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Factors Contributing to Variability in Club Cell Investment in a North American Cyprinid Fish Species

David G. Lonzarich[1], Megan Meller[1,2], Rebecca Frank[1]

[1] Department of Biology, University of Wisconsin – Eau Claire, Eau Claire, Wisconsin, USA
[2] Gunderson Health System, LaCrosse, Wisconsin, USA

Abstract: In many fish species, particularly those from the superorder Ostariophysi, individuals contain club cells in their epidermal tissue. These cells contain a chemical substance, which when released by injury during predation events, lead to stereotyped anti-predatory behaviors in nearby fish. Recent evidence concerning the evolutionary origins of this system have suggested that club cells are associated with innate immunity, and may serve to protect fish from parasite infestations and injury. In this study, we explored factors associated with variability in club cell investment from several populations of a North American cyprinid fish species (Creek Chub, Semotilus atromaculatus), with a primary goal to test the anti-parasite hypothesis in a natural setting. Using a path model approach, we evaluated the relative effects of fish length, mucous cell densities, epidermis thickness and parasite burden on club cell investment. Our model, which included all four independent variables, explained most of the variability in club cell densities for our fish ($R^2 = 0.80$), and that the variable, fish length (acting either directly or indirectly on the other variables) explained most of this variability. Club cell densities were positively associated with parasite burden when examined in isolation of other factors, but in the path model, the effect of parasite burden on club cell investment was non-significant. Although we could not find support for the anti-parasite hypothesis in this study, our model indicates that, at least in this species, most of the variability in club cell investment is associated with characteristics of individual fish, and not the conditions of the environments in which they occur.

Keywords: Alarm Response; Fish; Club Cells

1. Introduction

Over the past century there has been considerable debate over the evolutionary origins of epidermal club cells in ostariophysan fishes. During much of this time, it was widely held that club cells primarily arose as an anti-predatory adaptation. This hypothesis derived from the pioneering work of von Frisch (1938), who first described a fright reaction in fish exposed to injured conspecifics, and was subsequently supported by many field and laboratory studies (reviewed in Smith 1992, Døving *et al*. 2005, Ferrari and Wisenden 2015). The substance associated with the fright response was later localized to specific epidermal cells (club cells) by Pfeiffer (1960) and has been found in nearly all ostariophysan fishes (Pfeiffer 1977). When club cell contents are released into the water, a range of anti-predatory behaviors result (reviewed in Ferrari *et al*. 2010).

Among the hypotheses that have been proposed to help understand the origins of the anti-predatory behavior in ostariophysan fishes, two have been most widely cited. One hypothesis is that the alarm substances and club cells evolved via kin selection. This view is based on the fact that the alarm response has been shown to be more pronounced when the substance is derived from conspecifics than from heterospecifics (e.g., Brown *et al*. 1995, Júnior *et al*. 2010).

However, no direct empirical support for this hypothesis has ever been generated. A second hypothesis derives

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from evidence that odors released during a predatory event may attract other predators (Wisenden and Thiel 2002), and that a subsequent struggle between competing predators can increase the likelihood that the injured fish will escape (Mathis *et al*. 1995, Chivers *et al*. 1996). Although field evidence has been presented to show that predators are attracted to odors from ostariophysan skin extracts (Wisenden and Thiel 2002), no field evidence has been generated to indicate the behavior of predators attracted to injured fish actually increases the prey's chance of survival.

A third and more recently developed hypothesis for the evolution of club cells relates to their putative role in the fish immune system. This hypothesis, which was first proposed by Smith (1982), suggests that the primary role of club cells is to provide protection against disease-causing organisms and environmental stressors through the release of antipathogenic substances. Under this scenario, the alarm response would have been secondarily derived. A series of experiments by Chivers *et al*. (2007) provided the first empirical support for this hypothesis. These authors found that the abundance of club cells increased in fish exposed to elevated parasite levels. Other laboratory experiments since have tested the anti-parasite hypothesis, with support for the hypothesis being reported in some studies (Michalak 2006, Päkk 2011) but not others (James *et al*. 2009, Pollock *et al*. 2012).

