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An Animal Model of Non-Descript and Exotic Breeds of Pigs to Study Relative Expression Profiling of Key Genes of Muscle Development and Body Growth in Postnatal *Longissimus Dorsi* Muscle

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ABSTRACT

Pork meat is considered to be one of the cheapest source of animal protein. The pork from non-descript pigs is considered to be tenderer and juicy. Yet the growth rate in non-descript pigs is low. Pig rearing in the socio-economically weaker section had been highly preferred as it provides insurance against agricultural loses. The present study focuses on to study the relative expression of key genes of growth *(ARID5B, COL2A1)*, muscle organ morphogenesis *(MyBPH, PDK4)* and protein phosphorylation *(PKM2 and POSTN)* in *Longissimus dorsi* muscle of Large White Yorkshire (LWY) and non-descript pigs. The qualitative and quantitative differential expression were performed by agarose gel electrophoresis and qRT-PCR. The expression of all genes showed a significant difference ($P<0.05$) between both the breeds except *PKM2* ($P>0.05$). The gene *MyBPH* was significantly ($P<0.05$) upregulated in non-descript while other genes i.e. *ARID5B, COL2A1, PDK4 and POSTN* were significantly ($P<0.05$) down-regulated in nondescript pig muscles. With the help of this data we can say that the pork from non-descript pig is tender and juicy due to higher expression of *MyBPH* in them but the body growth rate is low due to low expression seen in *ARID5B, COL2A1, PDK4 and POSTN* genes. Therefore the present study can be used as a pioneer data to understand the genetic basis of differences between the meat quality of non-descript and LWY breeds of pig.

HIGHLIGHTS

- We studied relative expression of genes involved in growth, muscle organ morphogenesis and protein phosphorylation.
- The study will help in understanding the genetic basis of differences relating to the meat quality.

Keywords: Non-descript, Large White Yorkshire (LWY), *ARID5B, COL2A1, MyBPH, PDK4, PKM2, POSTN*

Asia stands out to be the largest producer of the pork meat, with an overall contribution of about 56% to the meat industry. Within Asia there is a difference in the preferences of the pig rearing practices and consumption. In South East Asia, pig is considered the most preferred livestock species while in South Asia it is not much preferred mainly due to cultural differences. Pig rearing in South and South East Asia is performed in a smallhouse system majorly and about 15-20% for large scale system (Deka *et al*., 2014). Pigs show quick growth rate and act as a cheaper source of animal protein with a better efficiency of feed conversion rate and has a lower cost of maintenance (Muhindro *et al*., 2019).

Pig rearing provides security of income with various direct and indirect employment potentials, insurance against adverse climate conditions like droughts, emergency funds

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in case of agriculture losses, cheap manure etc. (Haldar *et al*., 2017). Due to religious constrains, the cumulative pork meat consumption in India is one of the least globally (Devi *et al*., 2014). Due to which, there is restricted import and export of pork meat.

As per the recent statistics, the pork consumption was further decreased from 354.1 thousand metric tons in 2013 to 295 thousand metric tons in 2021 (Statista, 2022). According to NBAGR, ten pig breeds are officially registered in India and according to the 20th livestock status of India, the total pig population stands at 9.06 million i.e. shows a reduction of about 12.03% compared to the previous census of 2012.

The non-descript pigs of India, as compared to the exotic breeds, provide only a menial amount of returns to the farmers due to their low yields. But the indigenous breeds are locally well adapted and show disease resistance potential. Therefore, it is suggested that with crossbreeding programs focusing on cross-breed generation of non-descript/ indigenous pigs and exotic pigs would provide an expansion to the piggery industry. This would further contribute towards the nutritional status stability of the poor farmers and the backward sections of the society within a low expenditure rate (Sulabh *et al*., 2017).

In the recent times, due to the introduction of the pure-bred exotic stock of pigs in Indian piggery market, the overall economy of the country due to piggery sector has boosted. Large White Yorkshire, which is an European exotic pig breed is one of the most commercially available pork in India due to their great feed conversion ratios and rapid growth rates (Ramesh *et al.*, 2009).

