric sites along the Huron River and six sympatric sites along the Kokosing River (Figs. 1-2, Table 1). Allopatric native *O. rusticus* were sampled from one site on Stillwater Creek and one site on Bokes Creek. Allopatric non-invaded *O. sanbornii* were sampled from one site along the Vermilion River, one on the Mohican River, and one on Salt Creek (Fig. 1, Table 1). From each site, we sampled 14 individuals (7 males and 7 females when possible). Crayfish were caught with hand nets and located by eye and by disturbing rocks. Species were identified by eye in the field based on morphology. We transported crayfish back to the lab on ice and stored them at -20 degrees C before removing abdominal tissue for DNA. We uniquely marked each individual within a population using binary coded tail clips (clipping a triangle out of a designated sequence of uropods). After tissue removal, we stored whole crayfish in 95% ethanol.

Morphology

In the field, we distinguished morphospecies using several characteristics: (1) Rusty crayfish have lateral red spots on the carapace that are not present in Sanborn's crayfish; (2) male gonopod shape and size are species-specific for both form 1 and form 2 males; and (3) the female's annulus ventralis (reproductive organ) has a distinct pocket shape for Rusty crayfish, and a shape appearing as a zigzag line for Sanborn's crayfish (Zuber *et al.* 2012). We examined

morphological variation for collected specimens through measuring a standard set of traits (Fig. 3) using digital calipers: areola length, areola width, carapace width, acumen length, rostrum length, dactyl length, chela length, palm width, and palm length. Male reproductive morphology is commonly used in species identification, thus for males we measured total length of the gonopod, length of the mesial projection and length of the central projection (Fig. 3). Because there is high variation in overall size in crayfish and many of these traits covary with size, we analyzed many of these measurements scaled to carapace length or chela length, as appropriate. We also counted number of carpus spines and, for females, we categorized the annulus ventralis shape as either "pocket" (Rusty-like) or "zigzag" (Sanborn-like).

DNA Isolation

Abdominal tissue samples were removed from each individual, placed in 300 µl Cell Lysis Buffer, homogenized with a microfuge pestle, and stored at -20ºC. We isolated all DNA samples using the Qiagen Puregene DNA Purification Kit (Qiagen, Germantown, MD). Proteins were broken down by adding 1.5 µl of Proteinase K, inverting 25 times, and incubating at 55 ºC for 60 minutes. Proteins were precipitated with two rounds of the following: 100 µl of Puregene Protein Precipitation Solution was added, the sample was vortexed at high speed for 20 seconds, and then centrifuged to form a protein pellet. The supernatant was poured into new tube and the protein pellet was discarded. DNA was precipitated by adding 300 µl 100% Isopropanol and centrifuging to form DNA pellet. Supernatant from this step was discarded and 300 µl 70% ethanol was used to wash the DNA pellet, and the sample was centrifuged again. The ethanol was discarded and tubes with DNA pellets were inverted to dry for 60 minutes to overnight. DNA was rehydrated with 50 µl of Puregene DNA Hydration Solution by disturbing the pellet

by pipetting, and incubating at 65 ° C for 60 minutes. Purification quality was verified by gel electrophoresis for each sample and concentrations were checked using a Qubit. The first round of DNA isolation yielded 34% (75/223) of our samples with high quality DNA (high molecular-weight band on gel and concentration measured above 16.7ng/uL).

A substantial portion of our original tissue samples yielded low quality (low molecular weight and low concentration) DNA as well as RNA contamination as seen by gel electrophoresis. Thus, we removed fresh abdominal tissues from the 147 poor-quality samples and added the following steps and modification to the procedure. Abdominal tissue was crushed in liquid nitrogen with a mortar and pestle before being placed in 300µL Cell Lysis Buffer. Only one Proteinase K step was completed, and the incubation step was extended to 1.5 to 3 hours at 55 ºC. 1.5µL To remove RNA contamination, RNase A was added and tubes were incubated at 37 ºC for 30-60 minutes prior to protein precipitation. To improve yield, 0.5uL glycogen solution was also added along with the isopropanol at the DNA precipitation step. Ethanol was made fresh before each use in the wash step and tubes were not left to dry for longer than 1.5 hours. In this second round of DNA isolation with the modified procedure, 95% (139/147) of the samples resulted in high-quality DNA. Complete isolation protocols can be found in the appendix. A total of 214 samples were sent to the McDaniel Lab at the University of Florida for library construction and RAD-sequencing.

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RESULTS

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We have sent samples for DNA analysis to the McDaniel Lab, but we have not obtained results yet, so genetic data will not be presented here.

The shape of the female's annulus ventralis was clearly bimodal, exhibiting either a pocket shape or a zigzag shape, with one exception: one individual from the sympatric

population at Lovers Lane Bridge (LLB) along the Huron River contains the features characteristic of both Rusty and Sanborn's crayfish: the pocket shape and the zigzag pattern respectively (Fig. 5). Aside from that individual, each female crayfish was confidently identified to morphospecies based on annulus ventralis shape.

When comparing among allopatric Sanborn sites, allopatric Rusty sites, Huron River populations, and Kokosing River populations, we found that allopatric Rusty populations had overall larger body size than native Sanborn populations (carapace length and width; Fig. 6). In addition, Rusty crayfish had relatively longer dactyls and a higher ratio of the difference between central and mesial projection length to total gonopod length in comparison to Sanborn populations (Figs. 7-8). Central projection to total gonopod length ratio, and the difference between central and mesial projection length had the greatest difference between Rusty and Sanborn populations, with sympatric rivers falling intermediate to the two species in allopatry (Fig. 8). Allopatric Rusty populations had slightly higher values than allopatric Sanborn populations for areola to carapace ratio, areola width, and dactyl length to chela length ratio (Figs. 6-7). Both sympatric rivers had intermediate measurements for dactyl length to palm length ratio, palm length to chela length ratio, central projection to total gonopod length ratio, the difference between central and mesial projection length, and the ratio of this difference to the total gonopod length (Figs. 7-8).

