REPRODUCTIVE BIOLOGY AND PHENOLOGY OF WESTERN PEARLSHELL MUSSELS IN MONTANA

by

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DEDICATION

“Men come and go, cities rise and fall, whole civilizations appear and disappear- the earth remains, slightly modified. The earth remains, and the heartbreaking beauty where there are no hearts to break.... I sometimes choose to think, no doubt perversely, that man is a dream, thought an illusion, and only rock is real. Rock and sun.”

— Edward Abbey, Desert Solitaire: A Season in the Wilderness

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ABSTRACT

The Western Pearlshell mussel is the only native freshwater mussel inhabiting trout streams of western Montana; it has been designated a state Species of Concern because of declines in abundance and distribution. Conservation of Western Pearlshells in Montana will require fundamental information on their reproduction and life-history traits that is currently lacking. We therefore estimated the age and length at sexual maturity and incidence of hermaphroditism in mussels using histology. We determined the timing of reproductive events (spawning, brooding, embryogenesis, larval release, and larval infestation of hosts) and their relationship to temperature by collecting gonadal and marsupial biopsies to identify gamete presence and embryo developmental stages, visually identifying brooding mussels, and examining captured fish for the presence of mussel larvae. We identified the hosts of Western Pearlshells in nature by quantifying the probability of infestation and infestation intensities among salmonid species. Mussels reached sexual maturity at an estimated 34 mm in length and 11.5 years of age. Of 31 mature mussels examined histologically, all but one were gonadal hermaphrodites. The reproductive phenology of Montana Western Pearlshells differed among populations and years. Mussel populations brooded for about 24 to 39 days in May and June. Embryogenesis was synchronous among individuals in all populations except one and was about two to three weeks in duration. The larval infestation period generally occurred in June and July and was 47 to 71 days in duration. Some larvae grew > 400% in length before leaving the host. Gonadal recrudescence was rapid whereby mussels possessed mature or nearly mature gametes by early autumn. Both photoperiod and temperature appear to influence the timing of reproductive events. Native Westslope Cutthroat Trout and nonnative Brook Trout were the most susceptible fish species to infestation of Western Pearlshell larvae. Nonnative Brown Trout were moderately susceptible to infestation in the Flint-Rock watershed. Nonnative Rainbow Trout and native Mountain Whitefish were least likely to be infested with mussel larvae. Our findings will inform future conservation and propagation efforts of Western Pearlshells in Montana.

REPRODUCTIVE BIOLOGY AND PHENOLOGY OF WESTERN PEARLSHELL MUSSELS IN MONTANA

Introduction

Freshwater mussels are in decline worldwide (Williams et al. 1993; Cowie et al. 2017); the Western Pearlshell (*Margaritifera falcata*) in Montana is no exception. Western Pearlshells have been eradicated from much of their historical range in the United States (Jepson et al. 2012). This species has earned conservation status in almost every state it occupies including Montana, Washington, California, Idaho, and Utah, and is extirpated from Nevada (Jepson et al. 2012). Western Pearlshells were eradicated from four watersheds in Montana in the 20th century and the number of "populations" (defined as aggregations separated from other aggregations by at least 1 km; NatureServe 2015; Stagliano 2015) and individuals continue to decline (Stagliano 2015). Montana has lost an estimated 19% of its populations, and those which remain are small, isolated, and exhibit low rates of recruitment (Stagliano 2015). Only about 20 of about 85 pearlshell populations are predicted to be viable in Montana 100 years from now (Stagliano 2015).

The decline of Western Pearlshells in Montana is particularly alarming considering the benefits freshwater mussels provide to aquatic ecosystems. Freshwater mussels filter and clean water (Welker and Walz 1998; Atkinson et al. 2013), excrete and recycle nutrients (Atkinson et al. 2014; Bril et al. 2014), play important roles in food webs by acting as primary consumers (Howard and Cuffey 2006; Vaughn et al. 2008), increase aquatic macroinvertebrate diversity and abundance (Vaughn and Spooner 2006), and act as bio-indicators (Bauer 1992; Nedeau et al.

2009; Santos et al. 2015). For example, Western Pearlshells increased the percent and rate of deposited organic material in the Eel River, California (Howard and Cuffey 2006). Freshwater mussels are bioturbators, mixing organic material in the water column (Bender and Davis 1984; van Duren et al. 2006) and sediment (Vaughn and Hagenkamp 2001) through their siphoning and burrowing activity. Western Pearlshells increased the growth of larvae of Pacific lamprey in California, a designated Species of Concern by the U.S. Fish & Wildlife Service, by concentrating local organic material consumed by the lamprey larvae (Limm and Power 2011). The Western Pearlshell mussel is the only freshwater mussel found in western Montana's trout streams. Western Pearlshells potentially provide these benefits to threatened and endangered native fish species in Montana including Bull Trout (*Salvelinus confluentus*), Westslope Cutthroat Trout (*Oncorhynchus clarkii lewisi*), and Arctic Grayling (*Thymallus arcticus*).

Future conservation efforts of Western Pearlshells may entail propagation to increase population abundances or to replace extirpated populations. Past efforts largely focused on documenting viable populations and translocating mussels (Pierce et al. 2010; Stagliano 2010; Stagliano 2013; Stagliano 2015); however, few populations left in Montana are large enough to support future translocation efforts (D. Stagliano, Montana Biological Survey, personal communication). Propagation is a commonly used strategy for the conservation of freshwater mussels and Montana Fish, Wildlife and Parks (MTFWP) is interested in incorporating Western Pearlshell propagation efforts into native trout conservation actions (Stagliano 2015; J. Olsen, MTFWP, personal communication). Propagation for augmentation or reintroduction requires knowledge of freshwater mussel reproductive biology. Propagation strategies include stocking juvenile mussels reared in captivity or stocking fish infested with larvae. Both strategies require

harvesting larvae from brooding females; however, the timing of brooding of Western Pearlshells in Montana was heretofore unknown.

The reproductive biology of freshwater mussels is complex (Figure 1). During spawning, female mussels move mature eggs from their gonad to the marsupium, a portion of the gills where eggs are fertilized and develop as embryos. Mature sperm is released by males into the water column, siphoned by females, and fertilizes the eggs held in the marsupium. Margaritiferid mussels brood the developing embryos for several weeks until they mature into larvae (Karna and Millemann 1978; Hastie and Young 2003; Scheder et al. 2014). The larvae of Western Pearlshells are obligatory parasites; they must attach to fish gills for several weeks to metamorphose into juvenile mussels (Karna and Millemann 1978). Margaritiferids release their larvae into the water current passively, and the larvae attach to and are encapsulated on the host's gills after inhalation (Murphy 1942; Ziuganov et al. 1994; Hastie and Young 2003). The larvae then become encapsulated by epithelial cells on the host's gills (Arey 1921; Bauer 1987; Ziuganov et al. 1994; Rogers-Lowery and Dimrock 2006). The relationship between the larvae and host is mostly phoretic whereby mussel benefit from upstream dispersal (Watters 2001; Barnhart et al. 2008). After metamorphosis, juvenile mussels are shed from the host as the capsule breaks downs and settle onto the streambed.

Life-History Traits

Conservation of Western Pearlshell in Montana will require fundamental information on reproduction and life-history traits that is currently lacking. Conservation efforts of native freshwater mussels should include increasing our understanding of basic life-history traits to inform management strategies (NNMCC 1998; FMCS 2016). For example, knowledge of length

and age at sexual maturity and the incidence of hermaphroditism, in which individuals develop both male and female gametes, would inform managers about the size classes and numbers of mussels needed for propagation. Generally, margaritiferid mussels can live 100 years and are considered sexually mature beginning between seven to twenty years of age (Bauer 1987; Toy 1998). When Western Pearlshell mussels become sexual mature in Montana, and whether older individuals become senescent with age was heretofore unknown. Age at maturity and reproductive lifespan are key life-history traits linked to fitness attributes and population dynamics. Improving our knowledge of Western Pearlshell life-history traits in Montana will increase the likelihood of conserving this species and restoring the ecosystem functions that mollusks provide to aquatic ecosystems (FMCS 2016).

Hermaphroditism in Western Pearlshells has not been explicitly studied; however, hermaphroditism has been observed in this species in other states (Heard 1970; Karna 1972; Toy 1998). Montana Western Pearlshells had positive inbreeding coefficients, high rates of self-fertilization, and high incidences of identical genotypes (Mock et al. 2013). Some other margaritiferids are capable of hermaphroditism (Bauer 1987; Grande et al. 2000; Geist and Kuehn 2005) but the cue that triggers hermaphroditism is unclear (Breton et al. 2018). Hermaphroditism in *M. margaritifera,* the closest relative of the Western Pearlshell, has been correlated with low population densities (Bauer 1987; Geist and Kuehn 2005). The incidence of hermaphroditism in populations that may be used as source populations for propagation should be studied as it could affect the number of adults within and among populations needed.

Reproductive Phenology

Knowledge of the timing of reproductive events such as spawning, brooding, and larval release is required for the propagation of freshwater mussels; however, the timing of these events for Western Pearlshells in Montana was heretofore unknown. Propagation strategies include stocking juvenile mussels reared in captivity or stocking fish infested with larvae. Both strategies require harvesting larvae from brooding females. The timeframe to harvest viable larvae from brooding mussels is short because Western Pearlshell is a short-term brooder and expel its larvae shortly after they are fully developed (Toy 1998; Scheder et al. 2011). Prior to our study, only one reproductive event in Western Pearlshells in Montana had been observed, in which an individual was observed releasing a conglutinate in the Boulder River in June 2015 (D. Stagliano, personal communication).

Reproductive phenology can vary greatly among populations of Western Pearlshell in coastal states (Murphy 1942; Karna and Millemann 1978; O'Brien et al. 2013; Allard et al. 2017). The timing of brooding in pearlshells ranges from April to July in coastal state populations and may last weeks or months (Murphy 1942; Karna and Millemann 1978; O'Brien et al. 2013; Allard et al. 2017). For example, some populations in California begin brooding in April whereas others begin brooding in July (Spring Rivers 2007). Western Pearlshells in Merrill Creek, Oregon, released larvae over a period of four months; however, other populations in the Oregon released larvae over a period of weeks (Karna and Millemann 1978; O'Brien et al. 2013).

The timing of reproductive events of Western Pearlshells in Montana may differ from populations in coastal states because of differences in environmental temperatures and temperature requirements. Reproductive phenology in freshwater mussels is largely driven by

temperature (Watters and Dee 2000; Hastie and Young 2003). Western Pearlshells experience different temperature patterns in Montana than in coastal states because of differences in topography and climate. Latitude, elevation, and climate patterns can affect water temperature and have been shown to vary with reproductive timing and success (Toy 1998; Spring Rivers 2007; Zajac et al. 2018). In Montana, the average daily temperature was about 13.5°C when the conglutinate was observed in the Boulder River (D. Stagliano, personal communication). Montana populations are also more geographically isolated than those found in coastal states. Geographic isolation may result in differences in temperature requirements due to local adaptations.

Knowledge of the timing and duration of larval infestation of hosts is essential for conservation management because mussel recruitment is dependent on the interaction between the larvae and hosts. The hosts must be available and susceptible to the infestation of the mussel larvae—two requirements that are temporally dynamic. Fish that have been previously infested with mussel larvae build an acquired immune response to the larvae (Rogers and Dimrock 2003; Dodd et al. 2006; Rogers-Lowery et al. 2007). Compatible hosts acquire resistance to larvae after one or more infestations and the success of metamorphosis of larvae is greatly reduced by the third exposure (Arey 1924; Rogers and Dimrock 2003). Additionally, young salmonids are particularly susceptible to larvae infestation because their innate immune system can take weeks to months to fully develop post hatch (Manning et al. 1982; Castillo 1993; Ellis 2001). Therefore, mussels benefit by releasing larvae when naïve or age-0 hosts are present because they are the most susceptible to infestation (Bauer 1988; Henrikson et al. 2009; Ieshko et al. 2016).

Host Fish

Information regarding host species will be necessary to sustain, augment, or reintroduce Western Pearlshell populations in Montana. Freshwater mussels exhibit host specificity, wherein the larvae require specific species of fish for metamorphosis. Host specificity is determined by the adaptations of mussel larvae to survive a fish's innate immune response to infestation (Rogers-Lowery et al. 2007). Generally, mussel species such as Western Pearlshell that passively release larvae tend to be host generalists whereby multiple fish species are compatible hosts (Karna and Millemann 1978; Barnhart et al. 2008). Knowledge of the host relationships of freshwater mussels is essential for propagating mussels, assessing mussel habitat, and making population comparisons (Geist 2010; Douda 2015; Ford and Oliver 2015). Host densities may regulate the population abundance of mussels and may be the most important factor in conserving mussel populations (Bauer 1988; Zuiganov et al. 1994; Österling et al. 2008; Henrikson et al. 2009; Haag and Stoeckel 2015; Ieshko et al. 2016). Identifying the fish species that act as hosts for Western Pearlshells is the first step to determining the minimal host densities they require.

Historically, Western Pearlshell mussels used Westslope Cutthroat Trout as hosts in Montana as inferred from the overlap of these animals' ranges (MTNHP 2018). Currently, viable, recruiting populations of Western Pearlshell exist in Montana where Westslope Cutthroat Trout are absent, indicating that Western Pearlshells are using another species, or several species, as hosts (J. Olsen, personal communication). The host requirements of Western Pearlshells have not been studied in Montana.

Western Pearlshells are known to use a variety of salmonid hosts in coastal states (Murphy 1942; Meyers and Millemann 1977; Karna and Millemann 1978). In the wild, Western

Pearlshell larvae have metamorphosed on Brown Trout, Chinook Salmon *Oncorhynchus tshawytscha*, Cutthroat Trout *Oncorhynchus clarkii*, and sea-run Rainbow Trout *Oncorhynchus mykiss* to varying degrees of success (Murphy 1942; Karna and Millemann 1978). The prevalence (percentage of infested fish) of Western Pearlshell larvae in western Oregon was highest on Chinook Salmon, followed by sea-run Rainbow Trout, Cutthroat Trout, and Coho Salmon *Oncorhynchus kisutch* (Karna and Millemann 1978). The infestation intensities on these species followed a similar pattern: Chinook Salmon had the highest number of larvae followed by Cutthroat Trout, sea-run Rainbow Trout, and Coho Salmon (Karna and Millemann 1978). The assemblage of salmonids available to Western Pearlshells in Montana differs from those in coastal states and different populations of Western Pearlshells may have different host requirements, further supporting the need to identify hosts for Western Pearlshells in Montana (Larsen 2002, cited by Karlsson et al. 2014; Larsen et al. 2012, cited by Karlsson et al. 2014; Karlsson et al. 2014; Clements et al. 2018; Wacker et al. 2019).

Substantial gaps in information on the life-history traits of Western Pearlshells in Montana are preventing the conservation of this species. We therefore addressed the following objectives specific to Western Pearlshells in Montana: (1) to estimate the age and length at sexual maturity, (2) to determine the incidence of hermaphroditism, (3) to determine the timing of reproductive events (spawning, brooding, embryogenesis, larval release, and larval infestation of hosts) and their relationship to temperature, and (4) to identify the hosts of Western Pearlshells in nature. Increasing our understanding of reproduction and basic life-history traits is necessary to inform management strategies and will be the first steps to conserving this species in Montana (NNMCC 1998; FMCS 2016).

Preliminary Data

We collected preliminary data in 2019 to generalize reproductive phenology at a watershed scale and to test sampling techniques. Preliminary data were necessary to determine when and how to rigorously characterize reproductive phenology at a fine, population scale. We sampled six Western Pearlshell populations in the Big Hole watershed weekly, and six in the Flint-Rock watershed biweekly, to roughly capture timing of reproductive events. The timing of spawning, brooding, and embryogenesis were approximated by visually examining mussels for brooding and collecting gonad and marsupium biopsies. The gonad biopsies were examined for the presence of gametes to determine if mussels were pre- or post-spawn. Marsupium biopsies were collected to determine if embryo developmental stages could be identified and used to characterize embryogenesis. The timing of larval release was estimated by collecting stream drift samples using plankton nets and examining the contents for the presence of mussel larvae. Host infestation was generalized by capturing and examining salmonids for the presence of mussel larvae. We used binomial regression models to describe the timing of brooding and infestation.

Preliminary data from 2019 provided insight into the sampling intensity needed to thoroughly capture reproductive events in 2020 (Figure 2). Most gonad biopsies collected from late May to early July in 2019 did not contain any gametes, indicating that the biopsies need to be collected earlier in the spring to detect spawning. Brooding largely occurred in June and lasted for about a month. The probability of a mussel brooding was greatest in mid-June among mussels in the Big Hole watershed and late-June among those in the Flint-Rock watershed. Surprisingly, brooding peaked only eight days later in the Flint-Rock watershed than in the Big Hole even though these watersheds drain to opposite sides of the Continental Divide and differ in

elevations, temperature regimes, and fish assemblages. The contents of the marsupium biopsies indicated that fertilization generally occurred in June and embryos matured in about 12 days. We changed our sampling methods for determining the timing of larval release in 2020 after processing a subset of stream drift samples from 2019 that were likely to contain mussel larvae but did not. We also learned that we needed to capture fish earlier and increase our fish sampling efforts in 2020 to describe the infestation period.

Preliminary data from 2019 also led us to investigate the incidence of hermaphroditism in Western Pearlshells. We expected 30 to 50% of a population to be brooding based on a previous study (Bauer 1987) and the assumption that half of a population is female; however, the probability of a mussel brooding in 2019 reached 92% in the Big Hole and 77% in the Flint-Rock watersheds, respectively (Figure 2). Such high probabilities, and a lack of sperm in the gonad biopsies, indicated either hermaphroditism or sampling only females by chance; we therefore sexed a subset of mussels histologically in 2020.

Methods

Study Area

The reproductive biology and phenology of Western Pearlshells were investigated in the Big Hole and Flint-Rock watersheds in southwest Montana (Figure 3). The Anaconda Mountain Range, part of the Continental Divide, separates these watersheds. The Big Hole watershed drains east of the Divide into the Upper Missouri River basin, which flows into the Gulf of Mexico on the Atlantic side of North America, whereas the Flint-Rock watershed drains west into the Columbia River basin, which eventually flows into the Pacific Ocean. The Big Hole watershed has the greatest number of streams (11) inhabited by Western Pearlshells in the state;

however, this number has declined by 15% since 2007 (Stagliano 2015). Western Pearlshells have consistently inhabited five streams in the Flint-Rock watershed since before 2010 (Stagliano 2015). We studied two Western Pearlshell populations in each watershed that showed evidence of recruitment (i.e., had densities > 0.5 mussels per square meter of stream, a wide range of sizes, and the presence of juvenile mussels < 30 mm in length; Master et al. 2012; Stagliano 2015).

Clam Creek is a second-order spring creek (Strahler 1952) located in the Big Hole watershed (Table 1). Clam Creek is the warmest of the four study sites and experiences high diurnal water temperature fluctuations because it is narrow and shallow. Clam Creek is remote and receives minimal human visitation because it is located in the Anaconda-Pintler Wilderness. The stream flows through a large open meadow and a forest dominated by lodgepole pine *Pinus contorta*, but most of the mussels occupy the forested section of the stream (Figure 4A). Clam Creek contains the most dense and abundant population of Western Pearlshells of the four study sites; they occupy all microhabitats including sand and gravel sediments, deep and shallow water, and vegetated as well as open substrates. This study site has an old culvert and is frequented by cattle in some years. Nonnative Brook Trout are the only fish species present in this section of Clam Creek.

Deep Creek is a fourth-order stream in the Big Hole watershed that is lined by willows, (*Salix* spp.), and surrounded by privately owned livestock pastures (Table 1). Irrigation diversions, Beaver, *Castor canadensis*, activity, and a nearby (30 m) paved highway are present (Figure 4B). Most of the mussels occupy deep pools or the thalweg or are beneath willows. Native Mountain Whitefish and nonnative Brook Trout, Rainbow Trout, and Brown Trout occupy the study site.

Upper Willow Creek is a third-order stream in the Flint-Rock watershed that is largely surrounded by privately owned agricultural lands and has several irrigation diversions (Table 1). The study site is located next to a dirt road in a sparsely wooded area and experiences high spring-snowmelt discharges and woody debris input (Figure 4C). The mussels are most common in deep pools and buried in fine sediment but can also be found underneath the bridge that crosses the site. Native Westslope Cutthroat Trout and Mountain Whitefish and nonnative Brown Trout inhabit this section of Upper Willow Creek.

West Fork Rock Creek is also located in the Flint-Rock watershed and is the largest and coldest of the four streams (Table 1). The creek is a fourth-order stream located in Pintler-Anaconda National Forest. The stream has no barriers to fish passage, flows through a forest dominated by lodgepole pines, and has undercut banks in several sections (Figure 4D). The Western Pearlshells are largely found in deep pools and deep stream edges. The study population is 25 m from a dirt road. West Fork Rock Creek is inhabited by native Bull Trout, Westslope Cutthroat Trout, and Mountain Whitefish and nonnative Brown Trout.

Life-History Traits

Western Pearlshells of various lengths were collected to determine their reproductive status and sex by histological examination. We collected nonbrooding mussels (n = 40) for histological examination in late April and early May in an attempt to catch them prior to spawning (i.e., depositing eggs). Most mussels (n = 26) were collected from Clam Creek. Lengths were measured with a digital caliper. All mussels were biopsied (as described in the Reproductive Phenology section) before fixation for histology to compare the two assessment methods. We then removed the bodies of the mussels from their shells and fixed them in 10%

neutral buffered formalin at a 1:10 ratio of mussel to formalin volume. The mussels were stored and periodically shaken in the formalin fixative for 24 hours. The mussels were then removed from the fixative, preserved in vials with 30 mL of 70% ethanol, and shipped to the California Department of Fish and Wildlife Shellfish Health Laboratory for processing.

Ten 5-µm cross-sections of the visceral mass were excised from animals > 35 mm in length (Moore et al. 2001; Moore et al. 2011); ten is a seemingly adequate number of sections to detect both ovaries and testes in ripe, hermaphroditic Western Pearlshells (Heard 1970). Fewer than ten cross-sections were excised from mussels less ≤ 35 mm in length. The tissue sections were stained with hematoxylin and eosin, and slides were encoded to prevent bias during assessment (Moore et al. 2001; Moore et al. 2011). The histologist (1) identified the presence of ovaries, testes, and precursor gonadal tissue, (2) determined reproductive status (unripe, ripe, or post-spawn), and (3) coarsely estimated the ratio of female to male gonadal tissue in hermaphrodites.

Sexual Maturity. We used the presence or absence of gonadal tissue and lengths and ages of the mussels to estimate the onset of sexual maturity. Mussels were first classified as sexually mature (gonadal tissue present) or immature (gonadal tissue absent). The probability of sexual maturity was modeled on length using a binomial logistic regression (Downs et al. 1997; DeMartini et al. 2000; Smith et al. 2004; Doi et al. 2007; Gerhart and Bert 2008; Segura and Delgado 2021). We estimated the length at sexual maturity as the length at which the probability of sexual maturity reaches 50% (L_{50}) (Downs et al. 1997; DeMartini et al. 2000; Smith et al. 2004; Doi et al. 2007; Gerhart and Bert 2008; Segura and Delgado 2021) and 95% confidence intervals using the dose.p function of the ‘MASS’ package in R version 4.0.2 (Venables and

Ripley 2002; Charpentier et al. 2014). The age at sexual maturity was calculated by estimating the age at which mussels were L_{50} in length using linear regression. Mussels were aged by coating the surface of their outer shell in mineral oil and counting the number of annuli under a dissecting scope (10×) (Neves and Moyer 1988). Only mussels ≤ 35 mm in length were aged because larger individuals had erosion at the umbo of the shell and annuli that were nearly contiguous or indistinguishable (Neves and Moyer 1988). We used another age-length model created from Western Pearlshells collected in northern Montana to corroborate our age estimate (D. Stagliano, unpublished data).

Hermaphroditism. Histological examination of the mussels provided information on the incidence of hermaphroditism. The presence of ovaries or testes or both was used to classify the sex of each individual. Unlike histology, gonadal biopsying is a nonlethal method for determining the sex of dioecious mussels. Whether biopsies can be used to accurately classify sex in hermaphrodites was heretofore unknown; therefore, we compared the results from the histological findings (presence of ovaries or testes or both) to the presence of eggs or sperm or both in the gonadal biopsies to determine if discrepancies existed. The presence of eggs (10×) or sperm (40×) or both in the biopsies was used to make an initial determination of the sex of individuals using a Leica DM 1000 LED clinical microscope. After receipt of the histology results, we determined if the gonadal biopsies misclassified the sexes of individuals, specifically hermaphrodites. The ratio of ovaries to testes present in hermaphrodites also provided insight into the likelihood of extracting both types of gametes from a hermaphrodite using gonadal biopsies.

Reproductive Phenology

We aimed to collect all reproductive phenology samples before, during, and after each reproductive event (spawning, brooding, embryogenesis, larval release, and larval infestation) to capture the timing of the entire series of events at a population scale. Reproductive phenology sampling occurred over a 20-week timeframe in 2020 from the last week in April until the second week of September (Table 2). At least 30 presumably mature mussels (≥ 40 mm in length; Toy 1998) were sampled weekly to document spawning and brooding, and biweekly after brooding ended (Scheder et al. 2011). Embryogenesis in all brooding mussels was assessed. Previously unsampled mussels were collected from different areas of the mussel beds on each visit, when possible, to ensure independence among sampling dates. Two stream drift samples were collected biweekly throughout the 20-week season, but weekly during and shortly after brooding (Allard et al. 2017). We sampled a minimum of 30 fish to assess larval infestation biweekly beginning before larval release, if flows allowed, and continuing until fish no longer appeared to be infested. These sample sizes proved adequate for modeling reproductive phenology using the preliminary data collected in 2019.

Gonadal Recrudescence and Spawning. The timing of gonadal recrudescence and spawning of Western Pearlshells was determined by identifying the presence of gametes (eggs or mature sperm) and measuring egg size. We extracted gametes from mussels by taking nonlethal, minimally invasive gonadal biopsies using a needle and syringe (Bauer 1987; Shiver 2002; Saha and Layzer 2008; Galbraith and Vaughn 2009; Galbraith and Vaughn 2011; Tsakiris et al. 2016). Gonad biopsies do not affect the survival or growth rates of mussels (Tsakiris et al. 2016). We gently pried the mussel open, placed a wedge between the bivalves, inserted a 20-gauge needle

into the gonadal tissue, and pulled the syringe back to extract the gonadal fluid. The gonad is in the visceral mass, which is easily identifiable based on color and texture. A maximum of 0.20 mL of gonadal fluid was collected. The biopsies were preserved in cryotubes with 10% neutral buffered formalin and kept cold until processing. The presence of eggs (10×) and mature sperm (40×) were identified using a Leica DM 1000 LED clinical microscope. Each gonad biopsy was classified twice: eggs either present or absent, and mature sperm either present or absent. For each biopsy containing eggs, we calculated the egg diameters of the first 50 eggs by averaging two perpendicular diameter measurements of each egg using a Leica ICC50W camera and Leica LAS EZ software (Galbraith and Vaughn 2009; Tsakiris et al. 2016). We estimated the mean egg diameter and standard error for each mussel population by collection date after checking for independence among biopsy samples (Haggerty et al. 2005; Galbraith and Vaughn 2009; Tsakiris et al. 2016).

