POPULATION DYNAMICS OF WOOLLY APHID, *ERIOSOMA LANIGERUM,* HAUSMANN (HEMIPTERA: APHIDIDAE) AND ITS NATURAL ENEMIES IN APPLE ORCHARD IN THIMPHU VALLEY, BHUTAN

SONAM DORJI N

MASTER OF SCIENCE (AGRICULTURE) IN PLANT PROTECTION

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE
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ACADEMIC ADMINISTRATION AND DEVELOPMENT MAEJO UNIVERSITY
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THIS THESIS HAS BEEN APPROVED IN PARTIAL FULFLLMENT
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IN PLANT PROTECTION

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ชื่อเรื่อง	พลวัตรประชากรของเพลี้ยอ่อน Woolly aphid, Eriosoma lanigerum Haumann (Hemiptera: Aphididae) และศัตรูธรรมชาติในสวนแอปเปิ้ล ในหุบเขาทิมพู ประเทศภูฏาน
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บทคัดย่อ

การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อประเมินการเปลี่ยนแปลงประชาของของเพลี้ยอ่อนแอปเปิล (woolly aphid (WA), *Eriosoma lanigerum*; Hemiptera: Aphididae) และแมลงศัตรูธรรมชาติในสวนแอปเปิล ณ ธิมพู วาล์เลย์ ราชอานาจักรภูฐาน ในช่วงระหว่างเดือน เมษายน ถึง ตุลาคม 2561 โดยสำรวจประชากรในสวนสองจุด ได้แก่สวน Hongtsho-2 และ Kausumche-3 (พิกัด 27 28′20″องศาเหนือ – 89 38′10″องศาตะวันออก, ระดับความสูงจากน้ำทะเล 2,248 - 2,648 เมตร, ช่วงอุณภูมิ 5-30 องศาเซลเซียส) ซึ่งมีการเก็บข้อมูลโดยการสุ่มตัวอย่างจากต้นแอปเปิ้ล 5 ต้นต่อสวนตรงบริเวณยอดที่ระดับความยาว 10 เซนติเมตร ในทั้งสี่ทิศของต้นทุกสองสัปดาห์ โดยบันทึกจำนวนและตรวจสอบความถูกต้องของชนิดของเพลี้ยอ่อนและแมลงศัตรูธรรมชาติที่พบ ทั้งในภาคสนามและห้องปฏิบัติการ พบว่าระดับทำลายของเพลี้ยอ่อนมีระดับสูงในช่วงฤดูปลูก (เดือนมิถุนายน ถึง กันยายน) โดยเฉพาะอย่างยิ่งบริเวณยอดอ่อน และจากการวิเคราะห์ข้อมูลโดยวิธี Independent sample t-test พบว่าการทำลายใน Hongtsho-2 สูงกว่าในสวน Kausumche-3 อย่างมีนัยสำคัญ ด้วยค่าเฉลี่ยประชากร 1.40 + 0.30 และ 1.27 + 0.38 ตัวต่อต้น ตามลำดับ (t= .94, P= 4.3410, P=0.0001) ทั้งนี้การปรากฏของแมลงศัตรูธรรมชาติรวม 7 ชนิดที่มีความเกี่ยวข้องกับเพลี้ยอ่อนแอปเปิล ซึ่งได้แก่ ตัวเบียน 1 ชนิด คือแตนเบียน *Aphelinus mali* (Hymenoptera: Aphelinidae) และตัวห้ำ 6 ชนิด ด้วงเต่าตัวห้ำ *Coccinella transversoguttata*, *Harmonia sedecimnotata* และ *H. dimidiata*; Coleoptera: Coccinellidae); แมลงวันดอกไม้สองชนิด (*Syrphus opinator* และ *Heringia calcarata*; Diptera: Syrphidae); และแมลงช้างปีกใส (*Chrysoperla* sp. ; Neuroptera: Chrysopidae) มีการเติบโตของประชากรในรูปแบบเป็นตามความหนาแน่นของประชากรเพลี้ยอ่อนแอปเปิ้ล นอกจากนี้ยังพบว่าแตนเบียน *A. mali* เป็นแมลงศัตรูธรรมชาติที่มีศักยภาพสูงในการควบคุมประชากรของเพลี้ยอ่อนในทั้งสองสวน โดยมีเปอร์เซนต์