Sympatric Sites: Huron River

Based on morphospecies, we found a much higher abundance of *O. sanbornii* in upstream populations than in downstream populations (Fig. 4a). Only the Sanborn's crayfish was detected in the three populations located furthest upstream (HUX, TMR, and HHT). We only

found *O. rusticus* in HRM, the second most downstream population, and only three Sanborn's crayfish were caught, out of 14 individuals, in the most downstream population, LLB. Looking at morphological measurements, the two sites located furthest downstream have much higher values for areola length to carapace length ratio, dactyl length to chela length ratio, central gonopod projection length to total gonopod length ratio, the difference between central gonopod projection and mesial gonopod projection, and the ratio of this difference to the total gonopod length (Figs. 9-11). HRM, the second most downstream population (located upstream only to LLB) had the highest values for carapace length and width, areola width, and dactyl length to palm length ratio (Figs. 9-10). Both of the furthest downstream populations (LLB and HRM) had the lowest values for palm length to chela length ratio (Fig. 10). For many, but not all traits, these two downstream populations were noticeably different from the three upstream populations, with measurements more similar to those expected for purely Rusty populations.

Sympatric Sites: Kokosing River

Based on morphospecies, we found a higher abundance of *O. sanbornii* further upstream than downstream, but there was a more rapid increase of *O. rusticus* as we sampled downstream (Fig. 4b). The most upstream population (KOB) was the only population where no Rusty crayfish were sampled. The next population along the river (KOK) contained approximately half *O. rusticus* and half *O. sanbornii*. In the next three populations along the river (KOC, KOK, and KDN) only *O. rusticus* was found. For the most downstream population (KGL), about half *O. rusticus* and half *O. sanbornii* were sampled. Looking at morphological measurements, we found that the two most downstream populations showed somewhat intermediate values between what would be expected for purely Rusty and purely Sanborn populations for values relating to

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gonopod length (central gonopod projection to total gonopod length and the ratio of the difference between central and mesial gonopod projection length to total gonopod length). Aside from those two populations, we found that traits relating to gonopod morphology increased in size as the populations were found further downstream up until KOC population. (Figs. 12-14).

Discussion

We found that the Rusty crayfish occurs in greater abundance at downstream locations along sympatric rivers (Fig. 4). This result agrees with sampling from 2015 along the Kokosing and the Huron Rivers (Roles Lab unpublished data). This finding in two separate rivers, presumably resulting from separate introduction events, suggests a mechanism by which Rusty crayfish outperform the Sanborn's crayfish in certain sites along a heterogeneous river environment. Perhaps the Rusty crayfish cannot easily migrate upstream after an introduction event, causing them to remain in downstream locations and perform better in these areas than the native Sanborn's crayfish, while allowing the Sanborn's crayfish to persist in upstream sites. However, given that *O. rusticus* was likely introduced many times through the use as live bait by humans, it is difficult to determine the specific introduction points for the species. Additionally, we did find individuals that were identified as *O. sanbornii* by morphospecies at the most downstream site in both sympatric rivers (three individuals on the Huron, and five on the Kokosing). This result was unexpected, given the data from 2015 sampling at these and other downstream sites, in which only Rusty crayfish were found. If there are unsampled locations downstream of our study area that still contain populations of the Sanborn's crayfish, our unexpected result could be caused by *O. sanbornii* moving upstream from unsampled downstream populations, and ending up in the sites that are downstream relative to the rest of our

study area. Alternatively, hybridization could be causing some individuals to exhibit phenotypes of *O. sanbornii* in these downstream sites. Analysis of incoming genetic data should help us distinguish between these possible explanations.

Morphological measurements that were taken for each individual were used to attempt to key out individuals to species. However, several traits were highly variable within and among populations and some ratios of measurements, such as central gonopod projection length/total gonopod length and mesial gonopod projection length/central gonopod projection length were so close to the specific cutoffs of the key that the species could not be confidently identified using the key. We found it especially difficult to key out individuals from sympatric sites, suggesting that, though overall phenotype appears to identify to one or the other species, measurements and ratios of trait lengths may be intermediate between the two species. This leads us to speculate that genetic data will uncover evidence of hybridization in both rivers, which could be causing individuals in these sites to have intermediate traits. Additionally, we expect to find upstream populations in both sympatric rivers to have genetic composition that more closely resembles allopatric *O. sanbornii* populations, while downstream populations should more closely resemble the genetic composition of allopatric *O. rusticus* populations. However, given that sympatric populations were especially difficult to key out to species, we expect all sympatric populations to have somewhat intermediate genetic composition between the two species.

In conclusion, we found that the *O. rusticus* invasion into native *O. sanbornii* range is characterized by the ability for the congeners to hybridize, and by landscape heterogeneity that modifies the progress of the invasion. We found that populations upstream of sites that contain the *O. rusticus* morphospecies contain *O. sanbornii* individuals that have an intermediate phenotype in body, chela, and gonopod measurements. This suggests that though Rusty crayfish

may not be migrating upstream, their genetic composition is traveling to upstream Sanborn populations through hybridization and introgression. These results bring into light a new type of invasion scenario that is caused by the synergistic effects of landscape heterogeneity and hybridization. It is important to recognize this potential effect in cases of introduced species, as at first glance, the severity of this and other invasions could be severely underestimated.

