ECOLOGY OF ONTOGENETIC BODY-MASS SCALING OF GILL SURFACE AREA IN A FRESHWATER CRUSTACEAN

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ABSTRACT

Several studies have documented ecological effects on intra- and interspecific body-size scaling of metabolic rate. However, little is known about how various ecological factors may affect the scaling of respiratory structures supporting oxygen uptake for metabolism. Our study is apparently the first to provide evidence for ecological effects on the scaling of a respiratory structure among conspecific populations of any animal. We compared the body-mass scaling of gill surface area (SA) among eight spring-dwelling populations of the amphipod crustacean *Gammarus minus*. Although gill SA scaling was not related to water temperature, conductivity or *G. minus* population density, it was significantly related to predation regime (and secondarily to pH). Body-mass scaling slopes for gill SA were significantly lower in four populations inhabiting springs with fish predators than for those in four springs without fish (based on comparing means of the population slopes, or slopes calculated from pooled raw data for each comparison group). As a result, gill SA was proportionately smaller in adult amphipods from fish vs. fishless springs. This scaling difference paralleled similar differences in the scaling exponents for the rates of growth and resting metabolic rate. We hypothesized that gill SA scaling is shallower in fish vs. fishless spring populations of *G. minus* because of effects of size-selective predation on size-specific growth and activity that in turn affect the scaling of oxygen demand and concomitantly the gill capacity (SA) for oxygen uptake. Although influential theory claims that metabolic scaling is constrained by internal body design, our study builds on previous work to show that the scaling of both metabolism and the respiratory structures supporting it may be ecologically sensitive and evolutionarily malleable.

INTRODUCTION

Metabolism fuels all biological processes. Despite its fundamental importance, the rate of metabolism varies considerably among organisms and environments for reasons incompletely understood. One major intrinsic factor that is strongly related to metabolic rate is body size. This relationship is often so regular that it can be described by a simple power function:

$$R = aM^b \tag{1}$$

This equation describes a linear relationship in log-log space, where R is metabolic rate, a is the scaling coefficient (antilog of the intercept of the log-linear regression line), M is body mass, and b is the scaling exponent (slope of the log-linear regression line). For decades, the scaling exponent was claimed to be 3/4 or approximately so, hence the proclamation of a "3/4-power law" (Kleiber, 1932, 1961; Schmidt-Nielsen, 1984, Savage et al., 2004). Furthermore, this law has been explained as being the result of resource (oxygen and nutrient) supply constraints, in particular those arising from the geometry and physics of internal resource-transport networks (West et al., 1997; Banavar et al., 2010; Banavar et al., 2014).

However, the 3/4-power law and the resource-transport network (RTN) theory used to support it have received severe criticism. First, the 3/4-power law is not universal: metabolic scaling relationships vary widely with exponents ranging from 0 to >1, but usually between 2/3 and 1 (reviewed in Glazier, 2005, 2010, 2014b; Agutter and Tuszynski, 2011; Hulbert, 2014; White and Kearney, 2014). Second, variation in metabolic scaling exponents has been linked to various environmental, physiological and developmental factors, thus showing that metabolic scaling cannot be a simple (sole) function of RTNs (Glazier, 2005, 2010, 2014b, c; White and Kearney, 2014). Third, RTN models apply only to a small fraction of existing animal species (mainly vertebrates) that have a closed circulatory system with a single central pump (heart) (Price et al., 2012; Glazier, 2014b). Fourth, no direct (e.g., experimental) evidence supporting predicted effects of RTNs on metabolic scaling yet exists (Glazier, 2014b), though future research may address this deficiency (see Tekin et al., 2016). Fifth, multiple lines of evidence contradict the predictions of RTN models. According to RTN models, the scaling exponent should not vary with activity level, but it does so, and in a systematic way that can be explained by changes in resource demand rather than resource supply (Glazier, 2005, 2010, 2014b, c). According to RTN models, oxygen and nutrients should be more limiting to cells of large versus small organisms, but currently available empirical evidence contradicts this prediction (Kozlowski and Konarzewski, 2004; Helm and Davidowitz, 2013; Harrison et al., 2014; Glazier, 2015a). In addition, current RTN models predict that the scaling exponents for metabolic rate should be lower in organisms that grow predominantly only in one or two dimensions than in organisms that grow isomorphically (West et al., 1999; Banavar et al., 2010; Dodds 2010), but the opposite has been found in many kinds of shape-shifting pelagic animals (Hirst et al., 2014; Glazier et al., 2015).

To fully understand the extensive variation in the body-mass scaling of the rates of metabolism and other associated biological processes that exists, it is necessary to consider the effects of both resource supply and demand (Glazier, 2014b, 2015b). Resource supply to metabolizing cells is affected not only by RTNs, but also by the areas and uptake capacities of various respiratory and digestive surfaces. In fact, the scaling of metabolic rate parallels the scaling of body surface area in skin-breathing pelagic invertebrate animals (Hirst et al., 2014; Glazier et al., 2015), and of pulmonary oxygen diffusion rates in various vertebrate animals (Gillooly et al., 2016). Rates of metabolic waste excretion also appear to be related to body surface area in pelagic invertebrates (Hirst et al., 2017). In addition, body volume-related changes in whole organism resource demand (as associated with growth, food processing, and locomotor activity) and other size-specific changes in functional energy expenditures and the relative proportions of tissues with different metabolic activity may significantly affect the scaling of metabolic rate (reviewed in Glazier, 2014b).

A key question regarding the causes of metabolic scaling is how size-specific resource supply and demand are interrelated, and what are the mechanisms involved. Although several studies have examined how the area of respiratory surfaces and their scaling with body size may be related to the intensity and body-mass scaling of metabolic rate in various aquatic animals (e.g. Brown and Shick, 1979; Shick et al., 1979; Johnson and Rees, 1988; Post and Lee, 1996; Rombough and Moroz, 1997; Wegner et al., 2010; White and Seymour, 2011; Hirst et al., 2014; Glazier et al., 2015; Killen et al., 2016), hardly anything is known about how these relationships may be affected by various environmental factors. Furthermore, to our knowledge, no study has examined how various ecological factors may affect the scaling of respiratory surface area among populations of a single species.