The muscle mass of livestock and fish species needed to sustain human needs is about 35-60% of their body mass. The striated skeletal muscles which are attached to the backbone and are voluntary in nature are involved in locomotion and posture control. The nutrition provided by the meat is dependent upon the amount of fats, carbohydrates and proteins that make it up. Meat which has good amount of proteins and good proportion of essential amino acids are considered superior (Maltin *et al*., 2003). Prenatal growth and myogenesis are controlled by various genetic and environmental factors besides hormonal and nutritional qualities (Rehfeldt and Kuhn, 2006).

The development of muscles is a quantitative trait and different genes contributes towards its development. Different genes contribute towards growth like *POSTN* and *MyBPH*. Periostin osteoblast specificity factor (*POSTN)* regulates fat deposition (Chen *et al*., 2011) and skeletal development (Voit *et al*., 2014). Myosin binding protein H (*MyBPH*) is responsible for taste, tenderness and marbling characters of pork meat (Ghosh *et al*., 2015). *COL2A1* and *ARID5B* are needed for the muscle organ morphogenesis. *COL2A1* gene encodes for collagen type-2 aplha-1 protein and is responsible for growth of cartilage and morphogenesis of skeletal system (Sodhi *et al*., 2014). *ARID5B* encodes for AT rich interactive domain 5B which is a DNA binding protein and is predominantly involved in adipogenesis by acting as a Fat Mass and Obesity Associated Protein (FTO) (Muñoz *et al*., 2018). Protein phosphorylation is important for the metabolism of fat and glucose in muscles. *PDK4* and *PKM2* are genes needed for protein phosphorylation and ATP generation. *PDK4* encodes Pyruvate dehydrogenase kinase-4 is a protein found in fat tissue where it complements the ketone metabolism and is related with the quality of meat by lowering the backfat content (Sodhi *et al*., 2014). Pyruvate kinase M-2 enzyme encoded by *PKM2* gene is vital for muscle glycolytic pathway. It is seen to be associated with the daily weight gain average, back fat thickness, lean cuts and feed: gain ratios (Fontanesi *et al*., 2008).

The indigenous breeds of Punjab are not well established. It is seen that the pork of non-descript pigs is tender and juicy, but not much notable work is reported yet. Thus, current study focuses on analyzing the transcriptome involved in skeletal development and body growth and their relative expression in muscles of adult non-descript and LWY breed of pig.

MATERIALS AND METHODS

The current study was carried out at Department of Animal Biotechnology, College of Animal Biotechnology, Guru Angad Dev Veterinary and Animal Sciences University (Ludhiana).

Collection of Tissues Samples

In the current study, the *longissimus dorsi* muscle samples (n=6) between 12th and 13th rib spaces from adult animals of Large White Yorkshire and non-descript pig of Punjab, were collected from the slaughter house immediately

after the slaughter, were transported immediately from slaughterhouse to lab in RNA later solution and dry ice and preserved for future use in – 80°C. Animals selected for sampling were 1 year old castrated males. The selected animals were thoroughly vaccinated and dewormed.

Tips and micro-centrifuge tubes were treated with Di-ethyl pyro-carbonate (DEPC). The RNA was extracted from *longissimus dorsi* muscle (120 mg) of adult animals of both the pig varieties. Total RNA isolation was done using TRIzol™ (QIAZEN) reagent. For this, the tissue was homogenized well using 1.0ml of TRIzol. Further in order to separate the different phase of the homogenate 200 µl of chloroform (HIMEDIA) was added and mixed well. Centrifugation at 13000 rpm was done to get upper aqueous phase containing the RNA. About 40% of upper aqueous layer was pipetted into a separate micro-centrifuge tube without disturbing the organic phase below. Further in order to precipitate down the RNA, 0.5 ml of isopropanol (HIMEDIA) was added and centrifugation was done at 13,000 rpm for 10 minutes. The supernatant was discarded and washing of the obtained pellet was performed using 1 ml of 75% ethanol. Ethanol was discarded and pellet was air dried and dissolved in 25 µl of autoclaved nuclease free water. The extracted RNA was stored at – 80°C for future use. To eliminate the genomic DNA impurities, a RNAse-free DNAse kit (QIAGEN, Hilden, Germany) was used. RNA purification was done using RNAasy mini kit as per the guidelines provided by the manufacturer. The quantity of RNA yield was assessed using the Thermo Scientific Nanodrop-One. The spectrophotometric absorbance ratios of A_{260}/A_{280} for the RNA samples ranged between 1.8 to 2.1 and the integrity of RNA ranged from 8.0 to 10.0. Further, cDNA was synthesized using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Catalog no. 4368814) as per manufacturer's guidelines.