We used logistic, quadratic logistic, or cubic logistic regressions to describe the timing of reproductive events (Finlay et al. 2020; Franzem et al. 2019; Haraldstad et al. 2018; Nielsen et al. 2017; Šlapanský et al. 2016; Thorne et al. 2006), including gonadal recrudescence and spawning. The probability of a reproductive event occurring was regressed on day of year using the binomial reproductive status of individuals. Specifically, the presence or absence of gametes in gonadal biopsies was used to describe gonadal recrudescence and spawning, mussels brooding or not brooding was used to describe brooding, and fish infested with mussel larvae or not infested was used to describe larval infestation. We calculated logistic, quadratic logistic, and cubic logistic regressions and 95% confidence intervals (Oksanen and Minchin 2002; Thorne et al. 2006; Li et al. 2017; Haraldstad et al. 2018) using generalized linear models (GLM) with a binomial family and logit link (Guisan et al. 2002; Oksanen and Minchin 2002; Venables and

Dichmont 2004; Keith et al. 2016; Šlapanský et al. 2016; Haraldstad et al. 2018; Pearson 2019; Finlay et al. 2020). The models for the regressions used were

Logistic:

$$P = \frac{\exp[a + b_1(x)]}{1 + \exp[a + b_1(x)]}$$

Quadratic logistic:

$$P = \frac{\exp[a + b_1(x) + b_2(x^2)]}{1 + \exp[a + b_1(x) + b_2(x^2)]}$$

Cubic logistic:

$$P = \frac{\exp[a + b_1(x) + b_2(x^2) + b_3(x^3)]}{1 + \exp[a + b_1(x) + b_2(x^2) + b_3(x^3)]}$$

where *P* was the probability of the presence of the reproductive status, exp was the base of the natural logarithm, *x* was the day of year, and a, b_1, b_2 were the model parameters. We determined whether logistic, quadratic logistic, or cubic logistic regressions best fit the reproductive statuses using AIC and the significance of terms. Only models where all terms were significant at $\alpha < 0.05$ were used (Table 3).

The probability of a reproductive event occurring was modeled on day of year instead of degree days because we were more interested in describing the timing of reproductive events than their relationship to water temperature. Arguably, the dates on which reproductive events occur may be more useful for managing Western Pearlshell populations, particularly when temperature or degree day data are lacking. Photoperiod may also play a role in reproductive timing of freshwater mussels (Galbraith and Vaughn 2009; Gascho Landis et al. 2012; Franzem et al. 2019) and day of year is representative of daylength and incorporates how daylength is changing. Additionally, using day of year instead of degree days to describe reproductive

phenology allowed us to make comparisons in the timing of reproductive events between years and among populations where water temperature data were lacking. Degree days were highly correlated with day of year and were reported on the phenology results graphs.

We defined critical points within reproductive events using methods used in plant phenology models. Binomial regression models commonly used to describe phenological events in animals often lack definitions for the critical points within events such as the onset, duration, magnitude, and end (Finlay et al. 2020; Franzem et al. 2019; Haraldstad et al. 2018; Nielsen et al. 2017; Šlapanský et al. 2016; Thorne et al. 2006). Regression models used in plant phenology commonly define these key points (White et al. 1997; Stockli and Vidale 2004; Fisher et al. 2006; Melaas et al. 2013; Liu et al. 2016; Li et al. 2017) and share several mathematical characteristics with ectotherm phenology models (Régnière and Powell 2013; Chuine and Régnière 2017) because ectotherms and plants share many environmental cues that trigger reproductive events (Watters and O'Dee 2000; Hastie and Young 2003; Fantinou et al. 2004; Caffara et al. 2011; Hand et al. 2016) and generally repeat reproductive events on annual cycles around the same calendar dates (Régnière et al. 2012; Allard et al. 2017; Stewart et al. 2020).

We described the timing of gonadal recrudescence by modeling temporal changes in the probabilities of the presence of gametes (eggs and mature sperm separately) and average egg diameter in each population (Figure 5). Western Pearlshells produce annual gametogenic cycles (Toy 1998). At the beginning of the gametogenic cycle, mussels first develop precursor gonadal tissue that ultimately matures into ovaries or testes (Lasiak 1986; Juhel et al. 2003; El-Deeb et al. 2018; Khafage et al. 2019). Next, immature gametes will appear in the ovaries and testes, and eventually increase in density and maturity (Galbraith and Vaughn 2009; Tsakiris et al. 2016). Accordingly, the probability of the gamete presence in gonad biopsies should increase as gonadal

recrudescence is occurring. Specifically, gonadal recrudescence peaks on the date at which the first derivative of the binomial regression model describing gonadal gamete presence is at the maximum value (See "GR Peak" on Figure 5; Melaas et al. 2013; Li et al. 2017). Gonadal recrudescence starts on the date in which the derivative is at the half-maximum (See "GR Onset" on Figure 5; Melaas et al. 2013; Li et al. 2017). The difference between the maximum probability of gamete presence and the minimum probability is considered the magnitude of the event (i.e., proportion of the population that experienced gonadal recrudescence) (White et al. 1997; Liu et al. 2016; Li et al. 2017). The model for the probability of gonadal egg presence does not describe the end of gonadal recrudescence because eggs may still be maturing as the probability reaches maximum. Because eggs increase in diameter as they mature, we used the average egg diameter of the biopsies to describe egg maturity during gonadal recrudescence (Galbraith and Vaughn 2009; Tsakiris et al. 2016). Mature eggs of Western Pearlshells are about 100 μm in diameter (Murphy 1942). The average egg diameter should drop drastically from the time spawning ends to the beginning of ovarian recrudescence (Tsakiris et al. 2016) because mature eggs are eventually replaced with immature eggs between gametogenic cycles. As eggs mature, the average egg diameter continues to increase until reaching a maximum (Galbraith and Vaughn 2009; Tsakiris et al. 2016).

We determined the timing of spawning by modeling the temporal changes in the probabilities of the presence of gametes in each population (Figure 5). Densities of mature eggs or sperm are high in sexually mature mussels before spawning occurs. The densities of gonadal eggs and sperm decline upon spawning (Galbraith and Vaughn 2009; Tsakiris et al. 2016) because eggs are moved to the marsupial gills for fertilization and sperm is broadcast into the water column. The gametogenic cycle ends after spawning when the gonadal tissues are resorbed

in situ and replaced with connective tissue (Bayne et al. 1978; Dorange and Le Pennec 1989; Toy 1998; Grande et al. 2000; Çek and Şereflişan 2006; Bignell et al. 2008; Lima et al. 2012). Accordingly, the probability of gamete presence in gonad biopsies should be high before spawning, decrease as spawning occurs, and nonexistent as gonadal tissue degrades. Specifically, spawning peaks on the date at which the first derivative (i.e., change rate of curvature) of the binomial regression model describing gonadal egg presence is at the minimum value (See "SP Peak" on Figure 5; Melaas et al. 2013; Li et al. 2017). Spawning ends on the date in which the derivative is at the half-minimum; the number of days from event peak to end is subtracted from the peak date to determine when the event starts (See "SP End" and "SP Onset" on Figure 5; Melaas et al. 2013; Li et al. 2017) and the duration of the event. The probabilities should remain low or near zero after the population is largely spawned-out and the gametogenic cycle has ended. The difference between the maximum probability of the gonadal egg presence (pre-spawning) and the minimum probability is considered the magnitude of the event (i.e., the proportion of the sampled population that spawned) (White et al. 1997; Liu et al. 2016; Li et al. 2017).

Brooding. The brooding period was defined by temporal changes in the probability of a mussel brooding in each population (Figure 6). We determined if Western Pearlshells were brooding by gently prying mussels open and examining them for the presence of swollen, opaque gills, which indicate that the mussel is brooding (Spring Rivers 2007; Scheder et al. 2011). The brooding period was described by modeling the probability of a mussel brooding through time using the methods described for spawning and gonad recrudescence. The brooding period of a population starts and ends on the dates at which the first derivative of the binomial regression

model is at maximum and minimum values, respectively (See "Onset" and "End" on Figure 6; White et al. 1997; Stockli and Vidale 2004; Fisher et al. 2006; Melaas et al. 2013; Li et al. 2017). The duration of the brooding period is defined as the timeframe between the maximum and minimum derivatives (Stockli and Vidale 2004; Fisher et al. 2006; Li et al. 2017). Brooding peaks when the probability of a mussel brooding is greatest (See "Peak" on Figure 6; Liu et al. 2016; Li et al. 2017; Franzem et al. 2019; Taylor 2019) and the difference between the highest and lowest probabilities is considered the magnitude of the event (i.e., the proportion of the population that were brooding) (White et al. 1997; Liu et al. 2016; Li et al. 2017). We can obtain a holistic view of the reproductive cycle of Western Pearlshells by modeling sequential reproductive events for a population (Denny et al. 2014; Taylor 2019; Thorne et al. 2006; van der Valk 2011), such as spawning, brooding, larval infestation, and gonadal recrudescence. The probability of brooding should increase as the probability of gonadal egg presence decreases, indicating eggs are being moved from the gonad to the marsupium gills (Figure 7). We also compared the probabilities of brooding in each population between 2019 and 2020 to explore annual differences.

Embryogenesis. Embryogenesis was characterized by tracking the progression of embryo development in each population. Embryos were extracted from the marsupium of brooding mussels using a similar biopsy technique as used for collecting gametes (Tsakiris et al. 2016; Barnhart 2015; Scheder et al. 2011). We inserted the needle into the inflated area of the gill and pulled back the syringe until we saw eggs or embryos (opaque pink-yellow clumps) in the syringe. The biopsies were placed in cryotubes with 10% neutral buffered formalin and kept cold until processing. We quantified the proportions of each biopsy that was made up of unfertilized

eggs and embryos in early (cleavage, blastula, gastrula), middle (adductor forming, ventral cilia), and late (ventral gape, larvae) developmental stages by examining the contents of a portion (50 – 100 eggs or embryos) of each biopsy at 10 or 20× with a compound microscope (Barnhart 2015; Scheder et al. 2011). We then averaged the proportions of each biopsy across all samples collected on the same date from each population (Jones et al. 1986; Gascho et al. 2016; Nielsen et al. 2017; Walker 2017). Tracking embryogenesis at a population level provides insights on the general timing of fertilization and duration of embryogenesis because margaritiferids tend to be synchronous in spawning (Bauer 1979; Hastie and Young 2003).

Three marked individuals from the coldest (West Fork Rock) and warmest (Clam) creeks were checked weekly for brooding to define the duration of embryogenesis at an individual level. When brooding, marsupium samples were collected to track the predominant embryo development stage present through time. We calculated degree days (as described in the Temperature section) from the date at which mussels were first brooding to the date at which the embryos were in late developmental stages (Scheder et al. 2011). Gonadal biopsies were also collected from these individuals to determine if gonadal gametes were absent at the start of brooding and to validate our methods for using the gonadal biopsies to determine the timing of spawning. Finally, we continued to check individuals for brooding weekly, even after brooding ended, to determine if double brooding (producing two broods in one season) occurred. These six individuals were excluded from all other analyses because of the lack of independence among sampling dates.

Larval Release. The timing of larval release was described by documenting overlapping reproductive events indicative of larval release. We attempted to describe the timing of larval

release by describing temporal changes in the presence of mussel larvae in stream drift; however, our attempt was deemed unsuccessful when a subset of samples likely to contain larvae did not. Alternatively, the timing of larval release by a mussel population was inferred by documenting overlap among declining brooding, presence of larvae in marsupium samples, and fish newly infested with larvae.

Larval Infestation of Hosts. The larval infestation period was described by capturing fish near the mussel beds and examining them for the presence of mussel larvae. Fish were captured within 100 to 400 m of mussel beds (Kneeland and Rhymer 2008; Reid et al. 2013) using a Smith-Root LR-20 Series backpack electrofisher. Salmonids were anesthetized, measured (total length, mm), weighed (g), and identified to species. Bull Trout were released immediately without processing because of their status as a threatened species. We examined the anterior hemibranchs of their wetted gills for the presence of larvae using binocular glasses (12×) and a flashlight (Hastie and Young 2001; Martel and Lauzon-Guay 2005; Kneeland and Rhymer 2008; Reid et al. 2013; Salonen et al. 2017a; Filipsson et al. 2017; Clements et al. 2018). Each fish was classified as infested with larvae or not. Fifty-five fish were lethally sampled throughout the season to verify larval presence under a Leica DM 1000 LED clinical microscope (4× or 10×). Fish gills were preserved in vials with 10% neutral buffered formalin. We submerged their gills in water in a petri dish and measured the lengths of the first 30 larvae or juvenile mussels observed using a Leica ICC50W camera and Leica LAS EZ software. Mean larval length was calculated by collection date for each mussel population after checking for independence among fish (Murphy 1942; Karna and Milleman 1978; Salonen 2016; Salonen et al. 2017a).

The timing and duration of larval infestation of host fish by each mussel population was determined by modeling the temporal changes in the binomial probability of a salmonid being infested with larvae (Figure 6). The methods used to fit binomial regression models and define important reproductive time points for brooding were also used to describe infestation. The probability of infestation should start and subsequently increase when the probability of brooding starts to decrease (Figure 7).

Temporal changes in mean lengths of larvae infesting fish were also used to describe the infestation period. Attached Western Pearlshell larvae increase in size as they develop from primary to secondary larvae and metamorphose into juvenile mussels (Murphy 1942; Karna and Millemann 1978; Salonen 2016; Salonen et al. 2017a). Encapsulated juvenile mussels (not larvae) should be present on fish near the end of the infestation period. Encapsulated juvenile mussels can be distinguished from larvae by the presence of adductor mussels (Araujo et al. 2002) and their lengths (Karna and Millemann 1978). Western Pearlshell larvae first attach to their hosts at about 70 µm in length and grow > 500% while attached (Murphy 1942; Karna and Millemann 1978; Allard et al. 2013; O'Brien et al. 2013; Scheder et al. 2014) and metamorphose at about 240 µm in length (Karna and Millemann 1978).

Temperature. Minimal threshold water temperatures and degree days were calculated to relate temperature to the timing of reproductive events. Water temperatures at the mussel populations were recorded hourly using Onset Hobo Pendant Data Loggers from spring 2019 to autumn 2020. The average daily temperatures on the date at which reproductive events peaked were considered the minimum threshold temperatures (Hastie and Young 2003; Galbraith and Vaughn 2009; O'Brien et al. 2013; Gascho Landis et al. 2016; Allard et al. 2017; Franzem et al.

2019). Degree days (DD) were calculated as the sum of daily mean water temperatures above 0°C from the date at which brooding peaked in 2019 to the date brooding peaked in 2020 for each population (Toy 1998; Hastie and Young 2003; Galbraith and Vaughn 2009; Allard et al. 2017; Franzem et al. 2019). Degree days and the average temperatures during the durations of brooding and infestation were also reported (Murphy 1942; Karna and Millemann 1978; Scheder et al. 2011). Missing hourly water temperatures were estimated using the linear relationship between air and water temperatures at each stream (Figure 8). All missing water temperatures were within the temperature range (5°C to 25°C) in which linear regression can be adequately used to model the relationship between air and water temperature (Mohseni and Stefan 1999; Erickson and Stefan 2000; Caissie et al. 2004; Chen and Fang 2015). Hourly air temperatures were obtained from the closest NRCS SNOTEL sites to the mussel populations: Mule Creek in Beaverhead County and Peterson Meadows in Granite County (NRCS 2020). We excluded water temperatures outside the temperature range of 5°C to 25°C and incorporated time lag, the delayed response of water temperature to air temperature, before modeling the linear relationship between air and water temperatures. Correcting for time lag can improve the temperature correlation at an hourly scale (Preud'homme and Stefan 1993; Webb and Nobilis 1995). The time lag for each stream was calculated as the average difference in the number of hours between the times in which air and water temperatures reached their daily maximum temperature (Stefan and Preud'homme 1993; Chen and Fang 2015). We used data from the SNOTEL station that produced the model with the highest r^2 value for each stream. We related temperature to phenological events by comparing the degree days of reproductive events among populations. Binomial regression models for the presence of gonadal eggs, brooding, and larval infestation were modeled on degree days for each population for comparisons.

Host Fish

The data collected for determining the timing and duration of larval infestation were used in the analyses for identifying the host fishes of Western Pearlshell populations. Fish captured outside of the infestation period (defined by the probability of infestation models) were excluded from the host fish analyses. Fish > 250 mm in total length were also excluded because we could not differentiate between clubbing of the lamellae (Futish and Millemann 1978; Karna and Millemann 1978) and encapsulated larvae on larger fish. Additionally, these larger fish had probably been exposed to mussel larvae in prior years and were therefore immune to infestation (Rogers and Dimrock 2003; Dodd et al. 2006; Rogers-Lowery et al. 2007).

The fish hosts of Western Pearlshells in Montana were determined by estimating the probability of larval infestation of each salmonid species. Whereas prevalence (the percentage of fish infested) is commonly used to describe host species (Zale and Neves 1982; Neves and Widlak 1988; Jansen 1991; Weiss and Layzer 1995; Martel and Lauzon-Guay 2005), we chose to use the probabilities of infestation because they allowed us to make statistical comparisons among species. The probability of infestation was modeled by fitting a GLM to the binomial data of whether a captured fish was infested with mussel larvae or not (Venables and Dichmont 2004; Šlapanský et al. 2016; Haraldstad et al. 2018). The first probability model included all variables (fish species, stream, fish length, date, etc.) and an analysis of variance using the Wald chi-square test was calculated to verify that fish species was the most significant variable for the probability of infestation. Next, the probabilities of infestation, and 95% confidence intervals, were calculated on fish species alone using a GLM for binomial data and the predict() function in R version 4.0.2. Finally, we tested for significant differences in the probabilities of infestation across fish species by using the Wald test to test combinations of two species at a time. The

Wald test was performed by testing for significant differences between two model coefficients (representing two species) while constraining the other three (essentially setting the other three species to zero). These same methods were also used to predict and assess the probabilities of larval infestation across streams.

Infestation intensities were compared among salmonid species to determine the relative efficiencies of examined host species in carrying larvae (Karna and Millemann 1978; Martel and Lauzon-Guay 2005; Klunzinger et al. 2012; Ieshko et al. 2016; Šlapanský et al. 2016; Marwaha 2020). While handling captured fish, the number of larvae present on the anterior side of the gill filaments under one operculum was counted using a tally counter (Hastie and Young 2001; Martel and Lauzon-Guay 2005; Scheder et al. 2014; Reid et al. 2013; Salonen 2016; Clements et al. 2018). Numbers of larvae under both opercula were counted for 40% of lethally sampled fish to verify that no significant differences existed in numbers of larvae between sides (Meyers and Millemann 1977; Karna 1972; Scheder et al. 2014). Larvae were counted on lethally sampled fish before euthanizing fish to keep methods consistent. We expected to underestimate the numbers of larvae in the field because we only examined the anterior side of gill filaments of the gills (Salonen et al. 2017a; Clements et al. 2018) and therefore counted the number of larvae on lethally sampled fish microscopically to estimate error and correct our estimates made in the field. Larvae on lethally sampled fish were counted under a compound microscope (4× or 10×) using a tally counter (Martel and Lauzon-Guay 2005; Hastie and Young 2001; Salonen et al. 2017a). The linear relationship between the estimated and actual numbers of larvae was used to correct our estimates. We tested for significant differences in numbers of larvae between left and right sides of the fish using a paired Wilcoxon matched-pairs test. The infestation intensities per fish were calculated by doubling the estimated number of larvae because estimates represented

the number of larvae under one operculum. The Kruskal Wallis test was used to test for significant differences in infestation intensities among fish species and streams (Martel and Lauzon-Guay 2005; Klunzinger et al. 2012; Marwaha 2020). Nonparametric statistical tests were used because infestation intensities were not normally distributed (Shapiro-Wilk normality test; $W = 0.64$, P-value < 0.001). The Dunn's test was performed post-hoc to make multiple comparisons of infestation intensities among fish species.

We compared length frequencies of larvae among fish species to explore differences in metamorphic success. Host species can sometimes be inferred from natural infestations of larvae on fish (Blažek and Gelnar, 2006; Österling 2011; Levine et al. 2012) but larval presence and numbers are not indicators of metamorphic success because incompatible hosts can slough off larvae days to weeks after attachment (Meyers and Millemann 1977; Karna and Millemann 1978; Rogers and Dimock 2003; Dodd et al. 2005; Rogers-Lowery and Dimrock 2006). Similar to most other margaritiferids and a few unionids (Barnhart et al. 2008), Western Pearlshell larvae grow > 500% in length while attached to hosts and metamorphosis occurs at about 240 µm in length (Murphy 1942; Karna and Millemann 1978; Scheder et al. 2014). If fish slough off larvae before metamorphosis, larval lengths (instead of presence or numbers) may be more indicative of metamorphic success when counting recovered juvenile mussels is not an option. We therefore compared length frequencies of larvae infesting Brook Trout and Westslope Cutthroat Trout using the Wilcoxon rank sum test. We expected larval lengths to be skewed towards smaller sizes on Brook Trout than on Westslope Cutthroat Trout if Brook Trout were sloughing off larvae before metamorphosis could occur.

We examined acquired immunity of hosts by testing for relationships between fish lengths and infestation intensities and larval lengths. Host fish become progressively resistant to

larval infestation (Hastie and Young 2001; Rogers and Dimock 2003; Thomas et al. 2014) and therefore, we expected larger fish to be less susceptible to infestation. We tested for a linear relationship between fish lengths and infestation intensities (Hastie and Young 2001; Thomas et al. 2014; Clements et al. 2018). We also tested for a relationship between mean larval lengths on individual fish and fish lengths. The range of larval lengths would increase as a function of fish lengths if older fish with acquired immunity sloughed off larvae before metamorphosis creating a wedge-shaped pattern of larval lengths relative to fish lengths. We therefore estimated the 90th quantile regression for mean larval lengths on fish lengths to determine fish lengths that may be limiting to larval growth (Terrell et al. 1996; Cade et al. 2008; Ranney 2018).

Results

Life History Traits

Sexual Maturity. Mussels reached sexual maturity at an estimated 34 mm in length and 11.5 years of age. Mussels examined histologically varied from 22 to 87 mm in length. The probability of sexual maturity reached 50% at 33.4 mm in length (95% CI = 27.7 – 39.0 mm; *G*-test = 24.64, df = 1, $P < 0.001$; Figure 9A). Mussels were an estimated 11.5 years old (95% CI = 9.7 – 13.2 years) at 33.4 mm in length ($r^2 = 0.58$; $F = 8.30$, df = 1, 6, $P = 0.028$; Figure 9B). All aged individuals < 11 years old were sexually immature ($n = 6$) and those ≥ 11 years old were sexually mature ($n = 2$). One individual < 33.4 mm in length was sexually mature; however, this mussel was 11.5 years old, came from the coldest site (West Fork Rock Creek), and was smaller than other mussels for its age (Figure 9B). Mussels from West Fork Rock Creek were smaller at age than expected from the linear relationship, suggesting that growth rates may vary among streams, which could result in differences in length at sexual maturity.

Hermaphroditism. A high incidence of hermaphroditism occurred in the mussels that were examined histologically (Figure 10). Seventy-four percent (n = 29) were hermaphrodites, one (3%) was female, and none were male. The single female had precursor gonadal tissue in addition to ovaries, which is indicative of new gonadal development and cannot be differentiated as ovaries or testes; we therefore could not rule out that it could be hermaphroditic. Three (8%) individuals (50 – 75 mm in length) only had undifferentiated precursor tissue present and therefore could not be sexed; six (15%; 22 – 33 mm in length) lacked gonadal tissue entirely indicating that they were sexually immature juveniles. One mussel was excluded from analysis because of contamination.

The gonadal biopsies were inadequate for determination of sex of hermaphroditic mussels because most of their tissue was ovarian. About 95% of the gonadal tissue of the hermaphrodites was ovary and only about 5% was testes (Figure 11). The biopsies therefore misclassified 86% (n = 25) of hermaphrodites as either females (45%) or lacking gametes (41%; Figure 10). The high incidence of missing eggs in ostensibly sexually mature mussels was caused by their post-spawn reproductive status at the time of sample collection and indicated that egg absence was a valid measure of spawning phenology. Some mussels were charging (eggs present in gonadal ducts), brooding (embryos present in marsupial gills), or spawned-out (ceroid present in hemocytes) at the time of collection.

Reproductive Phenology

Gonadal Recrudescence. Gonadal recrudescence started in mid- to late summer in the four mussel populations as judged by gonadal biopsies (Table 4). The low probabilities of the presence of gonadal eggs and sperm coupled with the large drops in mean egg diameters

indicated mussels were between gametogenic cycles for about a month, generally from early June to early July (Figure 12). The subsequent increases in mean egg diameters and probabilities of the presence of gonadal eggs and sperm in all populations indicated gonadal recrudescence occurred from late June to mid-July. Immature eggs were first observed in mid-July in gonadal biopsies of Clam Creek (mean diameter = 41 µm), Upper Willow Creek (mean diameter = 36 µm), and Deep Creek (mean diameter = 38 µm) mussels and in early August in West Fork Rock Creek mussels (mean diameter = 49 µm). Mean egg diameters had increased to 63 to 82 µm by late August and early September, indicating that eggs probably matured before winter. The probabilities of the presence of eggs (93 – 100%) and mature sperm (15 – 31%) were greatest in each population at the end of sampling in late August and early September (Figures 13 – 16). All gonad biopsies that contained sperm in August and September also contained eggs.