การเบียน ระหว่าง 12-34 เปอร์เซนต์ ดังนั้นแตนเบียนชนิดนี้ควรมีการศึกษาเพิ่มเติมในอนาคต สำหรับการนำมาควบคุมเพลี้ยอ่อน *E. lanigerum* โดยชีววิธี แบบการแผ่ขยายเพิ่มพูน (augmentative biological control) และ แบบการอนุรักษ์ (conservative biological control) ต่อไป

คำสำคัญ : การควบคุมโดยชีววิธี, แมลงศัตรูธรรมชาติ, เพลี้ยอ่อนแอปเปิ้ล

Title	POPULATION DYNAMICS OF WOOLLY APHID, *ERIOSOMALANIGERUM,* HAUSMANN (HEMIPTERA: APHIDIDAE)AND ITS NATURAL ENEMIES IN APPLE ORCHARDIN THIMPHU VALLEY, BHUTAN
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Degree	Master of Science (Agriculture) in Plant Protection
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ABSTRACT

This study aimed to evaluate the population change of woolly aphid (WA), *Eriosoma lanigerum* (Hemiptera: Aphididae) and associated insect natural enemies in apple orchards located at Thimphu valley, Kingdom of Bhutan from April to October 2018. The study was conducted in two apple orchards, Hongtsho-2 and Kausumche-3 (coordinates: 27°28′20″N - 89°38′10″E, altitude from 2,248 to 2,648 meters, Temp. 5-30 °C. Data was collected randomly from five trees comprising sample unit of four terminal shoots (approx. 10 cm) each tree. Species confirmation and population growth of woolly aphid and its natural enemies were examined at both field and laboratory levels twice a month. The aphid densities were observed higher during the growing season (June-September) when tender shoots and plants resource are abundant. During the apple growing season significantly an Independent sample t-test revealed that abundance of WA infestation was higher in Hongtsho-2 than Kausumche-3 averaging 1.40 + 0.30 and 1.27 + 0.38 aphis per tree respectively (t= .94, P= 4.3410, P=0.0001). Appearance was noted of seven species insect natural enemies associated with WA, including one parasitoid, *Aphelinus mali* (Hymenoptera: Aphelinidae); and six predators, including coccinellids, *Coccinella transversoguttata*, *Harmonia sedecimnotata* and *H. dimidiata* (Coleoptera: Coccinellidae); syrphids, *Syrphus opinator* and *Heringia calcarata* (Diptera: Syrphidae); and a green lacewings,

Chrysoperla sp. (Neuroptera: Chrysopidae). These were revealed according to WA population growth in density dependent manner. Additionally, in both the orchards *A. mali* was the most effective natural enemy with 12 - 34 percent parasitism. This parasitic insect should be subjected to further investigation for augmentative and conservative biological control of *E. lanigerum* in apple orchards.

Keywords : biological control, Insect natural enemies, woolly aphid

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Sonam Dorji N

TABLE OF CONTENTS

List of tables

List of figures

List of Abbreviations

BC	Biological control
CABI	Centre for Agriculture and Bioscience International
EPF	Entomopathogenic fungi
EPN	Entomopathogenic nematode
IPM	Integrated Pest Management
MoAF	Ministry of Agriculture and Forests
MM	Malling merton
NPPC	National Plant Protection Center
NCHM	National Center for Hydrology and Meteorology
RD	Royal delicious
WA	Woolly aphid

Chapter 1
Introduction

1.1 Background and rationale

Apple (*Malus domestica* Borkhausen) (Rosales: Rosaceae) is an economically important fruit crop grown for commercial exports massively contributing to the economy of the country. In 2005, China exported about 24,000 MT of apples standing at No.1 rank in the world contributing to 30% of the world's total production. The average apple production was 15.6 MT per ha in China. Aspects such as traditional agriculture practices and insect pest and diseases affected the apple yields. Aphid, spider mites, scales, leaf miners, leaf rollers, eucleid, caterpillars are some of the principal pest of apples. Natural enemies including spiders, pathogenic organisms, and insectivorous birds are considered crucial in suppression of apple pest in the orchards (Zhou *et al.,* 2014).