Qualitative Expression

The qualitative analysis was done using agarose gel electrophoresis (Agilent Technologies Ireland, Dublin, Ireland). For this a normal PCR was performed for each of the gene primer with the constructed cDNA. The conditions for which were specific to each primer set for the gene i.e. initial denaturation at 95°C for 2 minute, denaturation at 95°C for 15 seconds, annealing temperature as given in Table 1 for 30 seconds, extension at 72°C for 1 minute and final extension 72°C for 5 minutes.

Then PCR product was run in 1.5% agarose gel at 100 volt/cm. The UV- illumination (Bio-Rad, USA) and gel doc (G:Box Syngene) were used to visualize and capture gel images with bands (Fig. 1).

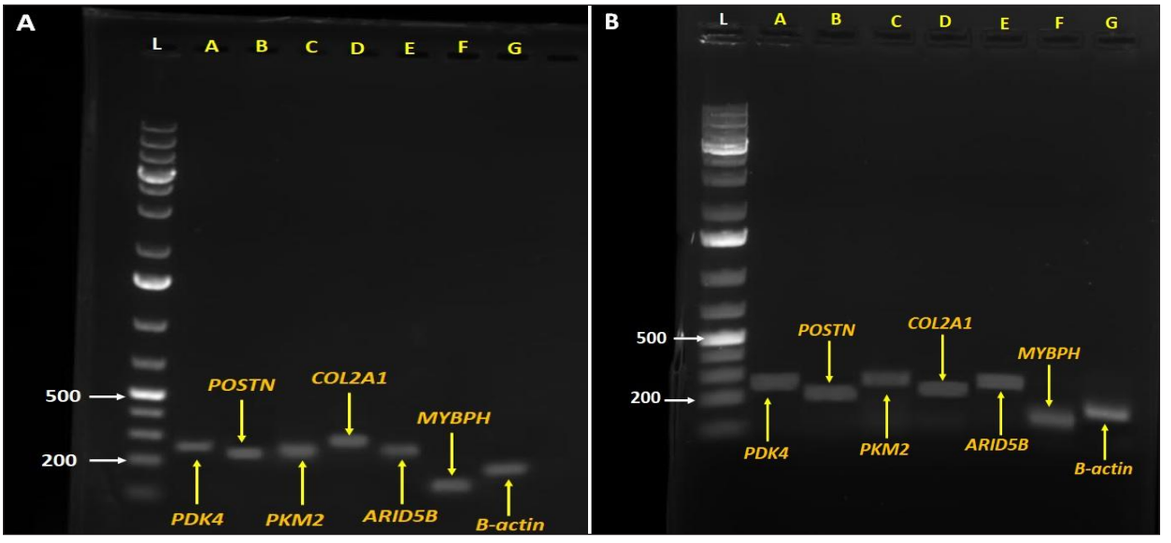

Fig. 1: The bands in gel-doc image showing ligation of primers in PCR product. Wells "L" – 1kb DNA ladder, well "A" – *PDK4* gene, well "B" - *POSTN* gene, well "C" – *PKM2* gene, well "D" – *COL2A1* gene, well "E" – *ARID5B* gene, well "F"- MyBPH gene and well "G"- β- *actin gene* respectively from (A)LWY and (B) Non-descript pig