Spawning. Gonadal biopsy results showed mussel populations spawned between late spring and early summer, depending on the population (Table 4). The decrease in the probability of the presence of gonadal eggs, coupled with the decrease in mean egg diameter, indicated that mussels were spawning (Figure 12). The population of mussels in Clam Creek was the first of the studied populations to spawn. The probability of the presence of gonadal eggs in biopsies was 14% in early May and dropped to 1% shortly thereafter, indicating that few mussels were still spawning in early May (Figure 12A; Table 4). The lack of gonadal eggs present in biopsies agreed with the histological results that most Clam Creek mussels collected in early and mid-May were either partially or fully spawned out. The Upper Willow Creek population spawned second from early to late May (Figure 12B; Table 4). Spawning peaked mid-May and was about 21 days in duration. The Deep Creek population spawned from mid- to late May (Figure 12C;

Table 4). Spawning peaked in late May and lasted 14 days in duration. The West Fork Rock Creek population spawned last in mid-June (Figure 12D; Table 4). Spawning peaked mid-June and the probability of presence of gonadal eggs decreased from 57% to 26% in five days. Three of the five West Fork Rock Creek mussels collected for histology in mid-June were partially spawned out; two had not yet spawned.

Sperm was released in West Fork Rock Creek during an eight-day period in mid-June; during which time the probability of the presence of gonadal sperm decreased from 31% to 9% (Figure 16; Table 4). The probabilities of the presence of gonadal sperm in Clam Creek and Upper Willow Creek mussels were lowest at the start of sampling and therefore indicated sperm release had already occurred. The presence of gonadal sperm in the Deep Creek mussel biopsies was sporadic throughout the entire sampling period; we were therefore unable to model the probability of the presence of sperm for this population.

Brooding. Brooding occurred for about one month in each mussel population sometime from late spring to mid-summer (Table 4). As expected, brooding peaked among populations in the same order as spawning. Clam Creek mussels brooded throughout the month of May (Figure 13). The probability of brooding was greatest (71%) in early May, at the start of sampling (2,537 annual degree-days), when eggs were absent from gonadal biopsies. Brooding ended in late May. The Upper Willow Creek population was brooding at the start of sampling in mid-May (Figure 14). Brooding peaked mid-May (79% probability) and ended in early June. The probability of the presence of gonadal eggs was 60% when brooding peaked, possibly because individuals had not fully spawned yet. The Deep Creek population brooded from mid-May to mid-June (Figure 15). Brooding peaked at 58% in late May (1,691 annual degree-days) coinciding with a low and

decreasing probability of the presence of gonadal eggs (48%). Brooding duration was 39 days (301 DD). The West Fork Rock Creek population brooded from mid-June to early July (Figure 16). The probability of brooding peaked at 87% in late June (1,286 annual degree-days), also coinciding with a 48% probability of the presence of gonadal eggs. Brooding lasted for an estimated 25 days (206 DD).

Brooding phenologies of some populations differed between 2019 and 2020 (Table 4; Figure 17). Mussels brooded earlier in Clam Creek (35 days earlier) and Upper Willow Creek (29 days) in 2020 than in 2019. Deep Creek mussels brooded about a week earlier in 2020 than in 2019. The brooding periods of the Clam Creek and Deep Creek populations were longer and lower in magnitude in 2020 than in 2019, suggesting that brooding was less synchronous among individuals in 2020. Brooding by the West Fork Rock Creek population peaked on the exact same day of year in both years. Interestingly, water temperatures were slightly cooler in 2020 than in 2019; the average monthly water temperatures for June were cooler by 1.4°C (Clam Creek), 0.9°C (Deep Creek), and 0.3°C (West Fork Rock Creek) in 2020 than 2019.

Embryogenesis. The transition of unfertilized eggs to embryos in the marsupium biopsies indicated fertilization occurred prior to early May in Clam Creek (Figure 18A), prior to mid-May in Upper Willow Creek (Figure 18B), in early to mid-May in Deep Creek (Figure 18C), and in mid-June in West Fork Rock Creek (Figure 18D). Fertilization appeared to occur before brooding peaked in all mussel populations. The timing of fertilization in West Fork Rock Creek mussels coincided with the timing of sperm release; brooded eggs transitioned to embryos in the same week that the probability of the presence of gonadal sperm decreased.

Embryogenesis was asynchronous among individual Clam Creek mussels but was synchronous and rapid in the other three mussel populations. Developmental stages of embryos brooded by Clam Creek mussels varied by date and did not clearly progress from early to late stages (Figure 18A). Also, late-stage embryos were present on almost every sampling date, suggesting that embryogenesis was asynchronous among individuals. Clam Creek mussels may also have been dribble spawning, in which individuals release relatively small amounts of gametes at a time (Figure 19). The succession from unfertilized eggs to late-stage embryos brooded by Upper Willow Creek, Deep Creek, and West Fork Rock Creek mussels indicated embryo development was fairly synchronous within each population (Figure 18). Predominantly early-stage embryos were present in marsupia of Upper Willow Creek mussels in mid-May (when the probability of brooding was greatest) and early June. Late-stage embryos first appeared in early June and increased in prevalence until mid-June, shortly after brooding had mostly ended. Predominantly early-stage embryos were present in marsupia of Deep Creek mussels from mid-May to late May. Late-stage embryos first appeared and were predominant after brooding peaked. Most embryos brooded by West Fork Rock Creek mussels were in early developmental stages until late June after brooding had peaked. Late-stage embryos were first brooded by mussels in early July and became predominant two days prior to the end of brooding.

Brooding and embryogenesis were synchronous among marked individuals in West Fork Rock Creek but not in Clam Creek (Table 5). The progression of embryo development in the three tracked individuals in West Fork Rock Creek agreed with the timing of embryogenesis at the population level. Two individuals brooded unfertilized eggs the same week in Mid-June and the embryos progressed to early, middle, and late-stages every subsequent week. The third mussel had gonadal eggs present in mid-June, started brooding unfertilized eggs one week after

the other two marked individuals started brooding, and aborted early-stage embryos the following week. Evidence of individual variation in brooding phenology was observed among the three marked individuals in Clam Creek; one individual appeared to have recently released larvae at the start of sampling and embryos brooded by the other two individuals were in different developmental stages throughout sampling. One of the marked mussels brooded early-stage embryos on two occasions seven days apart, which could indicate dribble spawning.

Embryos developed faster at warmer temperatures (Table 5). Clam Creek embryos matured from early to late developmental stages in seven days (77 DD) in one individual and from middle to late developmental stages in seven days (56 DD) in another. West Fork Rock Creek embryos matured from unfertilized eggs to mature embryos in 18 days (159 DD). In comparison, West Fork Rock Creek embryos took six days longer (+ 45 DD) to mature from early to late stages than Clam Creek embryos.

Larval Release. Larval release probably occurred sometime in May, June, and July, depending on the mussel population. Larvae were not present in the processed plankton samples (n = 4), but the timing of larval release was inferred by the presence of larvae in the marsupia of mussels and the coinciding decreases in probabilities of brooding. Larvae were probably released throughout May and June in Clam Creek; larvae were present in marsupia of mussels during this time, though brooding had largely ended by late May. Larval release of Upper Willow Creek mussels probably occurred from early to mid-June. Mussels exclusively brooded larvae in mid-June when the probability of brooding had decreased to 14%. Larvae were probably released from late May to early July in Deep Creek. Larvae were first brooded by mussels late May and generally increased in presence throughout June into early July, but brooding had largely ended

by mid-June. Larval release of West Fork Rock Creek mussels probably occurred briefly from early to mid-July; larvae were present in marsupia of mussels during the first half of July, though brooding had largely ended early July.

Larval Infestation of Hosts. Larvae infested Clam Creek salmonids from mid-May to late July (Figure 20A; Table 4); however, larvae may have been sloughed off before metamorphosis during the first half of the infestation period. Infestation started four days after brooding peaked in May (Figure 13) and the probability of infestation peaked at 78% in late June. Attached larvae did not increase in length on average until early July, even though infestation started more than a month earlier. The lack of increase in mean larval length on Clam Creek salmonids indicated that larvae were probably being sloughed off fish before metamorphosis. The increase in the average larval length first seen in early July may be a result of age-0 trout emerging in late June. Attached larvae on average grew from 80 to 300 µm from early to late July (21 days). Infestation ended in late July after 71 days (808 DD).

Larval infestation of salmonids in the other three streams generally occurred in July (Table 4). Larvae infested Upper Willow Creek salmonids sometime before late May; high water flows prevented us from capturing fish until 35 days after brooding ended (Figure 14). The probability of infestation was at 71% and the mean larval length was 262 µm at the start of sampling in early July (Figure 20B). Larval infestation ended late July, at which time the mean larval length was 372 µm. The infestation period was a minimum of 32 days in duration. Larvae infested Deep Creek salmonids from mid-June to late July and infestation lasted an estimated 47 days (515 DD; Table 4). Larval infestation started 10 days after brooding peaked (Figure 15). The probability of infestation peaked at 74% in early July, two weeks after brooding ended. The

mean larval length increased from 82 µm in late June to 290 µm in mid-July (Figure 20C). Larvae infested West Fork Rock Creek salmonids starting sometime before late July; high water flows preventing us from capturing fish earlier than late July, 15 days after brooding had ended (Figure 16). In late July, the probability of infestation was 91% and the mean larval length was 95 µm (Figure 20D). The mean larval length increased rapidly to 362 µm by mid-August. Larval infestation ended in late August and was a minimum of 29 days in duration (339 DD; Table 4).

Temperature. Water temperature regimes of Clam, Deep, and West Fork Rock Creeks differed (Figure 8). Mean water temperatures during the study period were 8.9°C (range = 0.9 – 23.6) in Clam Creek, 6.3°C (range = -0.2 – 21.6) in Deep Creek, and 4.8°C (range = 0.0 – 16.8) in West Fork Rock Creek. Degree days at Upper Willow Creek could not be calculated because temperature data was only obtained there from May 29, 2019 to October 7, 2019; however, the mean water temperature of Upper Willow Creek was slightly cooler (- 0.4°C) than that of Clam Creek and slightly warmer (+ 0.3°C) than that of Deep Creek during this same timeframe. Degree days were highly correlated with day of year at Deep (r = 0.89) and West Fork Rock (r = 0.86) creeks except during winter months when water temperatures were near 0°C. Degree days at Clam Creek were highly correlated with day of year (r = 0.93) year-round due to warmer winter water temperatures associated with groundwater influence.

A minimum threshold temperature may have influenced the timing of spawning in some populations. The average daily temperatures when spawning peaked were similar between Deep Creek (6.8°C) and West Fork Rock Creek (6.5°C; Table 4), though possibly coincidental. Deep Creek and West Fork Rock Creek first reached these temperatures six and eight days prior to peak spawning, respectively, indicating that 6.5°C may be a minimal threshold temperature for

this reproductive event (Figure 21). The temperatures at the time of spawning were unknown for Clam and Upper Willow creeks; however, Clam Creek mussels were first exposed to an average daily water temperature of 6.5°C in 2020 in early March, two months prior to finding most mussels were already spawned out. The average daily temperatures when larval infestation peaked were similar (10.4 – 10.9°C) among three of the streams (Table 4); however, they first reached these average daily temperatures from one day to one month earlier, indicating that these temperatures are probably not a minimal threshold temperature for infestation. The average daily temperatures at the time of brooding and gonadal recrudescence varied greatly among populations.

Reproductive phenologies among populations varied less on day of year than on degree days (Table 4; Figure 22). Brooding peaked among populations between 130 – 176 days of year (range = 45 days) or 1,286 – 2,537 annual degree-days (range = 1,251 annual degree-days). Larval infestation peaked among populations within a 25-day range (271 to 481 degree-days from 2020 brooding), and the presence of immature eggs peaked among populations within a 19-day range (648 to 1,172 degree-days from 2020 brooding). The degree days for Clam Creek reproductive events are roughly double of those for West Fork Rock Creek, indicating streams may have different temperature requirements, or photoperiod or other factors beyond degree days may influence reproductive events.

Host Fish

Brook Trout and Westslope Cutthroat Trout were the fish species most likely to be infested by mussel larvae (Figure 23A). Fish species was the most significant variable for the probability of infestation (df = 4, $P < 0.001$). The estimated probabilities of infestation were

greatest for Brook Trout (77%), followed by Westslope Cutthroat Trout (71%), Brown Trout (52%), Rainbow Trout (17%), and Mountain Whitefish (11%). All pairwise probabilities among fish species differed, except for those between Brook Trout and Westslope Cutthroat Trout and Rainbow Trout and Mountain Whitefish (Table 6). We documented juvenile mussels (determined by their length and the presence of adductor muscles) on several Westslope Cutthroat Trout, Brook Trout, and Brown Trout, one Rainbow Trout, and one Mountain Whitefish (Figure 24). One of two Bull Trout unintentionally processed was infested. The probabilities of infestation of any salmonid were from 55% to 72% across the four streams (Figure 23B). Salmonids in Upper Willow Creek were less likely to be infested than those in Clam or West Fork Rock creeks (Table 6). No other significant differences in the probabilities of infestation among streams existed (Table 6).

Infestation intensities were higher on Westslope Cutthroat Trout and Brook Trout than on the other species examined. As expected, the number of larvae on an individual fish (underneath one operculum) was underestimated in the field ($r^2 = 0.82$; $F = 242$, df = 1, 54, $P < 0.001$; Figure 25) and did not differ between the left and right sides of the fish (Wilcoxon matched-pairs test: $P = 0.73$). Infestation intensities differed among fish species (Kruskal Wallis test: $\chi^2 = 53.6$, df = 4, $P < 0.001$), but not stream ($\chi^2 = 0.2$, df = 3, $P = 0.97$; Figure 26). The estimated infestation intensities were ranked greatest among Westslope Cutthroat Trout (median = 76 larvae, maximum = 1,034) and Brook Trout (median = 42 larvae, maximum = 1,630), intermediate among Brown Trout (median = 26, maximum = 326), and lowest among Rainbow Trout (median = 0, maximum = 116) and Mountain Whitefish (median = 0, maximum = 26; Table 6). One Bull Trout was infested with an estimated 62 larvae and the other had none.

The difference in larval length distributions between fish species throughout the infestation period (Wilcoxon rank-sum test: $W = 139{,}860$, $P < 0.001$) may indicate Brook Trout were sloughing off most larvae before metamorphosis could occur (Figure 27). The lengths of larvae attached on Brook Trout were negatively skewed towards larval lengths at which they first attach on fish (median = 77 µm). In comparison, the lengths of larvae attached on Westslope Cutthroat Trout were more uniformly distributed from lengths at attachment to lengths at drop-off (median = 288 µm).

The relationship between acquired immunity of hosts and fish lengths was unclear. No linear relationship existed between fish lengths and numbers ($r^2 = 0.01$, $F = 2.48$, df = 1, 326, $P = 0.12$; Figure 28A); however, the negative relationship between fish lengths and mean larval lengths (90^{th} quantile goodness of fit = 0.21, df = 43, $P < 0.01$) may indicate that older fish were sloughing off newly attached larvae (Figure 28B). The 90^{th} percentile of larval lengths at metamorphosis (240 µm) were limited to fish < 166 mm in total length. The 90^{th} percentile of larval lengths greater than the size at which they first attach on fish (86 µm) were limited to fish < 232 mm in total length.

Discussion

Montana Western Pearlshells matured at similar ages and lengths as populations elsewhere. Our estimates of age (11.5 years) and length (34 mm) at maturity were similar to those estimated for Western Pearlshells in two streams in Washington (Toy 1998) and one stream in California (Murphy 1942). Western Pearlshells in the Truckee River in California appeared to become mature at 32 mm in length (Murphy 1942). On average, mussels in Washington matured between nine and 12 years of age and 46 to 49 mm in length there (Toy

1998). Male Western Pearlshells in those streams matured one to two years earlier than females but could be mature at ages as young as 6 years and at 37 mm in length (Toy 1998). Males of many mollusk species commonly mature earlier than females (Coe 1943). However, we found no males in western Montana. Females from the Washington streams matured at about 12 years of age, similar to the hermaphrodites in our study. In contrast, Western Pearlshells in British Columbia matured at age five (Fung et al. 2016), but this estimate was based on the age at which the growth rate first slowed and not on the presence of gonads. Water temperatures and other environmental factors can affect margaritiferid growth rates (Toy 1998; Hastie et al. 2000; Fung et al. 2016) and mussels with higher growth rates may mature at younger ages (Toy 1998).

Information gained in our study about reproductive lifespan and fertilization success may indicate reproductive output is probably not a limiting factor for declining Western Pearlshell populations in Montana. Much the same as Western Pearlshells in California (Murphy 1942) and *M. margaritifera* (Bauer 1987; Bauer 1992), Montana Western Pearlshells did not appear to senesce as they age; many large (> 90 mm in length) mussels contained gonadal eggs, including our largest and presumably oldest mussels (96 mm in length, estimated 90 years of age; D. Stagliano, unpublished data). Unfertilized eggs were a rare occurrence (< 1% of marsupia contents) on the last dates of brooding, indicating a high incidence of fertilization. Additionally, even mussels in populations with low densities ($< 0.1/m^2$) were brooding embryos, not unfertilized eggs, in 2019. Western Pearlshells appear to have a substantial reproductive output based on their long reproductive lifespan, successful fertilization of eggs, and the several million larvae per year that Margaritiferids typically produce (Murphy 1942; Young and Williams 1984 cited in Geist 2010).

Gonadal biopsies can be a nonlethal method for determining sex of monomorphic mussels (Saha and Layzer 2008; Tsakiris et al. 2016) but were inaccurate for identifying hermaphroditic Western Pearlshells in Montana. We detected sperm in only 13% of the biopsies collected from individuals histologically determined to be hermaphrodites because of the preponderance of ovaries to testes. The chaotic distribution of male and female gonadal tissues in hermaphroditic Margaritiferids (Grande et al. 2000) also decreases the reliability of gonadal biopsies. However, identification of hermaphroditic Western Pearlshells in western Washington using biopsies may be possible because individuals there had roughly equal proportions of ovaries and testes (Toy 1998), which would increase the probability of sampling both gonad types. Genotyping the mitochondrial DNA of Western Pearlshells may be a useful alternative method for sexing individuals nonlethally and at a large scale (Breton et al. 2011) in Montana. Histology is accurate but lethal and may therefore not be appropriate for sampling small, declining populations.

The high incidence of hermaphroditism among Western Pearlshells in Montana suggests that they are monoecious. Tissues of only one of the mature mussels examined histologically were identified exclusively as ovarian but undifferentiated tissue in this gonad precluded ruling out that the possibility that the individual was also a hermaphrodite. The high probabilities of the presence of gonadal eggs (93 – 100%) across all populations provided additional evidence of an unusually high incidence of hermaphroditism. Additionally, the probabilities of gonadal sperm (15 – 31%) in histologically examined individuals were higher than expected given the low detectability (13%) of sperm in the respective gonadal biopsies. The high incidence of hermaphroditism we observed was similar to that of Western Pearlshells in the Blackfoot River, Idaho (100%; Heard 1970), and is supported by the high estimated inbreeding coefficients and

high incidences of identical genotypes found across Montana Western Pearlshell populations (Anderson 2002; Chong et al. 2009; Mock et al. 2013).

The high incidence of hermaphroditism in Montana Western Pearlshell populations was surprising because hermaphroditism is thought to be a rare occurrence among margaritiferids (van der Schalie 1970; Karna 1972; Toy 1998; Bauer 1987; Grande et al. 2000) that is triggered by low population densities (Bauer 1987). Dioecy is considered typical in margaritiferids, but facultative hermaphroditism, in which dioecious mussels become hermaphroditic, can occur. Indeed, the low incidences of hermaphroditism in Western Pearlshell populations in Oregon (< 1%: Toy 1998), Washington (< 1%: Karna 1972), and Wyoming (< 10%: van der Schalie 1970) support this theory. However, sexual differentiation is not species-specific and can vary greatly among populations (Downing et al. 1989). Dioecious populations can transition to hermaphroditism under certain conditions, such as low population densities, that favor hermaphrodites because of increased opportunities to mate (Ghiselin 1969; Charnov et al. 1976; Downing et al. 1989). Hermaphroditic margaritiferids are more common when population densities are low (Bauer 1987; Geist and Kuehn 2005). Specifically, population densities of < 30 mussels upstream of individuals may trigger hermaphroditism in *M. margaritifera* (Bauer 1987) or < 10 individuals/m^2 may trigger hermaphroditism in other facultative hermaphroditic species (Downing et al. 1993). However, the mussels we collected came from populations exceeding these densities (380%) or with more than 30 mussels upstream of them.

Environmental variables may be responsible for the vastly different incidences of hermaphroditism between Montana and coastal state populations. Water body size, water velocity, temperature, food availability, and pollution may influence sex determination (Breton et al. 2018). The prevalence of hermaphroditism may be negatively related to stream size (Morton

1991) and our study streams were probably much smaller than those in which hermaphrodism was rare (Karna 1972; Toy 1998). For example, bivalves inhabiting small rivers in Hong Kong had a higher incidence of hermaphroditism than those inhabiting large rivers and lakes, which were almost exclusively dioecious (Morton 1991). Additionally, the incidence of hermaphroditism may be higher at low water velocities (Hinzmann et al. 2013) or at unnatural flow regimes (Galbraith and Vaughn 2011). The flow regimes during the spawning seasons in Montana and coastal states probably differ. Low or high water velocities may inhibit fertilization success regardless of population densities due to limited sperm dispersal (Quinn and Ackerman 2011). Hermaphroditism may be a better reproductive strategy when sperm dispersal is limited (Hinzmann et al. 2013).

Although Western Pearlshells are considered facultative hermaphrodites, their mitochondrial genome suggests a functional switch from dioecy to hermaphroditism (Breton et al. 2011). Mitochondrial genomes appear have a direct role in sex determination of bivalves through doubly uniparental inheritance (DUI) (Breton et al. 2007; Breton et al. 2011; Zouros 2013). Dioecious freshwater mussel species inherit gender-specific (female or male type) mitochondrial DNA (mtDNA) from each parent (Breton et al. 2007; Breton et al. 2011; Breton et al. 2014) whereas some hermaphroditic species have evolved a divergent hermaphroditic mitochondrial genome from the female mtDNA and have lost the male mtDNA altogether and hence, hermaphroditic species lack DUI (Breton et al. 2011; Breton et al. 2018). The rapid evolutionary divergence of the hermaphroditic mitochondrial genome has been independently derived in several hermaphroditic species of freshwater mussels (Breton et al. 2011) implying the function of the hermaphroditic type of mtDNA may be the reproductive switch from dioecy to hermaphroditism (Breton et al. 2011, 2014, 2018). Mussels with DUI (male and female type

mtDNA) can be capable of hermaphroditism (Breton et al. 2011); however, all tested individuals with hermaphroditic type mtDNA have been gonadal hermaphrodites (S. Breton, Université de Montréal, personal communication; Breton et al. 2011). Whereas Western Pearlshells and *M. margaritifera* are closely related and are both considered facultative hermaphrodites, *M. margaritifera* possessed DUI and Western Pearlshells possessed only the hermaphroditic type mtDNA (Breton et al. 2011), though which populations these individuals came from is unclear. Comparisons of mitochondrial genome types across dioecious and hermaphroditic Western Pearlshell populations across Northern America may provide insight into the life histories of these populations. Reproductive strategies are not species-specific (Downing et al. 1989) and the possibility that the mtDNA type could differ among populations within a species exists, though has not been observed (S. Breton, personal communication).

Hermaphroditic Western Pearlshell mussels and other Margaritiferids may or may not be self-fertilizing (Bauer 1987; Anderson 2002; Mock et al. 2013). Self-fertilization puts populations at risk for extinction in the long term because of reduced survival (Ibarra et al. 1995) and growth rates of larvae (Beaumont and Budd 1983; Ibarra et al. 1995), and inbreeding (Charlesworth and Charlesworth 1987; Ibarra et al. 1995; Brook et al. 2002), and mechanisms generally exist for preventing self-fertilization in freshwater mussels (Coe 1943; Gosling 2015). However, self-fertilization may be immediately advantageous when sexual partners are not available (Tsitrone et al. 2003) and whether mechanisms preventing self-fertilization exist in Western Pearlshells is unknown. One mechanism preventing selfing in hermaphrodites that produce eggs and sperm simultaneously is the temporal delay of spawning eggs after releasing sperm (Coe 1943; Gosling 2015); however, we found at least some individuals that had deposited their eggs before releasing sperm (Figure 21). Also, intrafollicular fertilization, in

which eggs are surrounded by sperm inside the ovary, has been documented in several species of freshwater mussels (Arujo and Ramos 1997; Lima et al. 2012) including Margaritiferids (Grande et al. 2000). The widespread heterozygosity deficiency seen in Montana populations (Anderson 2002; Chong et al. 2009; Mock et al. 2013) and high estimates of self-fertilization and inbreeding (Mock et al. 2013) may indicate self-fertilization is occurring. The high incidence of hermaphroditism we observed is not evidence of self-fertilization, but mussels in populations with low densities ($< 0.1/m^2$) were brooding developing embryos in 2019 (Stagliano et al. 2020). Also, our observations of low proportions of testes to ovaries are consistent with the sex allocation theory, which posits that animals switching from cross-fertilization to self-fertilization should reduce their male allocation (Johnston et al. 1998; Winkler and Ramm 2018). Indeed, a negative relationship exists between self-fertilization rates and the proportion of testes in hermaphroditic *Utterbackia imbecillis* mussels (Johnston et al. 1998).

Hermaphroditism and self-fertilization can have a profound effect on the genetic diversity within and among Western Pearlshell populations (Chong et al. 2009; Mock et al. 2013). Isolated populations that self-fertilize have reduced gene flow, which may increase divergence among populations (Lesica and Allendorf 1995). Balancing inbreeding and outbreeding depression in future propagation or translocation efforts may be particularly difficult for a facultative hermaphroditic species (Geist 2010). Moreover, self-fertilization and hermaphroditism could influence the effective population size necessary for a population to persist. The mechanisms driving hermaphroditism in Montana Western Pearlshells should be identified because the prevalence of hermaphroditism can be plastic among populations and years (Coe 1943; Bauer 1987; Downing et al. 1989).

The gametogenic cycle of Montana Western Pearlshells differed from that of conspecifics in western Washington even though they spawned at similar times of the year. Female Western Pearlshells in Washington lacked ovaries for about four months after spawning (Toy 1988). Their ovaries started developing in November or December and eggs reached maturity sometime in March, April, or May and were deposited in marsupia shortly thereafter (Toy 1998). Ovarian recrudescence was more rapid in Montana populations. Immature eggs were first detected in biopsies from 38 to 50 days after spawning had ended in three of the four populations, indicating that the resting phase for ovaries was probably less than a month in duration. Mature eggs occurred in Montana mussels by late August to early September, indicating that eggs matured about five months earlier in Montana than in Washington. Timing of sperm recrudescence was similar in Washington males (Toy 1998) and Montana hermaphrodites.

