1 **Title**
2 Transcriptomic support for the Immunocompetence Handicap Hypothesis but not the Oxidation
3 Handicap Hypothesis
4
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11
12 **Abstract**
13
14 Sexually selected traits are hypothesized to be honest signals of individual quality due to the
15 costs associated with their development or expression. Testosterone, a sex steroid known to
16 influence the production of sexually selected traits, has been proposed to underlie the costs
17 associated with sexually selected traits via its immunosuppressive effects (i.e., the
18 Immunocompetence Handicap Hypothesis) or by influencing an individual's
19 exposure/susceptibility to oxidative stress (i.e., the Oxidation Handicap Hypothesis). Previous
20 work testing these hypotheses has primarily focused on physiological measurements of immunity
21 or oxidative stress, but little is known about the molecular pathways by which testosterone could
22 influence immunity and/or oxidative stress pathways. To measure the molecular consequences of
23 experimentally elevated testosterone, we used previously published RNA-seq data from studies
24 that measured the transcriptome of individuals treated with either a testosterone-filled or an
25 empty (i.e., control) implant. Two studies encompassing two species of bird and three tissue
26 types fit our selection criteria. We found strong support for the Immunocompetence Handicap
27 Hypothesis, but no support for the Oxidation Handicap Hypothesis. More specifically,
28 testosterone-treated individuals exhibited strong signatures of immunosuppression,
29 encompassing both cell-mediated and humoral immunity. Our results suggest that testosterone
30 enforces the honesty of sexually-selected traits by influencing an individual's
31 immunocompetence rather than their exposure or susceptibility to oxidative stress.
32
33 **Keywords**
34 Androgens, testosterone, sexual selection, immunity, oxidative stress, transcriptomics
35
36 **Introduction**
37
38 There is a long-standing interest in understanding why sexually selected traits have evolved and
39 one hypothesis suggests that mates have selected for traits that are costly to develop or bear (i.e.,
40 the handicap hypothesis; Zahavi, 1975). An important assumption of the handicap hypothesis is
41 that an individual's investment in sexually selected traits correlates with their investment in other
42 traits that also influence their reproductive success or survival (Grafen, 1990; Andersson, 1994).
43 Individuals face tradeoffs when fitness-related traits exhibit negative correlations and, as a result,
44 individuals can incur survival costs from their reproductive investments (Stearns, 1992). These
45 costs arise because the development and/or expression of traits important for reproduction (e.g.,
46 sexually selected traits) and traits important for survival (e.g., immune function) are dependent