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Code	Name	Watershed	Site type	Latitude	Longitude
BOK	Bokes Creek	Bokes Creek	Allopatric O.rusticus	40.361992	-83.373389
CRM	Mohican River	Muskingum River	Allopatric O.sanbornii	40.747816	-82.636881
HHT	Huron River	Huron River	Sympatric	41.179121	-82.700602
HRM	Huron River	Huron River	Sympatric	41.241843	-82.692867
HUX	Huron River	Huron River	Sympatric	40.993113	-82.661327
KDN	Kokosing River	Kokosing River	Sympatric	40.46923	-82.574243
KGL	Kokosing River	Kokosing River	Sympatric	40.366965	-82.474524
KOA	Kokosing River	Kokosing River	Sympatric	40.537342	-82.761433
KOB	Kokosing River	Kokosing River	Sympatric	40.543603	-82.75182
KOC	Kokosing River	Kokosing River	Sympatric	40.514703	-82.751133
KOK	Kokosing River	Kokosing River	Sympatric	40.460741	-82.628488
LLB	Huron River	Huron River	Sympatric	41.286489	-82.642999
SCL	Salt Creek	Salt Creek	Allopatric O.sanbornii	39.4737	-82.741435
STW	Stillwater Creek	Great Miami River	Allopatric O.rusticus	40.207813	-84.600974
TMR	Huron River	Huron River	Sympatric	41.149721	-82.717854
VSG	Vermilion River	Vermilion River	Allopatric O.sanbornii	41.3321	-82.34964

Table 1: Population code, name, watershed, latitude and longitude for each population sampled in summer, 2016.

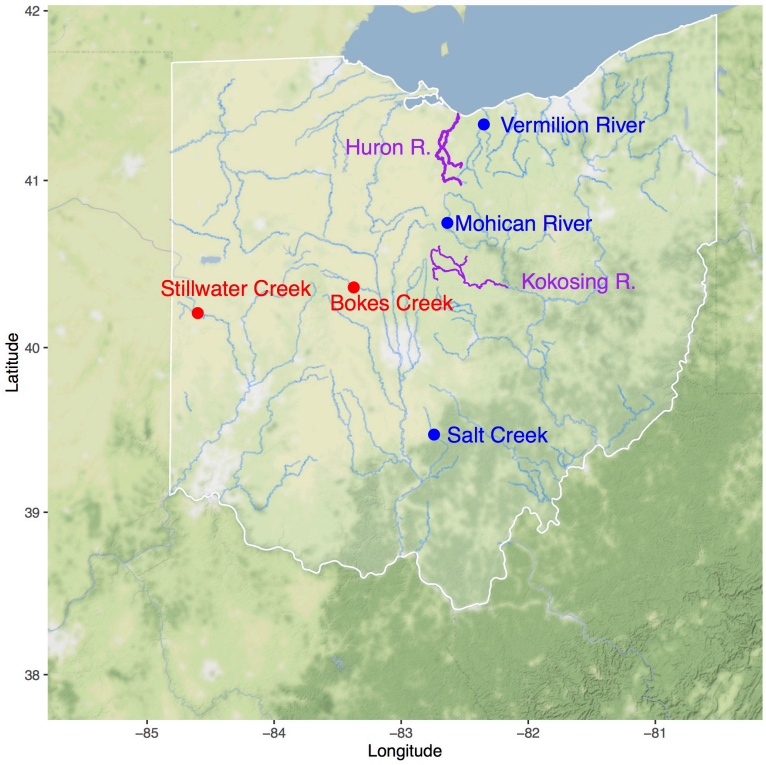

Figure 1: Locations of sampling sites for *Orconectes rusticus* and *O. sanbornii* in Ohio. Red=allopatric *O. rusticus* sites, blue=allopatric *O. sanbornii* sites, purple=sympatric rivers: native *O. sanbornii* range that has been invaded by *O. rusticus*.

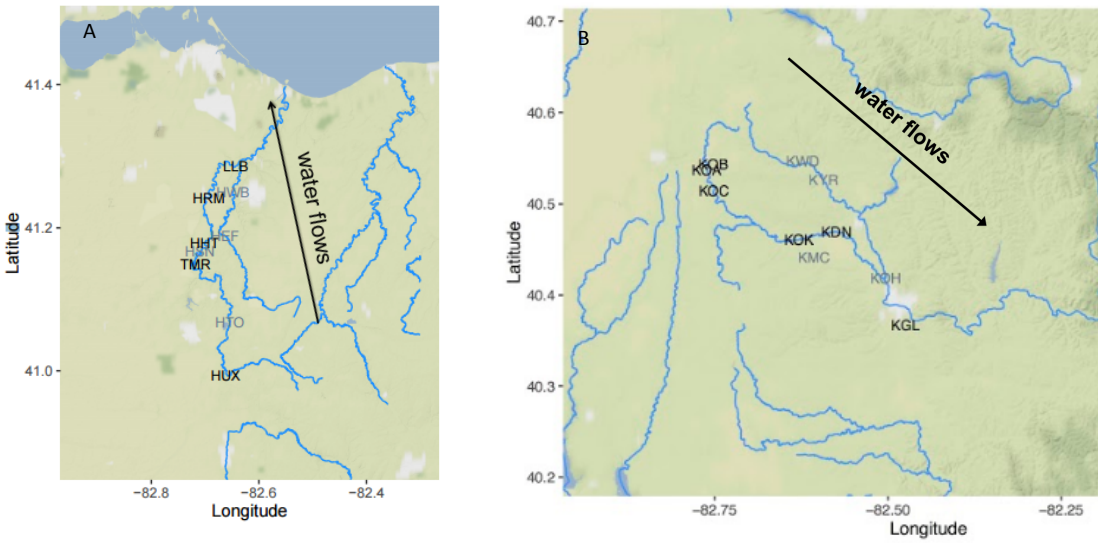

Figure 2: A) Population codes for sites sampled along the Huron river. B) Population codes along the Kokosing River. Populations in black were sampled in 2016 and used in this study. Populations in gray are from unpublished 2015 sampling (Roles Lab).