There have to date been no tests of the anti-parasite hypothesis in natural fish populations, and therefore our primary goal in this study was to examine the association between parasite burden and club cell densities in fish under natural conditions. The fish species we chose for this study was the Creek Chub (Semotilus atromaculatus, family Cyprinidae), which is a common intermediate host for the trematode Neascus pyriformis (Trematoda, Diplostomatidae). The larval life stage of this parasite embeds below the fish epidermis and appears as a visible black spot (metacercarial cyst) on the body surface (Evans and Mackiewicz, 1958, Vaughn, 1962, Berra and Au, 1978, Quist *et al*. 2007). We chose this fish-parasite association in part because the parasite burden can be characterized relatively easily in infected individuals, but also because Chivers *et al*. (2007) have shown previously under laboratory conditions that club cell densities were elevated in fish exposed to increasing concentrations of metacercariae larvae. Because club cell densities in laboratory fish also have been shown to vary with fish condition (Wisenden and Smith 1997), reproductive state (Smith 1976) and epidermal thickness (e.g., Wisenden and Smith 1997, James *et al*. 2009, Stabell and Vegusdal 2010), we recognized that any assessment of a link between parasite burden and club cell investment must be viewed in relation to other factors. Therefore, our more general goal in this study was to characterize through path analysis both the independent and combined effects of four variables (i.e., parasite burden, fish size, epidermal thickness and mucous cell densities) on club cell densities in Creek Chub.

2. Materials and methods

One hundred and thirteen Creek Chub (ranging in size from 2.9-20.1 cm, total length) were collected from streams, and from archives of University of Wisconsin-Stevens Point and the Bell Museum at the University of Minnesota-Twin Cities. All fish originated from streams in Wisconsin and Minnesota. This effort yielded samples from nine streams and at least nine fish per stream (Table 1). Fresh specimens were euthanized in accordance with University of Wisconsin – Eau Claire IACUC guidelines and preserved in a 4% buffered formaldehyde solution. From each fish, we counted black spots from the entire body surface (including fins) and recorded total length (cm). Following this, epidermal samples were extracted from the nape and histologically prepared using a standard protocol (Humason, 1979). The samples were cut into 8-µm thick sections using a microtome ("820" Spencer Microtome; American Optical Corporation), and six cross-sections were mounted onto each of six microscope slides produced per specimen (36 sections total). The tissues then were stained with Schiff's reagent (periodic acid) and counterstained with Mayer's Hematoxylin (PAS-H). Using these stains, we were able to identify two target cell types: 1) large mucous cells, which usually stained purple and 2) club cells, which stained clear with the exception of the nucleus (blue) and cell membrane (light pink). Mucous cells were widely dispersed throughout the epidermis whereas club cells tended to be more often found near the basal layer. Variability in this pattern was associated with epidermal thickness. These two cell types were contained in a matrix of smaller epidermal filament cells, which usually were stained a dark shade of red. We consulted Chivers *et al*. (2007) for club cell identification.

Stream Name	County, State	N	Fish length (range, TL, cm)	Black-spot densities (range, mm^{-2})
Daschum Creek	Eau Claire, WI	10	4.8-19	0-1.62
Rock Creek	Eau Claire, WI	10	8.5-14.7	0-0.57
Little Niagara	Eau Claire, WI	9	6.1-9.2	0-8.06
Duncan Creek	Chippewa, WI	12	3.9-8	0-1.39
O'Neil Creek	Chippewa, WI	16	4.8-13.1	2-5.56
Namekagon River	Sawyer, WI	9	7.1-15.1	0.18-5.8
Buffalo River	Wilken, MN	14	4.1-15.3	0
Black River	Taylor, WI	15	4-5.6	11.31-52.96
South Fish Creek	Bayfield, WI	20	2.9-20.1	14.48-363.39

Table 1. Stream origins, sample sizes and characteristics of fish used in this study. Fish from streams in Eau Claire and Chippewa counties, WI were collected by the authors. All other fish were museum specimens.

Epidermal cross-sections were imaged at 200X magnification using Olympus BX60 scope, Spot II camera and Spot imaging software v2.1 (Diagnostic Instruments, Inc. 1998). Club cell density (per mm^2), mucous cell density (per mm^2), epidermal thickness (mm) and epidermal cross-sectional surface area (mm2) were determined from up to six cross-sections for each fish (range: 4 – 6), and a total of 664 images were generated from the 113 fish specimens. Images were obtained from near the midline of each section, and only one image was generated per slide. Most cell counts, and all cross-sectional area and epidermal thickness measurements from tissue images were completed using the image analysis software IMAGEJ (Rasband, 1997-2001). Cell counts were made in duplicate by two observers. Where the deviation between two counts of the same slide was less than 4 cells/section (<10%), we took the average value of the two. If the deviation was greater than 4 cells/section, we recounted the cells using the original microscope slides. Samples that still yielded deviations greater than 4 cells/section after this second count were not included in subsequent analyses. From the collection of 664 images, we removed only 22 because of discrepancies in club cell counts. The average deviation between the duplicate counts of club cells for the 113 fish was 5% (+/- 4.5% s.d.). Unlike club cells, mucous cells often were difficult to accurately count due to uneven staining across samples. From the 664 sections, we excluded 44 due to count discrepancies or poor staining. The average deviation between duplicate mucous cell counts from the 113 fish was 8.5% (+/- 11% s.d.). Density estimates for mucous and club cell counts were calculated using area measurements obtained in IMAGEJ from the counted section of the images. We also used IMAGEJ to calculate the average epidermal thickness (mm) from five locations along the length of each tissue section. Mucous cell, club cell and epidermal thickness data used in all inferential analyses were obtained from the averages of all slides examined from individual fish.