Among the pest of concerns, aphid are the major pest of agriculture colonizing over 3,000 species of herbaceous plants and shrubs (Boivin *et al*., 2012). Despite immense pressure from other pest and diseases problem particularly, woolly aphid (WA), *Eriosoma lanigerum* (Hausmann) (Hemiptera: Aphididae), native to eastern North America and now distributed worldwide as major pest of apple (Asante, 1994) is an emerging pest in the orchards of Thimphu valley, Bhutan. *E. lanigerum* has turn out to be the predominant pest of apple in India, the middle East, the Far East, South America, Australia and New Zealand (CABI, 2018).

E. lanigerum is polymorphic and described as apterous, alate sexuparae and sexuales and confined to apple trees in Australia (Asante, 1994). They are anholocyclic on apple (Blackman and Eastop, 1984) and reproduced round the year without alteration of host (CABI, 2018). WA overwinter as adult females in the subterranean and canopy of apple tree in Australia (Nicholas *et al.,* 2005). The breeding of overwintered aphid commenced from March-April in USA producing over 100 nymphs by female. Usually, 3-4 generations may take place in USA and other parts of northern America and Europe (CABI, 2018). WA occurred round the year however, severe cold weather inhibits (Ateyyat and Al-Antary, 2010).

Their occurrence and damages are studied both on the aerial and subterranean parts of apple trees (Bergh and Stallings, 2016). It is reported that the optimal apple yields are limited by pest and disease and its impacts are escalating over the years (Zhou *et al.,* 2014) affecting apple yield (Abu-romman and Ateyyat, 2014). Studies has confirmed yield loss of 2.4 kg (13 apples) per tree and gross loss of $465.18/ha due to root infestation by WA (CABI, 2018).

In addendum, due to protective white, waxy, cottony substance characteristics of WA, suppression of infestation by pesticides application are thought to be ineffective. It required massive volume of insecticides to penetrate and adequately control the aphids escalating the production cost. Thus, biological control has been widely adopted for its efficiency and economic advantages than chemical control (Van lenteren *et al.,* 2003). Nonetheless, with the increasing concerns on the negative effects of pesticides on natural enemies, the environment and human health, a research interest has been shifted to the use of biological control agents such as predator and parasitoids (Quarrell *et al.,* 2017).

In Bhutan, farmers grow apple for both commercial exports and domestic consumption bolstering their livelihood. According to Agriculture Statistics (2016) Bhutan produced about 6,587 MT in 2016 and Thimphu valley alone produced approximately 3,025 MT accounting to 50% of the total production. Thimphu is one of the major apple growing district in the country. Common apple varieties grown are Royal Delicious, Red Delicious and Golden Delicious grown in the temperate regions (2,000 - 2,600 masl.) mainly traded with Bangladesh and India.

However, apple production has been confronted with insect pest damages over the years resulting in poor yield and quality deterioration.

The major insects prevalent in the apple orchards of Bhutan are *Popillia sp.*, *Protaetia neglecta,* apple twig borer (*Linda rubescens*), apple fruit borer (*Argyresthia sp.*), Green apple weevil (*Myllocerus sp. nr chloris*), Woolly aphid (*Eriosoma lanigerum*), and list of diseases are apple scab (*Venturia inaequalis*), Lichens on apple, Premature leaf fall (*Alternaria mali*). *E. lanigerum* is reiterated to be an emerging serious pest of apple orchards in Bhutan (NPPC, 2017).

Likewise, as mentioned earlier woolly aphid is a pest of concern in the apple plantation with increasingly heavy infestation occurring in canopies unfortunately yield impact assessment was lacking. Currently, limited work and information are available on biological control of WA aphid in Bhutan although, extensive studies were conducted elsewhere around the world. Furthermore, the vision of the country in gearing towards going organic agriculture has further necessitated for an alternative management strategy of pests as chemical use are restricted in organic farming. Woolly aphid and its natural enemies on apple production and impact of associated predators to suppress WA are not documented adequately in orchards of Thimphu valley. Specifically, natural enemy *A. mali* has never been reported in Bhutan which is a popular specialist natural parasitoid of woolly aphid used across the globe as biological control agent.