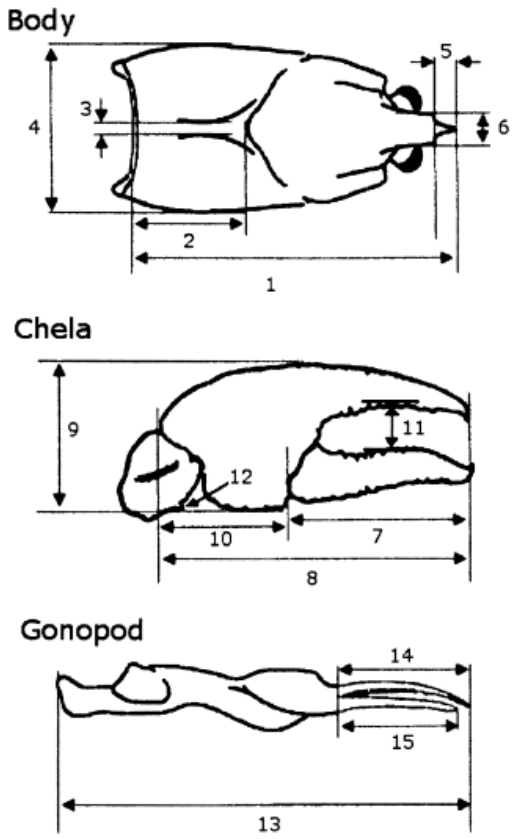

Figure 3: Diagram modified with permission from Perry et al. (2001). Body measurements: 1, carapace length; 2, areola length; 3, areola width; 4, carapace width; 5, acumen length; 6, rostrum width. Chela measurements: 7, dactyl length; 8, chela length; 9, palm width; 10, palm length; 11, chela gap width; 12, number of carpus spines. Gonopod measurements (males only): 13, gonopod total length; 14, central projection length; 15, mesial projection length.

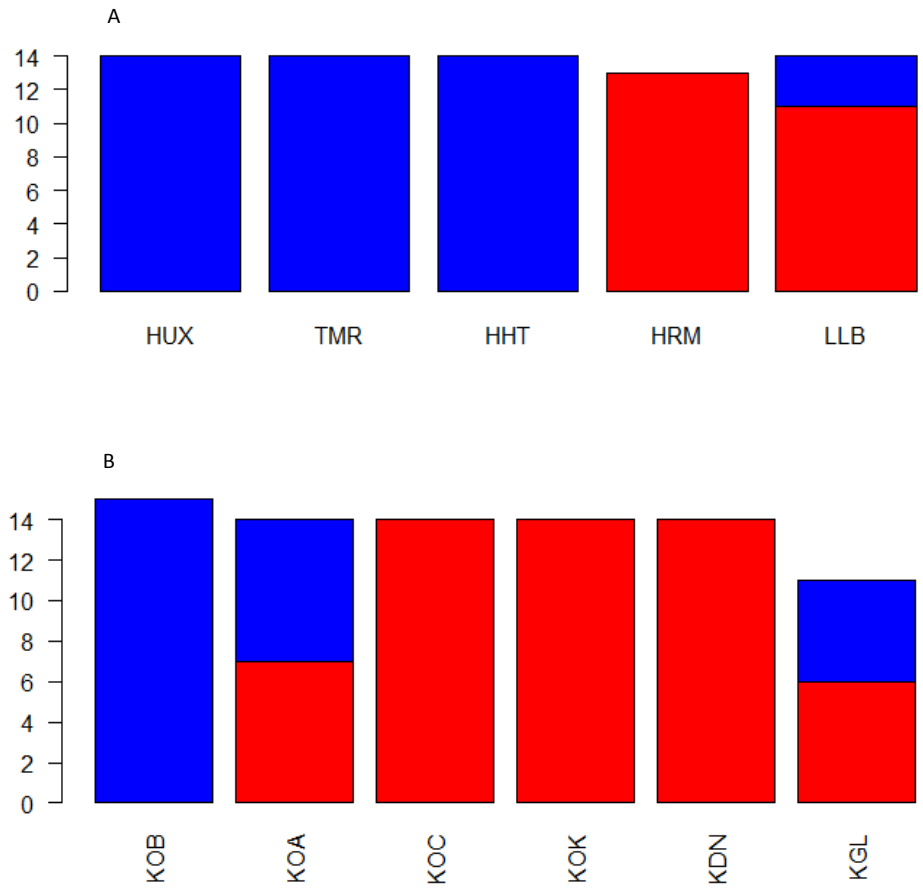

Figure 4: A) Species distribution along the Huron River. Blue=Sanborn's, Red=Rusty. Water flows from left to right (HUX→LLB). B) Species distribution along the Kokosing River. Blue=Sanborn, Red=Rusty. Water flows from left to right (KOB→KGL).

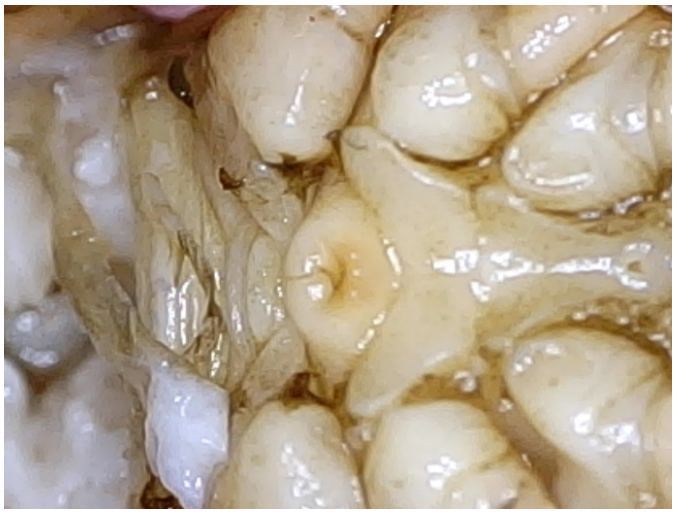

Figure 5: Individual from sympatric population along Huron river. The only individual we found with an intermediate annulus ventralis, having a zigzag suture below the pocket.