Most mussels examined histologically showed evidence of dribble spawning. Individuals contained multiple gametogenic stages of gonadal tissues (precursory, ripe, spent, or reabsorbing) in the late spring and early summer. Different gametogenic stages occur in ovaries of several long-term brooders (Villalba 1995; Lima et al. 2012) but were unexpected in a short-term brooder that typically reproduces synchronously (Hastie and Young 2003; Smith et al. 2003). However, some Western Pearlshell females in western Washington spawned partially in March and again in June (Toy 1998). Most of the mussels we examined histologically came from Clam Creek; dribble spawning may have been unique to that population. One marked Clam Creek individual brooded early developmental stage embryos during two consecutive weeks, which was also indicative of dribble spawning.

Brooding phenologies, but not degree days needed to initiate brooding, were similar among Montana and coastal state populations. Brooding generally occurred in May and June,

which is similar to that of populations in California, Washington, and Oregon (Karna and Millemann 1978; Toy 1998; Spring Rivers 2007; O'Brien et al. 2013; Allard et al. 2017). Brooding occurred after 1,286 to 2,537 degree days had accumulated since brooding peaked in the prior year. Fewer degree days accumulated between annual brooding events among Montana Western Pearlshells than among Western Pearlshells in Oregon (3,670 – 3,793 DD, Allard et al. 2017) or by *M. margaritifera* (3,000 – 3,600 DD, Hastie and Young 2003), possibly because of a lower thermal requirement of mussels in snowmelt-driven systems. Montana populations brooded for about 24 to 39 days, a duration which is similar to that reported for some populations (2 – 4 weeks, Murphy 1942; Karna 1972; Karna and Millemann 1978), but brooding duration can vary greatly (12 days – 7 weeks, Murphy 1942; Karna 1972; Meyers and Millemann 1977; Karna and Millemann 1978). The prolonged duration of brooding by the Clam Creek population was probably caused by variation in timing of brooding among individuals.

Causes of the observed differences in brooding phenologies of Clam Creek mussels between 2019 and 2020 are unclear. Brooding occurred about a month earlier, was less synchronous, and was longer in duration in 2020 than 2019. The evidence of dribble spawning and variation in timing among individuals observed in 2020 may explain the prolonged and asynchronous brooding that year, but reasons why these phenomena would occur in one year but not the other are unknown. Clam Creek is a small stream, and its temperatures are highly influenced by snowpack and air temperature, but no substantial differences in climate variables existed between years. The later brooding observed in the slightly warmer year (2019) suggests mussels may have produced an earlier brood that year before sampling started. Margaritiferid populations can reproduce twice in a season at warm temperatures, but whether individuals do so was not assessed (Scheder et al. 2011; Spring Rivers 2007). Water temperatures in 2019 and

2020 probably did not differ enough to cause a difference in the number of brooding events; June water temperatures were only 35 DD warmer in 2019 than in 2020. Similarly, Western Pearlshells in Merrill Creek, Oregon, released larvae for six to eight weeks starting in mid-April in three consecutive years but started releasing larvae several weeks earlier and for about three months in duration in a fourth year (Allard et al. 2017). Inconsistencies in reproductive timing also occurred among years in populations of Western Pearlshells in California (Spring Rivers 2007).

Attempts to capture Western Pearlshell larvae in stream drift were unsuccessful because concentrations of larvae in stream drift were probably insufficient for detection. Western Pearlshell release larvae in fragile conglutinates—aggregates of larvae held together by membranes—that may (Murphy 1942) or may not (O'Brien et al. 2013) break up readily in water currents. The likelihood of capturing larvae may be lower when margaritiferids release millions of larvae contained in a single conglutinate (Murphy 1942; Young and Williams 1984 cited in Geist 2010) than if they released separate larvae. Additionally, individual Western Pearlshell larvae are exceptionally small (Murphy 1942; Barnhart et al. 2008; Allard et al. 2013; O'Brien et al. 2013) and are therefore difficult to detect in stream drift. Larval concentrations in Montana may have been insufficient to detect using methods successfully used to capturing Western Pearlshell larvae elsewhere (O'Brien et al. 2013; Allard et al. 2017) because of low adult mussel densities in Montana (Culp et al. 2011). Larval concentrations also vary significantly as a function of turbulence, water velocity, time of day, and distance downstream from mussel beds (Haag and Warren 2000; Schwalb et al. 2010), which may have influenced our success.

Montana Western Pearlshell larvae infested fish at similar times and for similar durations as in other populations. Infestation generally occurred in June and July and lasted about 47 – 71

days (515 – 808 DD). Durations of larval infestation were comparable to those of other Western Pearlshells populations (32 days, Murphy 1942; 52 days, Meyers and Millemann 1977; 12 weeks, Fustish and Millemann 1978). Encapsulated larvae grew substantially (from 60 to 539 µm in length), as elsewhere (Murphy 1942; Karna and Millemann 1978). Juvenile mussels were shed from gill filaments at a minimum length of 300 µm. Size at drop-off (Karna and Millemann 1978) and duration of infestation (Karna and Millemann 1978; Dodd et al. 2005) may be partially dependent on host species. For example, juvenile Western Pearlshells were shed from Cutthroat Trout and Chinook Salmon at greater lengths than from other host species (Karna and Milleman 1978).

Photoperiod may influence the timing of Western Pearlshell reproductive events more than temperature. Many reproductive events in freshwater mussels appear to be thermally driven (Watters and O'Dee 2000; Hastie and Young 2003; Blažek and Gelnar 2006; Galbraith and Vaughn 2009; Gascho Landis et al. 2012; Schneider et al. 2018; Franzem et al. 2019) and reproductive events in Montana Western Pearlshells occurred slightly earlier in warmer streams; however, phenologies among populations varied less by day of year than on degree days, indicating photoperiod may be a stronger driver of reproduction than temperature. The degree to which photoperiod influences reproduction is generally thought to be secondary to temperature (Wayne and Block 1992; Fabioux et al. 2005; Galbraith and Vaughn 2009; Gascho Landis et al. 2012; Franzem et al. 2019) but can be a primary mechanism in some bivalves (Paulet and Boucher 1991; Mallet and Carver 2009).

An environmental cue such as a threshold temperature or a sudden increase in temperature may also influence the timing of spawning, though evidence is scarce. Thermal summation probably does not trigger spawning by Montana Western Pearlshells given that

mature gametes were present months before spawning occurred. Similarly, *Unio crassus* individuals brooded viable larvae for more than three weeks until a sudden temperature increase seemed to trigger larval release (Schneider et al. 2018). Sudden increases in water temperature have been associated with *M. margaritifera* reproductive events (Hastie and Young 2003) and are a common occurrence in the spring in snowmelt-driven streams in Montana. Minimum threshold temperatures may have in part triggered spawning in Western Pearlshells because mussels spawned shortly after average daily water temperatures first reached 6.5°C and 6.8°C in two streams with different thermal regimes. The timing of spawning may drive the phenologies of all other reproductive events in freshwater mussels (Haggerty et al. 2005) and therefore the similarity in average daily temperatures at the time of spawning may be particularly important. However, evidence of a minimal threshold temperature was scarce and could have been coincidental.

Additional investigations of the abiotic and biotic factors influencing reproductive phenology of Western Pearlshells in Montana can help identify conditions necessary for successful recruitment. For example, the timing of sperm release can affect fertilization rates (Quinn and Ackerman 2011), the timing of larval release can affect larval survival (Akiyama and Iwakuma 2007; Schneider et al. 2018), and the duration of encapsulation can affect juvenile mussel survival (Eybe et al. 2015; Marwaha 2020) and fitness (Marwaha 2020). The role of temperature in triggering reproduction in Western Pearlshells is unclear and discriminating the effects of temperature and photoperiod warrants further investigation. Additionally, the timing of reproductive events in freshwater mussels may be affected by other factors such as discharge (Hastie 1999; Walker 2017; Franzem 2019), food availability and quality (Borcherding 1995;

Wacker and von Elert 2003; Galbraith and Vaughn 2009), and the presence of hosts (Jokela and Palokangas 1993; Haag and Warren 2000).

Westslope Cutthroat Trout and Brook Trout are probably the most suitable hosts for Western Pearlshells in Montana as evidenced by probabilities of infestation and infestation intensities. Native Westslope Cutthroat Trout were the primary host for Western Pearlshells in the Flint-Rock watershed. Westslope Cutthroat Trout are considered the historical host for Western Pearlshells in Montana and wild Coastal Cutthroat Trout are one of several species of hosts to Western Pearlshells in western Oregon (Karna and Millemann 1978). Nonnative Brook Trout were the primary host in the Big Hole watershed. The native ranges of Western Pearlshell mussels and Brook Trout do not overlap, but evidence exists that Western Pearlshells can metamorphose on Brook Trout under artificial conditions (Murphy 1942) and in the wild. Specifically, we found Brook Trout infested with juvenile mussels and juvenile Western Pearlshells inhabiting streams where Brook Trout are the only fish species present.

The probabilities of infestation and infestation intensities on Westslope Cutthroat Trout and Brook Trout were similar to those on salmonids in Oregon (Karna and Millemann 1978). The probabilities of infestation of Brook Trout (71%) and Westslope Cutthroat Trout (77%) were slightly lower than the prevalences of Western Pearlshell infested salmonids (sea-run Rainbow Trout, Coho Salmon, Chinook Salmon, Coastal Cutthroat Trout) captured in Oregon (86 – 100%: Karna and Millemann 1978). The higher prevalence on Oregon salmonids may have been an artifact of examining only alevins (Karna and Millemann 1978). The infestation intensities on Brook Trout (median = 42) and Westslope Cutthroat Trout (median = 75) were similar to those on Western Pearlshell infested sea-run Rainbow Trout (mean = 88) and a single Coastal Cutthroat Trout (n = 102) in Oregon (Karna and Millemann 1978) as well as those of most

natural infestations by freshwater mussels (Neves and Widlak 1988; Kneeland and Rhymer 2008).

Whereas the probabilities of infestation and infestation intensities did not differ between Westslope Cutthroat Trout and Brook Trout, metamorphic success may have been greater on Westslope Cutthroat Trout. More larvae were reaching the juvenile mussel stage (> 240 µm in length) on Westslope Cutthroat Trout (65%; 20 larvae per individual fish) than on Brook Trout (14%; 3 larvae per individual fish). The short median larval length (median = 77 µm) and skewed larval length frequency on Brook Trout indicated that most mussel larvae sloughed off before metamorphosis. The difference in the length frequencies of attached larvae between the host species could be caused by differences in their acquired immune response to larvae. For example, Brook Trout may have more exposure to larvae than migratory fish species.

The presence of nonnative hosts may be beneficial to Western Pearlshells in the absence of their native host but may have long term consequences. The presence of nonnative fish may be advantageous to mussels when they increase the availability of potential hosts or opportunities for infestation (Garner et al. 1999; Clements et al. 2018). Westslope Cutthroat Trout have been extirpated in many streams in western Montana because of the invasion and displacement by Brook Trout (Shepard et al. 2002; Dunham et al. 2003; Peterson et al. 2004; Shepard et al. 2004) and none of the study sites from 2020 or 2019 (n = 12) had both Westslope Cutthroat Trout and Brook Trout present. Western Pearlshells may have to rely on a less suitable host in the absence of Westslope Cutthroat Trout. The presence of Brook Trout may allow recruitment of juvenile mussels, but nonnative fish are usually not suitable hosts for native freshwater mussels because of the lack of co-evolutionary adaptations (Watters and O'Dee 1998; Strayer 2008; Salonen et al. 2017b) and can act as larval sinks in which they reduce the number of larvae available to infest

suitable hosts (Poulin et al. 2011; Tremblay et al. 2016; Moore et al. 2021). The co-evolutionary relationship between Western Pearlshells and Westslope Cutthroat Trout could be lost when mussels rely on a novel host species (Taraschewski 2006; Douda et al. 2012; Modesto et al. 2018) or adaptions for host preference exist (Taeubert et al. 2010; Larsen et al. 2012, cited by Karlsson et al. 2014; Karlsson et al. 2014; Wacker et al. 2019). Additionally, the differences in movement patterns between Westslope Cutthroat Trout and Brook Trout may result in lower mussel dispersal and higher inbreeding. Margaritiferids rely on salmonids to disperse them (Morrissey and Ferguson 2011; Terui et al. 2014) and migratory Westslope Cutthroat Trout and resident Brook Trout have different movement patterns, particularly during larval release and when juvenile mussels are shed. Fluvial Westslope Cutthroat Trout migrate long distances (> 100 km, Schmetterling 2003; Muhlfeld et al. 2009), particularly in the spring to reach spawning tributaries (Muhlfeld et al. 2009) when host-larvae interactions occur. They also do not exhibit fidelity to the pre-spawning sites (Schmetterling 2001), possibly providing increased opportunities for juvenile mussel dispersal. In contrast, resident Brook Trout move little (< 0.02 km) in the spring and summer (Hansberger et al. 2010; Petty et al. 2012; Kanno et al. 2014; Ritter 2015) and exhibit even less movement as temperatures increase in late summer (Hansberger et al. 2010; Petty et al. 2012) when juvenile mussels are shed. Interestingly, Clam Creek mussels only interact with Brook Trout and this mussel population had a high estimated inbreeding coefficient of 0.92 (Mock et al. 2013).

Brown Trout were moderately susceptible to larval infestation by Western Pearlshells in the Flint-Rock watershed. The relationship between Western Pearlshell larvae and Brown Trout is poorly understood (Murphy 1942). In California, Western Pearlshell larvae were able to metamorphose on caged Brown Trout, but 52% of fish infested with 100 – 300 larvae died

within 50 days of exposure (Murphy 1942). Brown Trout had a 52% probability of infestation in our study, and their infestation intensities (median = 26) were similar to those reported for Coho Salmon in Oregon (mean = 24, Karna and Millemann 1978). No evidence of Brown Trout sloughing off larvae before metamorphosis existed and 89% of larvae on Brown Trout were greater than 240 µm in length, indicating that successful metamorphosis was occurring. Notably, the Flint-Rock watershed was the only watershed where we observed Western Pearlshell larvae on Brown Trout (Stagliano et al. 2020), even though Brown Trout are widely distributed throughout western Montana. The compatible relationship between Western Pearlshell larvae and Brown Trout in Montana may be unique to the Flint-Rock watershed because host requirements can vary among populations of a species (Karlsson et al. 2014; Taeubert et al. 2010; Salonen et al. 2017b; Clements et al. 2018). For example, two overlapping populations of *M. margaritifera* in Norwegian rivers have different host requirements—one population relies exclusively on Brown Trout and the other relies on Atlantic Salmon (Larsen 2002, cited by Karlsson et al. 2014; Larsen et al. 2012, cited by Karlsson et al. 2014; Karlsson et al. 2014; Wacker et al. 2019). The two populations are genetically distinct inferring that a functional adaptation exists between the host taxa (Larsen et al. 2012, cited by Karlsson et al. 2014; Karlsson et al. 2014; Wacker et al. 2019). Examining more Brown Trout, particularly juveniles, for larval infestation across watersheds inhabited by Western Pearlshells in Montana would be useful to determining if the use of Brown Trout as hosts occurs outside the Flint-Rock watershed.

Rainbow Trout were less suitable hosts for Western Pearlshells in our study sites than most other salmonids examined. Only one of the four study sites (Deep Creek) was inhabited by Rainbow Trout, but Brook Trout were more likely to be infested (83%) than Rainbow Trout (17%). Notably, the short average total length of Rainbow Trout (77 mm) in Deep Creek during

the infestation period indicated that the fish were age 0 and therefore lacked acquired immunity to Western Pearlshell larvae. Juvenile Rainbow Trout in the Truckee River in California were naturally infested at a much higher prevalence (Murphy 1942). Evidence that Western Pearlshells were metamorphosing on Rainbow Trout was observed in 2019 and 2020.

The discrepancies in the prevalences of infested Rainbow Trout among Montana and coastal state Western Pearlshell populations may be related to the native range of Rainbow Trout (Larson et al. 2002, cited by Karlsson et al. 2014; Clements et al. 2018) or the strain of Rainbow Trout (Taeubert et al. 2010). The low prevalence of infested Rainbow Trout observed in our study could have been specific to Deep Creek and other Montana watersheds where Rainbow Trout are nonnative. The Redband Rainbow Trout strain is native to the upper Kootenai River drainage in the northwest corner of Montana and this strain was observed to be heavily infested with Western Pearlshell larvae in four streams in 2019 and 2020 (Stagliano et al. 2020). In coastal states where Rainbow Trout are native, sea-run (Murphy 1942; Karna and Millemann 1978) and Redband (M. Steg-Geltner, Yakama Nation Fisheries, personal communication) Rainbow Trout were suitable hosts for Western Pearlshells, though appeared to be less susceptible to infestation than Coastal Cutthroat Trout and Chinook Salmon (Karna and Millemann 1978). The suitability of Rainbow Trout as a Western Pearlshell host may therefore depend on their natural distribution. Similarly, some populations of *M. margaritifera* only use Atlantic Salmon as a host within the natural distribution salmon; outside of this distribution, only native Brown Trout serve as hosts (Larson et al. 2002, cited by Karlsson et al. 2014; Clements et al. 2018). Additionally, host requirements may be specific to certain strains of a fish species (Taeubert et al. 2010) such that native Redband Rainbow Trout may be more compatible hosts than nonnative strains. For example, European populations of *M. margaritifera* used different

strains of Brown Trout to varying degrees of success (Taeubert et al. 2010), but native strains were most susceptible to infestation (Geist et al. 2006; Taeubert et al. 2010).

Mountain Whitefish did not appear to be compatible hosts for Western Pearlshell larvae. The low probability of infestation was a result of one Mountain Whitefish (age 0) infested with larvae. A single juvenile mussel fell off from its gills while being processing under the microscope, but which was probably a rare occurrence. Similarly, zero Western Pearlshell larvae were attached on gills of captured Mountain Whitefish in other watersheds in Montana (Stagliano et al. 2020). Mountain Whitefish were completely resistant to Western Pearlshell infestation in California (Murphy 1942).

The suitability of Bull Trout as hosts to Western Pearlshell warrants investigation. Bull Trout are native to many of the drainages Western Pearlshells inhabit in Montana, but their relationship has never been investigated because Bull Trout are designated as a Threatened Species by the U.S. Fish & Wildlife Service. We did nevertheless observe a Bull Trout infested with Western Pearlshell larvae. Similarly, Bull Trout in the headwaters of the Klamath Basin in south-central Oregon were once observed to be infested with Western Pearlshell larvae over a decade ago (M. Steg-Geltner, personal communication). Additionally, the estimated number of larvae on the infested Bull Trout was similar to that on Westslope Cutthroat Trout and Brook Trout, possibly indicating comparable susceptibility to infestation. The migratory life-history and large-scale movements of Bull Trout (Fraley and Shepard 1989; Swanberg 1997; Dunham and Rieman 1999; Schmetterling 2003; Muhlfeld and Marotz 2005) could promote substantial dispersal of Western Pearlshell mussels (Terui et al. 2014; Morrissey and Ferguson 2011) if they are suitable hosts. Furthermore, this larvae-host relationship should be investigated because conservation efforts that improve conditions for one species may also benefit the other.

Recruitment of juvenile mussels in Clam Creek may be dependent on the interaction between larvae and age-0 Brook Trout. The mean larval length did not increase above the size at which larvae first attach to hosts until age-0 trout emerged, indicating older fish were probably sloughing larvae before metamorphosis. Age-1 fish were not naïve to infestation in 2020 because larvae were released much later in 2019 when that cohort of fish had already emerged. Brook Trout older than age-0 had gills characteristic of immune reactions of incompatible hosts to mussel larvae (Reuling 1919; Fustish and Millemann 1978; Rogers and Dimrock 2003; Dodd et al. 2005); their gills were pale in color, had unusual cyst formation and extensive fusing of lamellae, and commonly had lamellae or entire gill filaments broken off. Age-0 Brook Trout in Clam Creek did not have these same physiological impairments, indicating the observed immune response of older trout was acquired. A disconnect in the timing of larval release and age-0 trout emergence in this system could result in failed recruitment of that year's cohort.

Evidence of an acquired immune response of hosts in other streams was sparse, possibly because the infestation intensities of naïve hosts was limited by the body size of the host and exposure to larvae was insufficient to cause complete immunity. The negative relationship between fish and larval lengths may be indicative of acquired immunity; the presence of juvenile mussels were limited to fish < 166 mm in total length. No relationship existed between fish lengths and infestation intensities, but the highest infestation intensities were present on fish from 161 to 210 mm in total length. The lower infestation intensities of larvae present on fish < 160 mm in total length may have been because body size of the host can be limiting to the number of larvae that can attach when the individual is naïve to infestation. A positive relationship between fish lengths and infestation intensities has been described when fish are naïve to infestation (Hastie and Young 2001; Klunzinger et al. 2012; Thomas et al. 2014; Ieshko

et al. 2016; Marwaha 2020). In fact, the fish with the highest infestation intensity of the study (815 larvae, mean larval length = 153 µm) was a Brook Trout greater than 200 mm in total length and was probably naïve to infestation or had incomplete immunity. The lack of relationship between fish lengths and number of larvae may also be explained in part by incomplete immunity to larvae. Complete acquired immunity to infestation requires multiple repeated exposures to mussel larvae (Reuling 1919; Rogers and Dimrock 2003; Dodd et al. 2005; Dodd et al. 2006) and the amount of exposure a fish receives may depend on the concentration and duration of larvae in stream drift. Salmonids from Clam Creek presumably had greater exposure to mussel larvae than those of our other study streams because Clam Creek had higher mussel densities and a longer larval release period. Naïvety or incomplete acquired immunity to larvae may explain why salmonids in other streams did not have gills that appeared characteristic of an immune reaction. Complete immunity may take years to develop when fish are only exposed to larvae for brief periods once a year.

The larvae-host relationship of Western Pearlshells should be further investigated to confirm host species in a laboratory setting, quantify host density requirements, and identify differences in host requirements among Montana populations. Laboratory studies are the most widely used approach to identify host species because the host's response and the number of recovered juvenile mussels can be monitored (Dodd et al. 2005; Dodd et al. 2006; Fritts et al. 2012; Ford and Oliver 2015; Haag and Stoeckel 2015). Pairing field and laboratory studies to identify host fish can provide ecologically relevant and robust results (Ford and Oliver 2015). Additionally, laboratory studies can identify more hosts than field studies (Keller and Ruessler 1997; Levine et al. 2012) and not all salmonids inhabiting streams occupied by Western Pearlshells were captured or examined in this study (e.g., Arctic Grayling and Bull Trout). Host

density requirements of Western Pearlshells should be quantified because they may regulate the population abundance and recruitment of mussels (Bauer 1988; Zuiganov et al. 1994; Österling et al. 2008; Henrikson et al. 2009; Haag and Stoeckel 2015; Ieshko et al. 2016). Because Montana Western Pearlshells use salmonids to varying degrees of success, host density requirements will probably vary based on fish species. Differences in host requirements of Western Pearlshells among watersheds should be considered when translocating mussels across watersheds or reintroducing mussels, especially if the source population was exclusively relying on nonnative hosts.

Propagation Implications

Results from our study will inform future propagation efforts of Western Pearlshells in Montana. Choosing which Western Pearlshell populations to use for broodstock may be challenging because populations relying on nonnative hosts may have lost the co-evolutionary relationship with Westslope Cutthroat Trout (Douda et al. 2012; Taraschewski 2006; Modesto et al. 2018) and may have unusually high rates of inbreeding (Mock et al. 2013). Hermaphroditism in this species could influence the numbers of source populations and brooding mussels needed for propagation and may make balancing inbreeding and outbreeding depression particularly difficult (Geist 2010). Biologists should use mussels > 34 mm in length for broodstock and expect that propagated and stocked mussels will not become reproductively active until about 11 years of age. Matching the brief timing of embryo or larval brooding to inoculate host fish may be particularly challenging for this species. Propagation entails either confining mussels brooding fertilized eggs with hosts for passive infestation or harvesting mature larvae from brooding mussels to actively inoculate fish. Both strategies require collecting mussels or larvae

during a brief period. In general, biologists would have to check embryo development frequently and would be limited to about two weeks to harvest larvae from a particular population for propagation.

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APPENDIX A

TABLES AND FIGURES

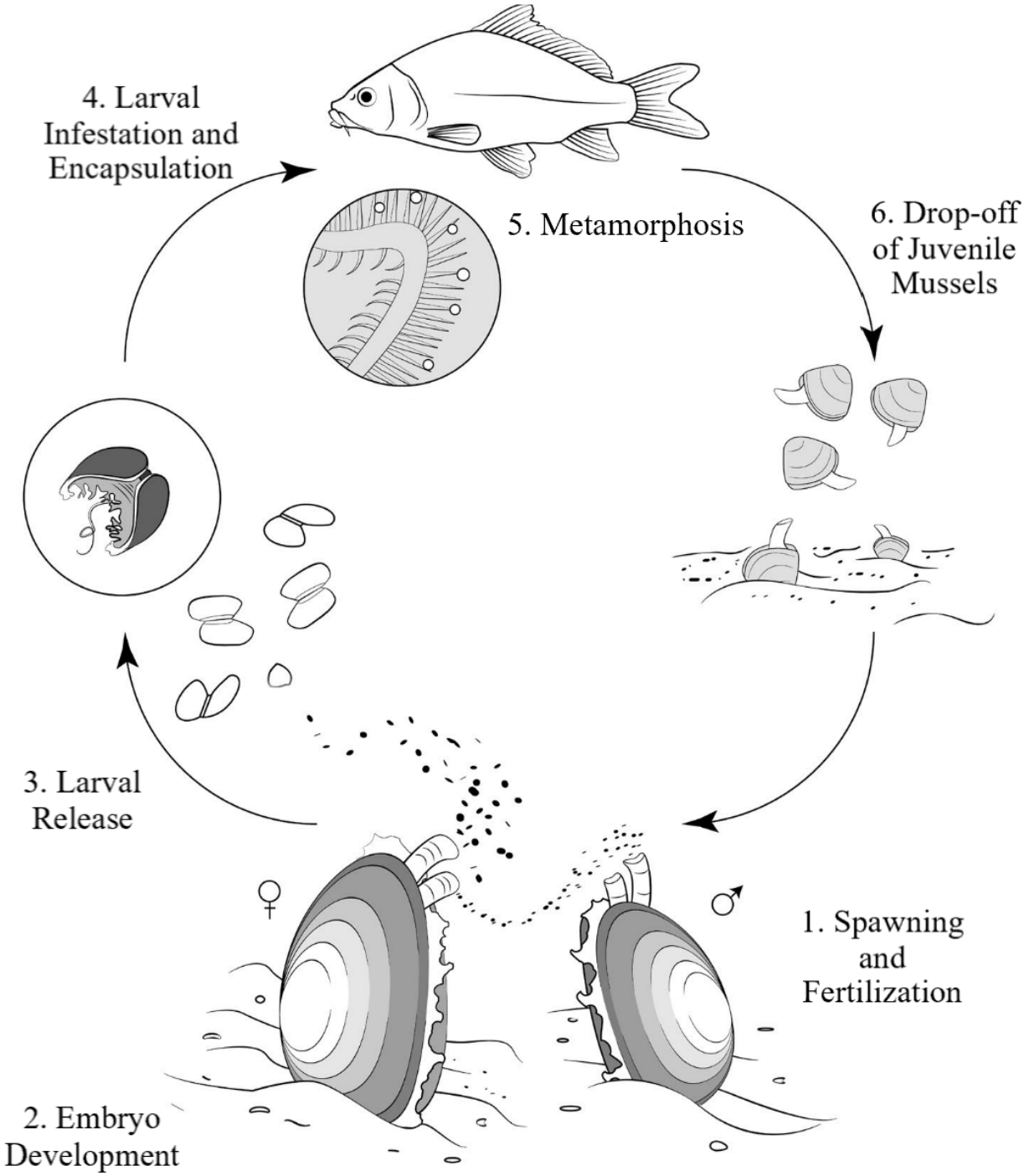

Figure 1. The life cycle of most freshwater mussels. Adapted from Modesto et al. 2018.