The aims of this study are investigating the seasonal occurrence of woolly aphid population density and exploring diversity of key associated predators and natural enemies of WA in apple orchards. The survey was designed and conducted in selected apple orchards in major apple growing areas with woolly aphid problem in Thimphu valley (Fig.1). This has furnished quantitative information to determine implementation of conservational biological control program in the context of an integrated pest management (IPM) system to suppress WA with the involvement of natural enemies.

1.2 Research objectives

The main objectives of the research are as follows;

i. This study focused on investigating and understanding the woolly aphid population abundance and fluctuation over apple growing season in orchards of Thimphu valley as limited information are available

ii. To explore list of potential natural enemies and predators associated with woolly aphid for future conservative biological control program in Integrated Pest Management (IPM)

Chapter 2
Literature Review

2.1 Woolly aphid

Woolly aphid (WA), *Eriosoma lanigerum* (Hausmann) (Hemiptera:Aphididae), is a key pest of apple trees which colonized both the roots and vegetative parts (Abu-romman and Ateyyat, 2014). It is native to eastern north America and a major pest of apple (Asante, 1994). They are small-medium size, reddish brown and the powdery waxy filaments provide woolly like characteristic form (CABI, 2018) which is a potential damaging pest of apple in southern hemisphere (Alspach and Bus, 1999). The aphid unveiled red-blood stain when crushed. They have piercing and sucking mouthparts and two cornical at back of abdomen (Lepaja *et al*., 2014). Comprehensive study on WA has been conducted in USA, New Zealand, Australia and south Africa (Lordan *et al.,* 2014).

Aphid (apterae) are small to medium size (1.2mm to 2.6mm) with purple, red or brown colored range covered with a thick flocculent wax appearing on roots, trunks and branches of host plants (Blackman and Eastop, 1984). Antennae are dusky brown, tibiae yellowish to slightly dusky brown and possess a relatively small cornicle pore (0.06-0.07 mm). Virginoparae are dark dusky green which hibernate in winter and occurred on the roots. They appear black particularly head and thorax and dingy yellowish-brown and lack white waxy covering in some other species (CABI, 2018).

Aphid (alatae) are 1.8 to 2.3mm (Blackman and Eastop, 1984) and Alatae virginoparae have reddish-brown abdomen with a covering of woolly white wax. Consist two or three sensoria on antennal segment with relatively small cornical pore about 0.05 mm. Oviparae are apterous, and rusty yellow to rusty brown. Males are apterous, and yellowish to dusky brown to dark green (CABI, 2018).

2.2 Distribution

Woolly aphid (WA), *E. lanigerum* is native to north America and also occurred in Australia (Asante, 1994). It is a cosmopolitan pest of apple and distributed in USA that colonized on apple roots and branches (Bergh and Stallings, 2016). WA are present in south Africa and associated with apple growing areas across the world (Stokwe and Malan, 2016). In addition, their presence are reported throughout Europe and Kosovo where adequate management of apple orchards are lacking and orchards with limited plant protection measures (Lepaja *et al.,* 2014). WA is a predominant pest of apples in India, the Middle east, the Far east, south America, Australia and New Zealand (CABI, 2018). WA infestations are also recorded in Bhutan as potential pest of apples (NPPC, 2017).

2.3 Biology and life history

Eriosoma lanigerum is polymorphic and described as apterous virginoparae, alate sexuparae and sexuales which are confined to apple trees in Australia (Asante, 1994). They are anholocyclic on apple (Blackman and Eastop, 1984) and reproduced round the year without the change of host (CABI, 2018). Aphid colonies overwintered in roots and bark crevices in plant host. The aphid being anholocyclic, an abortive sexual phase prevailed as unviable sexuales and eggs are produced on apples which do not hatch (CABI, 2018). WA overwinter as adult females in the subterranean and canopy of apple tree in Australia (Nicholas *et al.,* 2005). The reproductive life stages under field environment are dependent on season (Asante, 1994).

According to Lordan *et al*. (2014) re-infestation of WA occurred from canopy and root colonies. Bergh and Stallings (2016) reported major outbreak of WA are difficult to forecast. WA densities attained peak soon after buds outbreak and in late season (Gontijo *et al.,* 2013). According to Lordan *et al.* (2014) peak counts of aphid reported from May to June with two annual peaks. Similarly, they also reported two annual peaks of WA abundance, one in early growing season (June) and another in late season (September) Gontijo, *et al.* (2015). WA are present round the year although severe cold weather inhibit and as no colonies were recorded during Jan-March (Ateyyat and Al-Antary, 2010). Lordan *et al.* (2014) also observed that aerial infestation was not destroyed by low winter temperature. Woolly aphid colonies commenced its emergence from March-April according to NPPC (2017). Although, emergence of WA colonies varied in different orchards and season (Pringle and Heunis, 2008). Climatic factor may significantly influence WA ecology according to Lordan *et al.* (2014).