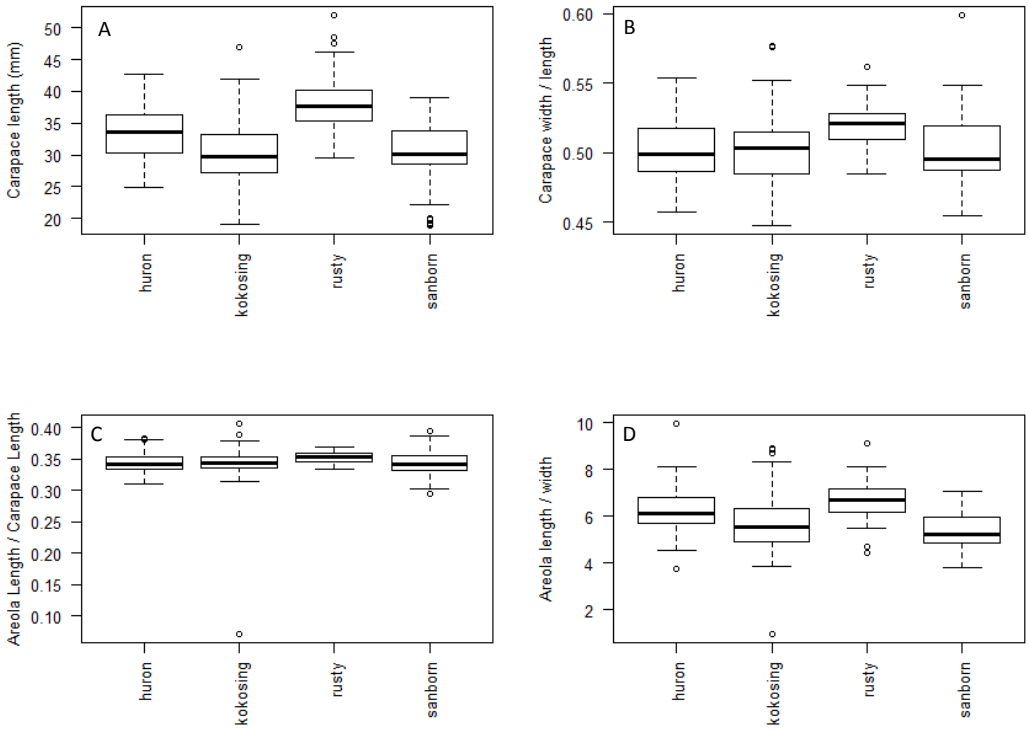

Figure 6: Boxplots of body measurement ratios comparing sympatric rivers with allopatric *O. rusticus* and *O. sanbornii* sites. A, carapace length; B, carapace length/width; C, areola length/carapace length; D, areola length/width.

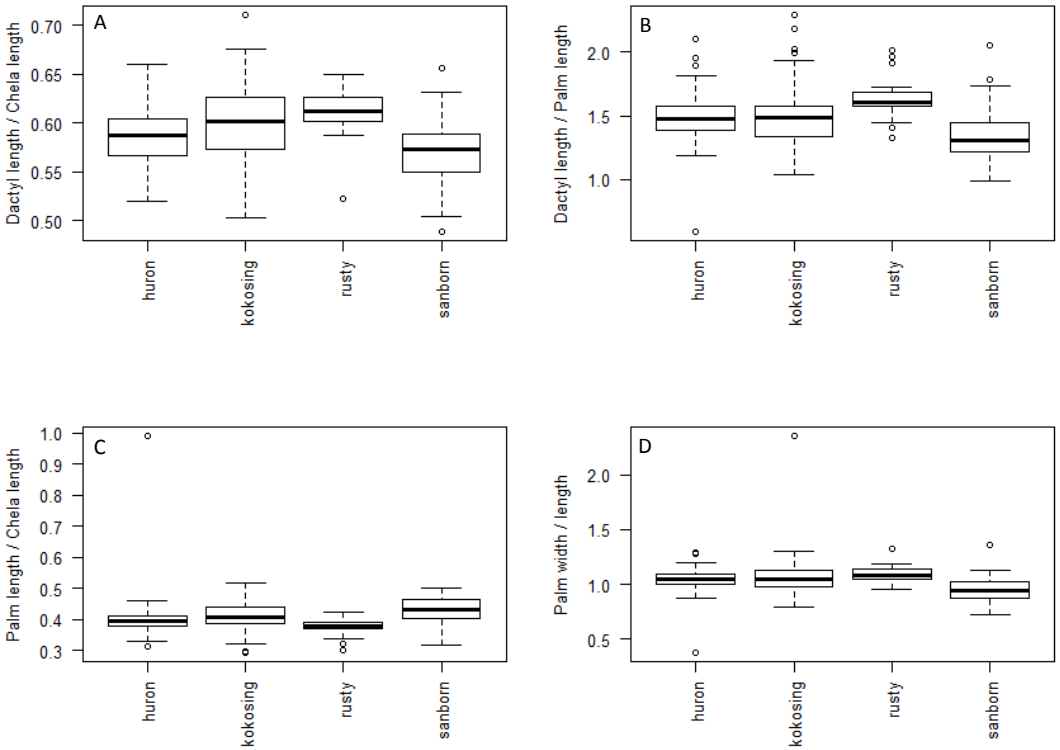

Figure 7: Boxplots of ratios of chela measurements comparing sympatric rivers with allopatric *O. rusticus* and *O. sanbornii* sites. A, dactyl length/chela length; B, dactyl length/palm length; C, palm length/chela length; D, palm width/length.