Therefore, the purpose of this study was to examine whether the ontogenetic scaling of gill surface area in eight populations of the amphipod *Gammarus minus* is related to differences in various abiotic and biotic environmental factors among the freshwater springs that they inhabit. Gill surface area varies among and within amphipod species from different habitats (Moore and Taylor, 1984; Spicer and Taylor, 1986; Swain and Richardson, 1993; Tsubokura et al., 1998; Roast and Jones, 2003), but environmental effects on the ontogenetic body-mass scaling of gill surface area among conspecific populations, and their possible relationship to ontogenetic metabolic scaling, have not yet been studied.

Spring-dwelling amphipods are useful for this kind of investigation because (1) they are abundant, easy to capture, and readily studied in the laboratory, (2) their leaf-like gills are thin and flat (Steele and Steele, 1991), thus facilitating the measurement of their surface areas, (3), they appear to have limited dispersal ability, as indicated by their significant inter-population genetic differentiation (Gooch, 1990; Gooch and Glazier, 1991; Culver et al., 1995), thus likely accentuating their adaptation to local environmental conditions via natural selection, as indicated by their significant inter-population differentiation in various physiological, morphological and life-history traits (Glazier et al., 1992; Culver et al., 1995; Glazier, 1998, 1999; Glazier and Sparks, 1997; Glazier and Deptola, 2011; Glazier et al., 2011), (4) data on metabolic scaling from five populations are available for comparison (Glazier et al., 2011), and (5) the numerous study springs exhibit remarkably constant intra-site environmental conditions (especially of substrate composition and water temperature, chemistry and flow rates), but several readily measured environmental factors vary considerably among sites, often independently of one another, thus facilitating incisive comparative analyses (Glazier and Gooch, 1987; Glazier et al., 1992; Glazier, 1999; Glazier et al., 2011). In general, springs are useful natural laboratories for ecological and evolutionary studies (Glazier, 1998, 2014d). Amphipods also deserve study because they are keystone species in springs and other aquatic habitats where they play important roles in ecosystem trophic dynamics and nutrient cycling (Glazier, 2014a).

We tested four, not mutually exclusive hypotheses. First, since the gills are the primary sites for oxygen uptake in *Gammarus* (Sutcliffe, 1984; Maltby, 1994; Roast and Jones, 2003), the body-mass scaling of gill surface area should parallel the scaling of metabolic rate, as estimated by rate of oxygen uptake. This hypothesis assumes that the intensity of oxygen uptake per unit gill area is invariant or nearly so (but see Johnson and Rees, 1988). Specifically, the scaling exponent for gill surface area should be less in populations inhabiting springs with versus without fish predators, thus matching the difference in metabolic scaling exponents reported by Glazier et al. (2011). Furthermore, since amphipods are less active in the presence of fish (Andersson et al., 1986; Holomuzki and Hoyle, 1990; Wooster, 1998; Åbjörnsson et al., 2000), they should require less gill surface area for oxygen uptake than more active amphipods living in the absence of fish.

Second, gill surface area should be greater in populations inhabiting warm versus cold springs, to support enhanced metabolic demand, while also compensating for reduced dissolved oxygen in warmer water (again assuming that oxygen uptake per unit gill area is invariant). Metabolic rate (and thus demand for oxygen) increases with increasing temperature, whereas oxygen availability decreases, both of which would favor larger gills with a higher capacity for oxygen uptake. In addition, according to the metabolic-level boundaries hypothesis (Glazier, 2010, 2014c; Killen et al., 2010), the scaling exponent for gill surface area should be inversely related to water temperature (assuming that gill surface area and metabolic rate are related).

Third, since crustacean gills are multi-functional, not only serving as respiratory gas-exchange organs, but also engaging in active ion transport required for ionic and osmotic regulation (Pequeux, 1995; Lingot et al., 2000; Brooks and Mills, 2003; Freire et al., 2008; Henry et al., 2012), gill surface area should be smaller in aquatic habitats with high versus low ion concentrations (conductivity), because a less steep ionic gradient between the inside and outside of the body entails a reduced rate of ion loss and thus a lesser demand for compensating ion uptake (assuming that ion uptake per unit gill area is invariant). The lower metabolic demand of ionic regulation in water with high ion concentrations (Sutcliffe, 1984; Glazier and Sparks, 1997) should also require less oxygen uptake, again favoring smaller gills.

Fourth, since amphipods are cannibalistic (larger individuals often prey on smaller individuals: MacNeill et al., 1997; McGrath et al., 2007; D. S. Glazier, personal observations), secretive behavior and associated reduced activity in small juvenile amphipods may require less oxygen uptake and gill surface areas in populations with high versus low densities. If so, higher population density should be associated with decreases in juvenile gill sizes, but no effect on adult gill sizes, thus causing steeper scaling of gill surface area.

MATERIALS AND METHODS

Study organism and sites

Gammarus minus is an omnivorous detritivore commonly found in springs and streams from the mid-Appalachians to the Ozarks in North America (Holsinger, 1976). Using dip nets, individuals ranging in body length from ~3 to 12 mm were collected within 10 m of each source of eight rheocrenes (lotic springs): Blue (BL), Ell (EL), Kanesatake (KS), Petersburg (PT) and Warm (WH) springs in Huntingdon County, Williamsburg (WB) spring in Blair County, Big Rock (BR) Spring in Mifflin County and Warm Spring (WP) in Perry County. These spring sites were selected to allow a factorial analysis of the effects of water temperature, conductivity, and predation regime on gill surface area (see Table 1). Qualitative estimates of *G. minus* population density were made because variation in substrate composition prevented the use of a single kind of quantitative sampler in all springs.

Spring environmental variables

At the times of amphipod sampling (summer, fall and winter seasons during 1 July 2013 to 3 March 2015), environmental measurements were made within 10 m of the source of each spring. Water temperature, conductivity and pH were measured with digital conductivity (YSI, Yellow Springs, OH) and pH (Markson LabSales, Henderson, NC) field meters (Table 1). These variables show little monthly variation in our study springs (Table 1; Glazier et al. 1992). Four of the study springs contain fish predators, three with *Cottus cognatus* (WB, BL, EL) and one with *Rhinichthys atratulus* (WH) (Table 1). WH also contains several other kinds of relatively large predators including Caudata, Decapoda, Megaloptera and Odonata. Additional environmental characteristics of the study springs are described elsewhere (Glazier & Gooch, 1987; Gooch & Glazier 1991; Glazier et al., 1992; Glazier 1998, 1999; Glazier et al., 2011).