During the warm growing season, WA exhibited rapid reproduction of 10-12 generations with apterous virginoparae producing average of 100 nymphs in lifetime (Quarrell *et al.,* 2017). Nymphs overwinter in roots 10-15 cm below the soil surface (Lepaja *et al.,* 2014). Young nymphs move up the canopy when the soil temperature attains about 10°C in late spring to early summer (Nicholas *et al.,* 2005). (Ateyyat and Al-Antary, 2010) observed similar trend that above 10°C, commenced aphid movement to the canopies in April. Climatic aspects, precipitation and temperatures may have contributed WA occurrence in apples (Bergh and Stallings, 2016).

In north America, the heteroecious woolly aphid spend their reproductive life parthenogenetically on apple and sexually on elm host whereas in Australia, life cycle was confined to apple trees in apterous form (Asante, 1994). The breeding of overwintered aphids begin from March-April in USA over 100 nymphs produced by individual female. Usually 3-4 generations may take place in Michigan, USA and other parts of northern America and Europe (CABI, 2018).

In Australia, alates emerged in early November producing only virginoparae, however that emerged in late November to late January produced a mixture of virginoparae and sexuales. Alates that appeared in February-April produce only sexuales. The continuity of the species are through parthenogenesis and overwinter as cold-resistant apterous virginoparae (CABI, 2018). In general, aphid tend to hover more on the terminal shoots and multiply on tender plants (Verghese and Jayanthi, 2002). Conversely, aphids are fond of dwelling in the lower parts of the canopy and trunk of the host plants (CABI, 2018).

In India, apterous virginoparae undergoes four moulting resulting in five instars. The reproduction process are longer in winter however, greater fecundity in summer. The highly mobile 1st instar nymphs scatter from the aphid crowd to establish fresh colonies and move from roots to shoot round the year (CABI, 2018). The 1st instar nymphs dispersed between the trees are transported in various means through orchard management practices, migration or wind (Lordan *et al.,* 2014). In India, the peak aphid movement from shoots and roots were observed during mid-June and October to November based on the fluctuation of ambient and soil temperature.

Aphids sheltering on aerial parts of plants including wounds and axils was probably to avoid natural enemies (CABI, 2018). WA overwinters on roots colonies and produces 1st instar nymphs (crawlers) in early spring. In spring, crawlers migrate up the canopies and initiate new colonies in protected parts of the tree (Abu-romman and Ateyyat, 2014). They also reported that WA overwinter as adult females on both aerial and edaphic parts of the tree (Nicholas *et al.,* 2005).

WA colonies persist throughout winter on roots of the host plants (Blackman and Eastop, 1984). In central Washington, crawler movement of WA was noted from early May to early July and peaked from early June to late July (Beers *et al.,* 2010).The aphid colonies developed on thin bark around pruning cuts or split caused by heavy cropping in the previous season. As the season progressed, colonies develop on the new season's growth. In autumn, nymphs migrate to the roots (Nicholas *et al.,* 2005).

2.4 Host plants

Apples, *Malus domestica* (Rosales: Rosaceae) are the main host of WA but it can also occur on other rosaceous plants such as hawthorn (*Crataegus spp.*) and coloneaster (*Coloneaster spp.*) rarely on quince (*Cydonia spp*.), pear (*Pyrus spp*.) and mountain-ash (*Sorbus spp*.) but not so injurious in these host plants (Blackman and Eastop, 1984). The life cycle of WA is confined to apple trees due to unavailability of American elm (Stokwe and Malan, 2016). Asante (1994) reported WA occurrence in species of *Malus, Coloneaster, Crataegus, Sorbus and Pyracantha*. WA are also observed on elm, pear, quince, hawthorn, mountain ash and cotoneaster (Van lenteren *et al.,* 2003).