47 on the same mechanism (Zera and Harshman, 2001). As such, our understanding of the evolution
48 of sexually selected traits is dependent upon our understanding of the pleiotropic nature of the
49 mechanisms that underlie their production (Kokko et al., 2003).
50
51   Testosterone is a sex steroid that is known to influence the development and/or
52 expression of sexually selected traits (Hau, 2007; Fusani, 2008; Ball and Balthazart, 2009). In
53 combination with its effects on other fitness related traits (e.g., immune function, Segner et al.,
54 2017), testosterone is thought to enforce the honesty of sexually selected traits (Ketterson and
55 Nolan, Jr., 1999, Buchanan et al., 2001, Wingfield et al., 2001, Reed et al., 2006). Two
56 prominent hypotheses have been proposed to explain how testosterone enforces the honesty of
57 sexually selected traits: the Immunocompetence Handicap Hypothesis (Folstad and Karter, 1992)
58 and the Oxidation Handicap Hypothesis (Alonso-Alvarez et al., 2007). The Immunocompetence
59 Handicap Hypothesis proposes that sexually selected traits remain honest because of
60 testosterone's antagonistic effects on an individual's immune function. Therefore, poor quality or
61 low condition individuals cannot maintain high levels of circulating testosterone due its
62 immunosuppressive effects (Folstad and Karter, 1992). A meta-analysis by Roberts et al. (2004)
63 revealed weak support for this hypothesis. However, a meta-analysis by Foo et al. (2017) found
64 that experimentally increasing testosterone results in suppression of both cell-mediated and
65 humoral immunity. Foo et al. (2017) also found positive trends between multiple measures of
66 immune function and naturally occurring levels of circulating testosterone. These results fit the
67 predictions of the Immunocompetence Handicap Hypothesis because individuals naturally
68 expressing high of testosterone represent high quality or high condition individuals that can
69 invest in sexually selected traits without compromising their immune system (Peters, 2000). The
70 Oxidation Handicap Hypothesis, on the other hand, states that sexually selected traits remain
71 honest because testosterone increases an individual's susceptibility and/or exposure to oxidative
72 stress (Alonso-Alvarez et al., 2007). In other words, testosterone may influence an individual's
73 ability to protect or repair cellular machinery from oxidative damage (e.g., an individual's
74 antioxidant defenses) or testosterone may influence the rate that reactive oxygen species are
75 produced (Alonso-Alvarez et al., 2007). Importantly, either one of these consequences may occur
76 independent of the other. Of the few studies that have directly tested the Oxidation Handicap
77 Hypothesis, some have found support (Mougeot et al., 2009; Hoogenboom et al., 2012) while
78 results from others did not find support for this hypothesis (Isaksson et al., 2011; Casagrande et
79 al., 2012; Taff and Freeman-Gallant, 2014; Baldo et al., 2015). Nonetheless, both hypotheses
80 have primarily been tested using physiological measurements of oxidative stress and immunity,
81 but less is known about the underlying molecular pathways. Given that sex steroids partly
82 function by binding to intracellular receptors and acting as transcription factors (Ketterson and
83 Nolan, Jr., 1999; Nelson, 2011), measuring the relationship between testosterone and
84 transcription can shed light on the proximate pathways that testosterone influences.
85
86   Modern sequencing approaches, like RNA sequencing (RNA-seq), allow for
87 comprehensive measurements of whole transcriptomes and the relative abundance of each
88 transcript (Wang et al., 2009). This approach assesses coordinated, large-scale transcriptional
89 responses rather than focusing on targeted candidate genes (e.g., via qPCR). RNA-seq
90 approaches have been used to investigate the role of androgens on gene expression, particularly
91 in the context of sex differences (Gao et al., 2015; Cox et al., 2017) and gonadal development
92 (Monson et al., 2017; Zheng et al., 2019). Similarly, RNA-seq based studies have been crucial in

93 providing a more comprehensive understanding of the complex and dynamic immune and stress
94 responses (e.g., Barshis et al., 2013; Huang et al., 2013; Kim et al., 2018). In the context of mate
95 choice, measuring the relationship between testosterone and transcription can shed light on the
96 pathways that testosterone influences to potentially enforce the honesty of sexually selected traits
97 (e.g., immune or oxidative stress pathways). Therefore, our understanding of the pleiotropic
98 nature of testosterone is partly dependent upon our understanding of the impact of circulating
99 testosterone on the transcriptomic signatures of immunity and oxidative stress. To date, these
100 hypotheses have rarely been tested using genome scale approaches (Wenzel et al., 2013). In red
101 grouse (*Lagopus lagopus scoticus*), testosterone treatment had little effect on overall gene
102 expression in the liver and spleen but did result in the down-regulation of genes related to
103 immune function in caecal tissue (Wenzel et al., 2013). Given that Wenzel et al. (2013) used a
104 microarray-based approach, testing these handicap hypotheses using RNA-seq represents a more
105 modern, robust test as RNA-seq provides many advantages over microarray technologies,
106 including higher sensitivity and no hybridization biases (Wang et al., 2009).
107
108   Here, we use published RNA-seq datasets to further examine the effects of testosterone
109 on the transcriptome. Specifically, we re-analyze studies that compared gene expression between
110 testosterone-treatment and control subjects in two bird species: golden-collared manakin
111 (*Manacus vitellinus*) and Japanese quail (*Coturnix japonica*). Golden-collared manakin males
112 produce brightly colored plumage ornaments and engage in elaborate courtship behaviors during
113 the breeding season (Day et al., 2007). Importantly, previous work on the golden-collared
114 manakin experimentally blocked androgen receptors to show that the expression of male
115 reproductive behaviors is dependent upon the interaction between testosterone and the androgen
116 receptor (Day et al., 2007; Schlinger et al., 2013). Japanese quail males also produce brightly
117 colored cheek feathers to attract females (Hiyama et al., 2018). Castrating males influences the
118 color of a male's cheek feathers and administering testosterone to castrated males causes cheek
119 patches of castrated males to match those of males that have not been castrated. In this study, we
120 use transcriptomic data from the foam gland of quail and muscular tissue of the golden-collared
121 manakin, tissues that are known to be express significant amounts of androgen receptors
122 (Adkins-Regan, 1999; Fuxjager et al., 2016). However, we re-analyze the data to explicitly test
123 the Immunocompetence Handicap Hypothesis and the Oxidation Handicap Hypothesis. We
124 constructed co-expression networks to identify gene networks that show correlated expression
125 patterns following testosterone treatment. If testosterone is immunosuppressive, then we predict
126 that testosterone treatment will cause consistent down-regulation (i.e. suppression) of genes with
127 annotated immune function in both species. Similarly, if testosterone influences an individual's
128 susceptibility or exposure to oxidative stress, then we predict that testosterone treatment will
129 cause a decrease in the expression of genes with annotated functions in antioxidant protection
130 and/or an increase in genes that are expressed in response to oxidative stress. An important
131 caveat is that no support for either hypothesis does not exclude the possibility that these
132 pathways enforce the honestly of sexually selected traits independent of testosterone's effects, as
133 has been suggested before (Metcalfe and Alonso-Alvarez, 2010; Weaver et al., 2017).
134
135 **Methods**
136 *Study Selection*
137