Gill surface area measurements

Juveniles and adult males (identified by enlarged gnathopods) were used for gill-area estimates. Adult females were not used because their body-mass estimates were affected by the mass of developing eggs and embryos. Individuals were maintained in native spring water with detrital leaf food in 1-L containers at 10^{o} C until body measurements were made (not more than 1 week after capture).

Before measurements were made, each specimen was anesthetized in carbonated spring water. After measuring the body length (± 0.1 mm; base of first antenna to base of telson), the fifth coxal gill was removed and photographed using a microscope-mounted camera (Sony CCD-Iris, Tokyo, Japan). This gill was selected because it is morphologically distinct from the neighboring gills, and located between the two longest legs of the organism, thus making it easy to identify and remove [*Gammarus* species and other gammaridean amphipods typically have six pairs of thoracic gills (Moore and Taylor, 1984; Sutcliffe 1984; Steele and Steele, 1991; Roast and Jones, 2003)]. We considered the surface area (SA) of the 5th coxal gill to be a useful relative index of total gill SA because the proportional relationships between the size of this gill and that of other gills is not significantly different from 1 (based on width measurements of the relatively large 4th, 5th and 6th coxal gills in 18 individuals with body lengths ranging from 2.5 to 9.5 mm from the PT and WH populations). Using a photographed ruler as a metric, gill SA was calculated as the area (± 0.01 mm^2) within a gill's outlined perimeter by using ImageJ photo analysis software (Fig. 1). Dry body mass, including the removed gill (DBM), was determined by desiccating each specimen in a 68° C oven (Hotpack, Philadelphia, PA) for 60 hours, and then weighing it (± 0.001 mg) using a Cahn C-31 microbalance (Cerritos, CA).

Statistical analyses

Least squares regression analysis was used to compare gill SA with dry body mass for each population (Fig. 2). Log_{10} values were used to normalize the data variation and to produce linear, proportional relationships (cf. Kerkhoff and Enquist, 2009; Glazier, 2013; Mascaro et al., 2014). Significant differences between specific scaling slopes were determined using the 95% confidence intervals. If a mean value was outside the 95% CI of another mean value and vice versa, they were considered significantly different ($P < 0.05$), following Smith (1997). The gill SA scaling relationships, including slopes, and calculated gill SA values at dry body masses of $\log_{10}$ -0.25 (0.631 mg), $\log_{10}$ 0 (intercepts at 1 mg), and $\log_{10}$ 0.25 (1.778 mg), were compared between springs with vs. without fish predators using t-tests, and among springs with different *G. minus* population densities and water temperatures, pH values and conductivities using Pearson's product-moment correlation analyses (Table 2). Gill SA values were calculated at the designated small and large body masses given above because they were substantially different and equally distant from the intercept, without being beyond the recorded body-mass range of any population (see Table 1; Fig. 3A).

RESULTS

Highly significant relationships between gill SA and dry body mass were found within each of the eight study populations of *G. minus* (Fig. 2). The mean scaling slope for gill SA was significantly lower for the populations in springs with fish (0.667 ± 0.034 SEM) vs. those without fish (0.763 ± 0.019; Table 2; Fig. 3B). Pooling samples from all populations in each type of spring also revealed that the scaling slope was significantly lower for the fish spring populations (0.628 ± 0.049 95% confidence intervals) than the fishless spring populations (0.749 ± 0.038) (Fig. 4). In addition, the mean calculated gill SA (mm^2) at the large (1.778 mg) dry body mass was significantly lower in springs with fish (0.323 ± 0.021 SEM) vs. those without fish (0.403 ± 0.020; Table 2; Fig. 3A). However, the intercept ($\log_{10}$) and mean calculated gill SA (mm^2) at the small (0.631 mg) dry body mass were not significantly lower in springs with fish (-0.660 ± 0.029 95% confidence intervals; 0.150 ± 0.011 SEM, respectively) vs. those without fish (-0.587 ± 0.022; 0.168 ± 0.009, respectively; Table 2).

Among all eight populations, the slopes, intercepts and calculated gill SA at small and large body sizes were not significantly related to water temperature, conductivity or *G. minus* population density (Table 2). Although the slopes and calculated gill SA at the small body size were not significantly related to pH, significant inverse relationships were found for the intercept and calculated gill SA at the large body size in relation to pH (Table 2).

DISCUSSION

We found highly significant allometric scaling relationships (slopes < 1) between gill SA and body mass in all eight study populations of *G. minus*, as has been observed in other *Gammarus* species (Moore and Taylor, 1984; Maltby 1995). Furthermore, these scaling relationships vary significantly among populations in relation to specific ecological factors. No other animal study has documented ecologically sensitive intraspecific variation of the body-mass scaling of gills or any other respiratory structure.

Effects of fish predators

Our results most strongly support the hypothesis that the scaling of gill SA should parallel that of metabolic rate, as mediated by the effect of size-specific predation by visually hunting fishes. As predicted, the gill SA scaling slopes tended to be lower for the populations in springs with fish vs. those without fish (Figs. 3), as was also observed for the scaling slopes of resting metabolic rate (Glazier et al., 2011). In fact, the scaling slopes for gill SA and metabolic rate are not significantly different (within each paired group the 95% CI of each mean overlaps the mean of the other) for the pooled data of all study populations in the fish springs (0.628 ± 0.049 vs. 0.598 ± 0.070) and in the fishless springs analyzed separately (0.749 ± 0.038 vs. 0.760 ± 0.080) (n = 125, 230, 115 and 190, respectively: data from Fig. 4 and Glazier et al. 2011). A predation effect is also supported by the observation that, among the fish spring populations, the gill SA scaling slope was lowest for the two populations with the highest density of fish predators (WB) or the most diverse assemblage of predators (WH), whereas the population (EL) exposed to a relatively low density of fish predators showed a gill SA scaling relationship most similar to that of the populations in springs without fish (Fig. 3). Similarly, the WB population showed a lower metabolic scaling exponent than the EL population (Glazier et al. 2011).