2.5 Damage on agricultural crops

About 5000 species of aphid recorded and considered as serious group of insect pest of crops in the temperate zone (Wieczorek, 2015). Aphids are primary pest of agricultural crops across the globe (Boivin *et al.,* 2012) which caused deformation in leaves and fruits (Abu-romman and Ateyyat, 2014). Particularly, woolly aphid damaged to apple trees by feeding on bark and colonizing both roots and vegetative parts (Asante, 1994; Bergh and Stallings, 2016). Woolly aphid is a key pest but not as vector of apple virus diseases (CABI, 2018). Depending on the severity and feeding site, WA can cause serious impact on the apple production (Stokwe and Malan, 2016). WA infestation varies with cultivars and orchard management practices (Wearing *et al.,* 2010).

During the high yielding period, numbers and weight of the fruits are reduced as consequence of galls formation. The galls infection can enormously retard the water conduction capacity of the roots and affect the shoot elongation (Ateyyat and Al-Antary, 2009). It destroyed developing buds in leaf axils (Stokwe and Malan, 2016).

Furthermore, WA can cause phytosanitary concerns for trade of fruits (Abu-romman and Ateyyat, 2014 ; Stokwe and Malan, 2016).

WA colonies established on pruning cuts and splits in early season that are caused by heavy fruiting in the previous season, however infestations occur on the current plant parts with commencement of new season (Nicholas *et al.,* 2005). WA infestation occurred on canopy and roots although prominent damages were observed on roots. The root damages are hard to detect and control (CABI, 2018) moreover, root infestations cause major damages (Ateyyat and Al-Antary, 2010). Due to hidden underground colonies, it causes major injury prior to revealing signs of aerial infestation (Stokwe and Malan, 2016). Root infestation resulted in the formation of galls impeding water conduction (CABI, 2018). Stokwe and Malan (2016) described root colonies damage and information are hard to furnish as sampling process was challenging.

The 1st instar nymphs (crawler) colonized leaf axils, pruning wounds and injured bark (Pringle and Heunis, 2008). WA destroyed buds in leaf axil and can infest fruit by entering the core through calyx (Stokwe and Malan, 2016). The nymphs and adults of WA resulted in hypertrophic galls and ruptured the roots and limbs facilitating pathogenic infections by impeding plant sap flow (Quarrell *et al.,* 2017). According to Asante (1994) WA damages the roots and shoots resulting in poor quality and yield. Ateyyat and Al-Antary (2009) also reported root damage are more severe and difficult to control than stem. Studies confirmed an average yield loss of 2.4 kgs (13 apples) per tree and gross loss of $465.18/ha due to root infestation (CABI, 2018).

In the situation of heavy infestation in the orchards, people deny to work in such undesirable condition (Nicholas *et al.,* 2005). WA infestation are nuisance to apple pickers causing respiratory issues due to inhalation of waxy filaments (Quarrell *et al.,* 2017). Gontijo *et al*. (2012) reported that change in pesticide program and interruption of the biological controls attributed to frequent outbreak of WA. Aphid obstructs the normal development of trees impacting the yield loss (Sood and Gupta, 2005).

2.6 Woolly aphid management strategies

2.6.1 Biological Control

Various techniques of woolly aphid management are discussed like use of resistant rootstocks, chemical and biological control or combination of all these measures (Stokwe and Malan, 2016). Biological control (BC) approach has been adopted for its efficiency and economic advantages against chemical to manage numerous insect pests across the globe (Van lenteren *et al.,* 2003). Massive focus on biological control of pest was due to demand for chemical free and safe agricultural commodities (Zhou *et al.,* 2014). This techniques are being practiced for about 100 years now using exotic natural enemies to control severe pest, disease and weed problems (Van Lenteren *et al.,* 2003). However, high cost of mass rearing of aphid parasitoids remained a constraint (Boivin *et al.,* 2012). There are limited reports available on negative impacts on environment and health compare to chemical solution. Conservative biological control are most eco-friendly and stable pest control technique which can avoid pesticide use (Boivin *et al.,* 2012).