138 To identify studies of interest, we first performed a literature search on both Scopus and Google
139 Scholar with the following search terms: "testosterone" AND "RNA-seq" or "transcriptome" or
140 "transcriptomics". This literature search produced 260 results. From this list of 260 studies, we
141 retained RNA-seq studies that measured gene expression in adult males from both testosterone-
142 manipulated and control groups. This process resulted in one study for re-analysis and we also
143 identified an additional dataset by searching within NCBI's Sequence Read Archive
144 (Supplemental Figure 1). Fuxjager et al. (2016) experimentally increased testosterone in golden-
145 collared manakins (*Manacus vitellinus*, "manakin") and performed RNA-seq on pectoralis and
146 scapulohumeralis caudalis tissue (n= 3 each testosterone and control for each tissue). Finseth and
147 Harrison (2018) experimentally increased testosterone in Japanese quail (*Coturnix japonica*,
148 "quail") experiencing short days and performed RNA-seq on the foam gland (n=6 each
149 testosterone and control).

150

151 *Data Re-analysis*

152

153 We downloaded the raw sequencing data from SRA with sratoolkit fastq-dump (quail:
154 PRJNA397592; manakin: PRJNA297576) and adaptor trimmed all reads with Trim Galore!
155 v0.3.8 (https://github.com/FelixKrueger/TrimGalore). We aligned trimmed reads to the
156 respective reference genome (*M. vitellinus* v2, *C. japonica* v2) for each species with STAR
157 v2.5.3 (Dobin et al., 2013) and quantified expression with htseq-count v0.6.0 (Anders et al.,
158 2015), specifying strand 'no'. We normalized counts to sequencing depth and variance
159 stabilizing transformed counts with DEseq2 (Love et al., 2014). Transformed counts were
160 visualized with a principal component analysis (PCA) using pcaExplorer v2.8.1 (Marini and
161 Binder, 2016).
162   To test for the effect of testosterone treatment on transcription, we performed network
163 analysis with the weighted gene co-expression network analysis (WGCNA) tool (Langfelder et
164 al., 2011; Langfelder and Horvath, 2008). We created modules independently for each species
165 with the following shared parameters: network type=signed, minimum module size=30, and
166 module dissimilarity=0.2. We used β=12 for quail and β=18 for manakin, which represents the
167 point the network reached scale free topology. We then tested for correlations between modules
168 and testosterone treatment using a $p < 0.05$ cutoff. We identified the hub genes of each module by
169 selecting the top five genes with the highest module membership (MM) score.
170   To test the Immunocompetence and Oxidation Handicap Hypotheses, we performed
171 ranked order gene ontology (GO) analyses with GOrilla (Eden et al., 2009, 2007). For each
172 module, we ordered the gene list by descending MM scores and input this entire list into GOrilla.
173 GOrilla then tests for enrichment and places greater weight on genes at the top of the list relative
174 to the bottom. GO categories were significantly enriched if the qvalue < 0.05. To find support for
175 the Immunocompetence Handicap Hypothesis, immune related GO categories (e.g., "immune
176 system process") had to be significantly enriched among down-regulated genes. To find support
177 for the Oxidation Handicap Hypothesis, oxidative stress related GO categories had to be
178 significantly enriched among up-regulated genes (e.g., "response to oxidative stress") or down-
179 regulated genes (e.g., "antioxidant activity").