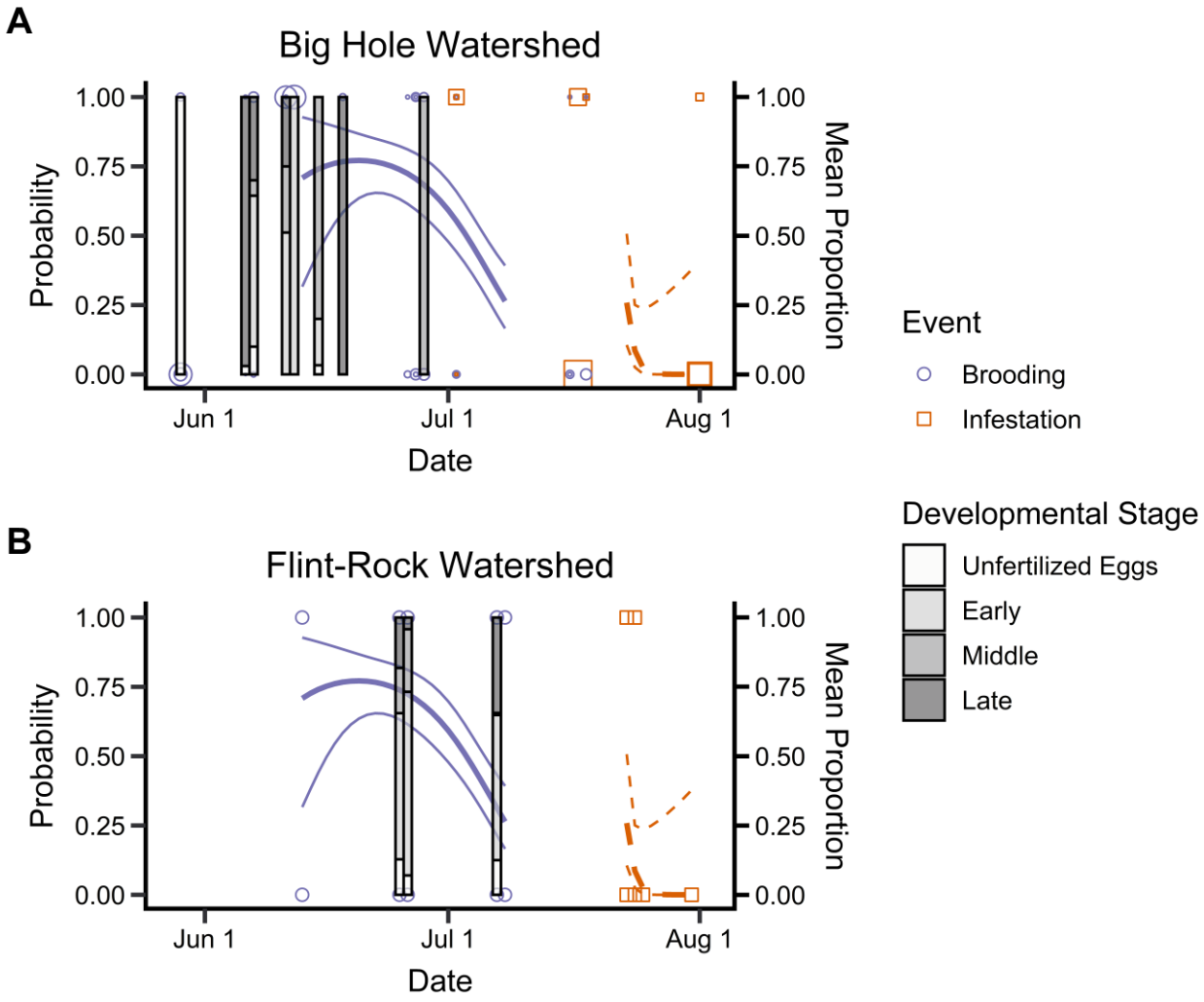

Figure 2. The relationships among probabilities of brooding (purple solid line) and infestation (orange dashed line) and embryo development stages (gray bars) in 2019 in the A) Big Hole and B) Flint-Rock watersheds. The sizes of the symbols represent the relative number of individuals with reproductive status at one or lacking reproductive status at zero. The probabilities for each reproductive event (thick lines) and 95% confidence intervals (thin lines), are modeled on day of year. The areas of the bars represent the mean proportion of marsupium samples made up of unfertilized eggs or embryos in early, middle, or late developmental stages.

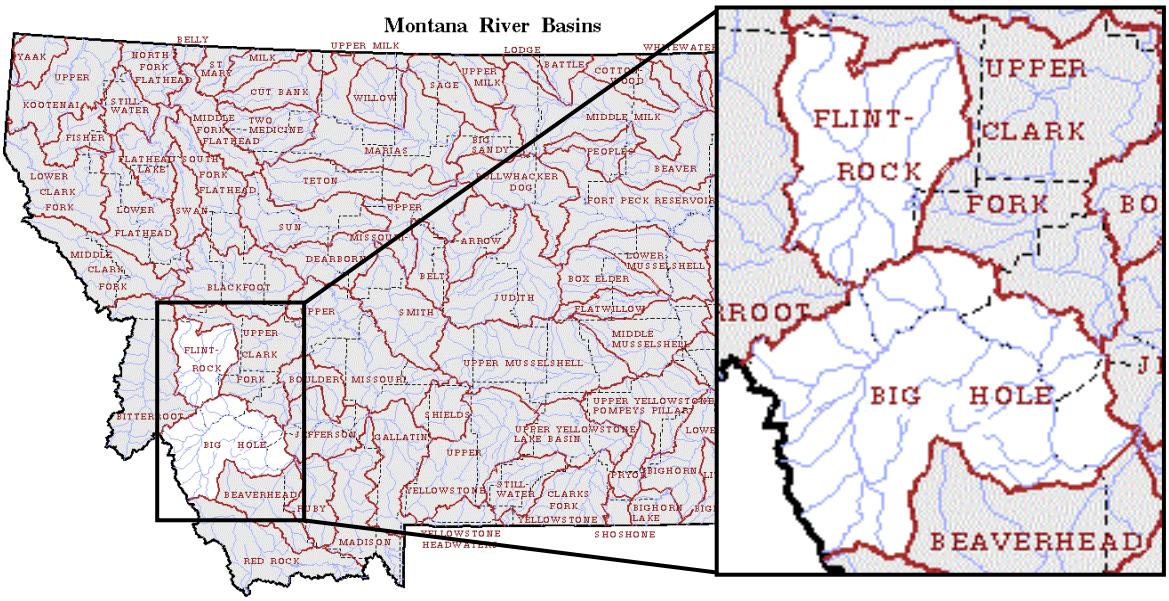

Figure 3. A map of Montana's watersheds. The Flint-Rock and Big Hole watersheds are highlighted.

Table 1. Characteristics of the four stream sites that we studied. The average wetted width, average depth, and median sediment grain sizes were estimated for the riffle closest to the mussel bed in August 2019 (Stagliano et al. 2020).

Stream characteristic	Clam Creek	Deep Creek	Upper Willow Creek	West Fork Rock Creek
Watershed	Big Hole	Big Hole	Flint-Rock	Flint-Rock
Starting coordinates (UTM)	12N 304001m E 5075013m N	12N 337183m E 5088198m N	12N 307393m E 5142840m N	12N 291824m E 5121230m N
Ending coordinates (UTM)	12N 304106m E 5074917m N	12N 337118m E 5088200m N	12N 307412m E 5142747m N	12N 291931m E 5121195m N
Reach length (m)	203	74	125	125
Elevation (m)	1,963	1,804	1,597	1,951
Average wetted width (m)	2.4	6.0	6.6	8.9
Average depth (m)	0.21	0.23	0.26	0.16
Median sediment size (d50; mm)	29.2	32.0	48.6	28.0

Figure 4. Photos of the four study sites. (A) Clam Creek in June 2019. (B) Deep Creek in October 2019. (C) Upper Willow Creek during high flows in late May 2020. (D) West Fork Rock Creek in late June 2019.

Table 2. Sample frequencies (Freq.) and sample sizes (*n*) used to model reproductive events in 2020.

	Gonad Biopsies			Brooding Surveys			Marsupium Biopsies			Fish Capture		
Creek	Freq.	*n*	Total *n*	Freq.	*n*	Total *n*	Freq.	*n*	Total *n*	Freq.	*n*	Total *n*
Clam	7	30 – 33	224	13	29 – 69	448	9	13 – 23	106	6	25 – 47	216
Deep	8	2 – 30	186	15	2 – 48	425	10	1 – 27	110	6	25 – 39	208
Upper Willow	8	14 – 38	241	10	14 – 38	294	5	2 – 16	43	5	25 – 66	226
West Fork Rock	7	25 – 38	225	10	25 – 51	359	6	1 – 33	119	5	46 – 62	270

Table 1. The probability of the presence of reproductive status (gametes, brooding, or larval infestation) was calculated as a function of day of year using logistic, quadratic logistic, or cubic logistic binomial models. The model parameters (a, b_1, b_2, and b_3) are reported using scientific notation (E).

					Model Parameters				
Event	Year	Creek	*n*	Model	*a*	b_1	b_2	b_3	*P*-values
Gonadal egg presence	2020	Clam	211	cubic	2.61E+02	-4.47	2.44E-02	-4.29E-05	< 0.006
	2020	Deep	186	cubic	9.60E+01	-1.45	6.96E-03	-1.05E-05	< 0.045
	2020	Upper Willow	241	cubic	3.57E+02	-5.99	3.25E-02	-5.68E-05	< 0.001
	2020	West Fork Rock	214	cubic	7.45E+02	-1.10E+01	5.35E-02	-8.50E-05	< 0.001
Gonadal sperm presence	2020	Clam	211	logistic	-12.05	0.04			< 0.001
	2020	Upper Willow	241	logistic	-4.53	0.01			< 0.011
	2020	West Fork Rock	214	cubic	4.20E+02	-6.16E+01	2.96E-02	-4.67E-05	< 0.002
Brooding	2019	Clam	175	cubic	-4.40E+03	7.52E+01	-4.26E-01	8.02E-04	< 0.007
	2020		448	logistic	11.36	-0.08			< 0.007
	2019	Deep	125	cubic	-2.55E+03	4.41E+01	-2.53E-01	4.80E-04	< 0.001
	2020		425	quadratic	-72.92	0.97	-0.003		< 0.001
	2019	Upper Willow	56	logistic	20.12	-0.11			< 0.026
	2020		295	logistic	13.15	-0.09			< 0.001
	2019	West Fork Rock	101	logistic	20.12	-0.11			< 0.001
	2020		360	quadratic	-415.00	4.73	-0.01		< 0.001

Table 3. Continued.

					Model Parameters				
Event	Year	Creek	*n*	Model	*a*	b_1	b_2	b_3	*P*-values
Infestation	2019	Clam	123	logistic	21.92	-0.11			< 0.001
	2020		216	quadratic	-81.24	0.92	-0.003		< 0.001
	2020	Deep	208	quadratic	-97.47	0.92	-0.003		< 0.001
	2020	Upper Willow	226	logistic	14.31	-0.07			< 0.001
	2020	West Fork Rock	270	logistic	19.76	-0.09			< 0.001

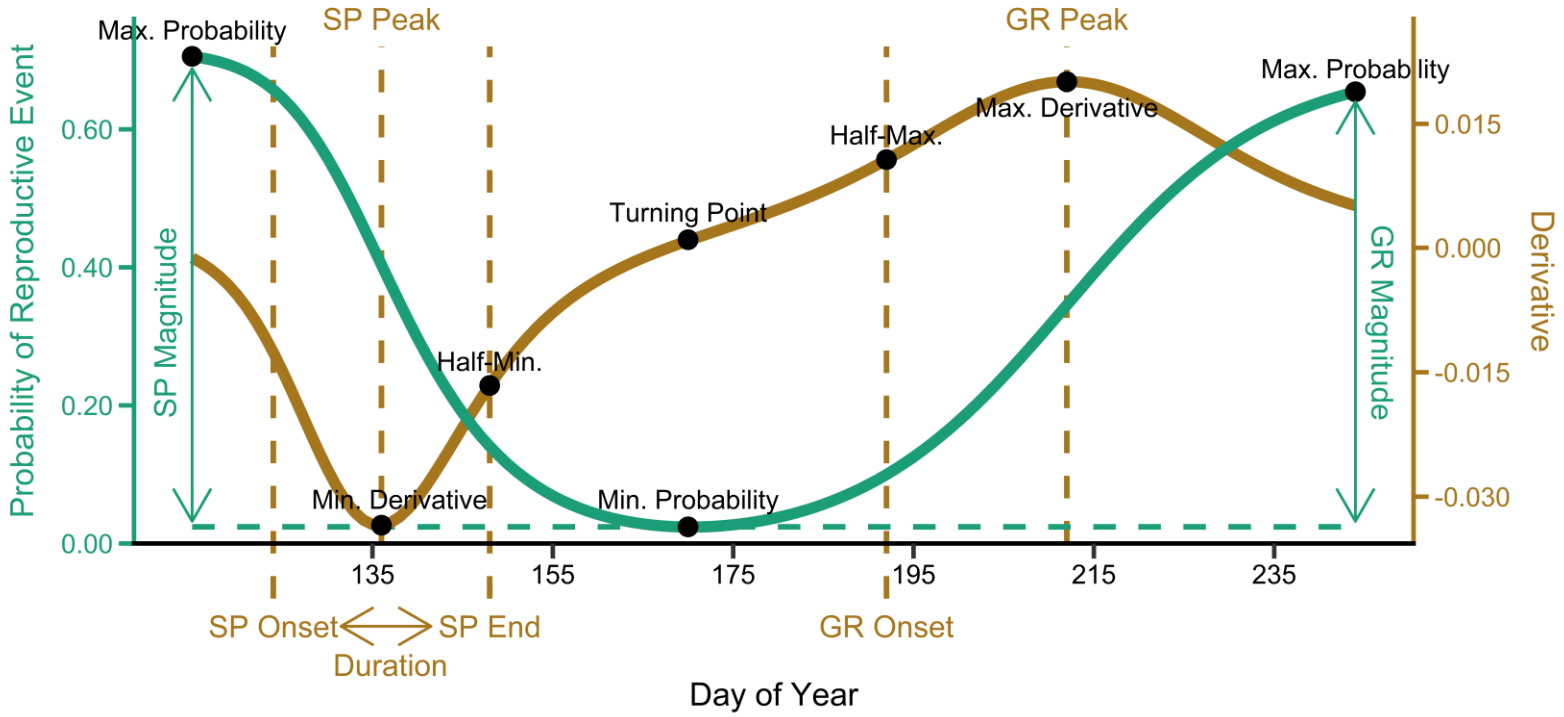

Figure 5. Conceptual figure of a binomial regression model used in phenology to define the timing and duration of reproductive events. The probability of gonadal egg presence (green line) and the first derivative of the regression model (rate of change curve; brown line) were used to define the timing of spawning (SP) and gonadal recrudescence (GR). The timing of the onset, end, duration, and peak of a reproductive event such as spawning or gonadal recrudescence were defined using the derivative of the regression model, whereas the magnitude of the event was defined using the regression model. Adapted from Li et al. 2017.

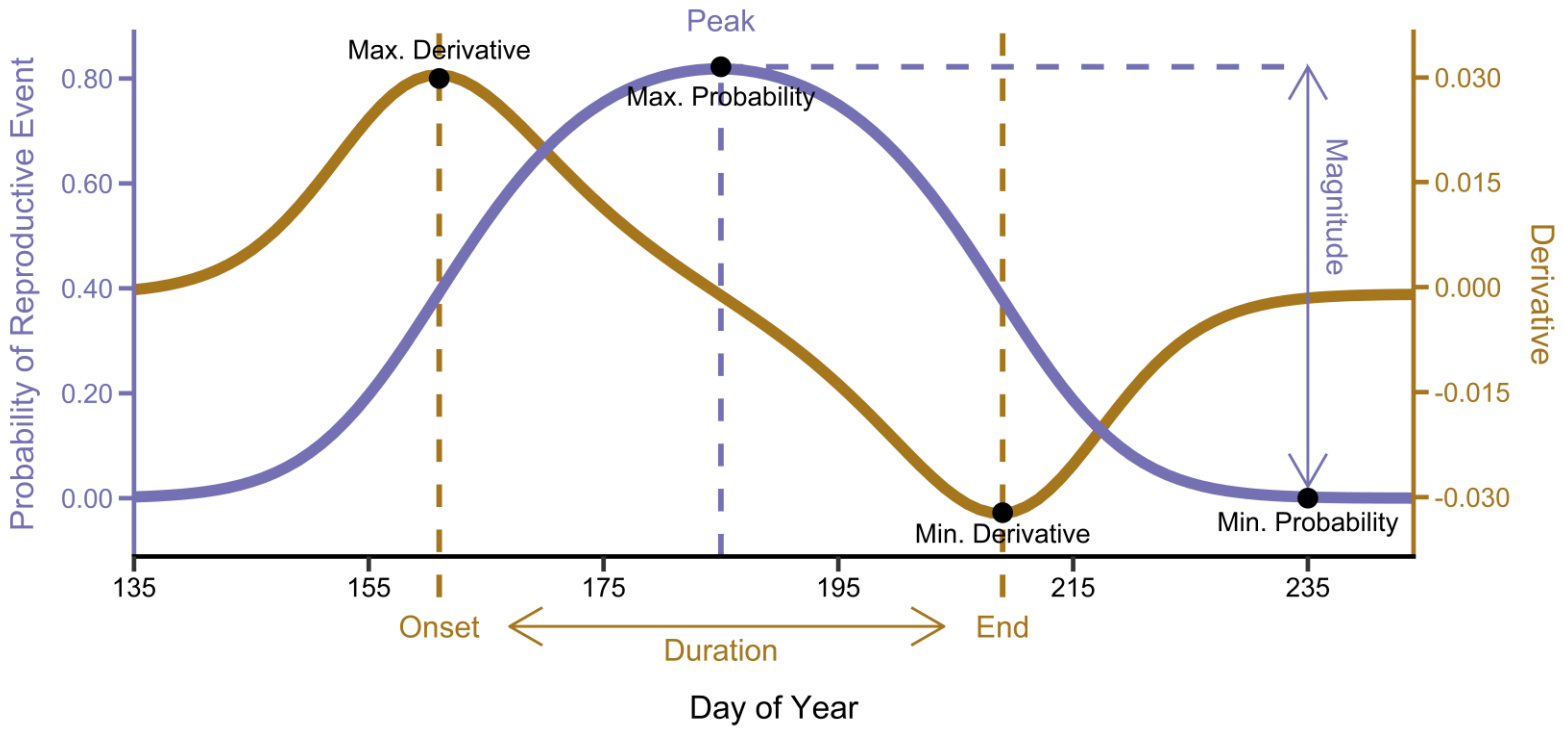

Figure 6. Conceptual figure of a binomial regression model used in phenology to determine the timing and duration of reproductive events. The probability of the event (green line) and the first derivative of the regression model (rate of change curve; brown line) were used to define the timing of brooding and larval infestation. The timing of the onset, end, and duration of a reproductive event such as brooding or infestation were defined using the derivative of the regression model, whereas the timing of the peak and the magnitude of the event was defined using the regression model. Adapted from Li et al. 2017.

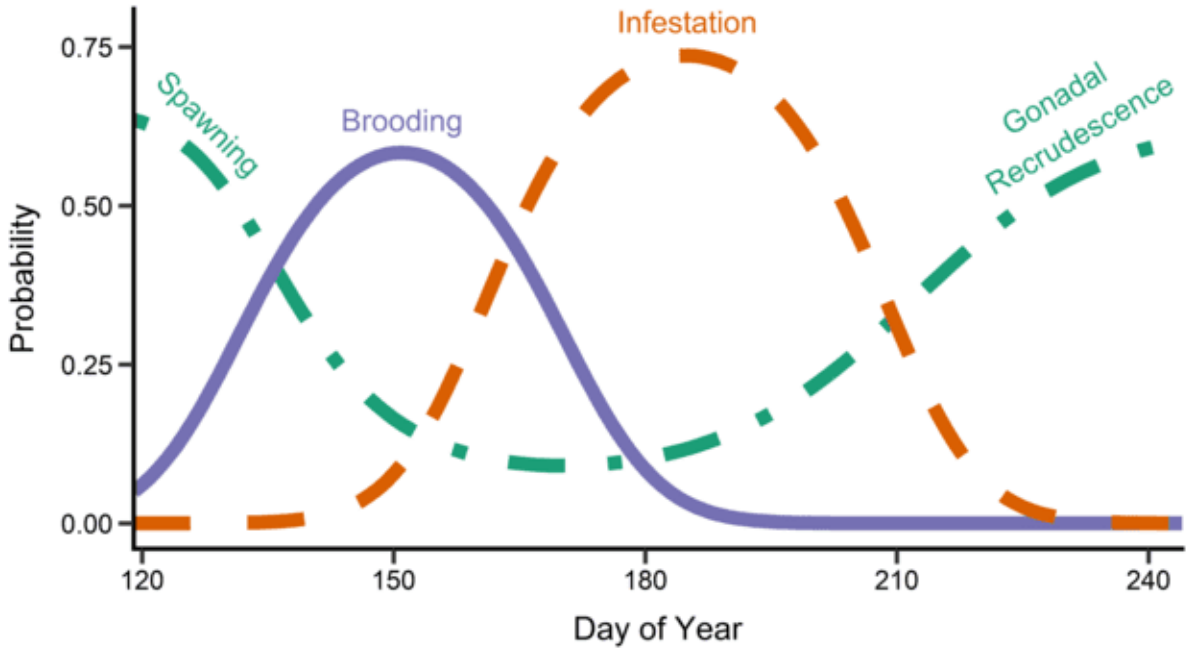

Figure 7. Conceptual figure of the overlap in the probabilities of successive reproductive events occurring in a population in which variation in timing among individuals exists. The probabilities of spawning (green line) and brooding (purple line) should overlap as mussel spawn. The probabilities of brooding and larval infestation (orange line) should overlap as mussels release larvae and the larvae infest fish hosts. The areas of overlap in probabilities would depend on the amount of variation in timing among individuals.

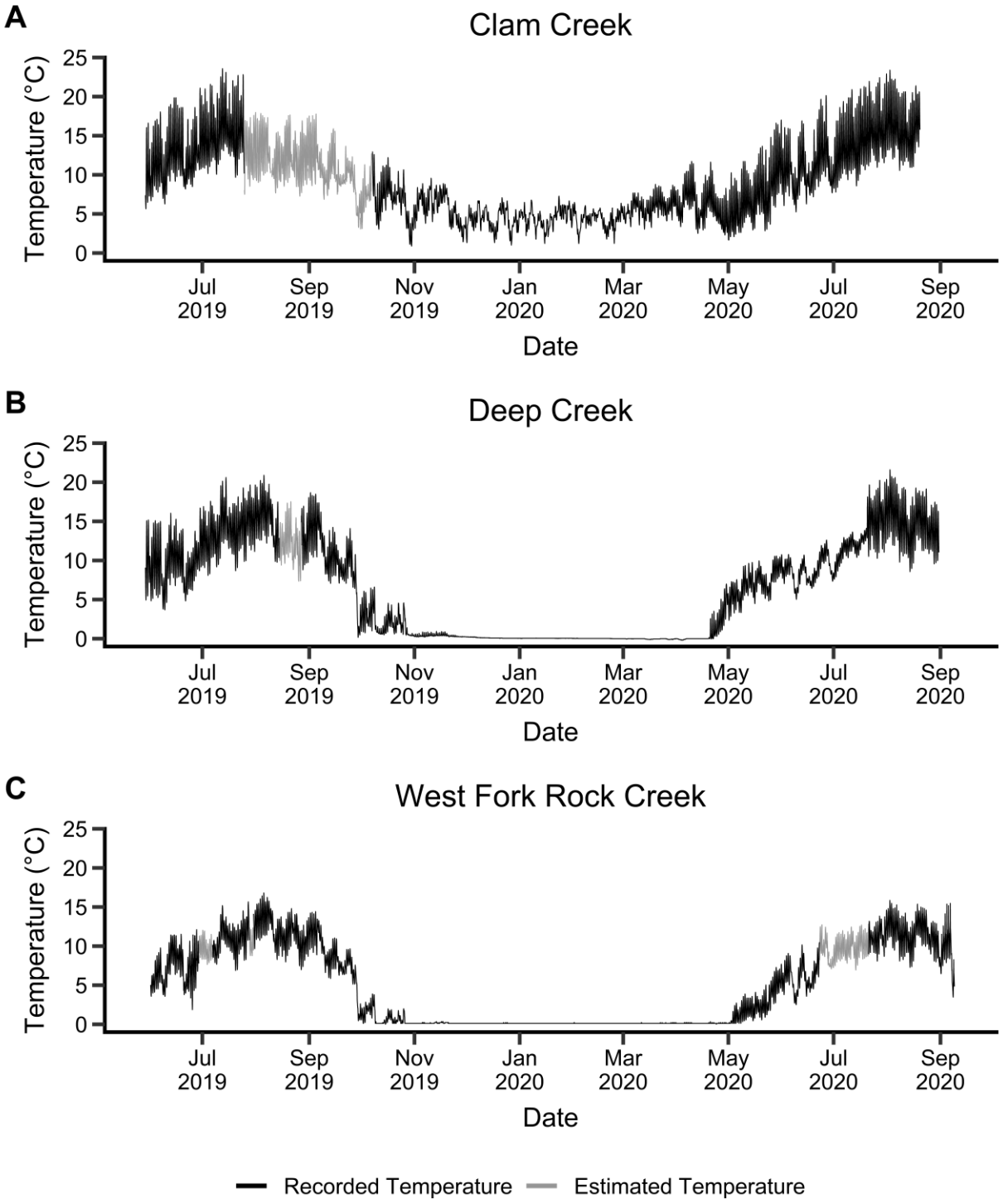

Figure 8. Recorded (black lines) and estimated (gray lines) hourly water temperatures used to calculate degree days for (A) Clam Creek from May 29, 2019 to August 20, 2020, (B) Deep Creek from May 29, 2019 to August 31, 2020, and (C) West Fork Rock Creek from June 1, 2019 to September 9, 2020.