Quantitative Real Time PCR (qRT-PCR) Expression

Primers for the qRT-PCR were constructed by the online Primer-3 software (Rozen and Skaletsky, 2000) and the information of the primers has been enlisted in Table 1. For the quantitative evaluation of mRNA transcript levels of *ARID5B*, *COL2A1*, *MyBPH*, *PDK4, PKM2* and *POSTN* genes and endogenous controls b-*actin* and *RPL-4*; in non-descript and LWY, real-time qRT-PCR was performed using BIO RAD model CFX96^{TM} Optics Module real time PCR. To determine the quantity of transcripts of target genes, SYBR Green (Promega, USA) dye was used. Two technical replicates of the samples were used for the quantification by following the amplification conditions i.e. 95 °C for 10 min (initial denaturation), and then 39 cycles of 95 °C for 15 sec (denaturation) followed by annealing at separate temperatures for each primers enlisted in Table 1 for 1 min (annealing and extending), followed by melt curve insertion. The efficiency of real-time PCR primers was defined by the standard curve method. The amplified transcript levels of the target genes were compared with that of the *β-actin* (Wang *et al*., 2003) and *RPL-4* (Nygard *et al*., 2007), as endogenous controls. The mRNA transcript levels were quantified by the relative C_T method. The results in terms of relative expressions have been expressed after normalizing with the transcript levels of the endogenous control genes *β-actin* and *RPL-4* (Erkens *et al*., 2006; Van Poucke *et al*., 2001).

STATISTICAL ANALYSIS

Statistical analysis and level of significance $P<0.05$ of the differential expression patterns was calculated using two sample paired t-test. The values have been expressed as mean ± SEM.

RESULTS AND DISCUSSION

Relative quantitative expression levels of *ARID5B, COL2A1, MyBPH, PDK4, PKM2* and *POSTN* was investigated by quantitative Real Time PCR with normalizing against transcript levels of endogenous reference *β-actin* and *RPL-4* genes. The expression of *ARID5B, COL2A1, PDK4,* and *POSTN* was significantly lower ($P<0.05$) in non-descript pig as compared to the LWY (Fig. 2).

Table 1: Primer sequences of for quantitative and qualitative analysis

Gene	Primers	Product Size (bp)	Annealing Temperature	GenBank ID
ARID5B	F: GTGGTCCAGTCCTTCGACAT R: GTCATCCCTCGCAATCAGTT	234	60ºC	XM_003359209
COL2A1	F: TCAAGGATTTCAAGGCAACC R: TGTGACCTTTGACACCAGGA	209	60ºC	NC_010447
MyBPH	F: AGTGCAGAAGGCAGACAAA R: AGACCCGGAAGGAGTAAGA	117	60℃	XM_001033014
PDK4	F: CCTCATTCCTCCACCAAGAA R: GAGAAATGCTCGACCTCTCG	244	61ºC	NC_010451
PKM2	F: TTCGCATCTTTCATCCGTAA R: CGCCCAATCATCATCTTCT	224	60 ºC	CN166623
POSTN	F: CAATTAGGCTTGGCTTCTGC R: GAAGACCCTGAGCTGTTTGC	213	60ºC	NC_010453
***β- actin* (Control)**	F: GACATCCGCAAGGACCTCTA R: ACACGGAGTACTTGCGCTCT	250	60° C	XM_003124280
***RPL-4* (Control)**	F: TTCCTGAACTTCCTTTGGTGGT R: CATTATAGATGATGCAGGGTCC	198	60 ºC	NM_000968.4