180

181 **Results**
182 *Overall Results*

183

184 After filtering, we used 13,509 manakin genes and 13,946 quail genes for PCA and WGCNA
185 network construction. Testosterone treatment had pronounced effects on gene expression and
186 individuals clustered by treatment in both comparisons (Figure 1).
187
188 *WGCNA – Quail*
189
190 WGCNA constructed 18 modules for quail, six of which were correlated with testosterone
191 treatment (Supplemental Figure 2). The yellow module (925 genes, r=-0.74) and dark green
192 module (88 genes, r=-0.67) were both strongly enriched for immune related GO categories
193 (Table 1). The yellow module was primarily enriched for broad immune categories, e.g.,
194 "immune system process" and "immune response", whereas the dark green module was
195 primarily enriched for lymphocyte and leukocyte related categories. This represents a significant
196 decrease in immune gene expression following treatment (Figure 2A). The yellow module hubs
197 were *SASH3*, *ITGB2*, *SLAMF8* (LOC107324444), *TRAF3IP3*, and *EVI2A*. The dark green hub
198 genes were *FBL*, *PIK3R6*, *STOML2*, *GPR157*, and *DNAL4*.
199   The black and purple modules were also negatively correlated with testosterone treatment
200 and were enriched for translation and muscle process GO categories respectively. Lastly, we
201 found two modules up-regulated following testosterone treatment. The turquoise module was the
202 most strongly correlated with testosterone treatment (4423 genes, r=0.98). GO enrichment was
203 largely driven by genes involved in the Golgi apparatus and endoplasmic reticulum functions
204 (Supplemental Table 1). The green module (795 genes, r=0.61) was primarily enriched for broad
205 metabolic activity and protein modification processes.
206
207 *WGCNA – Manakin*
208
209 WGCNA constructed 34 modules for manakin, 12 of which were correlated with testosterone
210 treatment (Supplemental Figure 3). Seven modules were correlated with muscle type. None of
211 these modules were also correlated with testosterone treatment, indicating no tissue specific
212 response at the network level. Of the 12 modules, 7 were negatively correlated and 5 positively
213 correlated. Like the quail, manakins also exhibited a significant decrease in immune gene
214 expression following testosterone treatment (Figure 2B, Supplemental Table 2). The dark
215 turquoise module (198 genes, r=-0.71) was strongly enriched for a broad range of immune
216 related GO categories (Table 1). The dark turquoise hub genes were *MHC1A* (LOC108639055),
217 *INPPL1* (LOC103767762), *CCL14* (LOC103758017), *CCL3L* (LOC103757995), and an
218 uncharacterized non-coding RNA (LOC108640668).
219   The remaining negatively correlated modules were primarily enriched for metabolism
220 (green, dark olive green), ribosomal components (dark red, pale turquoise), and mitochondria
221 related categories (steel blue, pale turquoise). Among the positively correlated modules, we also
222 found enrichment of cellular metabolism, catabolism, and mitochondrial related GO categories
223 (Supplemental Table 2).
224

Discussion

226 In this study, we quantified transcriptional responses to experimentally increased circulating
227 testosterone in two species of bird. Our gene network analysis revealed that both manakin and
228 quail exhibit immunosuppression following testosterone treatment, supporting the