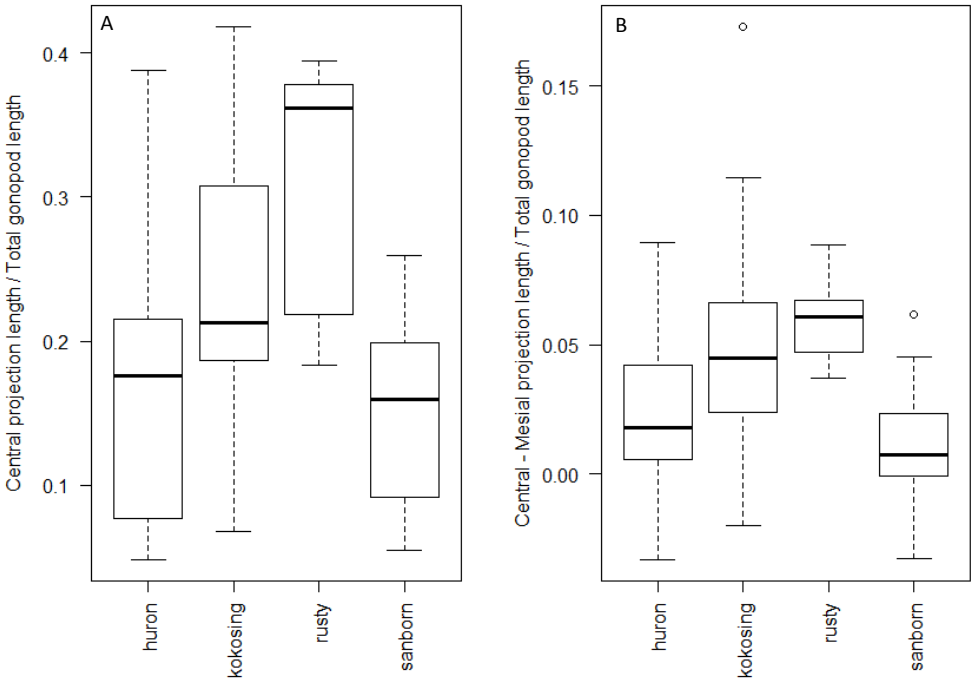

Figure 8: Boxplots of gonopod length ratios comparing sympatric rivers with allopatric *O. rusticus* and *O. sanbornii* sites. A, central gonopod projection length/total gonopod length; B, (central – mesial gonopod projection length)/total gonopod length.

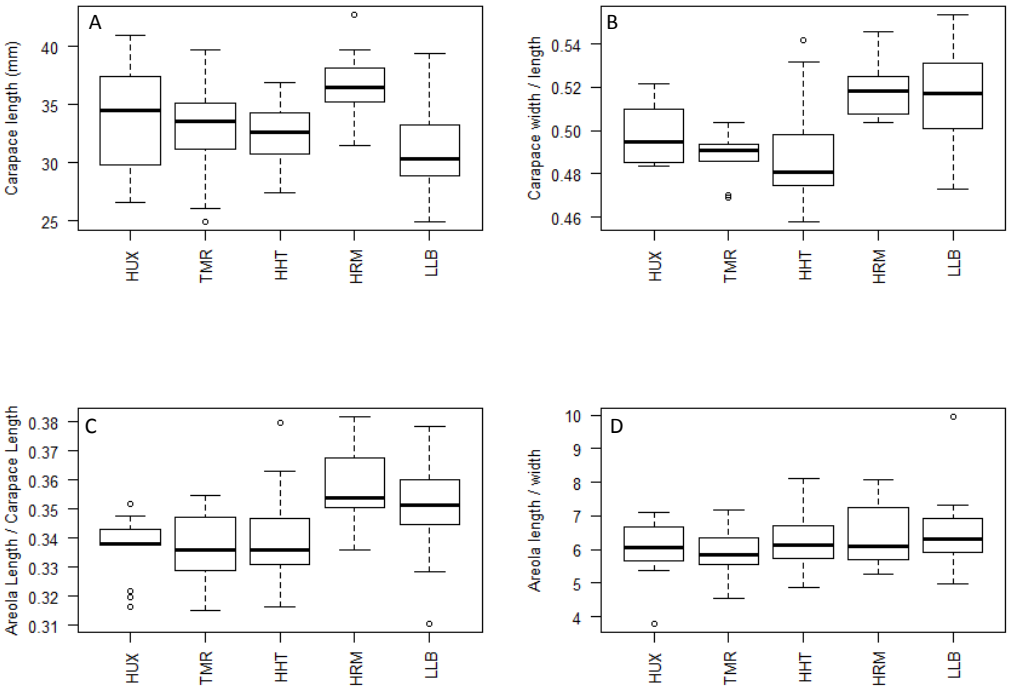

Figure 9: Boxplots of body measurement ratios comparing populations along the Huron River. A, carapace length; B, carapace length/width; C, areola length/carapace length; D, areola length/width.