Data were analyzed first by creating bivariate plots examining the relationships between the dependent variable (club cell density) and each of the four independent variables (i.e., total length, black-spot density, mucous cell density and epidermal thickness). We also created bivariate plots examining the effect of fish length on mucous cell densities, black-spot densities and epidermal thickness. Simple linear regression analyses were performed for all relationships (alpha = 0.05). Where relationships deviated from linearity, we performed log transformations (ln) of the data.

Based on the findings of bivariate analyses, we performed a path analysis using Amos 16.0.1 (Amos Development Corporation, 2007) to characterize the combined direct and indirect effects of the four independent variables on club cell density. Our choice of path model was evaluated using several fit indices. We evaluated model-data consistency with a chi-square test ($P > 0.05$ indicates good model fit) to determine whether the chosen model significantly deviated from the data (Grace *et al*. 2010). We also report the goodness-of-fit index (GFI), for which values above 0.90 suggest a good model fit to the data (Hooper *et al*. 2008). Finally, we report the root mean square error of approximation (RMSEA), which favors model parsimony. An RMSEA value below 0.08 indicates a good model fit (MacCallum *et al*. 1996). As another measure of model fit, we report the standardized root mean square residual (SRMR) where a value < 0.08 is considered a good fit (Hu and Bentler 1999). Lastly, we report P -values and the associated path coefficients for each path in our illustration of the SEM where $P < 0.01$. All other paths ≥ 0.10 are indicated as "NS" for nonsignificant.

Parameter values for the path analysis model were identified using maximum-likelihood estimation, and Model fit was determined from an examination of two model outputs (i.e., goodness-of-fit index (GFI), Root Mean Square Error of Approximation (RMSEA)). The GFI assesses the proportion of the variance in the sample variance-covariance

matrix that is accounted for by the model (Byrne, 2009). Acceptable values for GFI do not exceed 0.9 for a good model. The RMSEA estimates lack of fit compared to the saturated model; values less than 0.05 are taken to indicate good fit (Kenny, 2000). We assessed the normality assumption (both Kurtosis and Skewness) and performed log transformations of the data where necessary. Partial coefficients were used to evaluate the strength of the relationships between variables.

3. Results

Our overarching goal was to understand causes for variability in club cell densities across different Creek Chub populations. We were fortunate, therefore, to uncover large variability in club cell densities both across the nine streams (average club cell densities from 184 - 860 cells/mm-2) and among the 113 individuals examined (average club cell densities from 36 – 1497 cells/mm-2). In an attempt to explore the basis for population level differences in club cell densities, we focused first on a possible association with black-spot densities. However, this effort was confounded by large differences in body length of fish taken from the different streams, and by the fact that both black-spot densities and club cell densities (Figure 2) were negatively associated with body length. Because average fish sizes ranged so widely across the nine streams (Table 1), we chose to pool the data from the different streams, recognizing that doing so would leave us unable to address the environmental contributions to club cell variability across populations. Our focus then turned towards explaining club cell variability from characteristics traceable to the fish themselves, namely black-spot densities, mucous cell densities, epidermal thickness and fish length.

As our first objective was to explore the relationship between black-spot parasite load and club cell abundance, we begin with this relationship. Measured on a log-log scale, club cell density increased with black-spot density (R^2=0.17, $P < 0.001$, Figure 1a). Fish with highest black-spot parasite load had club cell densities four times higher than fish with the lowest parasite load. Although statistically significant, this relationship between club cell densities and parasite load was much weaker than the relationships measured for the other three variables. Club cell densities decreased dramatically with increasing mucous cell densities (R^2=0.64, $P < 0.001$, Figure 1b), epidermal thickness (R^2=0.52, $P < 0.001$, Figure 1c) and fish length ($R2 = 0.65$, $P < 0.001$, Figure 1d). Fish length also explained some of the measured variability in mucous cell densities (R^2=0.44, $P < 0.001$, Figure 2b) and epidermal thickness (R^2=0.62, $P < 0.001$, Figure 2c), with values for both of these traits increasing with body length.