We suggest that the gill SA scaling difference between fish vs. fishless spring populations may have resulted from different size-specific net effects of growth and activity on oxygen demand, and thus the amount of oxygen uptake needed by the gills. The importance of growth is revealed by shallower ontogenetic scaling of growth rate in relation to body mass in the fish vs. fishless spring populations (Glazier et al. 2011), thus paralleling that observed for metabolic rate and gill SA. Prematurational growth is as high or higher in small young amphipods inhabiting fish vs. fishless springs, presumably because of strong selection to reach maturity and reproduce before being eaten. By contrast, postmaturational growth is significantly lower in large older amphipods inhabiting fish vs. fishless springs, presumably because of the selective advantage of remaining as small and inconspicuous as possible to visually hunting fish predators (Glazier et al., 2011). Fish (e.g. *Cottus cognatus*) prefer to feed on large vs. small amphipods (Newman and Waters, 1984).

In addition, the presence of fish favors secretive and relatively sedentary behavior in amphipods (Andersson et al., 1986; Holomuzki and Hoyle, 1990; Starry et al., 1998; Wooster 1998; Åbjörnsson et al., 2000; Glazier et al., 2011; Szokoli et al., 2015), especially those that are large and relatively conspicuous (Andersson et al., 1986). If so, the presence of fish should influence relative oxygen demand more in large older vs. small younger amphipods. In fish springs, oxygen demand for both growth and activity should be reduced in large older amphipods, but only for activity and probably to a lesser extent in small younger amphipods. In addition, a possibly higher metabolic demand of growth in small, young amphipods in fish vs. fishless springs may partially or wholly counterbalance a reduced activity demand for oxygen. The net effect should be a shallower scaling of oxygen demand in populations from fish vs. fishless springs, which should favor a similar scaling difference for oxygen-uptake capacity, as indexed by gill SA (Fig. 5).

An extrapolation of the linear regression line for the populations in the fish springs beyond the range of measured data points suggests that a crossover of the gill SA scaling relationships may occur (Figs. 4, 5), and if so, this pattern would parallel that observed for metabolic rate (Glazier et al. 2011), thus further supporting our hypothesis that both of these scaling relationships should respond similarly to size-specific predation. Additional data on the gill SA of very small amphipods in both fish and fishless springs are needed to test this inference, which we found difficult to obtain because of problems in extracting their gills without damage, and thus accurately measuring their surface areas.

Effects of other ecological factors

Contrary to the other hypotheses proposed in the **INTRODUCTION** section, water temperature, conductivity and *G. minus* population density showed no significant correlations with the body-mass scaling of gill SA, including slopes, intercepts, and calculated gill SA at small and large body sizes (Table 2). Perhaps the differences in water temperature (and associated oxygen demand and availability) were not great enough to necessitate the evolution of changes in gill SA. In the two warm springs of this study, dissolved oxygen concentrations were never observed to be below 5 mg l^{-1} (Glazier and Gooch, 1987; D. S. Glazier, personal observations), above which only small adjustments in oxygen uptake (metabolic rate) and ventilation rate in *Gammarus* are observed (Sutcliffe, 1984; Maltby 1995). However, *G. duebeni* had significantly larger gills in a hypoxic sewage treatment area compared to its native estuarine habitat (Roast and Jones, 2003). Therefore, larger gills may facilitate oxygen uptake when oxygen availability is very low.

No apparent effect of conductivity on gill SA scaling was observed possibly because of limited differences in conductivity among the study springs, and because gills are not the only sites for ion-regulation in crustaceans (Kikuchi and Matsumasa, 1997). However, among *Gammarus* species, freshwater species tend to have larger gills than marine species (Moore and Taylor, 1984). This habitat difference is consistent with the hypothesis that amphipods in water with lower ionic concentrations should have larger gills to facilitate ion uptake.

An unexpected result was that the intercept and calculated gill SA at large body size were both negatively related to pH (Table 2). However, we believe that these associations are probably coincidental rather than causal. First, the pH range among the study springs was small (only 6.69 to 7.07: Table 1) and thus probably not biologically meaningful. The intra-site variation in pH often encompassed the inter-site range in pH (Table 1). Second, no significant effect of pH on the calculated gill SA at small body size was observed (Table 2). If pH has truly influenced gill SA, then one would expect it to have had this effect on amphipods of all sizes, and especially those with small sizes and thus large surface area to volume ratios. Third, the pH correlation is likely due to a coincident association of relatively high pH with the presence of fish predators ($t = 3.11$, $P = 0.021$: mean pH = 7.025 ± 0.027 SEM in fish springs vs. 6.801 ± 0.064 in fishless springs: data from Table 1). The primary effect of fish predators would explain why the pH correlation was only observed for large amphipods and not small amphipods. However, a possible direct effect of pH on amphipod gill SA requires more study.

Gill surface area and oxygen uptake

The hypotheses proposed in this study all assume that oxygen or ion uptake is proportional to gill surface area. Although this is probably true at least approximately, oxygen or ion uptake may also be increased by enhancing the permeability, transport ability or ventilation of the gills, thus increasing the rate of oxygen or ion uptake per unit area of gill surface. Johnson and Rees (1988) showed that oxygen uptake per unit gill SA varies among crab species. In the blue crab, ion uptake and water permeability per unit gill SA may also vary with salinity (Li et al. 2006). Therefore, future research should examine the effects of various ecological factors on both gill SA and the intensity of oxygen or ion uptake per unit gill SA, and whether they are due to phenotypic acclimation vs. adaptive genotypic evolution (cf. Harrison, 2015).

Benefits and costs of enlarging gill surface area

Our study raises further questions requiring investigation. In particular, what are the benefits and costs of enlarging gill surface area? Our results suggest that adult amphipods with high levels of metabolic demand (growth and behavioral activity) in fishless springs benefit from enlarged gills because they enable increased oxygen uptake. To further test this hypothesis, measurements of active (including maximal) metabolic rates and their scaling with body mass are required for populations in both fish and fishless springs. Presumably, gill surface area relates to not only resting metabolic rate (including oxygen demand for growth), as indicated by their similar body-mass scaling, but also active metabolic rate (including oxygen demand for behavioral activity) (Fig. 5). However, why do adult amphipods in fish springs have smaller gills? Presumably there is a resource cost to producing and maintaining large gills, and thus if metabolic demand is routinely low, selection should favor smaller gills. Large gills may also entail higher osmoregulatory costs than small gills, as observed in fish (e.g. Brill 1996). Further studies are needed to measure these costs in amphipod gills with different sizes.