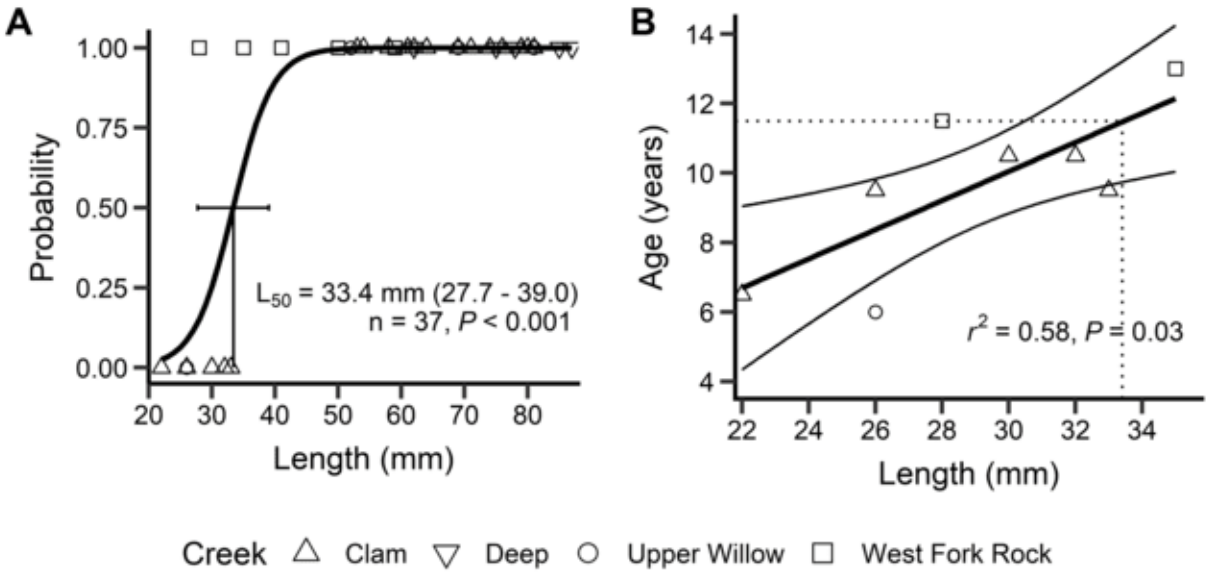

Figure 9. Estimated length and age at sexually maturity. The symbols represent the populations from which the individual mussels came from. (A) Binomial probability of sexual maturity as a function of length with sexually mature mussels at one and immature mussels at zero. The thick, solid line denotes the logistic regression. The "T-post" represents the estimated length at which 50% of individuals first achieved sexual maturity (L_{50}, vertical line) and its 95% confidence intervals (horizontal line). (B) The relationship between age and length. The thick and thin solid lines represent the linear regression and 95% confidence intervals, respectively. The dashed lines indicate the estimated age (11.5 years) at which mussels reached the estimated length of sexual maturity (L_{50}, 33.4 mm).

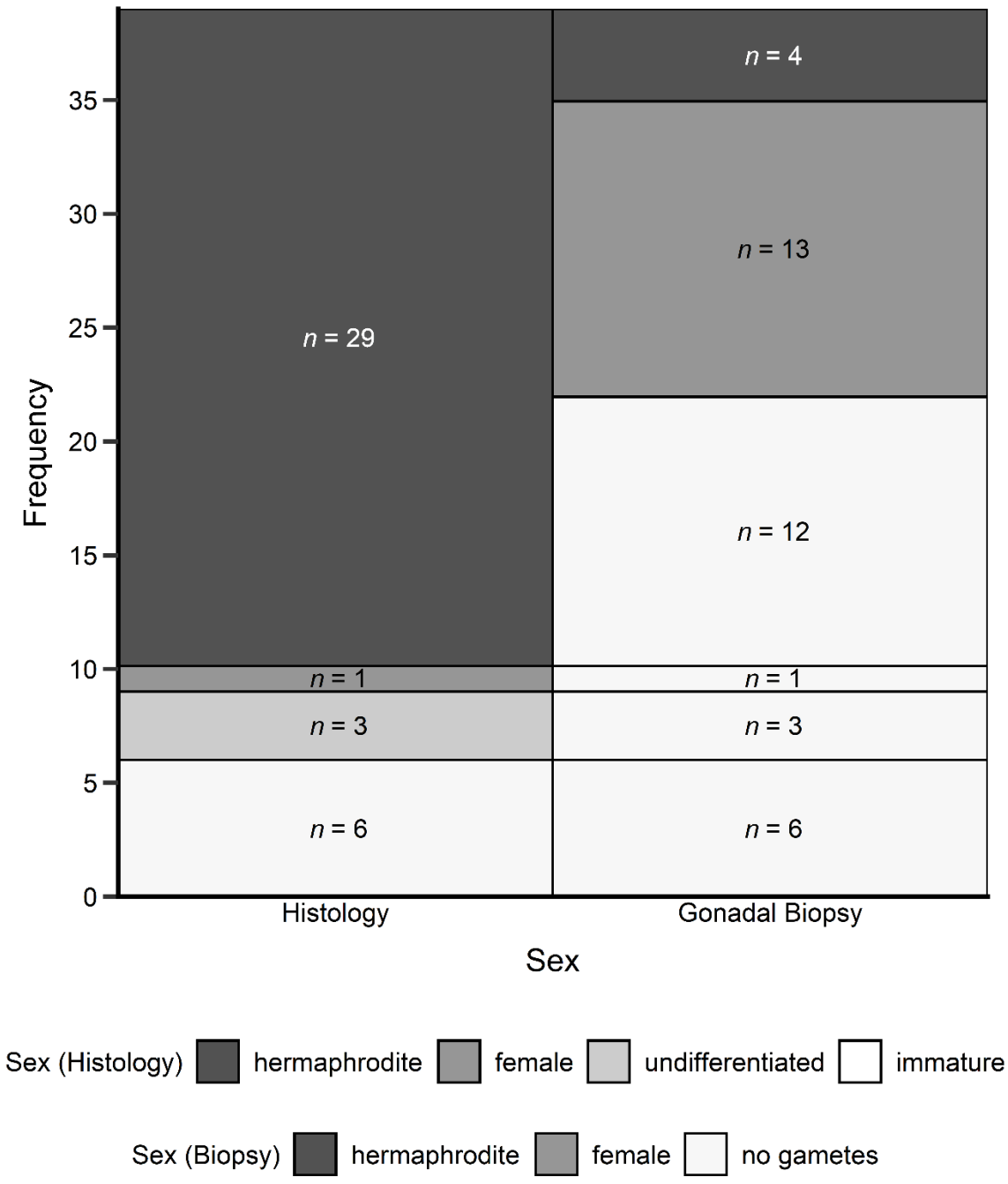

Figure 10. Sexes of 39 mussels determined using histology and gonadal biopsies. The frequencies of mussels that were deemed hermaphrodites, female, had undifferentiated gonadal tissue, or were sexually immature using histology are reported on the left. The frequencies of individuals from each histological classification that were identified as hermaphrodites (eggs and sperm present), females (only eggs present), or lacking gametes based on the gonadal biopsies are reported on the right. No males were found.

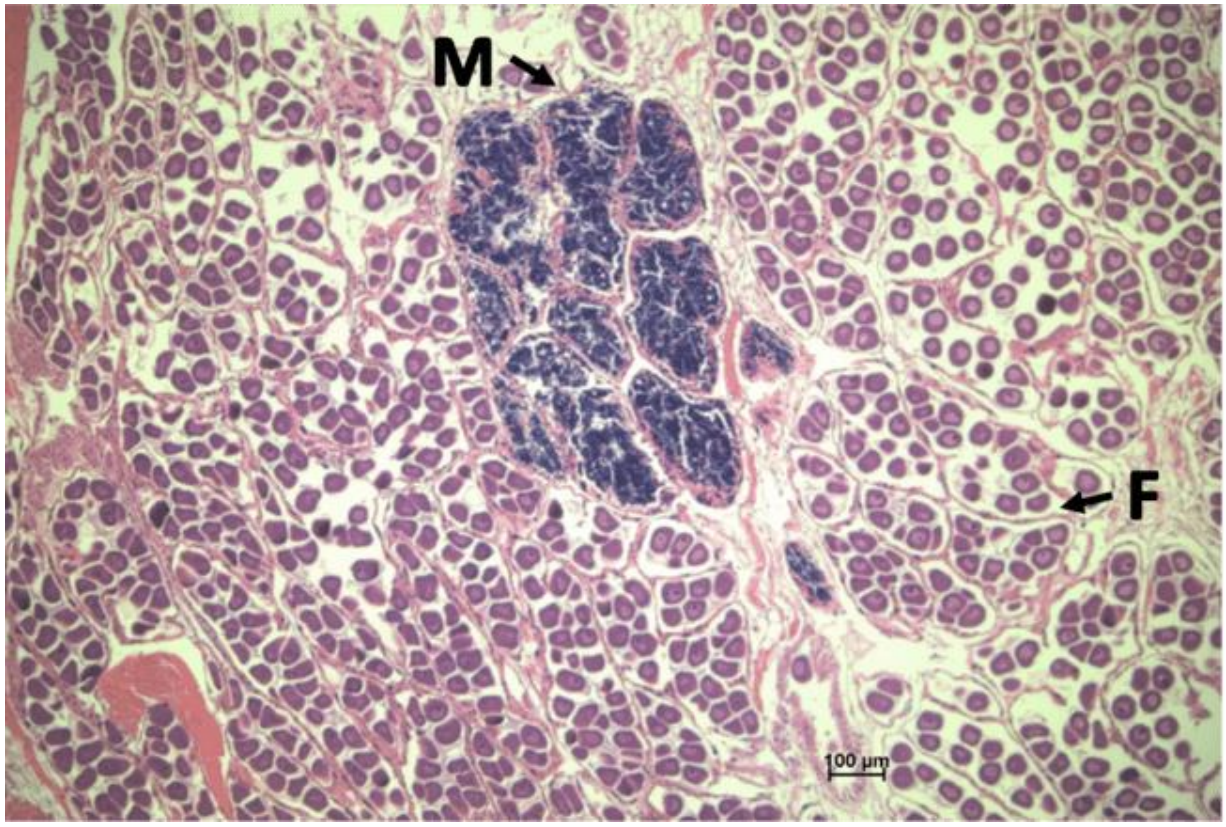

Figure 11. Gonadal tissue from a hermaphroditic Western Pearlshell. Hermaphrodites had a small ratio of male:female gonadal tissue, where abundant ovaries (“F”) surrounded rare testes (“M”).

Table 4. The estimated timing and duration of reproductive events determined using binomial regression models. The annual degree days (DD) reported for the event peaks were accumulated from the date at which brooding last peaked in that population, and therefore started accumulating in 2019 for 2020 spawning and brooding events and in 2020 for 2020 infestation and gonadal recrudescence events. The days, degree days, and mean temperatures reported for the event durations represent the number of days, the accumulated degree days, and the mean temperatures, respectively, from the event start to end dates.

			Event Onset	Event Peak				Event End	Event Duration	
Reproductive Event	Year	Creek	Date	Date	DD	Mean Daily Temperature	Magnitude	Date	DD	Mean Temperature
Egg recrudescence	2020	Clam	24-Jul	8-Aug	1091	14.7℃	95%	-	-	-
	2020	Deep	10-Jul	30-Jul	794	15.3℃	93%	-	-	-
	2020	Upper Willow	23-Jun	1-Jul	-	-	100%	-	-	-
	2020	West Fork Rock	18-Jul	27-Jul	325	11.5℃	96%	-	-	-
Sperm recrudescence	2020	Clam	31-Jul	20-Aug	1269	19.0℃	15%	-	-	-
	2020	Upper Willow	23-Jun	1-Jul	-	-	28%	-	-	-
	2020	West Fork Rock	24-Jul	8-Aug	472	10.8℃	31%	-	-	-
Spawning	2020	Deep	3-May	15-May	1567	6.8℃	84%	27-May	164	8.9℃
	2020	Upper Willow	7-May	13-May	-	-	> 56%	19-May	-	-
	2020	West Fork Rock	6-Jun	11-Jun	1189	6.5℃	> 52%	16-Jun	62	5.7℃
Sperm release	2020	West Fork Rock	7-Jun	11-Jun	1189	6.5℃	28%	15-Jun	50	5.6℃

Table 4. Continued.

			Event Onset	Event Peak				Event End	Event Duration	
Reproductive Event	Year	Creek	Date	Date	DD	Mean Daily Temperature	Magnitude	Date	DD	Mean Temperature
Brooding	2019	Clam	5-Jun	15-Jun	-	13.7°C	97%	29-Jun	309	12.4°C
	2020	Clam	-	10-May	2537	7.0°C	71%	28-May	-	-
	2019	Deep	30-May	10-Jun	-	8.2°C	98%	27-Jun	272	9.4°C
	2020	Deep	12-May	31-May	1691	9.5°C	58%	19-Jun	301	7.9°C
	2019	Upper Willow	-	13-Jun	-	16.2°C	87%	2-Jul	-	-
	2020	Upper Willow	-	14-May	-	-	79%	3-Jun	-	-
	2019	West Fork Rock	-	25-Jun	-	6.7°C	84%	4-Jul	-	-
	2020	West Fork Rock	13-Jun	25-Jun	1286	9.8°C	87%	8-Jul	206	8.3°C
Infestation	2020	Clam	14-May	28-Jun	481	10.6°C	78%	24-Jul	808	11.4°C
	2020	Deep	10-Jun	4-Jul	300	10.4°C	74%	27-Jul	515	11.0°C
	2020	Upper Willow	-	8-Jul	-	-	71%	29-Jul	-	-
	2020	West Fork Rock	-	23-Jul	271	10.9°C	91%	21-Aug	-	-

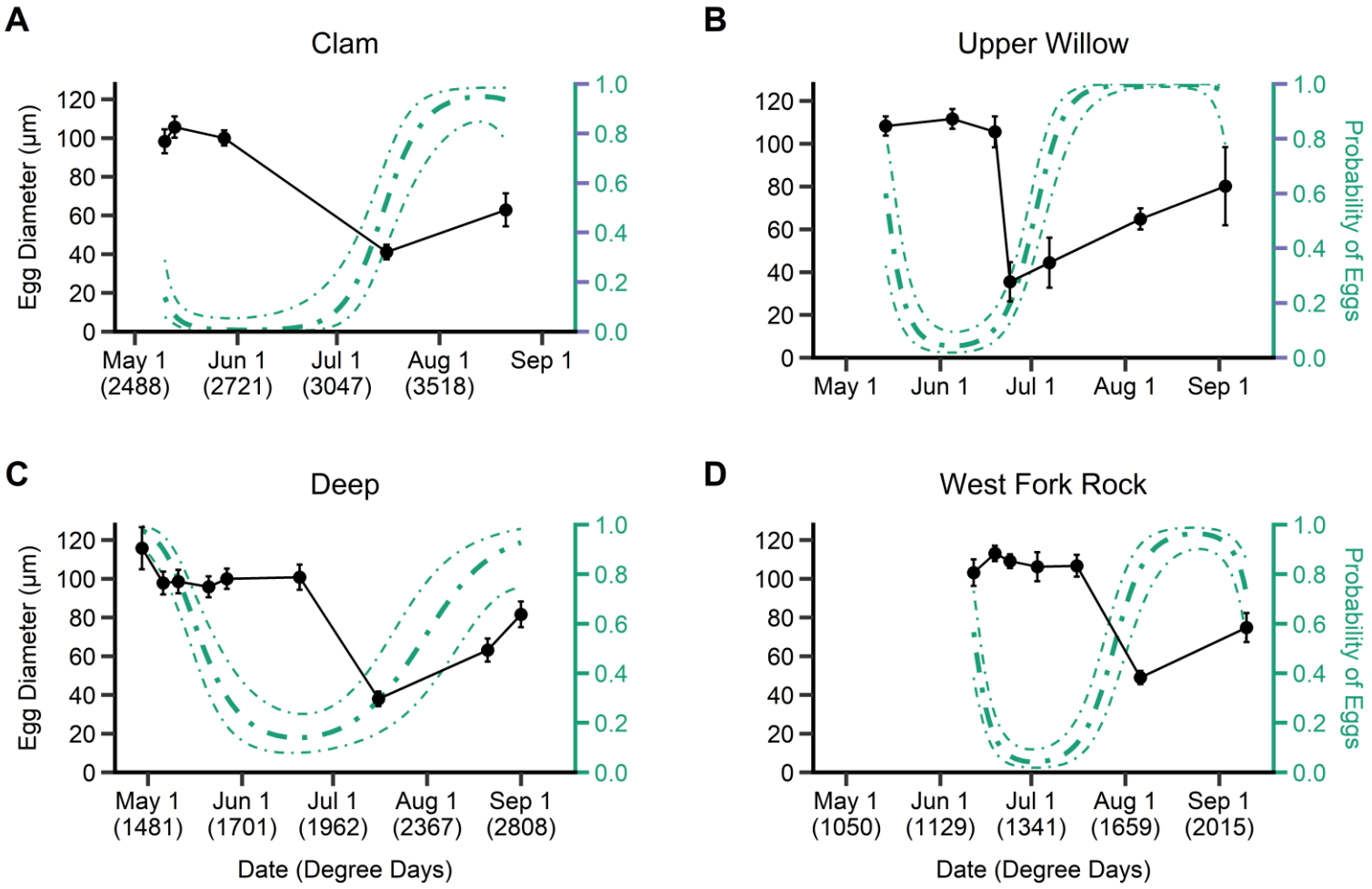

Figure 12. Gonadal egg development during spawning and gonadal recrudescence in 2020 in A) Clam Creek, B) Upper Willow Creek, C) Deep Creek, and D) West Fork Rock Creek. The average gonadal egg diameters (black circles) with standard error (black bars) for each sampling date were overlayed on top of the probabilities of the presence of gonadal eggs (thick green line) and 95% confidence intervals (thin green lines) modeled on date.

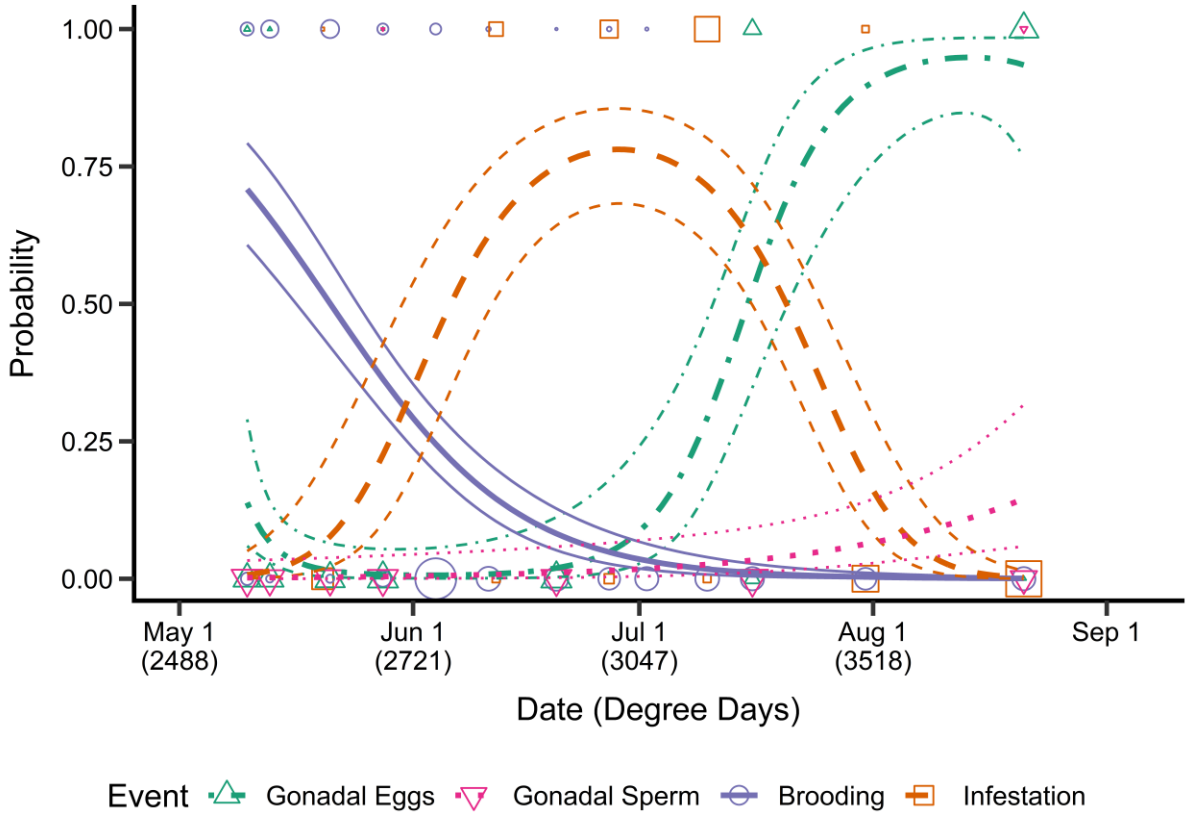

Figure 13. The binomial probability of a reproductive event occurring in Clam Creek mussels in 2020. The sizes of the symbols represent the relative number of individuals with reproductive status at one, or lacking reproductive status at zero. The probabilities for each reproductive event (thick lines) and 95% confidence intervals (thin lines), were modeled on day of year with the corresponding degree days reported in parentheses.

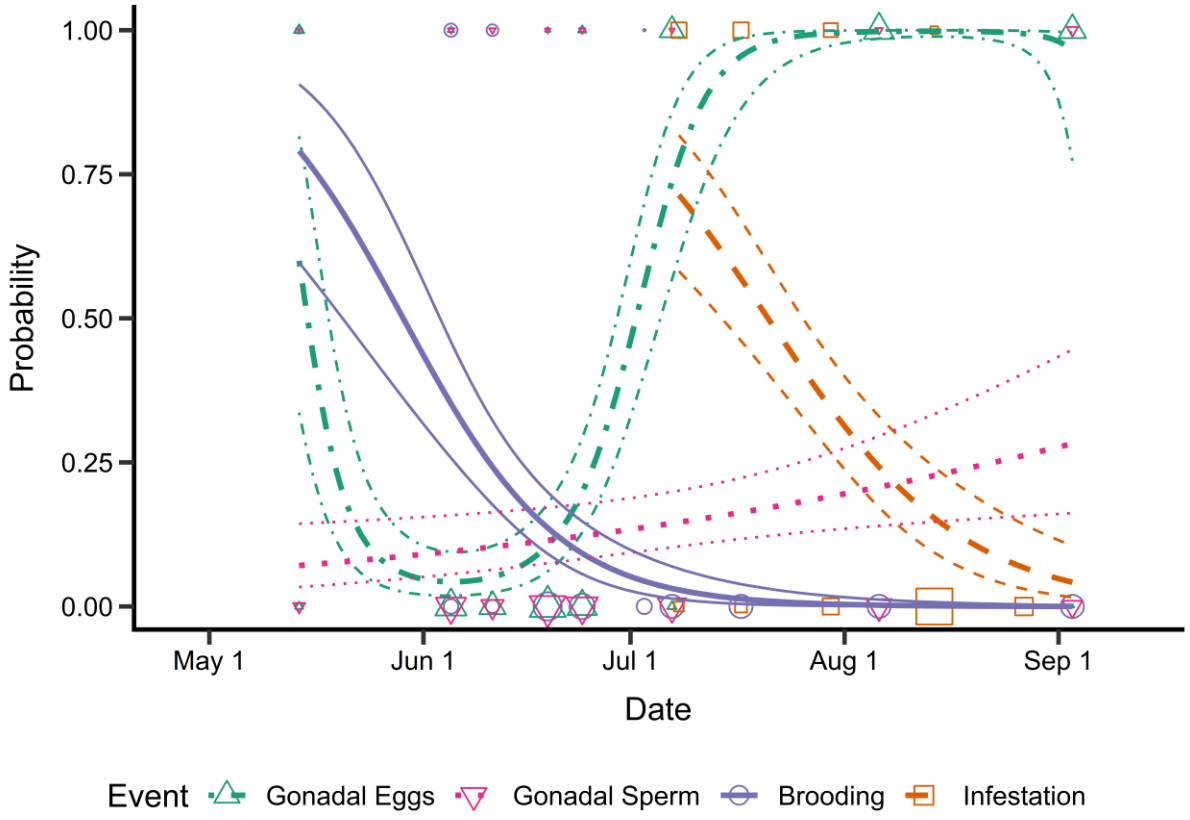

Figure 14. The binomial probability of a reproductive event occurring in Upper Willow Creek mussels in 2020. The sizes of the symbols represent the relative number of individuals with reproductive status at one, or lacking reproductive status at zero. The probabilities for each reproductive event (thick lines) and 95% confidence intervals (thin lines), were modeled on day of year with the corresponding degree days reported in parentheses.