Because all four of the measured fish traits yielded significant effects on club cell densities, and because three of these traits varied significantly with respect to fish length, we developed a path analysis model to measure the relative contributions of each of the four fish characteristics and to characterize the direct and indirect effects of fish length on club cell variability. In this exercise, we made specific assumptions about the nature of all pairwise relationships. The first was that club cell densities varied in response to black-spot densities, mucous cell densities, epidermal thickness and fish length. The second was that black-spot densities, mucous cell densities and epidermal thickness all varied in response to fish length. The path model that we constructed collectively explained 80% of the observed variability in club cell densities (Figure 3, model chi-square = 0.7; $P = 0.69$; GFI=0.997; RMSEA <0.001; SRMR = 0.009). Fish length predicted most of this variability through its combined direct and indirect effects (standardized partial coefficient = -0.80); however, much of the effect of fish length was indirect, that is, through the effect of length on the other three variables (Table 2). Even after accounting for size, mucous cell densities (standardized partial coefficient = -0.48) and epidermal thickness (standardized partial coefficient = -0.26) both retained significant negative relationships with club cell densities, although for both of these independent variables, these relationships were weaker than the total effect of length. By contrast, after accounting for the effect of size, the relationship between black-spot densities (standardized partial coefficient = 0.012) and club cell densities almost completely disappeared. (Figure 3).

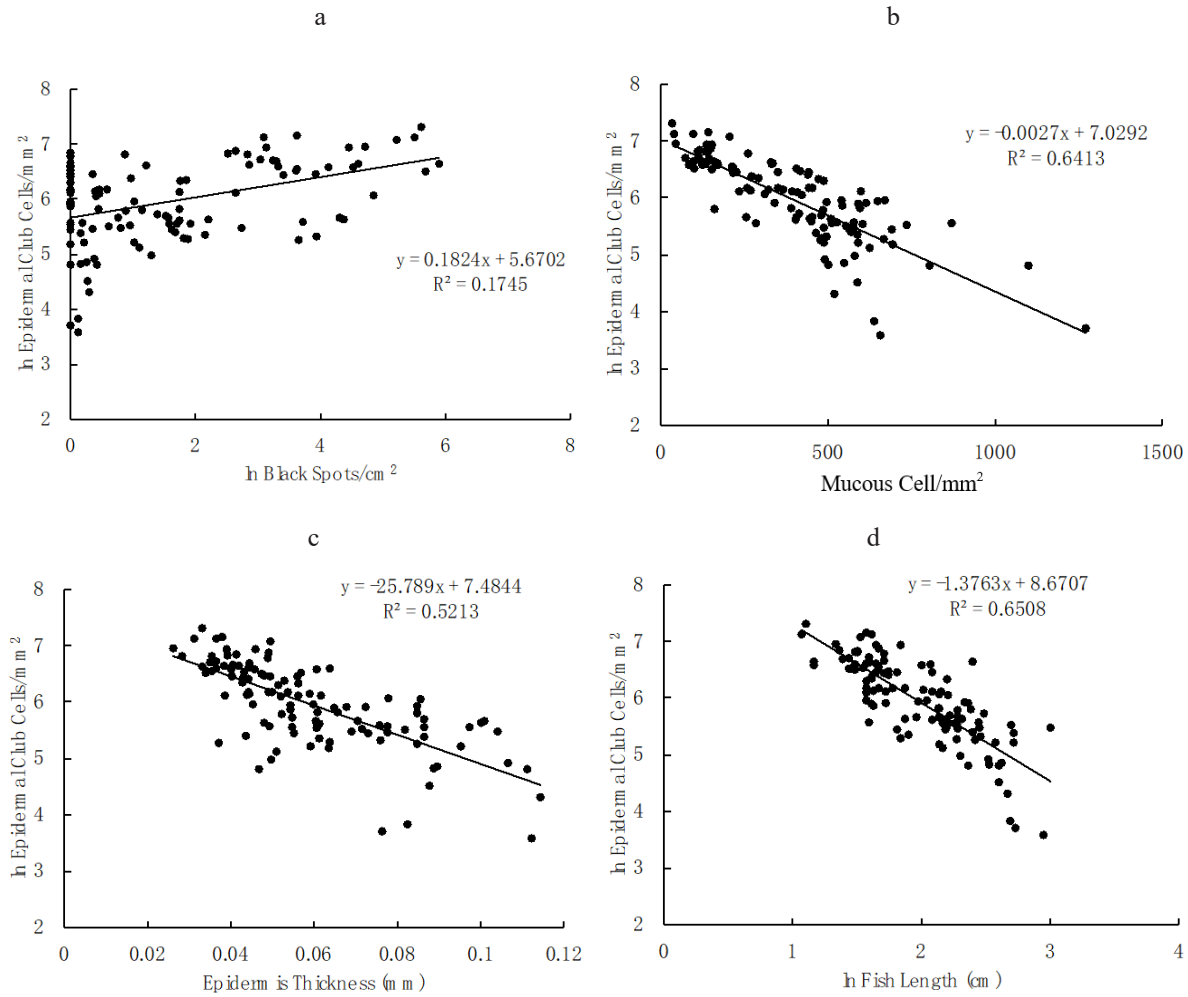
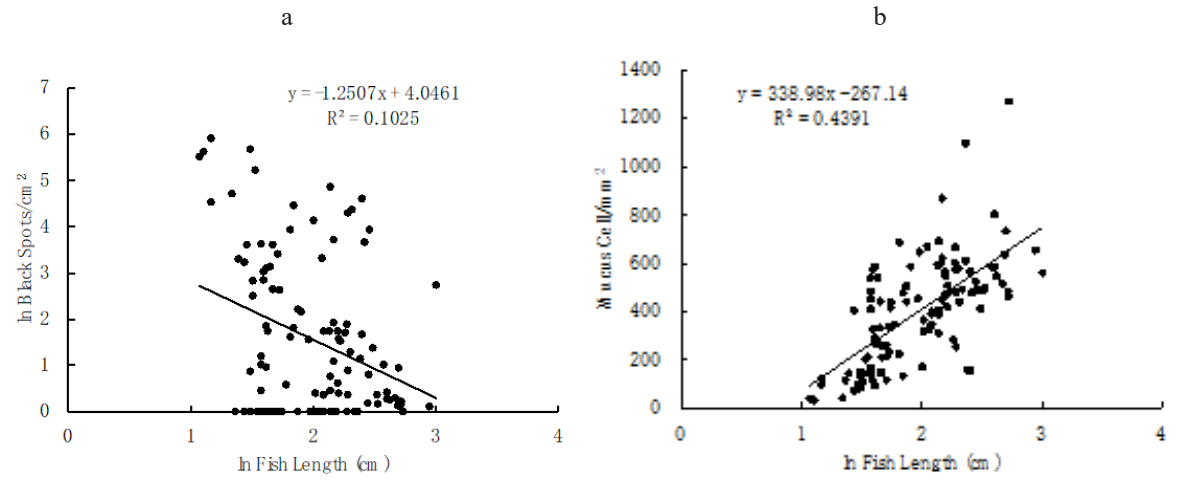