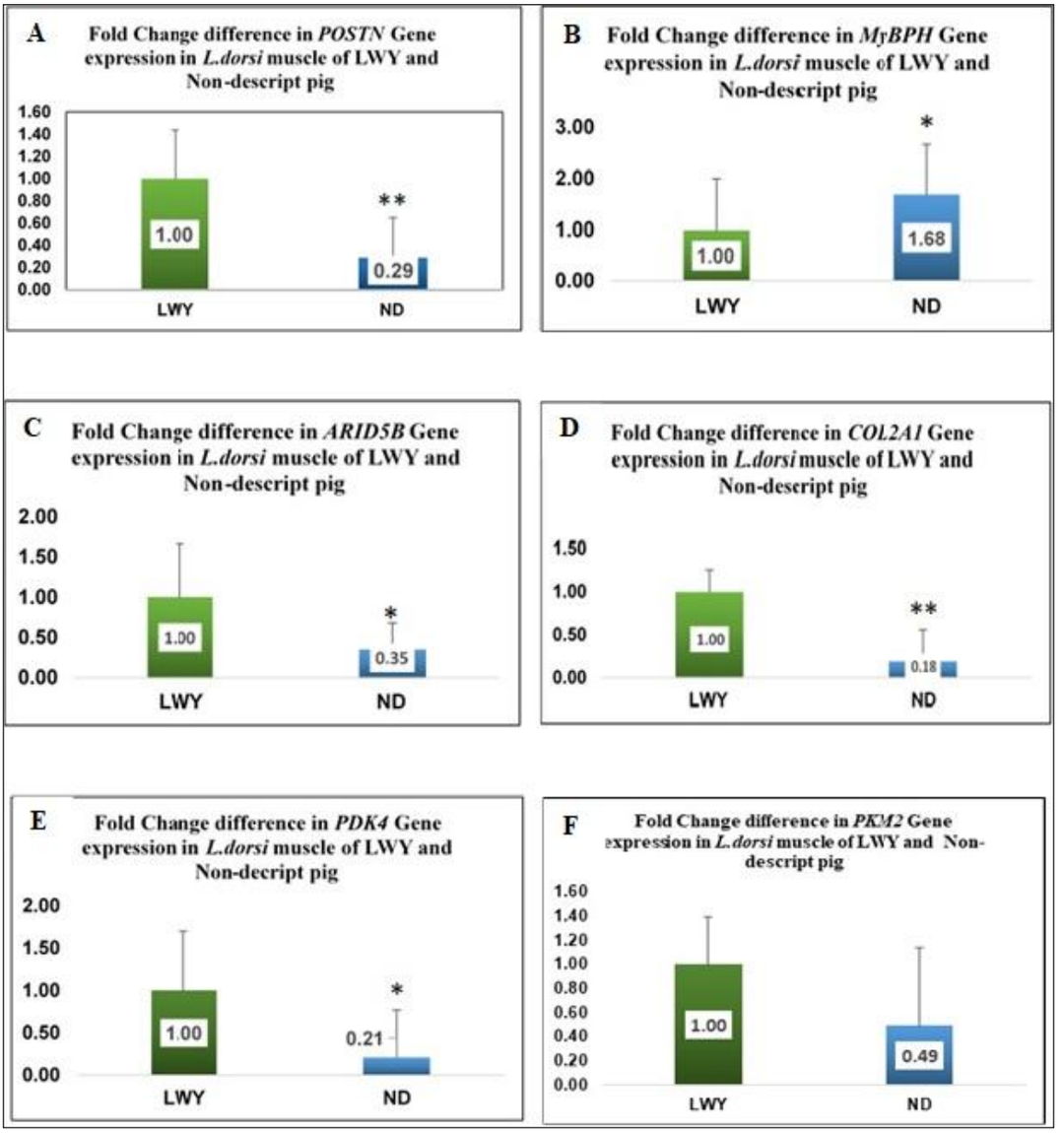

Fig. 2: The relative mRNA fold change expression of genes **(A)** *POSTN*, **(B)** *MyBPH*, **(C)** *ARID5B*, **(D)** *COL2A1*, **(E)** *PDK4* **(F)** *PKM2* in non-descript pigs of Punjab and Large White Yorkshire (LWY). The target genes were normalized with *β-actin* and *RPL-4* as endogenous control. The ** & * superscripts on bars signifies that transcript levels differs highly significantly *(P < 0.01)* & significantly *(P<0.05)* respectively

The expression of *MyBPH* was seen to be significantly higher (*P<0.05*) in non-descript as compared to LWY (Fig. 2). There was no statistically significant difference observed in the transcript levels of *PKM2* between both the postnatal muscle samples of non-descript and LWY pigs.

Expression of genes involved in growth

The expression of two genes relating to overall body growth was assessed i.e. *POSTN* and *MyBPH*. Their expression levels were found to be contrasting. The *POSTN* expression was significantly lower (*P<0.05*) in non-descript pig muscles as compared to LWY (Fig.