Conclusions

A growing number of studies show that the body-mass scaling of metabolic rate is ecologically sensitive and evolutionarily malleable (e.g., Killen et al., 2010; Glazier et al., 2011; and others reviewed by Glazier 2005, 2014b, c). Our study extends this conclusion to the scaling of respiratory structures. The scaling slope for gill SA is significantly different in *G. minus* populations in springs with vs. without fish predators. Our study also provides a mechanistic explanation for this difference. We hypothesize that size-selective fish predation has driven the evolution of size-specific changes in metabolically expensive growth and activity, that have in turn altered the body-size scaling of oxygen use (metabolic rate) and uptake (a function of gill SA). However, empirical support for this mechanism is entirely correlational, and experimental selection studies are needed to further test it (following Garland and Rose, 2009).

Some popular explanations of metabolic scaling have invoked internal resource-supply constraints as the primary cause (e.g. RTN models of West et al., 1997; Banavar et al., 2010), but our study provides support for an alternative view that metabolic scaling is the result of a co-adjustment between resource supply and demand (Glazier, 2014b, 2015b). The parallel effects of fish predators on the body-mass scaling of growth, metabolism and gill SA can be more readily explained as being the result of changes in resource demand and the secondary adjustment of resource supply, rather than vice versa. Although constraints on resource supply may sometimes have important effects on metabolic scaling (Glazier 2010, 2014b, c; Hirst et al. 2014; Glazier et al. 2015), adaptive, size-specific resource demand may often be a primary driver of metabolic rate and its scaling with body mass, with resource supply playing a secondary supporting role (Glazier, 2014b, 2015b).

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COMPETING INTERESTS

The authors declare no competing or financial interests.

AUTHOR CONTRIBUTIONS

D.S.G and D.A.P. conceived the study. D.A.P collected the data. D.S.G. and D.A.P. conducted data and statistical analyses. D.S.G. drafted the paper, and both D.S.G. and D.A.P. contributed to revising and finalizing the text.

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DATA AVAILABILITY

Data on surface area of the 1st coxal gill in relation to body mass of the amphipod *Gammarus minus* in eight populations will be submitted to Dryad.

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Figures

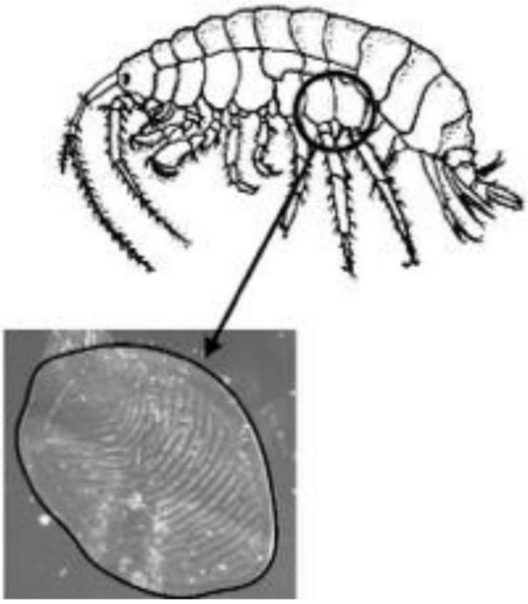

Fig. 1. Location of the fifth coxal gill in the amphipod *Gammarus minus*, and an example of an outlined gill used to estimate surface area.