The wasp parasitoid, *Aphelinus mali* (Hymenoptera: Aphelinidae) is native of USA which was introduced to western USA, Europe and other apple producing countries. It is a host specific endoparasitoid (Zhou *et al.,* 2014) mainly used for controlling woolly aphid in apples (CABI, 2018). Gontijo *et al*. (2015) mentioned that maintaining diversity of predators and parasitoids enhanced effectiveness in regulating WA. However, as reported by Beltrán *et al.* (2017) there lacked parasitism by parasitoid, *A. mali* under broad-spectrum insecticide applications and declined in the number of predators. Ateyyat and Al-Antary (2010) found parasitism by *A. mali* could be influenced by heavy applications of pesticides impeding the parasitoid densities. Parasitism was evident from late June-mid September with peak parasitism in late July (Bergh and Stallings, 2016).

A. mali is a specialist of WA (Gontijo *et al.,* 2013) and most predominant natural enemies for effective control of the above ground trees (Zhou *et al.,* 2014). Specialist *H. calcarata* (Diptera: Syrphidae) native of US (Gresham *et al.,* 2013) reported to attack WA colonies in roots (Gontijol *et al.,* 2012). Laboratory study confirmed that *A. mali* parasitized all stages of WA however, preferred the 3rd instar nymphs (CABI, 2018). Nevertheless, Lordan *et al*. (2014) mentioned that control of WA in the aerial part of host plants by *A. mali* is arguable. It is reported to have ineffective suppression of WA by *A. mali* as it occurred in late season and not able to control initial infestation (Stokwe and Malan, 2016).

The effectiveness of parasitoid *A. mali* diminished in cold climatic condition (Stokwe and Malan, 2016; Wearing *et al.*, 2010). Bergh and Short (2008) also reported that species of hoverflies like *Heringia clacarata, Eupeodes americanus*, and *Syrphus rectus* abetted in regulating WA population. Gresham *et al.* (2013) found *H. calcarata* species greatly aided in the biological control of WA infestation. Zhou *et al.* (2014) studied technique of conserving overwinter aphid mummies with management of hay mulch to maintain adequate parasitoid densities in early season for managing WA.

According to (Bergh and Stallings, 2016), *H. calcarata* and *A. mali* are the main natural enemies of WA, and former known to have more impact on aphid colonies. The parasitism level reduced with increased in colony size as parasitoid may have difficulties in reaching core of the colonies (Stokwe and Malan, 2016). It is advantageous when generalist and specialist predators are both present for effective WA management due to their diverse feeding habit and occurrence at different time

Gontijol *et al.* (2012). Furthermore, maintaining several group of natural enemies, both predators and parasitoids is an effective means for WA control and other aphid species (Gontijo *et al.,* 2015). The acceptable level of WA suppression is problematic when adverse climatic aspects prevailed for development of natural enemies (Nicholas *et al.,* 2005). Gresham *et al.* (2013) recommended more studies on reproductive biology and ecology of *H. calcarata*. The crucial role of predators was acknowledged lately for WA population control (Bergh and Stallings, 2016).

In India, success of 98% WA management by *A. mali* was recorded. It was supplemented by release of predators like the lacewing *Brinckochrysa scelestes* (Neuroptera: Chrysopidae) and the hover fly *Eupeodes confrater* (Diptera: Syrphidae) in Australia and New Zealand. The popular wasp parasitoid, *A. mali* can be adopted for biological agent to control WA however, it is not reported in Bhutan (NPPC, 2017). In New Zealand, reported success of 80% suppression with *A. mali* by averting aphidicide. In the Netherlands, beside *A. mali*, the most common predatory coccinellid, *Exochomus quadripustulatus* (Coleoptera: Coccinellidae) support WA suppression in apple orchards (CABI, 2018). Study found that there was reduction or complete demolition of aphid population when accessed to both parasitoid and predators (Gontijo *et al.,* 2015). Conversely, the population of WA increased when natural enemies are not present (Bergh and Stallings, 2016). Pesticides are highly toxic to parasitoids, thus use of more bio-pesticides are alternatives to control aphid pest (Zhou *et al.,* 2014).