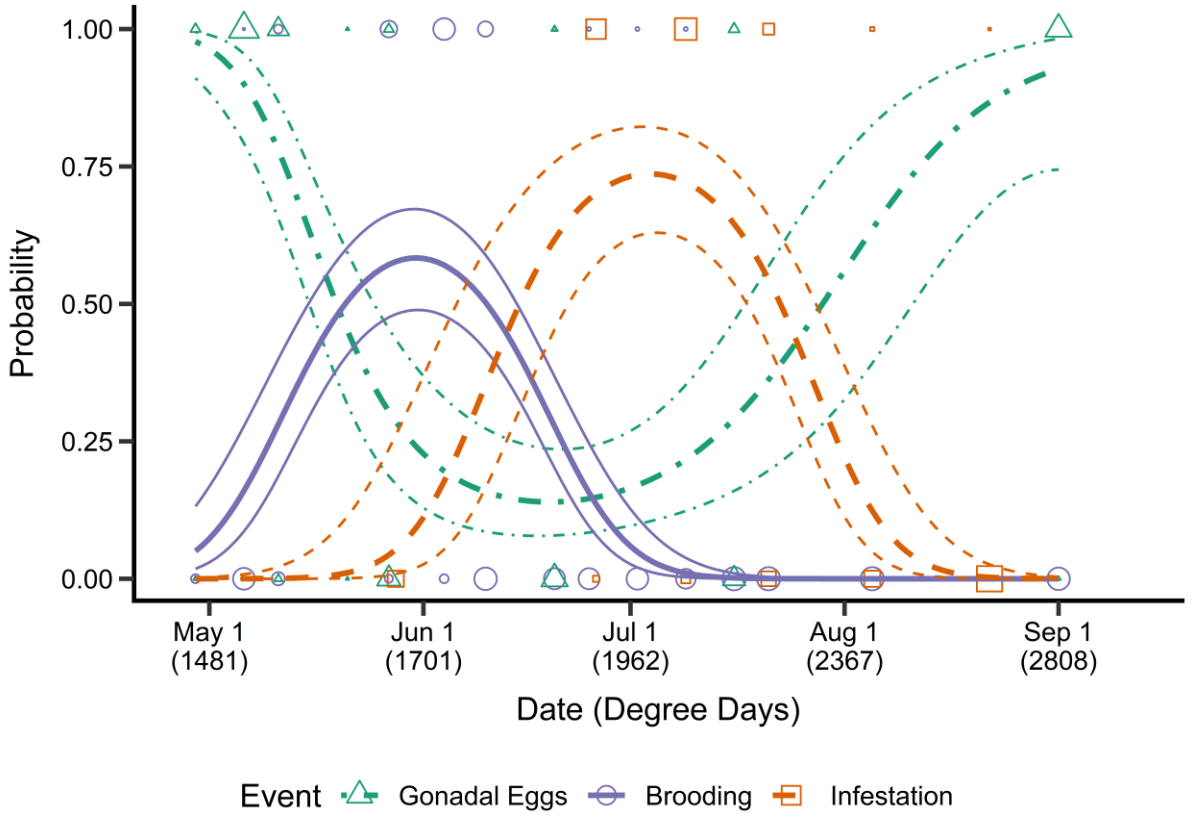

Figure 15. The binomial probability of a reproductive event occurring in Deep Creek mussels in 2020. The sizes of the symbols represent the relative number of individuals with reproductive status at one, or lacking reproductive status at zero. The probabilities for each reproductive event (thick lines) and 95% confidence intervals (thin lines), were modeled on day of year with the corresponding degree days reported in parentheses.

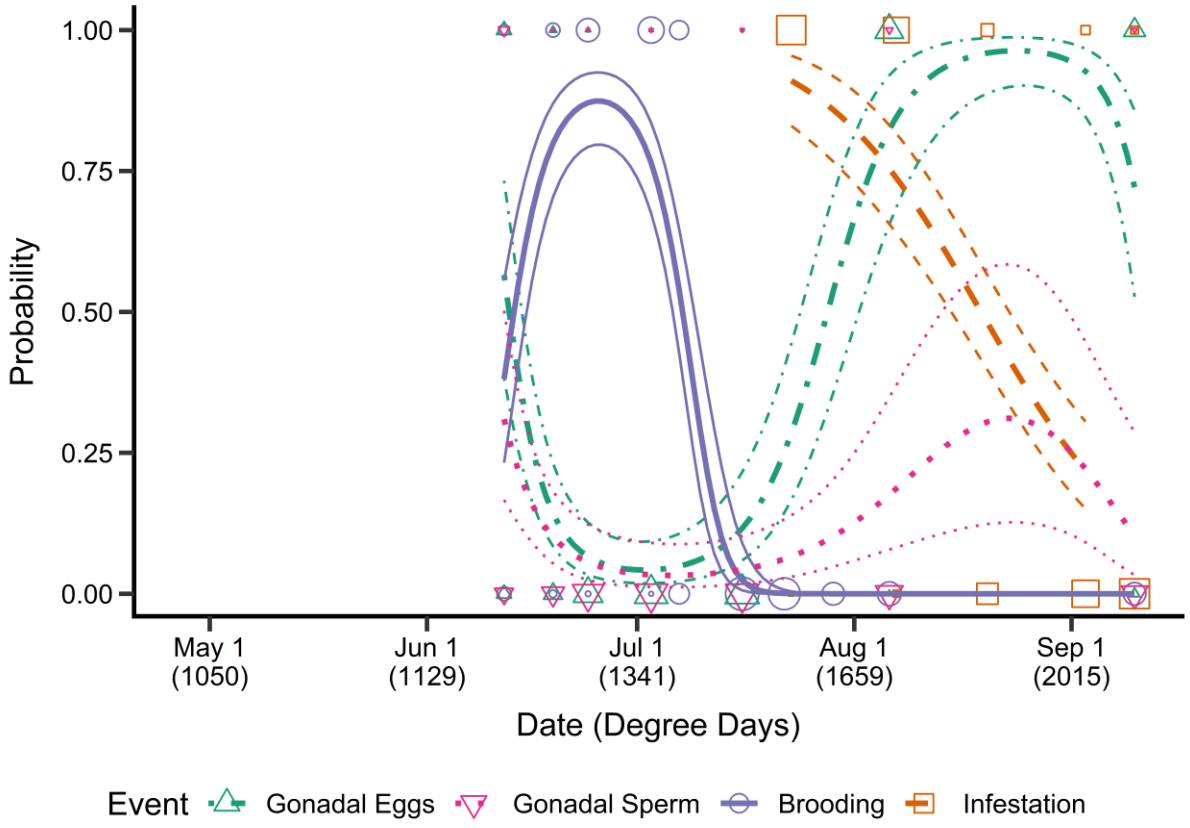

Figure 16. The binomial probability of a reproductive event occurring in West Fork Rock Creek mussels in 2020. The sizes of the symbols represent the relative number of individuals with reproductive status at one, or lacking reproductive status at zero. The probabilities for each reproductive event (thick lines) and 95% confidence intervals (thin lines), were modeled on day of year with the corresponding degree days reported in parentheses.

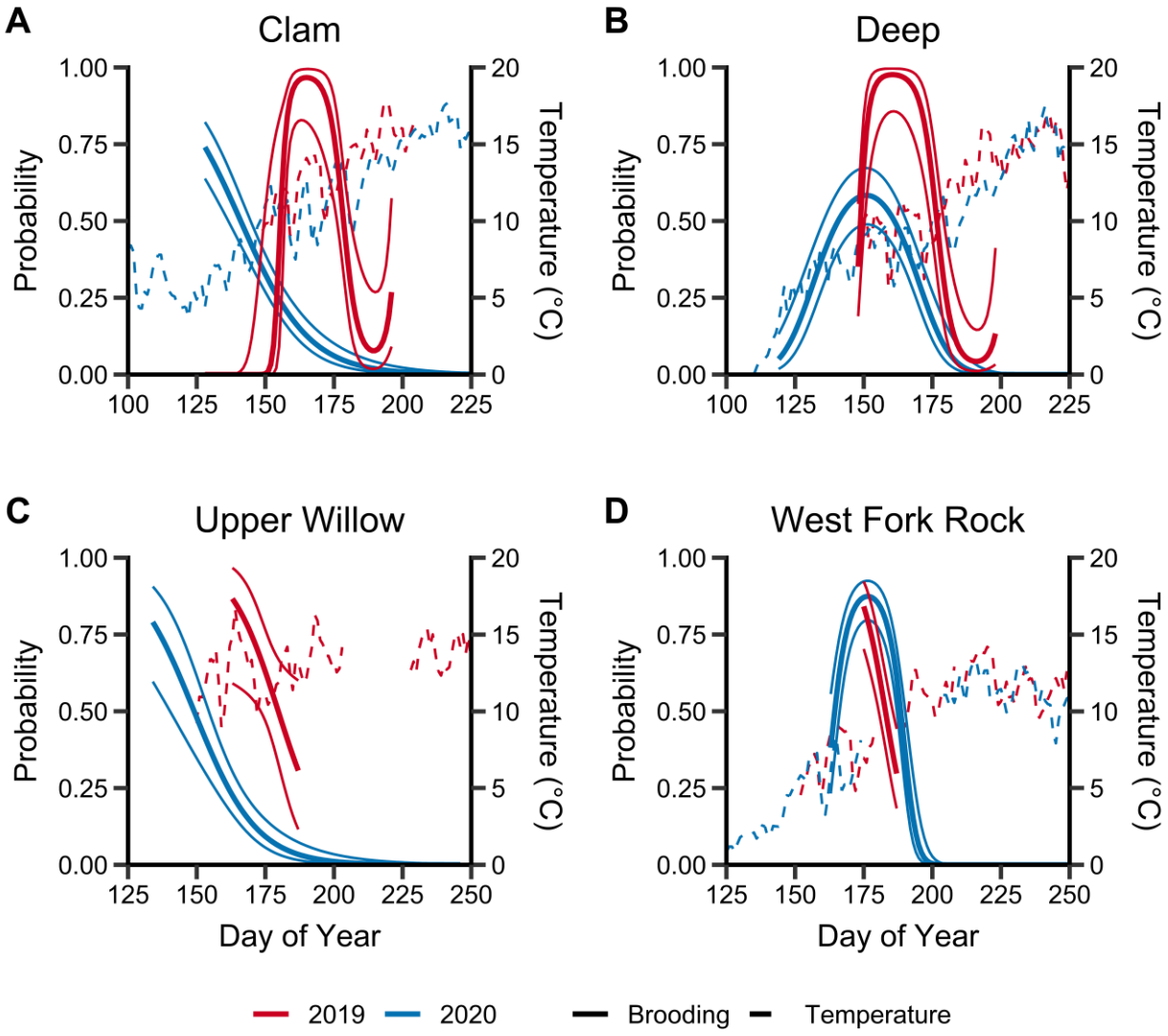

Figure 17. Brooding phenologies in 2019 and 2020 for A) Clam Creek mussels, B) Deep Creek mussels, C) Upper Willow Creek mussels, and D) West Fork Rock Creek mussels. The probabilities of brooding (solid lines) and the average daily temperatures (dashed lines) are modeled on day of year. The 2019 data (slightly warmer year) are in red, and the 2020 data (colder year) are in blue.

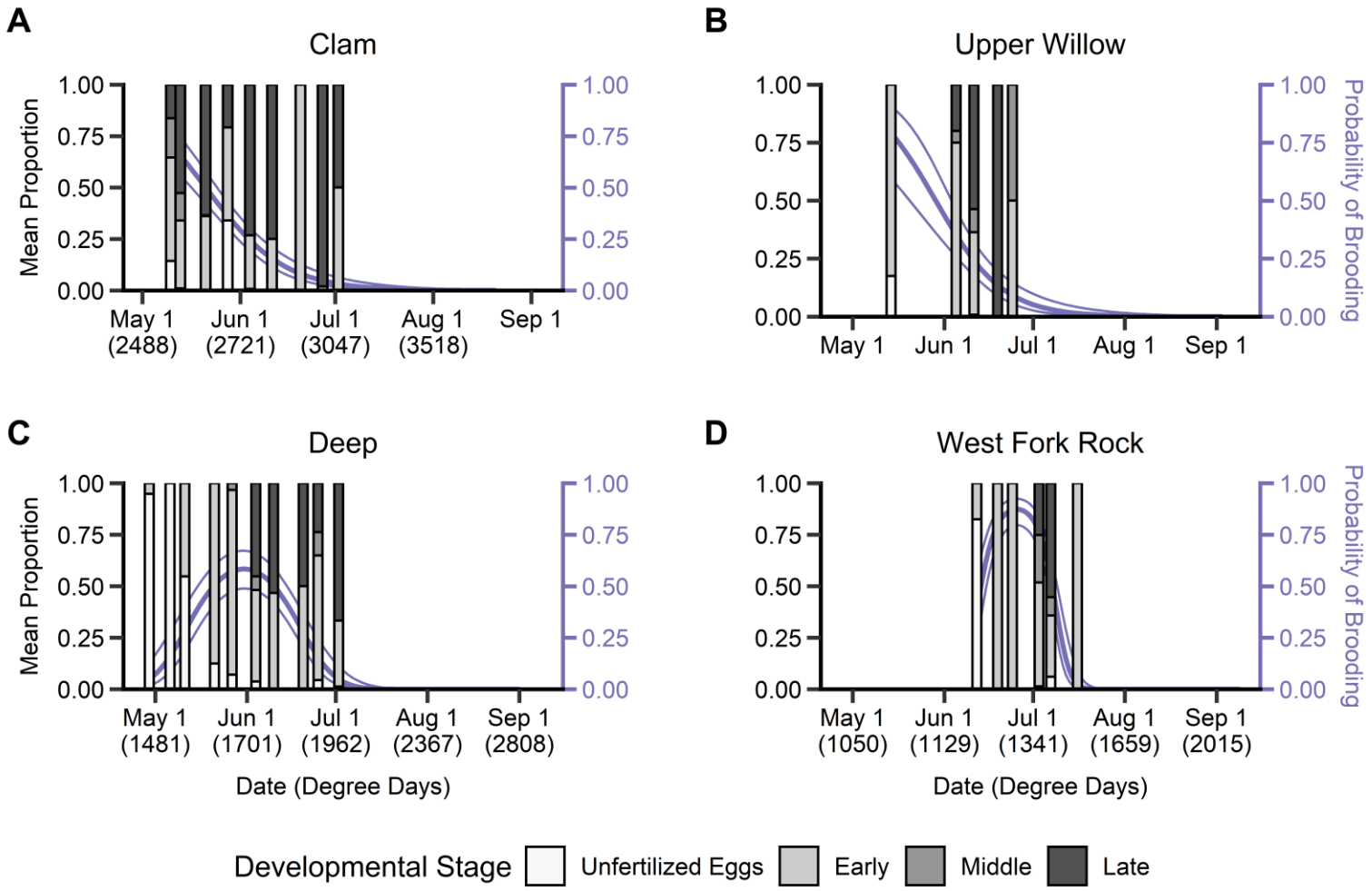

Figure 18. Embryo development (gray bars) during the brooding period in 2020 in A) Clam Creek, B) Upper Willow Creek, C) Deep Creek, and D) West Fork Rock Creek mussels. The area of the bars represent the mean proportion of marsupium samples made up of unfertilized eggs or embryos in early, middle, or late developmental stages. The probabilities of an individual mussel brooding (thick purple line) and 95% confidence intervals (thin purple lines) were modeled on date.

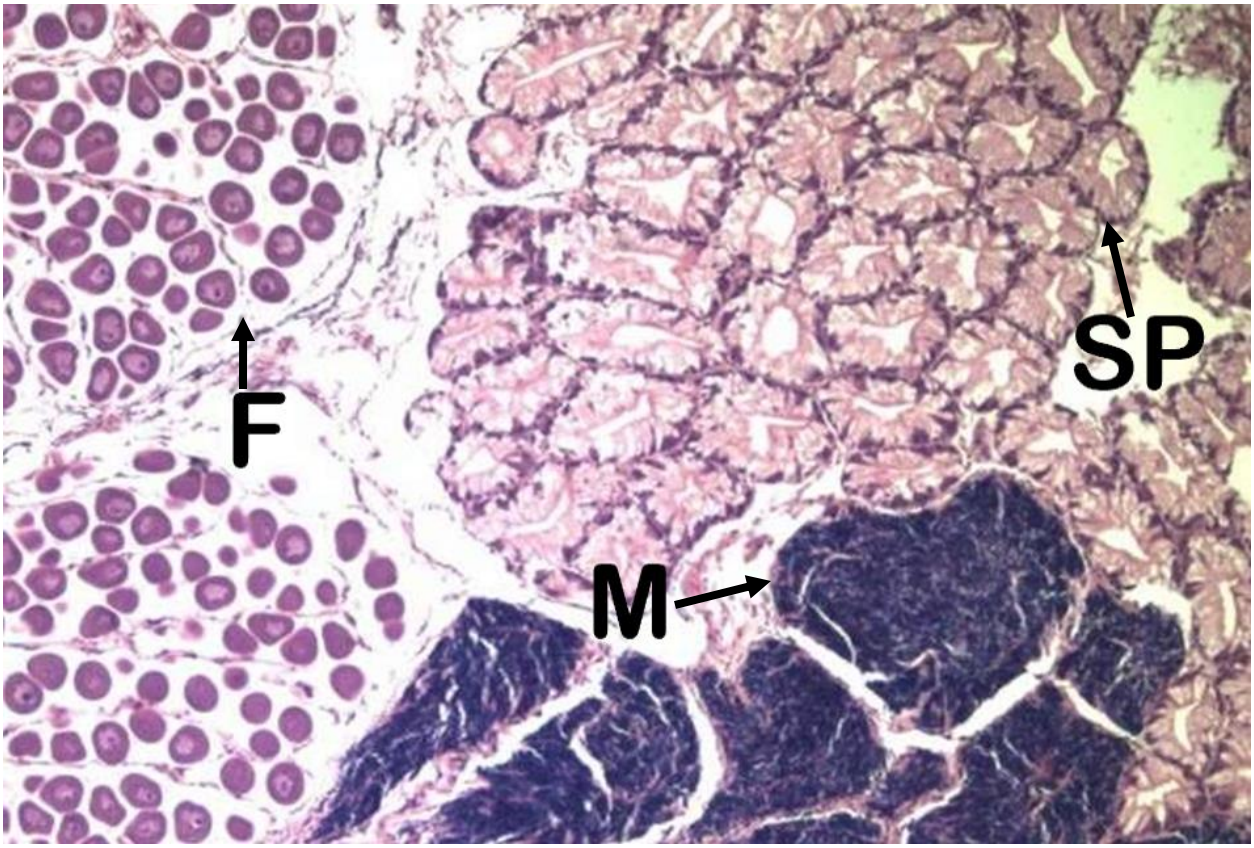

Figure 19. Gonadal tissue from a hermaphroditic Western Pearlshell that had ovaries present in different stages of the gametogenic cycle, indicating it may have been dribble spawning. This individual had ovaries that contained mature eggs ("F") and were recently spawned out ("SP") and testes that contained mature sperm ("M").

Table 5. Reproductive status of six marked mussels in Clam and West Fork Rock creeks. The degree days accumulate from the date mussels were first marked. The presence or absence of gonadal eggs and signs of brooding are designated as a one or zero, respectively. Embryo developmental stages are categorized as unfertilized eggs or embryos in early, middle, or late developmental stages.

		Clam Creek								
		Individual no. 1			Individual no. 2			Individual no. 3		
2020 Date	DD	Gonadal Eggs	Brooding	Embryo Stage	Gonadal Eggs	Brooding	Embryo Stage	Gonadal Eggs	Brooding	Embryo Stage
20-May	0	0	0		0	1	early	0	1	middle
27-May	56	0	0		0	1	early	0	1	late
3-Jun	133	NA	NA		NA	1	late	NA	NA	
10-Jun	201	0	0		0	0		0	0	
19-Jun	294	0	0		NA	NA		0	0	
26-Jun	383	0	0		NA	NA		0	0	

		West Fork Rock Creek								
		Individual no. 1			Individual no. 2			Individual no. 3		
2020 Date	DD	Gonadal Eggs	Brooding	Embryo Stage	Gonadal Eggs	Brooding	Embryo Stage	Gonadal Eggs	Brooding	Embryo Stage
18-Jun	0	0	1	unfertilized	1	0		0	1	unfertilized
23-Jun	37	NA	1	early	0	1	unfertilized	NA	1	early
2-Jul	121	0	1	middle	0	1	early	0	1	middle
6-Jul	159	NA	1	late	NA	0		NA	1	late
15-Jul	246	0	0		NA	NA		NA	NA	
21-Jul	306	NA	0		NA	NA		NA	0	

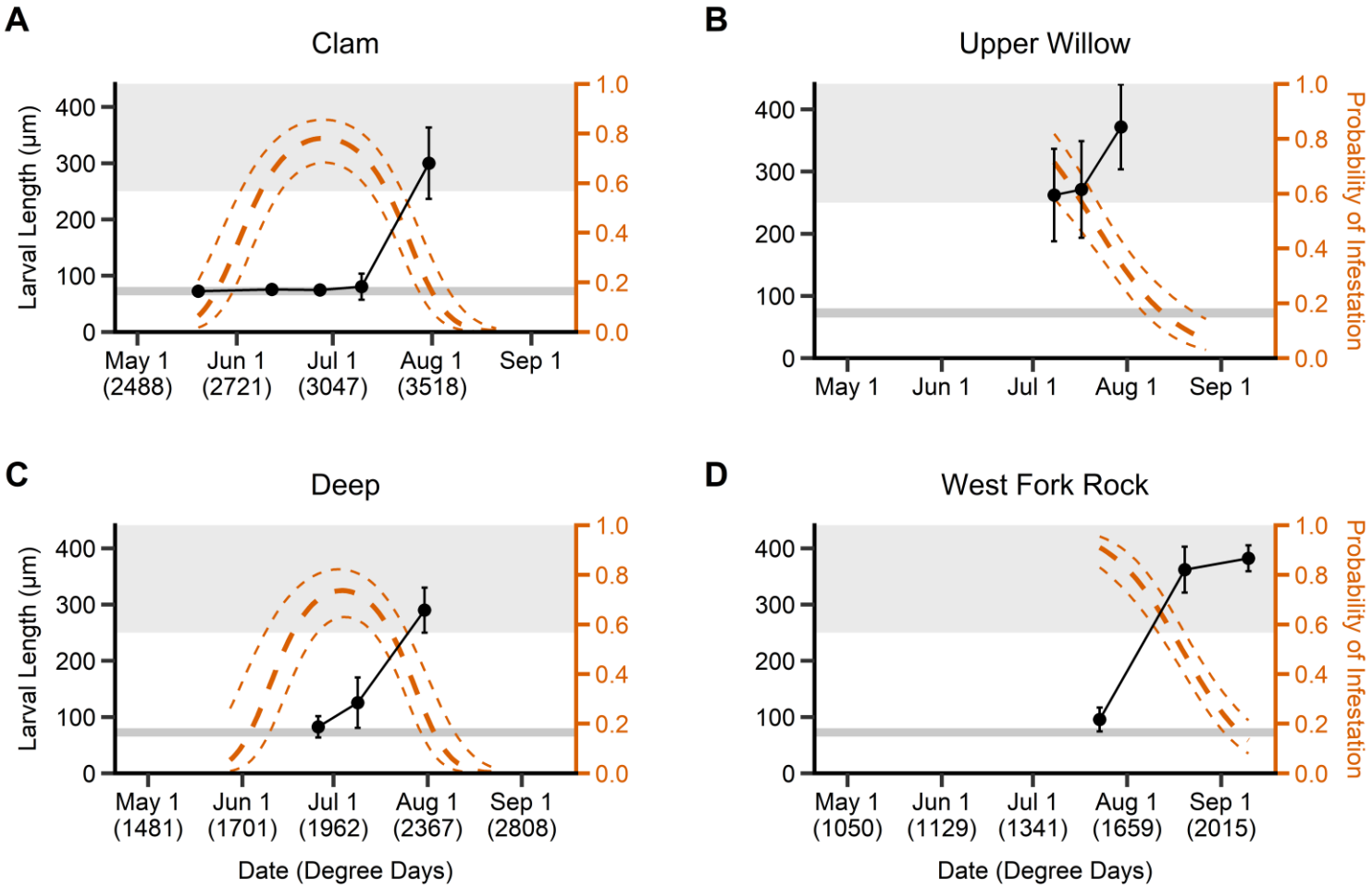

Figure 20. Larvae development during the infestation period in 2020 in A) Clam Creek, B) Upper Willow Creek, C) Deep Creek, and D) West Fork Rock Creek. The average larval lengths (black circles) with standard error (black bars) for each sampling date were overlayed on top of the probabilities of an individual fish being infested with larvae (thick orange line) and 95% confidence intervals (thin orange lines) modeled on date. The gray areas represent the lengths of larvae when they are released from mussels (65 to 80 µm) and lengths of juvenile mussels (minimum of 240 µm).

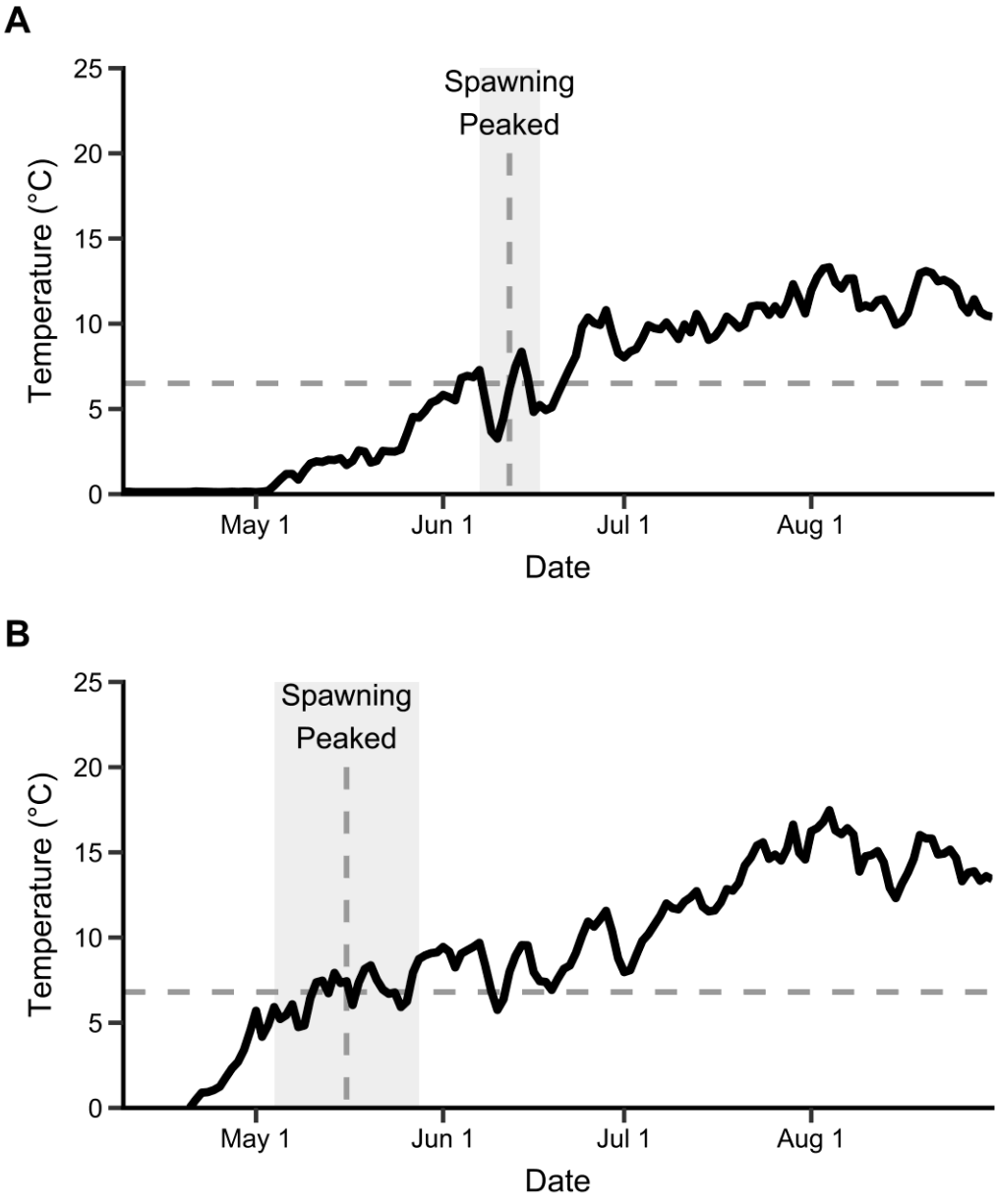

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Figure 21. The mean daily water temperature (black lines) during spawning in 2020 in (A) West Fork Rock Creek and (B) Deep Creek. The gray boxes denote the durations of the spawning periods and the dashed lines indicate the dates on which spawning peaked and the mean daily temperatures on those dates.