229   Immunocompetence Handicap Hypothesis. However, we did not find support for the Oxidation
230   Handicap Hypothesis, as there was no enrichment of genes expressed related to oxidative
231   damage, nor suppression of genes related to antioxidant defenses in either species. These results
232   suggest that high levels of circulating testosterone can be costly to maintain partly due to their
233   potential negative effects on an individual's immune response and not the individual's
234   susceptibility or exposure to oxidative stress. Importantly, oxidative stress could still be involved
235   in enforcing the costs of reproduction or sexually selected traits; however, our results suggest
236   that this cost is not borne out via molecular pathways that are sensitive to testosterone, at least in
237   the tissues and species examined here.
238
239   Our analyses revealed that transcriptomic immunosuppression was broad, encompassing
240   aspects of both innate immunity (e.g., leukocyte activation and cytokine signaling) as well as
241   adaptive immunity (e.g., antigen processing and presentation) across both species (Table 1). The
242   observed effect of testosterone could occur through both genomic and non-genomic pathways,
243   but regulation of the immune system by androgens receptors likely plays an important role
244   (Trigunaite et al., 2015; Segner et al., 2017; Gubbels Bupp and Jorgensen, 2018). More
245   specifically, while testosterone exposure and subsequent androgen receptor activity can promote
246   innate immune cell differentiation and development, testosterone also reduces activity of these
247   cells (Gubbels Bupp and Jorgensen, 2018). As such, the hub genes of the immune related
248   modules highlight broad suppression of innate immune signaling (quail yellow: *SASH3*,
249   *SLAMF8*, *TRAF3IP3*; manakin dark turquoise: *INPPL1*, *CCL14*, *CCL3L*, ncRNA; (Beer et al.,
250   2005; Veillette, 2010; Dauphinee et al., 2013; Sokol and Luster, 2015; Zou et al., 2015; Thomas
251   et al., 2017; Wang et al., 2018). Similarly, testosterone exposure had substantial effects on the
252   regulation of the adaptive immune system. Testosterone exposure greatly reduces T cell activity
253   (Lin et al., 2010; Kissick et al., 2014), which is a prominent signature in both quail
254   (Supplemental Table 1) and manakin (Table 1). In addition to suppression of T cell activity in
255   manakin, we also identified MHC class IA as a hub gene in the manakin dark turquoise module.
256   MHC class IA binds and presents viral peptides to CD8+ T cells, which is a critical component
257   of the adaptive immune response (Neefjes et al., 2011). Previous work has shown suppressive
258   effects of testosterone on CD4+ T cells/MHC class IIB (Lin et al., 2010) and CD8+ T cells (Page
259   et al., 2006). However, our study is the first to describe suppression of genes involved in T cell
260   activity as well as MHC class I.
261
262   We were also interested in whether the changes in gene expression as a result of
263   experimental testosterone treatment were consistent between manakin and quail. Despite
264   evidence of immunosuppression in both species, the immune related gene networks are not
265   preserved between the species (Supplemental Figure 4). These results suggest either a species
266   specific and/or tissue specific response to testosterone treatment, both of which have previously
267   been documented in transcriptomic data (Breschi et al., 2016). Given that muscle tissues used in
268   the manakin study are very distinct from the foam gland tissue used in the quail study, it should
269   not necessarily be surprising that the response to testosterone treatment was not preserved.
270   Nonetheless, we identified these immunosuppression signatures in muscle and foam gland,
271   tissues which are not traditionally studied in avian immunology (Rose, 1979; Schat et al., 2014).
272   Thus, our results are likely conservative, and we may expect to see a stronger signature in
273   immune tissues, such as broader suppression of adaptive immune response. This highlights the
274   sensitivity of RNA-seq to detect functional signatures in non-traditional tissues (e.g., Louder et

275   al., 2018) In both species, testosterone is necessary to produce secondary sexual characteristics
276   for mating (Schlinger et al., 2013, Hiyama et al., 2018). Our results detail the potential molecular
277   pathways underlying the trade-off between the expression of sexually selected traits and immune
278   function.
279
280   Given that we found strong support for immunosuppression in both studies, multiple
281   experiments should be conducted to continue to broaden our understanding of testosterone's
282   immunosuppressive effects. First, studies should focus on performing testosterone manipulations
283   and examining transcriptomic responses in a wider range of tissues and species. Moreover,
284   studies should prioritize conducting experimental infections and/or immune challenges in
285   combination with RNA-seq analyses to examine how transcriptomic signatures relate to immune
286   function. Novel endocrine-based experiments, similar to (Goymann et al., 2015; Goymann and
287   Flores Dávila, 2017), paired with RNA-seq analyses can also shed light on how acute changes in
288   testosterone levels influence transcription over shorter timeframes. When possible, studies
289   should also prioritize measuring testosterone's effect on gene expression using a within-
290   individual sampling approach as this allows for a more robust test of testosterone's effect on
291   gene expression (Williams, 2008). Overall, these integrative, mechanistic approaches will
292   ultimately provide novel insights into the evolution of sexually selected traits.
293