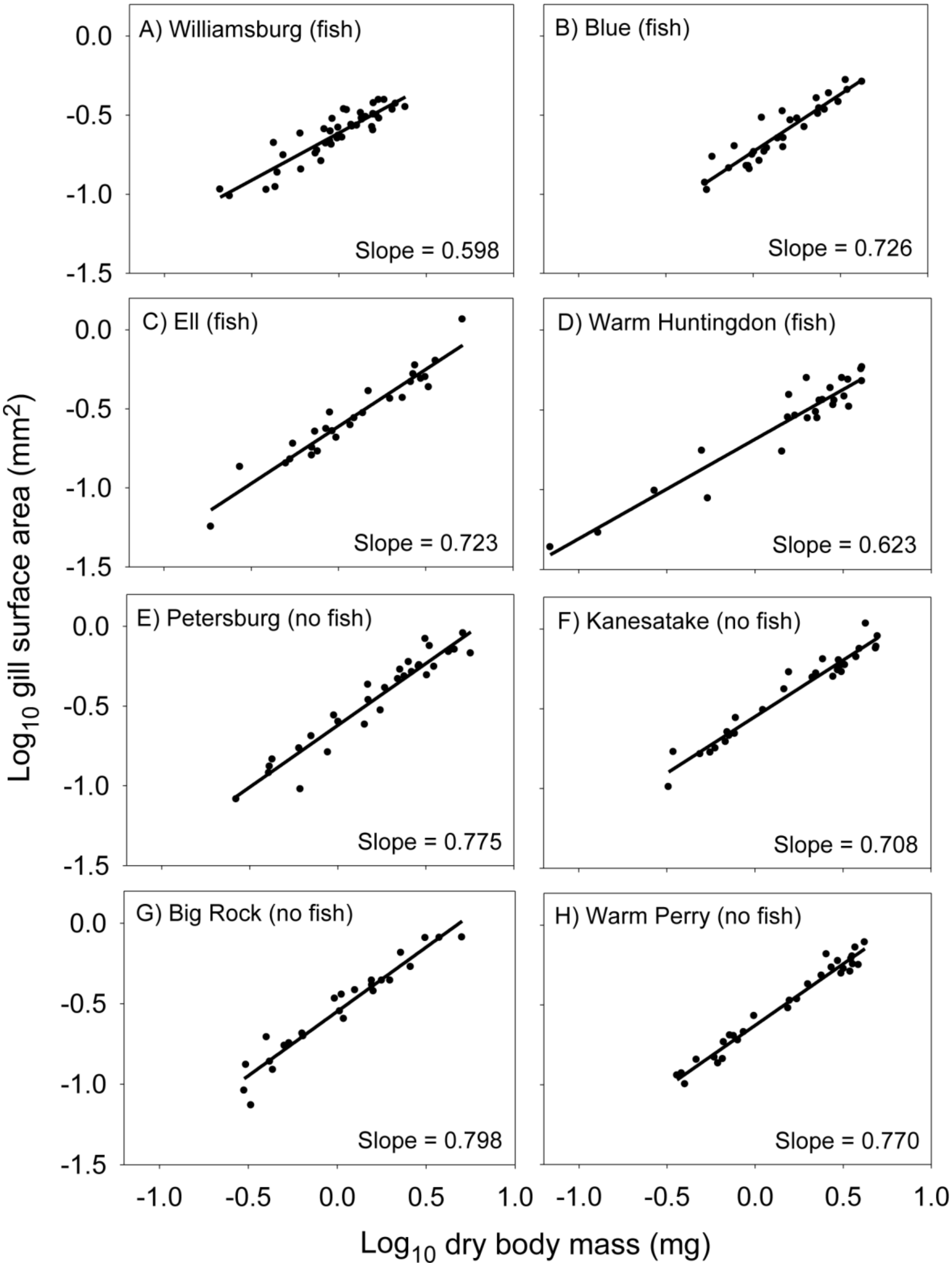

Fig. 2. Surface area of the fifth coxal gill in relation to dry body mass, both $\log_{10}$-transformed, in eight populations of the amphipod *Gammarus minus*. Least-squares regression lines and slope values are shown for four populations in springs with fish predators (A-D) and four in springs without fish (E-H). Additional regression statistics are presented in Table 1.

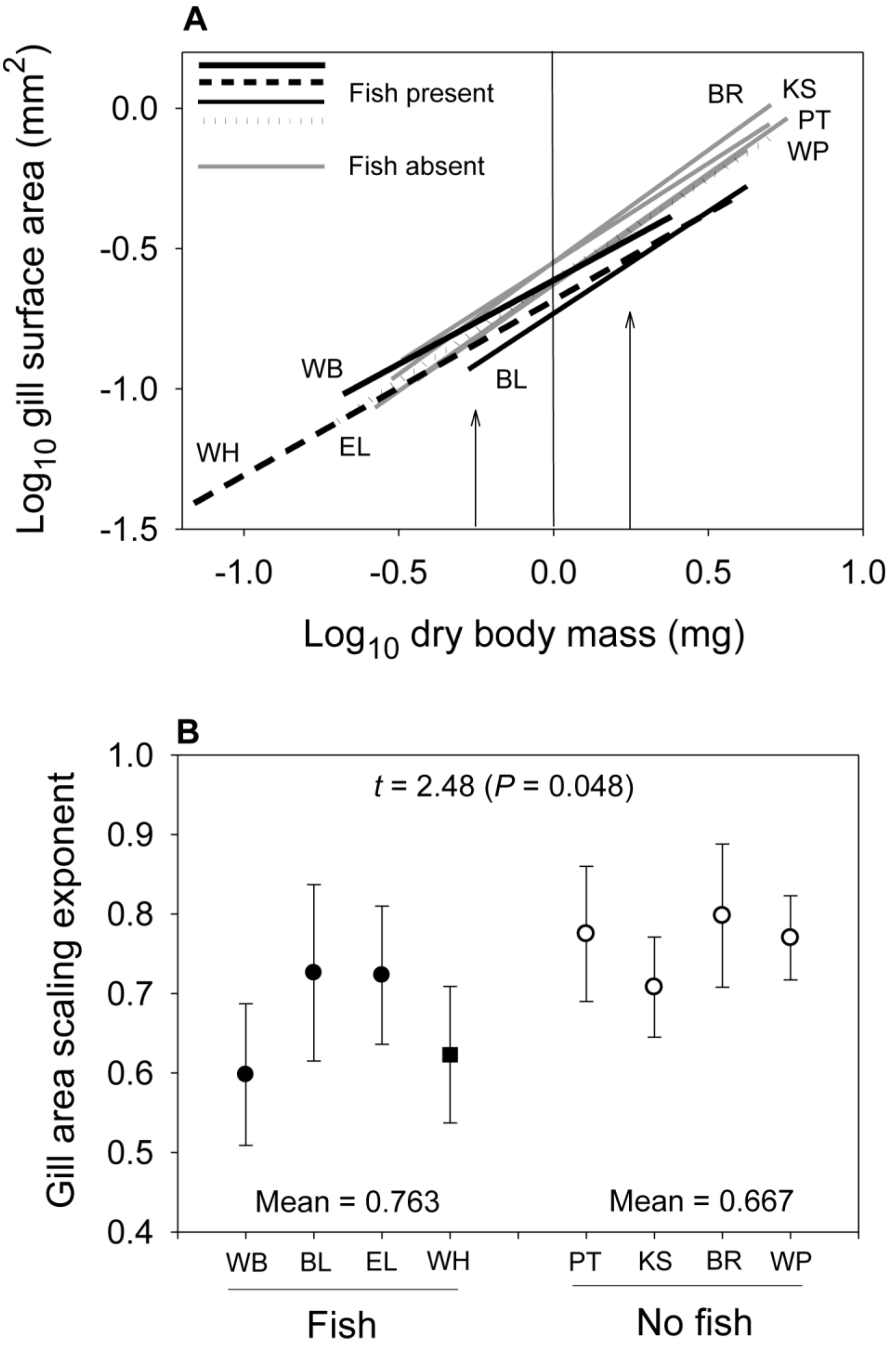

Fig. 3. Comparison of the scaling relationships between the surface area of the fifth coxal gill and dry body mass in eight populations of the amphipod *Gammarus minus*. Data points are shown in Fig. 1. (A) Scaling relationships from populations in springs with fish predators are designated with black lines (WB = Williamsburg Spring: thick solid line; BL = Blue Spring: thin solid line; EL = Ell Spring: dotted line; WH = Warm Spring Huntingdon County: thick dashed line), whereas those from populations in springs without fish are designated with grey lines (PT = Petersburg Spring; KS = Kanesatake Spring; BR = Big Rock Spring; WP = Warm Spring Perry County). The vertical line transects the intercept values of gill surface area (at 1 mg dry body mass), whereas the vertical arrows point toward the

values of gill surface area in relatively small (0.631 mg) and large amphipods (1.778 mg) (also see Table 1). (B) Scaling slopes (± 95% confidence intervals) of gill surface area in relation to dry body mass in four springs with fish predators (solid circles: *Cottus cognatus*; solid square: *Rhinichthyes atratulus*) and four without (open circles). The populations from springs with fish predators show a significantly lower mean scaling slope than that of the populations from springs without fish (t-test statistics shown here and in Table 2). Note that the WB population, which is exposed to an especially high population density of the fish predator *C. cognatus* (Glazier et al., 2011), and the WH population, which is exposed to numerous kinds of predators (including not only the fish *R. atratulus*, but also several species of Caudata, Decapoda, Megaloptera and Odonata: D. S. Glazier, personal observations) exhibit the lowest scaling slopes for gill surface area. By contrast, the EL population is exposed to a low density of *C. cognatus* (Glazier et al., 2011) and shows a scaling relationship most similar to that of the populations in springs without fish (also see graph A).