Figure 1. Bivariate relationships between epidermal club cell densities (cells/mm^2) and the four independent variables; (a) black spot densities (spots per fish epidermal surface cm^2), (b) mucous cell densities (cells/mm^2), (c) epidermis thickness (mm) and (d) fish length (ln, cm). All relationships were statistically significant ($P < 0.001$, linear regression analysis).



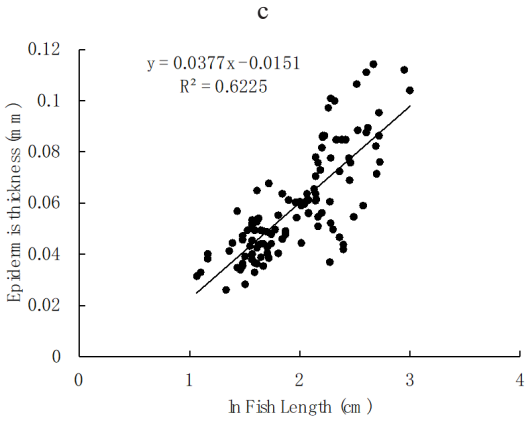


Figure 2. Bivariate relationships between each of the three independent variables and fish length; (a) black spot densities (spots per fish epidermal surface cm^2), (b) mucous cell densities (cells/mm^2) and (c) epidermis thickness (mm). All relationships were statistically significant ($P < 0.001$, linear regression analysis).