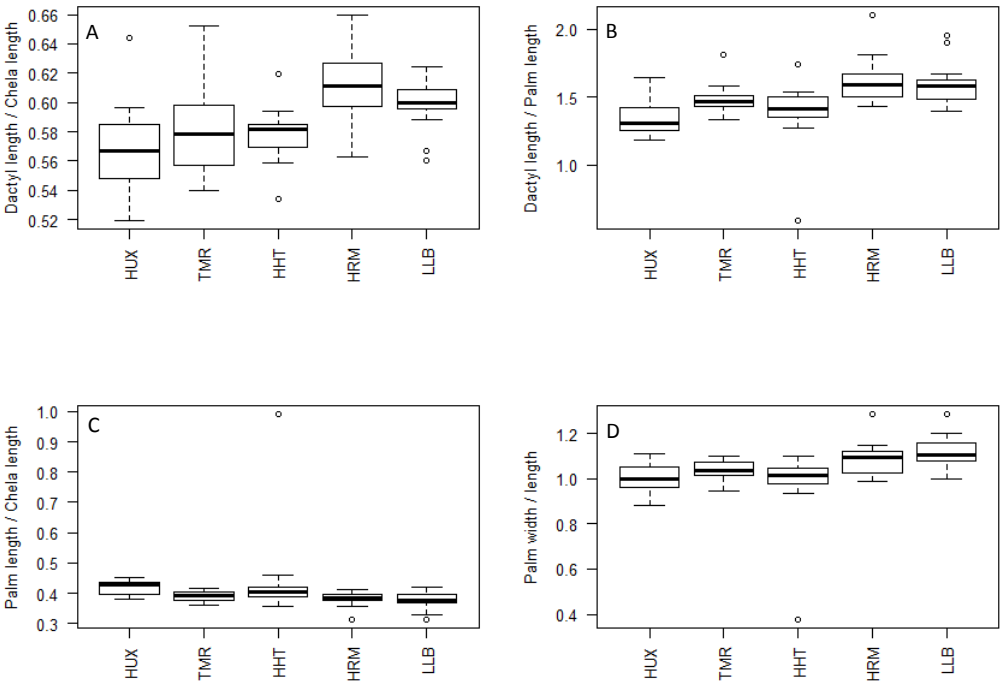

Figure 10: Boxplots of ratios of chela measurements comparing populations along the Huron River. A, dactyl length/chela length; B, dactyl length/palm length; C, palm length/chela length; D, palm width/length.

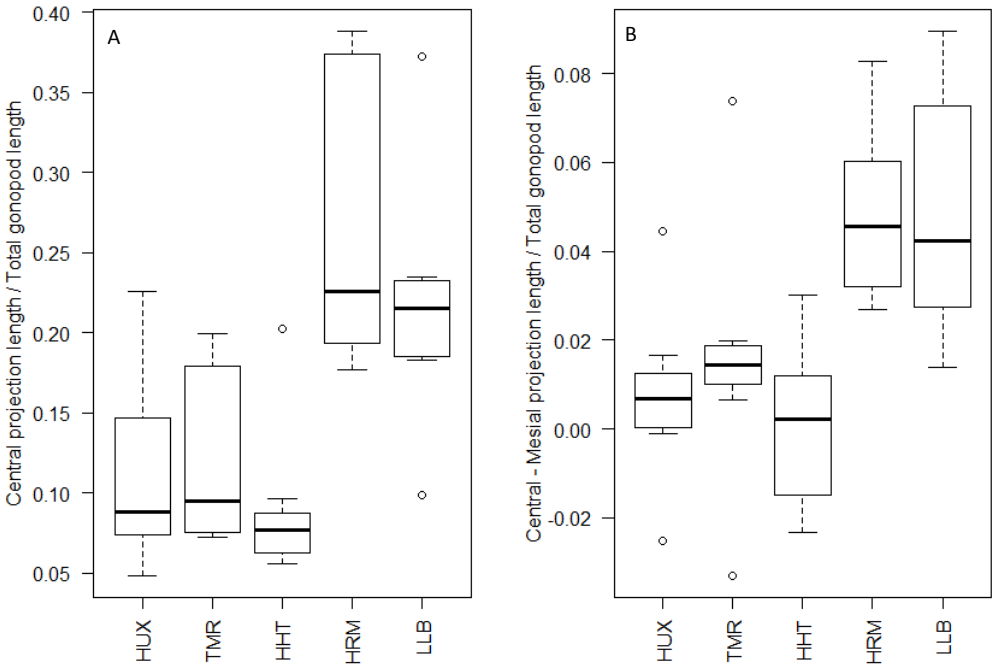

Figure 11: Boxplots of gonopod length ratios comparing populations along the Huron River. A, central gonopod projection length/total gonopod length; B, (central – mesial gonopod projection length)/total gonopod length.