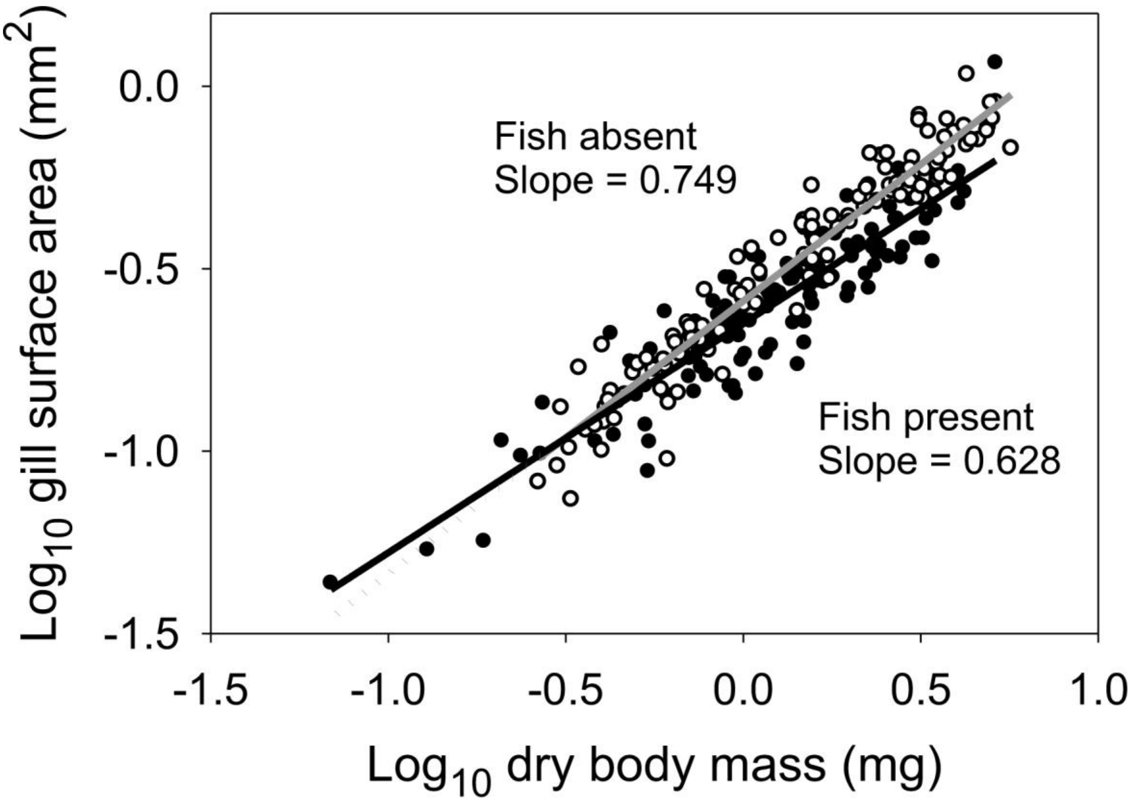

Fig. 4. Scaling relationships between the surface area of the fifth coxal gill and dry body mass for pooled samples from populations of the amphipod *Gammarus minus* in springs with vs. without fish predators. Data pooled from four fish spring populations (solid line and circles) and four fishless spring population (grey line and open circles). Equations for regression lines (including 95% confidence intervals for intercepts and slopes), Pearson's product-moment correlation coefficients (r), sample sizes (n) and P values are respectively $Y = 0.628$ (± 0.049)(X) -0.650 (± 0.017); $r = 0.917$; $n = 125$; $P < 0.00001$; and $Y = 0.749$ (± 0.038)(X) - 0.589 (± 0.015) +; $r = 0.965$; $n = 115$; $P < 0.00001$. The dotted grey line represents an extrapolation of the linear regression for the populations in fish springs beyond the range of measured data points.