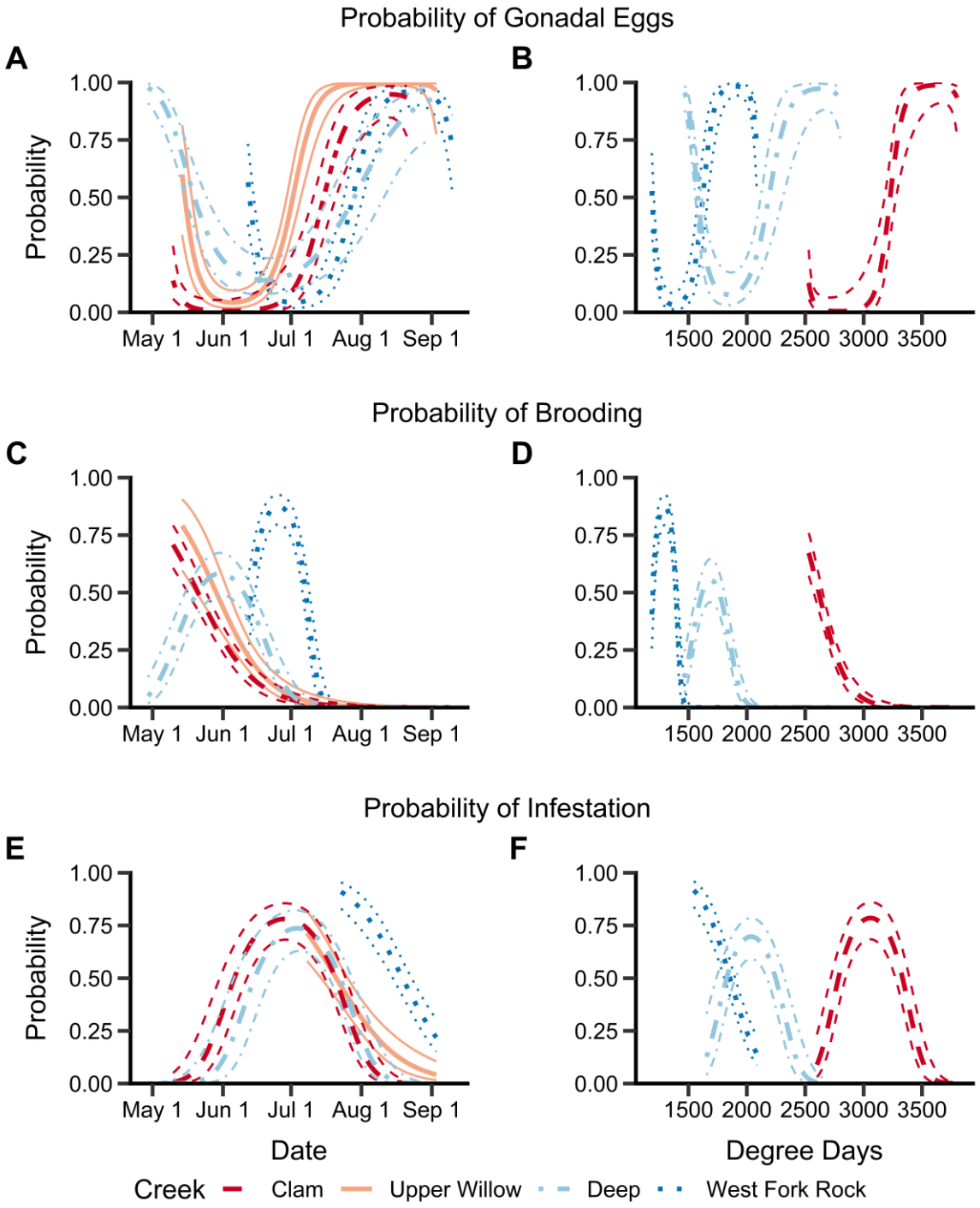

Figure 22. The probabilities of reproductive events occurring in 2020 modeled on day of year (left) and degree days (right). Degree days started accumulating on the date which brooding peaked in each population in 2019. The colors of the streams indicate their relative water temperatures; Clam Creek is the warmest, followed by Upper Willow Creek, Deep Creek, and West Fork Rock Creek.

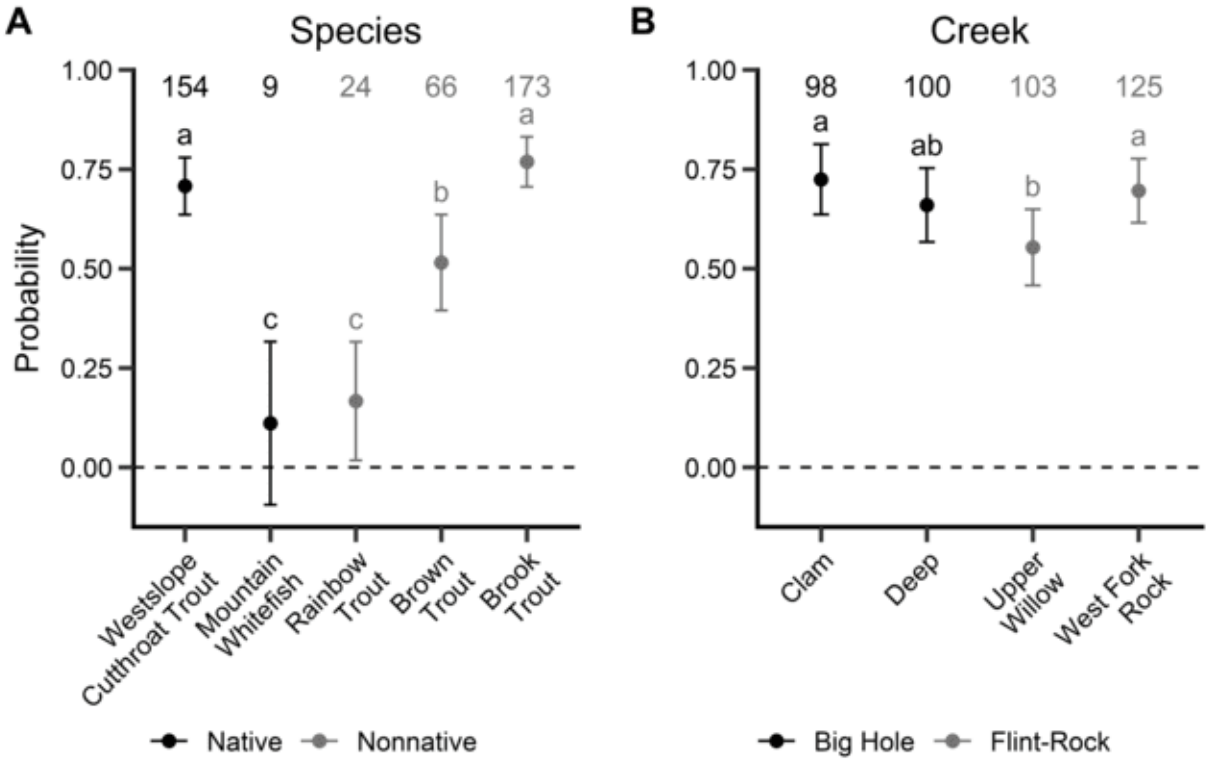

Figure 23. The estimated probabilities (circles) of larval infestation of an individual fish and 95% confidence intervals (bars) based on A) species and B) creek. Significant differences among probabilities are denoted by different letters. The number of fish examined during the infestation period are reported at the top of the graphs.

Table 6. Differences in the probabilities of infestation and infestation intensities between A) fish species and B) creeks. Differences in the probabilities of infestation (Probability) were tested for using the Wald test where the test statistics (χ^2), degrees freedoms (df), and *P*-values are reported for each paired comparison. Differences in the infestation intensities (Median number of larvae) were tested post-hoc using the Dunn's test in which the *Z*-test statistic and adjusted *P*-values (using Holm's procedure) are reported.

					Probability Comparisons			Infestation Intensity Comparisons	
A.	Fish Species	Probability	Median	Species Comparison	χ^2	df	*P*-values	*Z*	*P*-values
	Brook Trout	77%	42	Westslope Cutthroat Trout	1.6	1	0.210	-1.090	0.552
				Brown Trout	14	1	< 0.001	3.240	0.006
				Rainbow Trout	23.8	1	< 0.001	4.950	< 0.001
				Mountain Whitefish	9.3	1	0.002	3.730	0.001
	Westslope Cutthroat Trout	71%	76	Brown Trout	7.4	1	0.007	-4.020	< 0.001
				Rainbow Trout	18.8	1	< 0.001	-5.470	< 0.001
				Mountain Whitefish	7.6	1	0.006	-4.090	< 0.001
	Brown Trout	52%	26	Rainbow Trout	7.7	1	0.005	2.570	0.040
				Mountain Whitefish	3.9	1	0.490	2.200	0.083
	Rainbow Trout	17%	0	Mountain Whitefish	0.16	1	0.690	-0.360	0.720
	Mountain Whitefish	11%	0						
B.	Stream	Probability	Median	Stream Comparison	χ^2	df	*P*-values	*Z*	*P*-values
	Clam	72%	33	West Fork Rock	0.96		0.330	-0.395	1.000
				Deep	6.3	1	0.012	-0.254	1.000
				Upper Willow	0.22	1	0.640	-0.448	1.000
	West Fork Rock	70%	43	Deep	2.4	1	0.120	-0.125	1.000
				Upper Willow	0.33	1	0.570	0.810	0.935
	Deep	66%	36	Upper Willow	4.9	1	0.027	-0.194	1.000
	Upper Willow	55%	32						

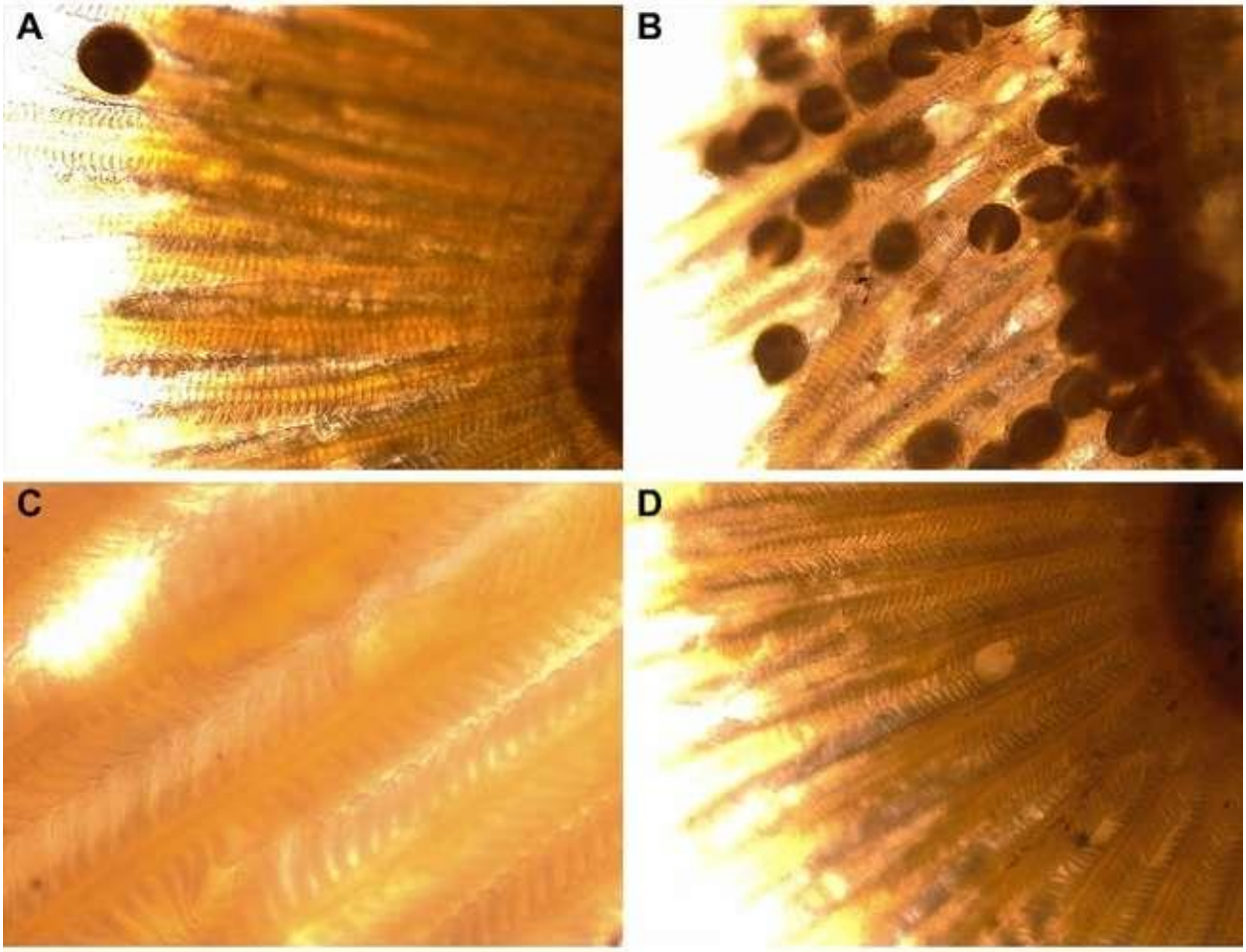

Figure 24. Metamorphic success of Western Pearlshells on various salmonids evidenced by mussel length and the presence of adductor muscles. Juvenile mussels on a A) Brown Trout and B) Brook Trout. Juvenile mussels became dislodged from the gills of a C) Rainbow Trout and D) juvenile Mountain Whitefish while handling the gills under the microscope.

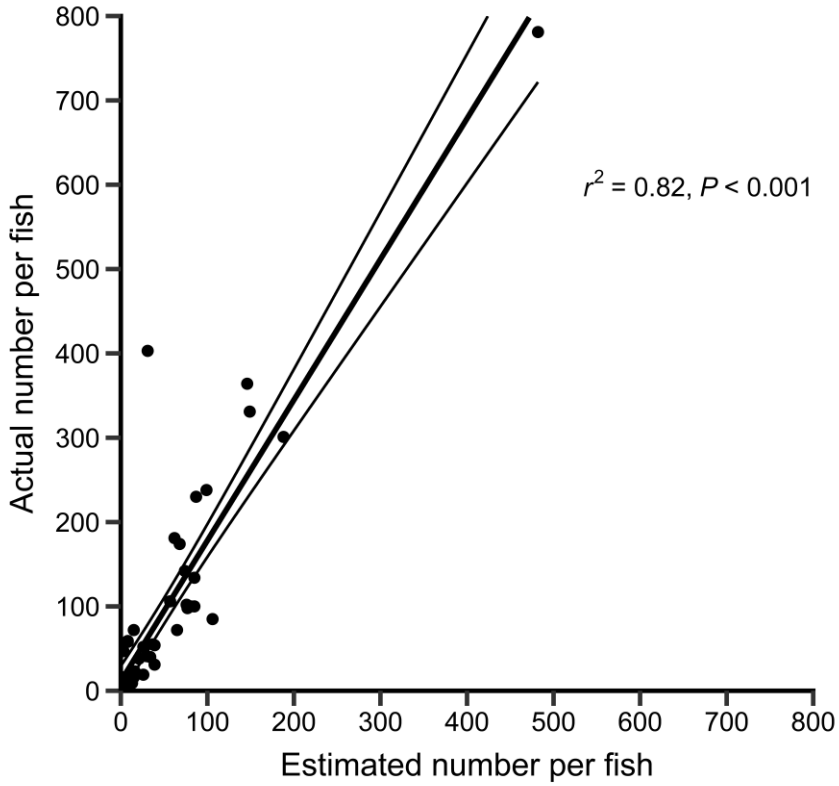

Figure 25. The relationship between the estimated (counted in the field) and actual (counted microscopically) larval abundances on individual fish, under one operculum. The linear regression model (thick, solid line) with 95% confidence intervals (thin, solid line) was used to correct the estimated larval abundance.

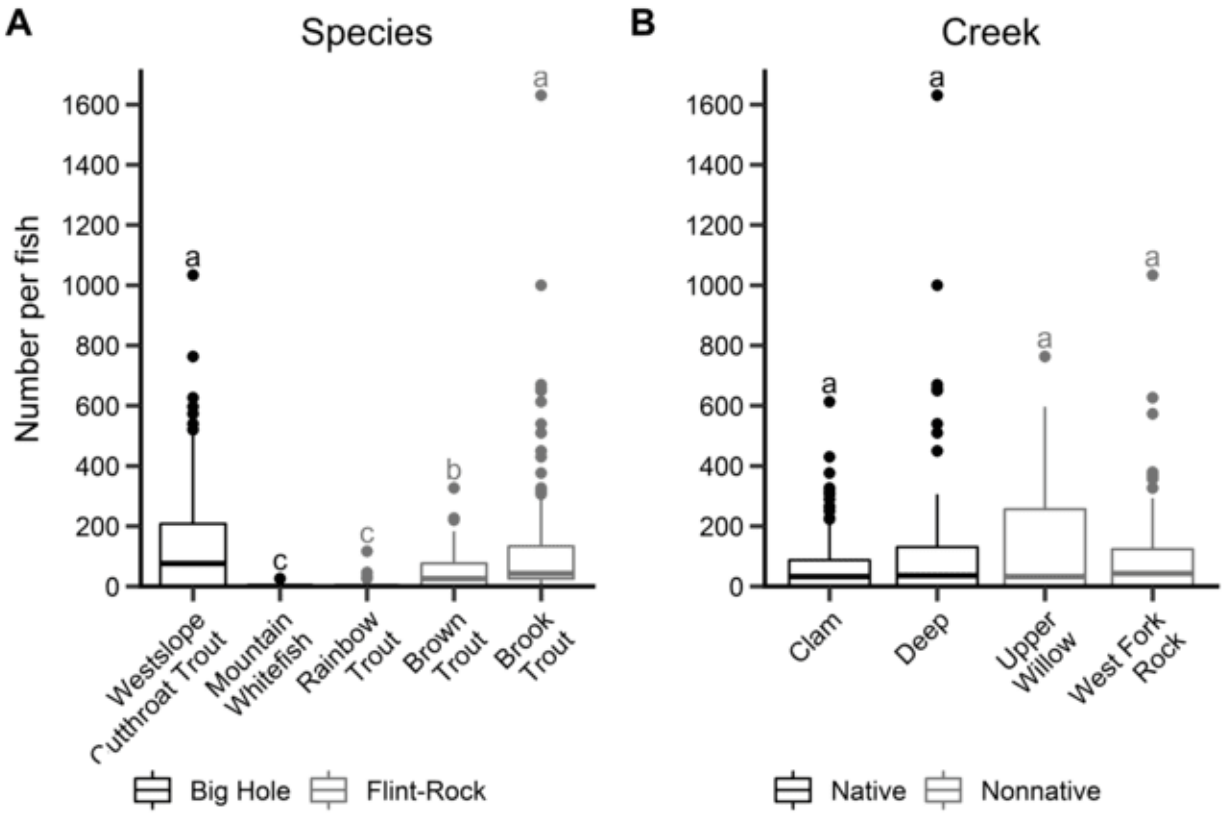

Figure 26. Infestation intensity among A) fish species and B) creek. The boxplots indicate median values, interquartile ranges, and outliers (circles). Significant differences in the numbers of larvae per fish among fish species are denoted by different letters. The numbers of larvae per fish did not significantly differ among streams.

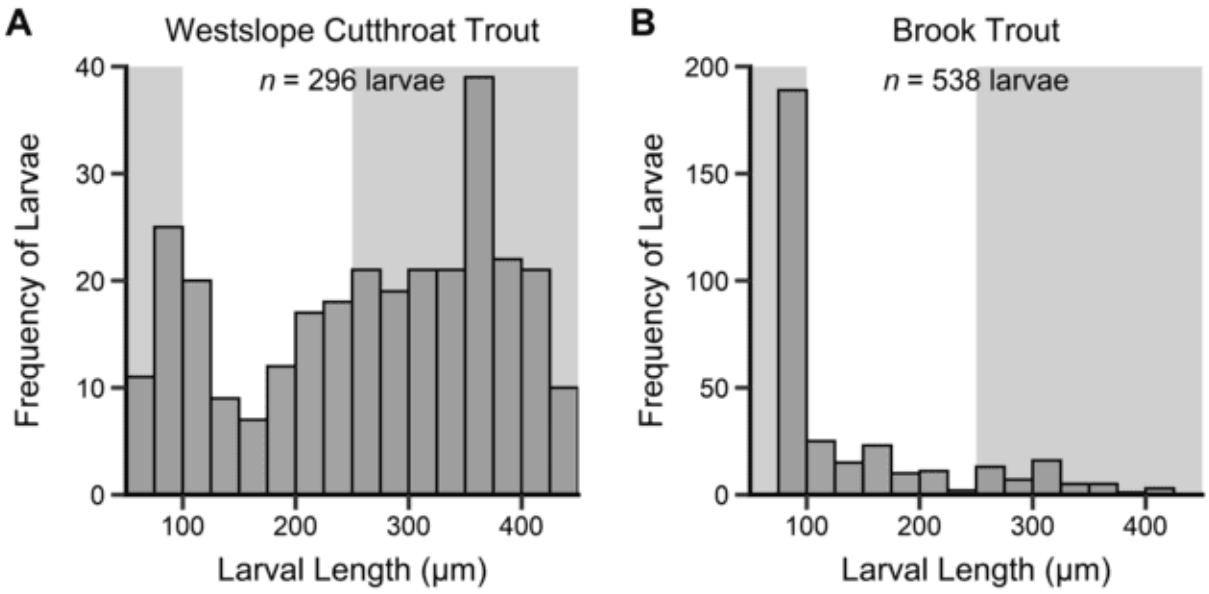

Figure 27. Length-frequency distributions of larvae attached on A) 10 Westslope Cutthroat Trout and B) 27 Brook Trout. The gray areas represent the lengths of larvae when they are released from mussels (65 to 80 µm) and lengths of juvenile mussels when they excyst from fish (minimum of 240 µm).

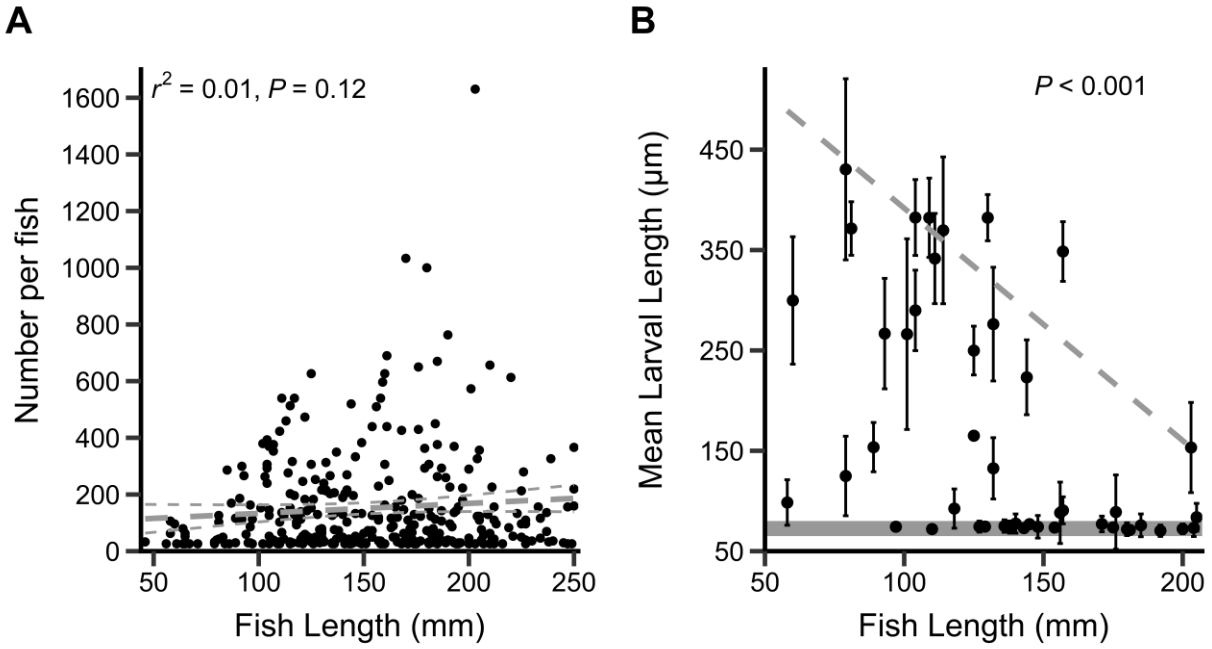

Figure 28. Relationships describing acquired immunity of hosts to larvae. A) The linear regression model and 95% confidence intervals (gray dashed lines) indicate no linear relationship existed between fish lengths and larval abundances (black circles). B) The 90th quantile regression model (gray dashed line) for the relationship between fish lengths and mean larval lengths (black circles) and standard error (black bars). The gray area at the bottom of the graph represents the larval lengths at which larvae first attach to hosts (65 to 80 µm). Fish > 250 mm in total length were excluded from both analyses.