2A, Table 2) while in contrast the expression *MyBPH* was found to be significantly higher (*P<0.05*) in non-descript pig muscles as compared to LWY (Fig. 2B, Table 3). *MyBPH* is majorly present in the myosin region of myofibrils and contributes towards the quality of muscle fiber. The muscle fiber type is responsible for the muscle type and therefore important for the water content, muscle texture, colour, flavor, quality of meat and its nutritional value (Kim *et al*., 2021). Ghosh *et al.* (2015) reported that *POSTN* gene induces skeletal development and bone development by inducing specific signaling pathways. The upregulation of *MyBPH* in non-descript could be a cause for juiciness and tenderness of the pork obtained from them as compared to LWY. While a lower expression of *POSTN* would correspond to the lower growth rate seen in the non-descript pigs.

Expression of genes involved in muscle organ morphogenesis

The expression of *ARID5B* (AT-Rich interactive domain containing protein 5B) and *COL2A1* (collagen type 2 aplha-1) was analyzed using the qRT-PCR. The levels of expression of both of these genes were seen to be significantly lower (*P<0.05*) and (*P<0.01*) respectively in non-descript as compared to LWY (Fig. 2C; Table 4; Fig. 2D; Table 5). Both of these are transcriptional regulator involved in skeletal system morphogenesis and development of cartilage (Lefebvre and Smits, 2005; Kim *et al*., 2007). Their lower expression in non-descript pigs could probably explain about declined body growth seen in them as compared to LWY which show great body growth and developmental characters.

Table 2: Validation of *POSTN* mRNA expression using real time qPCR (SYBR green)

Group	*POSTN* (Average Ct)	*β-actin* (Average Ct)	*RPL-4* (Average Ct)	Geometric Mean (GM) of *β-actin* and *RPL-4* Average Ct	ΔCT (Ct *POSTN* - Ct *β-actin*) – GM	ΔΔCT (ΔCt ND - ΔCt LWY)	Fold change
LWY	32.73 ± 0.45	25.83 ± 0.21	34.35 ± 0.21	29.78 ± 4.25	4.36 ± 0.44	0.00 ± 0.00	1.00
Non-descript (ND)	34.61 ± 0.45	26.72 ± 0.48	32.63 ± 0.38	29.53 ± 2.95	6.14± 0.36	2.13 ± 0.53	0.29 **

Values are Mean ± SE. Mean having superscript ** shows high significant difference at *P<0.01*

Table 3: Validation of *MyBPH* mRNA expression using real time qPCR (SYBR green)

Group	*MyBPH* (Average Ct)	*β-actin* (Average Ct)	*RPL-4* (Average Ct)	Geometric Mean (GM) of *β-actin* and *RPL-4* Average Ct	ΔCT (Ct *MyBPH* - Ct *β-actin*) – GM	ΔΔCT (ΔCt ND - ΔCt LWY)	Fold change
LWY	28.26 ± 0.47	26.44 ± 0.12	25.58 ± 0.08	26.44 ± 0.43	0.25 ± 0.21	0.00 ± 0.00	1.00
Non-descript (ND)	27.86 ± 0.48	26.82 ± 0.28	26.93 ± 0.77	26.82 ± 0.05	-0.50 ± 0.30	-0.75 ± 0.62	1.68 *

Values are Mean ± SE. Mean having superscript * shows significant difference at *P<0.05*.

Table 4: Validation of *ARID5B* mRNA expression using real time qPCR (SYBR green)

Group	*ARID5B* (Average Ct)	*β-actin* (Average Ct)	*RPL-4* (Average Ct)	Geometric Mean (GM) of *β-actin* and *RPL-4* Average Ct	ΔCT (Ct *ARID5B*- Ct *β-actin*) – GM	ΔΔCT (ΔCt ND - ΔCt LWY)	Fold change
LWY	29.42 ± 0.71	25.68 ± 0.52	29.30 ± 0.59	27.43 ± 1.81	0.77 ± 0.67	0.00 ± 0.00	1.00
Non-descript (ND)	29.73 ± 0.35	25.40 ± 0.1	29.13 ± 0.11	27.20 ± 1.86	2.27 ± 0.33	1.25 ± 0.96	0.35 *

Values are Mean ± SE. Mean having superscript * shows significant difference at *P<0.05*.