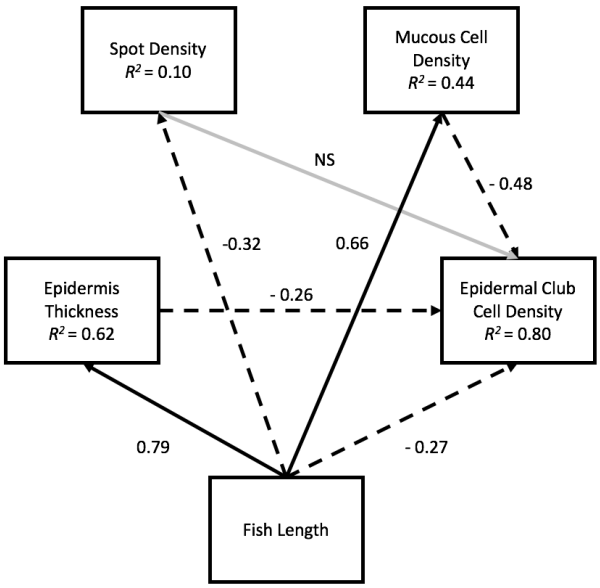


Figure 3. Path model representing the relationships between epidermal club cell densities, fish length, epidermis thickness, and black-spot and mucus cell densities in Creek Chub. Solid arrows indicate positive relationships, and dashed arrows indicate negative relationships. NS = non-significant ($P > 0.5$).

Variable	Direct Effect	Indirect Effect	Total Effect
Fish Length	-0.27	-0.53	-0.80
Black-spot Density	0.012	-	0.012
Mucous Cell Density	-0.48	-	-0.48
Epidermis Thickness	-0.26	-	-0.26

Table 2. Standardized direct, indirect and total effects of four independent variables on variability in epidermal club cell densities (cells/mm^2) in Creek Chub.

4. Discussion

At the outset of this study, our principle objective was to explore the relationship between club cell densities and black-spot parasite burden in natural populations of Creek Chub. While analyses of fish from nine streams yielded results that were consistent with predictions of the anti-parasite hypothesis (Chivers *et al*. 2007), the relationship uncovered was weak, and was not as strong as the apparent effects of mucous cell densities, epidermal thickness or body size. Moreover, when all four of these variables were examined simultaneously in a path model, the effect of black-spot parasite burden no longer remained a significant predictor of club cell densities. Collectively these findings lead us to the conclusion that the observed association between black-spot parasite burden and club cell densities in Creek Chub

was not causal, but rather that black-spot parasite burden was correlated with fish length, which itself was correlated with variability in club cells.

The path model yielded two additional insights that contribute towards an understanding of the causes for variability in club cell densities among fish from these different populations. The first is that the four attributes measured (i.e., black-spot parasite burden, mucous cell densities, epidermal thickness and fish length) explained nearly all of the observed variability in club cell densities ($R2 = 0.80$). Although we could not directly evaluate the contribution of stream conditions on variability in club cell densities, these path model results suggest that any direct environmental contribution would be relatively minor. The second insight gained from the path analysis is that fish length had the strongest effect on club cell densities and that this effect was primarily indirect, acting through the three other independent variables. Taken together, these model outputs clearly point to a strong ontogenetic basis for variability in club cell densities in Creek Chub, and that much of this effect appears to be driven by size-based variability in mucous cell densities and epidermal thickness and to a much lesser extent, parasite burden.

Interpreting the study findings first from the perspective of the anti-parasite hypothesis, our results when viewed through the lens of the path model indicate that club cell production in the epidermis of Creek Chub does not appear to be responsive to black-spot parasite load. This finding conflicts with the experimental work of Chivers *et al*. (2007) who detected an increase in club cell investment in fathead minnows exposed to the black-spot parasite and the pathogenic water mould (Saprolegnia sp.). Further evidence supporting the anti-parasite hypothesis has been presented by Michalak (2006) and Päkk *et al*. (2011). Michalak (2006) experimentally treated Fathead Minnow (Pimephales promelas) and reported an increase in club cell production following fish exposure to trematode cercariae and Saprolgenia. Meanwhile, in a study of Common Carp (Cyprinus carpio), Päkk *et al*. (2011) found that club cell investment increased following injection of a protist ecto-parasite, and that epidermal tissue with elevated club cell densities protected fish from reinvasion of the parasite. Halbgewachs *et al*. (2009) provided indirect support for the anti-parasite hypothesis by demonstrating a reduction in club cell investment in fish with suppressed immune systems. By contrast, an experiment by James *et al*. (2009) found no change in club cell investment in Fathead Minnows exposed to the trematode metacercariae, Ornithodiplostomum sp, and another experiment by Pollock *et al*. (2012) using Fathead Minnow found no change in club cell investment following exposure to two species of pathogenic water mould.