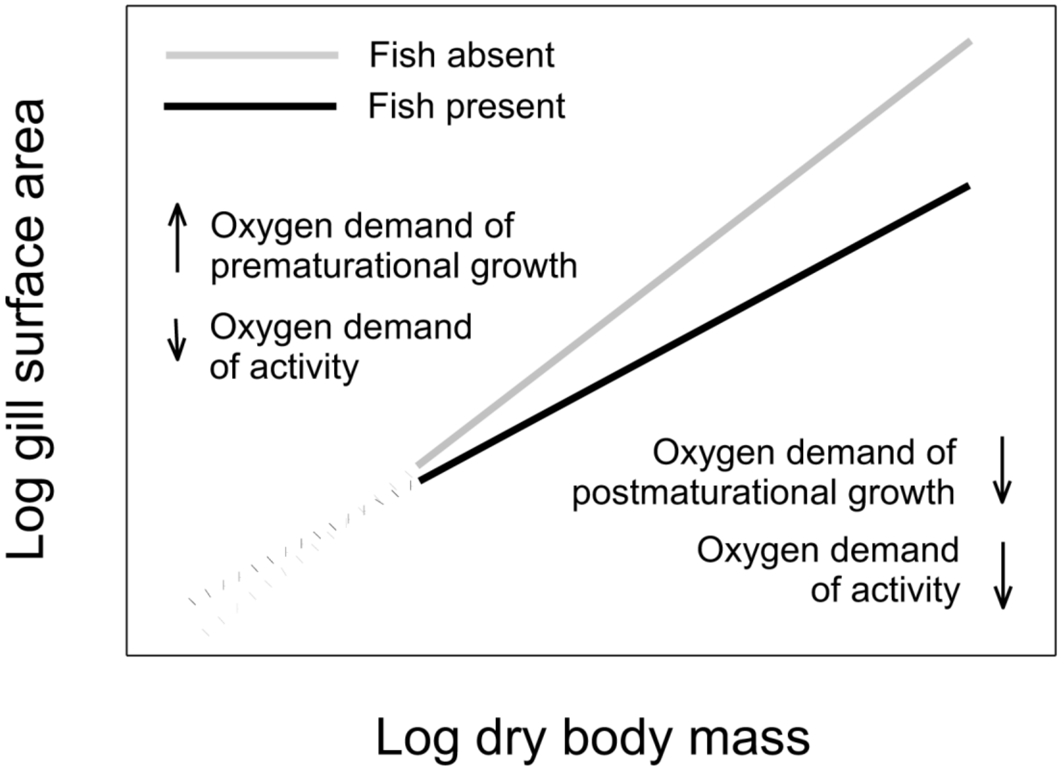

Fig. 5. Schematic representation of hypothetical causes of the differences in gill surface area scaling observed between *Gammarus minus* populations in springs with vs. without visually hunting fish predators. The lower scaling slope of the populations in the fish springs (black line) relative to that of the populations in the fishless springs (grey line) based on data depicted in Fig. 4 may result from different size-specific net effects of a combination of factors that affect oxygen demand, and thus the amount of oxygen uptake needed by the gills. For example, existing growth and metabolic data suggest that the oxygen demand for prematurational growth is as high or higher in small young amphipods inhabiting fish versus fishless springs, but the reverse occurs for postmaturational growth in large older amphipods (Glazier et al., 2011). In addition, predation may select for more secretive and sedentary behavior, thus lowering oxygen demand, especially in large conspicuous adults. The net effect may be greater selection for relatively small gills in large vs. small amphipods in the fish springs, as compared to the fishless springs. For more details, see the **DISCUSSION** section.

Tables

Table 1. Slopes, intercepts and other aspects of scaling relationships between $\log_{10}$ surface area (mm^2) of the 5th coxal gill and $\log_{10}$ maternal dry body mass (mg) in eight populations of the amphipod *Gammarus minus*. Values of various ecological variables (based on 1-5 samples) in the corresponding spring habitats (codes given in Materials and Methods) are also shown.

Spring	Slope	Intercept	Small	Large	*r*	*n*	T	pH	Cond.	Pop.	Fish
WB	0.598 (±0.089)	-0.613 (±0.022)	0.173	0.344	0.904	43	10.6	7.06	266.8	L	*C.t.*
BL	0.726 (± 0.111)	-0.731 (±0.032)	0.122	0.282	0.931	30	10.5 (±0.1)	7.07 (±0.09)	367.2 (±7.2)	M	*C.t.*
EL	0.723 (± 0.087)	-0.612 (±0.032)	0.161	0.371	0.960	27	9.8 (±0.0)	6.95 (±0.11)	338.2 (±0.4)	M	*C.t.*
WH	0.623 (± 0.086)	-0.684 (±0.043)	0.145	0.296	0.952	25	17.0 (±0.1)	7.02 (±0.05)	169.1 (±1.5)	M	*R.a.*
PT	0.775 (± 0.085)	-0.620 (±0.036)	0.154	0.375	0.961	31	10.4 (±0.0)	6.76 (±0.24)	146.2 (±0.6)	H	None
KS	0.708 (± 0.063)	-0.550 (±0.026)	0.188	0.424	0.976	29	11.0 (±0.3)	6.79 (±0.04)	652.0 (±8.0)	H	None

BR	0.798 (± 0.090)	-0.548 (±0.032)	0.179	0.448	0.968	25	13.7 (±0.1)	6.69 (±0.31)	666.0 (±0.0)	H	None
WP	0.770 (± 0.053)	-0.629 (±0.021)	0.151	0.366	0.985	30	18.3 (±0.0)	6.99 (±0.02)	230.7 (±0.4)	L	None

Small and Large = surface areas (mm^2) of the 5th coxal gill of amphipods with 0.631 and 1.778 mg dry body mass (log$_{10}$ -0.25 and 0.25), respectively, calculated from the scaling relationship of each population (regression lines and data points shown in Fig. 1); *r* = Pearson's product-moment correlation coefficient; *n* = sample size; P values all < 0.00001; T = water temperature: °C; Cond. = conductivity: µS; Pop. = relative population density of *G. minus*: L (low), M (medium), H (high); Fish = presence or absence of the fish predators *Cottus cognatus* (*C. t.*) or *Rhinichthyes atratulus* (*R. a.*)]. The error terms are ± 95% confidence intervals (scaling slopes and intercepts) or ± 1 standard error of the mean (environmental variables).

Table 2. Statistical tests of relationships between the slopes, intercepts and calculated surface areas of the 5th coxal gill at small and large dry body masses (0.631 and 1.778 mg, respectively) based on scaling relationships between $\log_{10}$ surface area (mm^2) of the 5th coxal gill and $\log_{10}$ maternal dry body mass (mg) in eight populations of the amphipod *Gammarus minus*.

Parameter	Factor	*t*	*r*	*P*
Slope	Fish present vs. absent	-2.476		**0.048**
Intercept	Fish present vs. absent	-2.014		0.091
Small	Fish present vs. absent	-1.225		0.267
Large	Fish present vs. absent	-2.809		**0.031**
Slope	Temperature		+0.041	0.924
Intercept	Temperature		-0.117	0.782
Small	Temperature		-0.162	0.701
Large	Temperature		-0.091	0.831
Slope	pH		-0.642	0.086
Intercept	pH		-0.755	**0.030**
Small	pH		-0.598	0.117
Large	pH		-0.858	**0.006**
Slope	Conductivity		+0.313	0.450
Intercept	Conductivity		+0.619	0.102
Small	Conductivity		+0.601	0.115
Large	Conductivity		+0.680	0.063

Slope	Population density	+0.473	0.237
Intercept	Population density	+0.409	0.314
Small	Population density	+0.307	0.460
Large	Population density	+0.524	0.183

t = t-test statistic; *r* = Pearson’s product-moment correlation coefficient; *P* = probability (**bold** values are significant at < 0.05).