Table 5: Validation of *COL2A1* mRNA expression using real time qPCR (SYBR green)

Group	*COL2A1* (Average Ct)	*β-actin* (Average Ct)	*RPL-4* (Average Ct)	Geometric Mean (GM) of *β-actin* and *RPL-4* Average Ct	ΔCT (Ct *COL2A1*- Ct *β-actin*) – GM	ΔΔCT (ΔCt ND - ΔCt LWY)	Fold change
LWY	27.85 ± 0.56	29.55 ± 0.56	28.50 ± 0.32	20.02 ± 0.52	-2.23 ± 0.25	0.00 ± 0.00	1.00
Non-descript (ND)	27.80 ± 0.58	28.01 ± 0.39	29.00 ± 0.47	28.49 ± 0.49	0.26 ± 0.38	0.48 ± 0.27	0.18 **

Values are Mean ± SE. Mean having superscript **shows significant difference at *P<0.01*.

Table 6: Validation of *PDK4* mRNA expression using real time qPCR (SYBR green)

Group	*PDK4* (Average Ct)	*β-actin* (Average Ct)	*RPL-4* (Average Ct)	Geometric Mean (GM) of *β-actin* and *RPL-4* Average Ct	ΔCT (Ct *PDK4* - Ct *β-actin*) – GM	ΔΔCT (ΔCt ND - ΔCt LWY)	Fold change
LWY	31.55 ± 1.77	31.72 ± 0.17	32.07 ± 0.04	31.89 ± 0.17	0.26 ± 0.70	0.00 ± 0.00	1.00
Non-descript (ND)	32.32 ± 1.45	34.13 ± 1.07	32.63 ± 0.38	33.37 ± 0.74	0.47± 0.56	0.21 ± 0.59	0.21 *

Values are Mean ± SE. Mean having superscript * shows significant difference at *P<0.05*.

Table 7: Validation of *PKM2* mRNA expression using real time qPCR (SYBR green)

Group	*PKM2* (Average Ct)	*β-actin* (Average Ct)	*RPL-4* (Average Ct)	Geometric Mean (GM) of *β-actin* and *RPL-4* Average Ct	ΔCT (Ct *PKM2* - Ct *β-actin*) – GM	ΔΔCT (ΔCt ND - ΔCt LWY)	Fold change
LWY	28.62 ± 0.82	27.72 ± 0.34	26.84 ± 0.20	27.27 ± 0.25	-0.38 ± 0.39	0.00 ± 0.00	1.00
Non-descript (ND)	29.55 ± 1.02	26.87 ± 0.20	26.80 ± 0.34	26.83 ± 0.03	0.64 ± 0.65	1.37 ± 1.06	0.49

Values are Mean ± SE. Mean having superscript * shows significant difference at *P<0.05*.

Expression of genes involved in protein phosphorylation

There was a significantly lower (*P<0.05*) expression of *PDK4* (Pyruvate dehydronase kinase 4) reported in non-descript pig muscles as compared to LWY muscle (Fig. 2E; Table 6). While *PKM2* (Pyruvate Kinase M-2) expression showed no significant difference between the expression among the muscle samples of both the candidate pig samples (Fig. 2F; Table 7). The *PDK4* is involved in lactate breakdown and lipid breakdown process (Manio *et al*., 2016). A lower *PDK4* activity could correspond to less fat consumption in non-descript pigs and therefore corresponds to more backfat percentage in them.

CONCLUSION

Enhancements in body growth rate and quality of pork are amongst the top urgencies of breeding plans. Non-descript pigs have good meat quality, high tenderness and juiciness but lags in body growth rate as compared to western breeds. Constant fall in the quality and carcass characteristics have guided the breeders to improve such parameters. LWY is a rapidly-growing breed with large heavy muscular body and higher muscle fibers in the carcass with respect to non-descript breeds.

Our study on relative expressions of different genes involved in overall development and muscle growth in non-descript versus LWY pigs can be used as one of the pioneer studies reported in context with pigs of Punjab as meager knowledge is presently available regarding this. The findings can be helpful in understanding the underlying reasons for the poor growth yet good quality of pork meat from non-descript pigs. This study can help in analyzing different pathways relating muscle growth

and selecting specific QTLs for crossbreeding programs in order to enhance the breed characters.

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