James *et al*. (2009) explored some of the reasons for the contrasting outcomes of experiments testing the anti-parasite hypothesis. Differences in experiment duration, fish age, fish condition and parasite source were identified as possible contributing factors, although these authors focused most of their attention on pathogen source. In the experiments of both Chivers *et al*. (2007) and Michalak (2006), parasites were used that remain for extended periods in the fish epidermis, while the experimental subject in James *et al*. (2009) was a parasite that encysts in the liver and body cavity, suggesting only a transitory exposure to the fish epidermis. While a link between club cell response and pathogen tissue specificity seems reasonable, this hypothesis cannot explain the findings of *et al*. (2012), who used the same skin-dwelling pathogenic water mould as Chivers *et al*. (2007), nor can it explain the findings of the present study, which focused on a link between club cell investment and black-spot parasite densities encysted in the skin of Creek Chub. *et al*. (2012) discussed the possibility that environmental stressors and population differences might confound efforts to identify any relationship between club cell investment and exposure to infectious agents. This also was the conclusion of Manek *et al*. (2013) who reported the persistence of population differences in club cell investment in Fathead Minnow even after fish had been held in captivity for 28 days.

Leaving behind the question of the role of club cells in fish immunity, the most striking findings of this study are those that link variability in club cell investment in Creek Chub so strongly to mucous cell densities, epidermal thickness and fish size. An association between epidermal thickness and club cell densities has been reported elsewhere (e.g., Wisenden and Smith 1997, James *et al*. 2009, Stabell and Vegusdal 2010), but our findings depart from these others in several important respects. First, we used epidermal tissue cross-sectional area in our density estimates whereas these other authors computed club cell densities in relation to epidermal length. However, what was most distinctive about our findings was the direction of the relationship between epidermal thickness and club cell density. In the studies cited above, all reported a linear increase in club cell densities with increasing epidermal thickness. Here that association was negative, with club cell densities varying in a strongly non-linear manner with increasing epidermal thickness. This

relationship was best explained by a power function having a scaling exponent of -1.6. However, as reported earlier in the results, this relationship was strongly confounded by the effects of fish length on both epidermal thickness and club cell densities. When we accounted for the length effect in the path model, the association between epidermal thickness and club cell densities was much weaker, though still statistically significant.

The earlier research demonstrating a relationship between epidermal thickness and club cell investment exhibited two other important similarities that may help to clarify our anomalous findings. First, the fish used in those studies, Fathead Minnow (Wisenden and Smith 1997, James *et al*. 2009) and Crucian Carp (Stabell and Vegusdal 2010), all were small and similar in size, ranging from 30 – 60 mm. Moreover, in at least two of these experiments (i.e., Wisenden and Smith 1997, Stabell and Vegusdal 2010), both epidermal thickness and club cell densities were positively correlated with fish condition. Therefore, it would seem reasonable to conclude that the positive association between epidermal thickness and club cell density results from a covariation of these two traits with fish condition. Moreover, because fish in those experiments exhibited little variability in body size, we can add that it would have been unlikely that an effect of fish size on the relationship between epidermal thickness and club cell densities would have been discovered.

What then can be made of this negative association between epidermal thickness and club cell densities in Creek Chub? One explanation, that this relationship is due to the covariance of both epidermal thickness and club cell densities with fish length, is not supported by the path model. In that model, epidermal thickness retains its negative association with club cell densities even after accounting for the effects of fish length. Another possible explanation is that the relationship is an artifact of our decision to measure club cell densities in relation to the cross-sectional surface area of the epidermis, rather than its length. In an effort to explore this possibility, we re-examined our data, focusing on the relationship between epidermal thickness and alarm cell density, measured in terms of cells per length of epidermal tissue (i.e., cells/mm). This effort yielded a much weaker, though still significant, negative relationship between epidermal thickness and club cell density (cells/mm, $R^2 = 0.14$). It appears then that while the relationship between epidermal thickness and club cells is conditional on how club cell density is measured, there is still likely to be a biologically meaningful, although unknown, contribution to its development.

On the question concerning the relationship between club cell and mucous cell densities, we are aware of only one other study that has described such an association (Päkk *et al*. 2011). By contrast, Wisenden and Smith (1997) found that both club cell and mucous cell densities increased with fish condition. Mucous cells are a conspicuous feature of the fish epidermis, both in their morphology and function. In histologically prepared cross-sections, mucous cells can be readily distinguished from other cells of the epidermis because of their staining properties, large relative size (along with club cells) and tendency to be located near the tissue's outer surface. An important function of mucous cells appears to be their participation in the fish immune response. Mucous cells produce anti-pathogenic compounds (Ingram 1980, Shephard 1994), impair the penetrance of parasites into the epidermis (Haas 1994, Buchmann and Bresciani 1998) and act as phagocytes during tissue healing (Iger and Abraham 1990).