Table and Figures

Table 1. GO enrichment for modules found in both species that support the Immunocompetence Handicap Hypothesis. The top 5 gene ontology (GO) categories are presented, along with FDR adjusted p-value and GOrilla enrichment score.

GO ID	Description	FDR	Enrichment
Quail, Yellow Module			
GO:0002376	immune system process	3.11E-45	4.53
GO:0006955	immune response	3.47E-35	5.53
GO:0002682	regulation of immune system process	4.30E-35	3.59
GO:0002684	positive regulation of immune system process	1.34E-32	4.25
GO:0046649	lymphocyte activation	1.08E-30	11.1
Quail, Dark Green Module			
GO:0002684	positive regulation of immune system process	4.09E-05	1.86
GO:1903706	regulation of hemopoiesis	5.89E-05	2.17
GO:0046649	lymphocyte activation	6.06E-05	2.45
GO:0038023	signaling receptor activity	6.38E-05	1.83
GO:0002682	regulation of immune system process	6.42E-05	1.61
Manakin, Dark Turquoise Module			
GO:0006955	immune response	8.81E-10	2.48
GO:0046649	lymphocyte activation	3.29E-08	7.44
GO:0042110	T cell activation	3.57E-08	10.58
GO:0002376	immune system process	4.34E-08	3.04
GO:0002521	leukocyte differentiation	3.31E-07	4.09

8

302   **Figure 1**. PCA of (A) manakin and (B) quail. Samples separate by treatment along PC3 for
303   manakin and PC1 for quail. Each circle represents a sample and is color-coded by treatment.
304   Manakin samples are labeled by muscle type. Ellipses represent 95% confidence intervals.
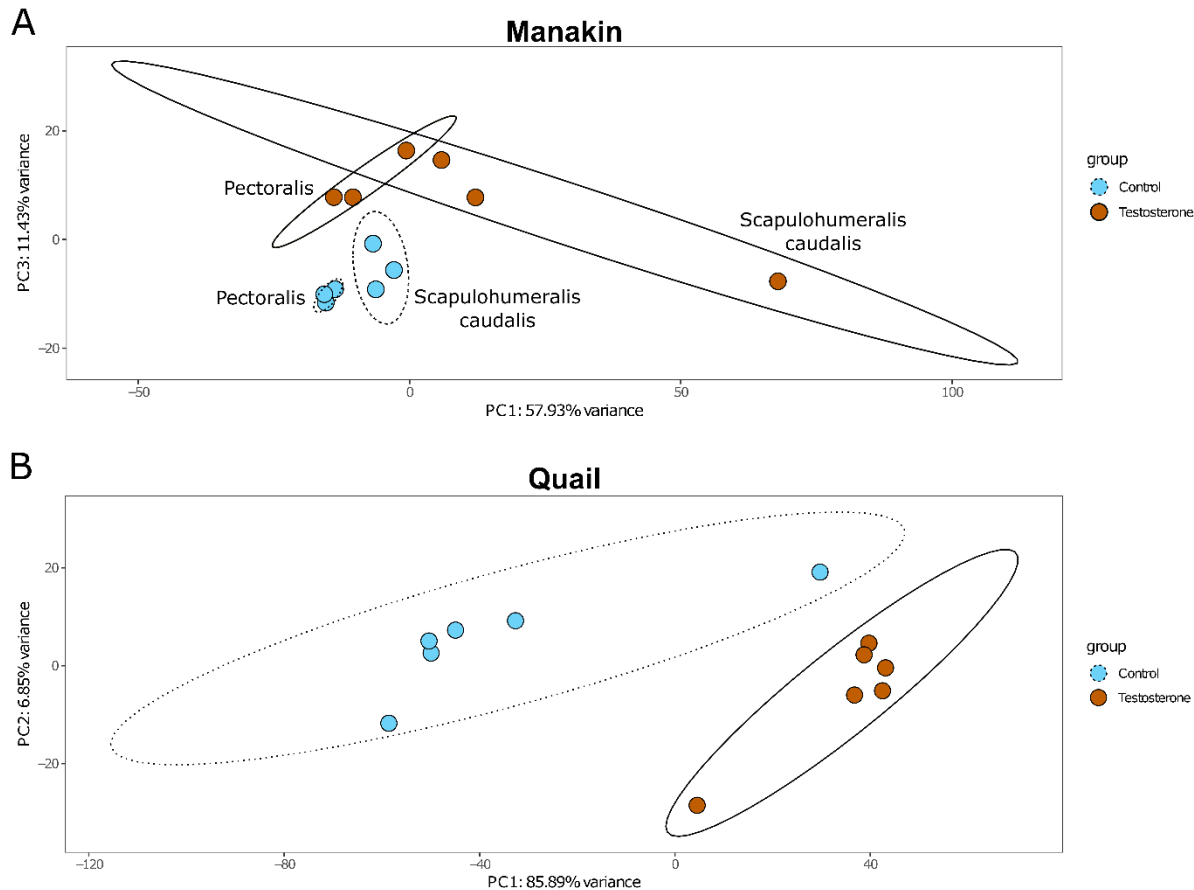