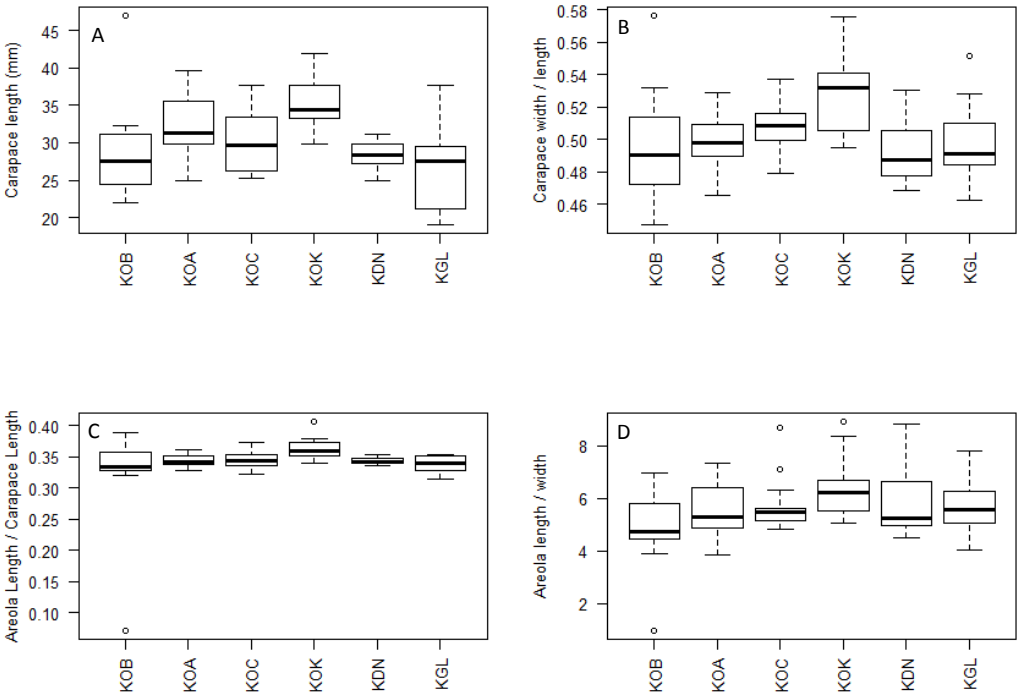

Figure 12: Boxplots of body measurement ratios comparing populations along the Kokosing River. A, carapace length; B, carapace length/width; C, areola length/carapace length; D, areola length/width.

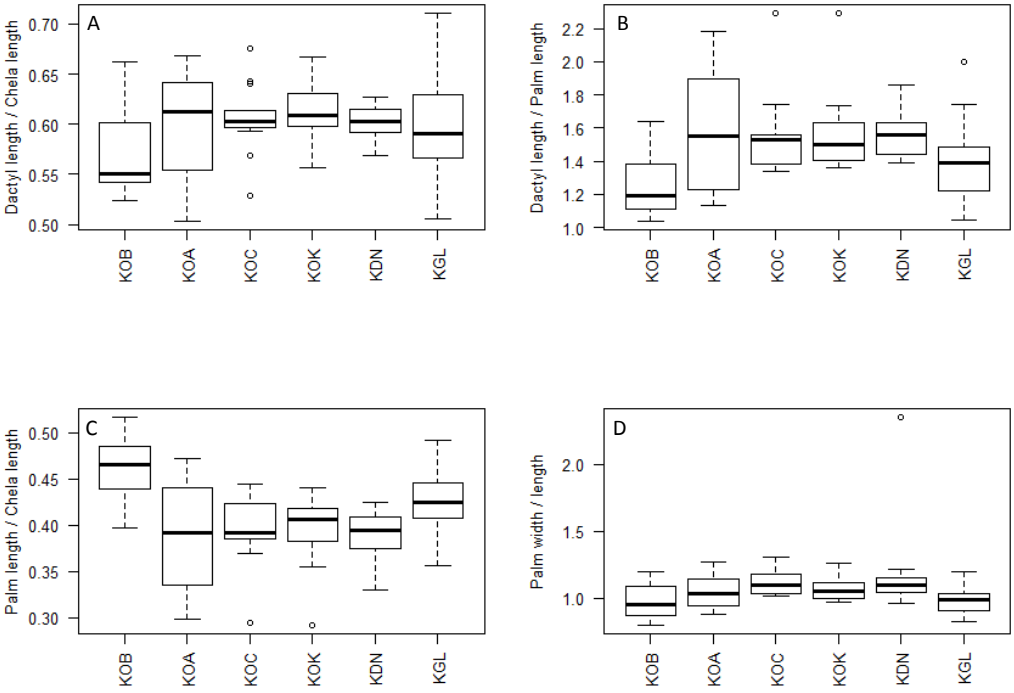

Figure 13: Boxplots of ratios of chela measurements comparing populations along the Kokosing River. A, dactyl length/chela length; B, dactyl length/palm length; C, palm length/chela length; D, palm width/length.

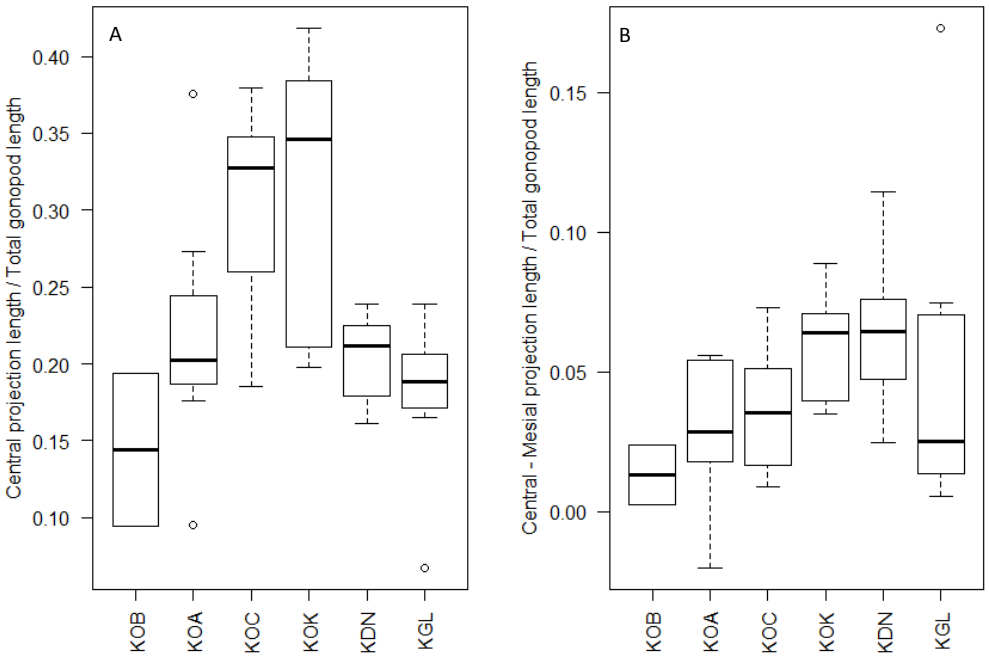

Figure 14: Boxplots of gonopod length ratios comparing populations along the Kokosing River. A, central gonopod projection length/total gonopod length; B, (central – mesial gonopod projection length)/total gonopod length.