Accepting that mucous cells do have an immune function, it would therefore seem reasonable to expect that their abundance should covary with those of club cells, given the apparent role of the latter in the fish immune response. Instead, we found a strong negative association. We admit then that our findings are perplexing, but as this pattern also has been reported elsewhere (i.e., Päkk *et al*. 2011), it merits some examination here. Possible explanations for this relationship would be categorized as either causal or coincidental. Regarding the former, we have not uncovered any work to suggest that an increase in either mucous or club cells would cause a decease in the abundance of the other cell type. However, there is evidence that mucous cells and club cells do not react consistently to steroid and peptide hormones (Pfeiffer *et al*. 1985). Among the findings of the study by Pfeiffer *et al*. (1985), two have relevance here: 1) that species appear to differ significantly in how they react to different hormone treatments and 2) that adrenaline and the antigonadotropic chemical Paroxypropione caused reductions in club cell and increases in mucous cell densities in the epidermal tissue of the European minnow (Phoxinus phoxinus). Pfeiffer *et al*. (1985) also found that androgens caused a reduction in club cell densities, but did not produce any change in the density of mucous cells. These authors suggested that the link between hormones and epidermal cell densities is likely associated with reproductive maturity. As all fish examined in our study were collected in fall, outside of the breeding season months, we can discount the possibility that difference in the reproductive state of small and large Creek Chub was responsible for the association we found between

mucous and club cells. What the findings of Pfeiffer *et al* (1985) do suggest, however, is that the size-based relationships that we detected for mucous (+) and club cells (-) are likely to be a product of age-based differences in hormone activity.

Perhaps the most remarkable finding of this study was, in fact, the very strong negative association between fish length and club cell investment. The nature and possible origins of this relationship became apparent in the path model, which revealed that much, but not all, of the effect of length was indirect (i.e., through the effect of length on mucous cell density, epidermal thickness and parasite burden). Therefore, a significant amount of the effect of length on club cell investment appears to have been based on the effect of length on these other variables. Nonetheless, length retained a significant direct effect on club cell investment in the path model, suggesting that some other factors associated with body size affect club cell investment in Creek Chub.

While the proximate cause for the association between body size and club cell investment documented here is likely to be associated with age-specific differences in hormone activity, the ultimate cause, or functional basis, for this association is not at all clear. One plausible explanation is that the risk of parasite infections is negatively associated with body size, and that club cell investment should vary in direct relation to infestation risk. Although this hypothesis is supported by the negative association we uncovered between black-spot infestation and body size, this relationship was nonetheless a weak one (Figure 2a), and much weaker than the relationship between body size and club cell investment (Figure 1d). Among other possible explanations for this relationship, we believe two, reproductive state and fish condition, lack merit. All fish from this study were taken in the fall months, following the reproductive season, and we found no association between body size and condition for fish used in this study (unpubl. data). There is evidence from the literature that club cell investment may increase with UV exposure (e.g., Blazer *et al*. 1997, Chivers *et al*. 2007), so perhaps the effect of size on club cell investment in Creek Chub reflects differences in the exposure of small and large fish to UV radiation. There is in fact evidence from a study of juvenile sea breams (Pagrus major and Acanthopagrus schlegeli), that individuals learn to avoid UV radiation as they grow (Fukunishi *et al*. 2006). However, because that study was limited to juvenile fish, it may have limited value in helping to clarify why club cell densities in Creek Chub decreased with body size (i.e., from juvenile to adult fish). Finally, we cannot discount the possibility that club cell investment declines with body size in Creek Chub because predation pressure on chub by piscine predators may also decrease with body size. However, in order to accept such a connection, we also must accept that a primary function of club cells is associated with the alarm response, and that individuals invest in the production of these cells in direct relation to predation pressure. With respect to the latter point, Chivers *et al*. (2007) found no evidence connecting predation pressure and club cell production in Fathead Minnow. This is reason enough to be skeptical of the predation risk hypothesis as an explanation for our results. However, in doing so we are left in a position of having not gained any understanding for the unexplained (direct) effects of body size on club cell investment in Creek Chub.

What we can claim from these and other findings of this study is that there is a strong ontogenetic basis for club cell investment in Creek Chub, and that most of the variability in club cell production for this species can be predicted by characteristics associated with the fish (and not their environmental context). Although there can be no doubt about the role played by hormones in controlling the production of both mucous and club cells in fish epidermis, it has been, and continues to be, much more difficult to determine the implications of this endocrine regulation on fish health and ecology.

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