305

306   **Figure 2.** Expression heatmaps of the (A) Manakin Dark Turquoise Module and (B) Quail
307   Yellow Module, which represent down-regulation of the immune system. Each column
308   represents a sample color coded by treatment or muscle type. Each row represents a module
309   gene. High expression is indicated by orange colors and low expression is represented by blue
310   colors.

311
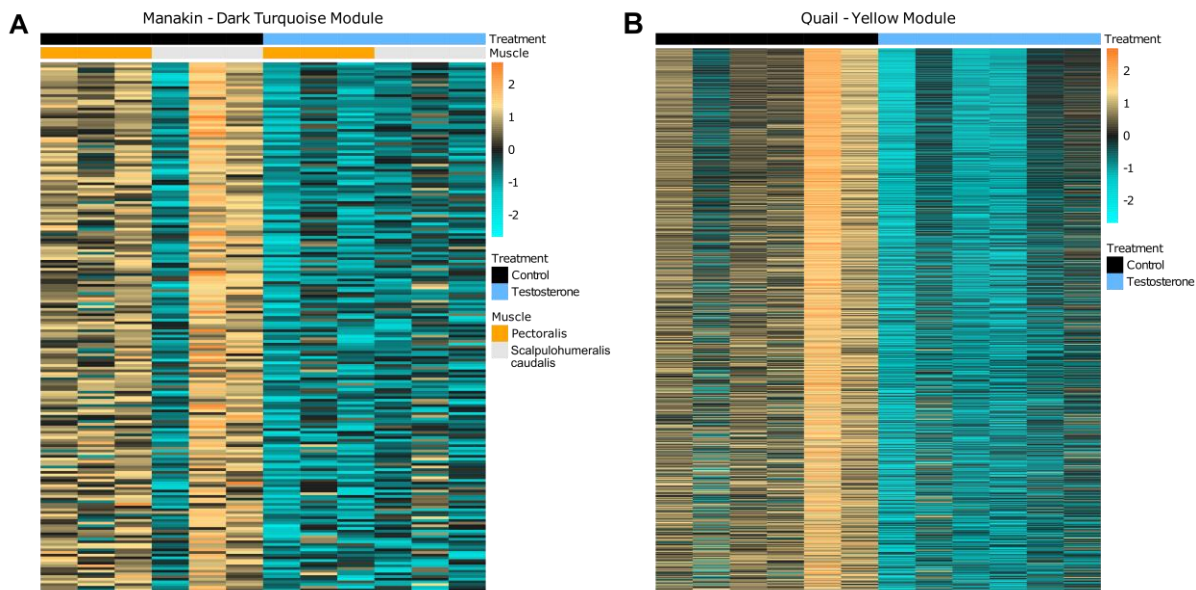
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