In Europe, supplementary bio-control of WA is earwig, *Forficula auricularia* (Dermaptera: Forficulidae). The generalist predator, earwigs are nocturnal (Gontijo *et al.,* 2013) and overwinter as adult in underground in late autumn. They undergo 5-6 generations during apple growing period and their abundance varies in different orchards and years. Earwigs dwelled in fruit bunches and on canopies during daytime. Several known literature mentioned different level of WA control by European earwigs (Quarrell *et al.,* 2017). Earwigs are efficient predators of woolly aphid (Gontijo *et al.,* 2013). WA infestations are severe when earwig populations are low (Quarrell *et al.,* 2017). Nicholas *et al.* (2005) mentioned earwigs as main agent in WA control which has the capacity to ingest 106 aphids per day. Earwigs are potential for WA management as they dwell in orchards regardless of presence or absence of WA in the orchards (Nicholas *et al.,* 2005).

Lordan *et al*. (2014b); Gontijol *et al.* (2012) mentioned regarded list of useful predators for controlling WA, like ladybird beetles, lacewings, hoverflies, earwigs and spiders (Araneae) and amongst, earwigs are most vital predator. Wearing *et al*. (2010) also reported some predators such as *Micromus tasmaniae* (Walker), *Sejanus albisignata* (Knight), *forficula auricularia* thought to support *A. mali* to control *E. lanigerum* although their impact remained unclear. Hoverflies are active during sunny and warm weather. Small and isolated WA colonies are observed when eggs or larvae of syrphid predators are present (Bergh and Short, 2008). Bergh and Stallings (2016) mentioned lacewings associations with woolly aphid colonies. They studied approaches to maintain diversity of natural enemies by managing crops and grasses in the orchards (Zhou *et al.,* 2014).

Their study uncovered impact of WA control was higher when both *A. mali* and predators are available and the natural enemy guild may differ in all apple growing areas (Bergh and Stallings, 2016). Regarded list of predators like ladybird, Neuroptera and Syrphidae and their abundance varies in orchards, between years and management practice (Quarrell *et al.,* 2017).

Conservation biological control has advantage in creating stable ecosystem suitable for the local orchard management (Zhou *et al.,* 2014). Limited documentation of natural coccinellid despite their massive role in pest management program (Obrycki and Kring, 1998). There are numerous predators and natural enemies that bolster WA suppression but admitted minimal merits on their usefulness (Bergh and Stallings, 2016). Aphid population monitoring are indispensable to determine necessary intervention for management (Pringle and Heunis, 2008). They suggested more study on disruptive and complementary effects of predators and parasitoid (Gontijo *et al.,* 2015).

2.6.2 Host-plant resistance

Utilization of resistant varieties and rootstock are significant techniques for control of WA infestation (Abu-romman and Ateyyat, 2014). Northern Spry and Golden Delicious cultivars showed some extent of resistance to WA. Malling Merton (MM) series of rootstocks derived from northern Spry are ideally selected. These rootstocks are popularly used in pest control as they significantly lower the infestation levels although there are no complete suppression. Abu-romman and Ateyyat (2014) also described list of resistant varieties such as Golden delicious, Delbarestivale, Golden smoothie, Red miracle and Harmony. They studied, WA infestation extremely varied among apple varieties (Nicholas *et al.,* 2005).

(CABI, 2018) reported WA management technique with the use of resistant cultivars. Thirty cultivars and eight *Malus sp.* breeding lines tested to WA resistance in which *Malus baccata* (Jin 67) was identified promising. It demonstrated less root damage, winter hardy and induced dwarfism in apple crosses (CABI, 2018).

In New Zealand, M.793 and MM.106 are resistant apple rootstocks used to suppress WA (Wearing *et al.,* 2010). Their priority remained for development of apple varieties and rootstocks of genetic resistance for WA (Alspach and Bus, 1999). In Jordan, Ateyyat and Al-Antary (2009) recommended Harmony cultivar rootstock owing their highly resistance feature against WA. They also reported aphid infestations are not similar in all varieties of apple. Ateyyat and Al-Antary (2010) found WA infestation varied between cultivars as Fuji sustained more injury than Golden Delicious.

2.6.3 Integrated pest management (IPM)

Countries like Australia, New Zealand, Romania and south Africa has recognized advantage of adopting integrated pest management (IPM) programmes. It is known that less use of broad-spectrum pesticides improved the diversity of predators and parasitoids, and high population of natural enemies especially *A. mali* are used in IPM. Wearing *et al.* (2010) reported significant increase of natural enemies in their studies shifting from conventional to integrated fruit production (IFP). According to Stokwe and Malan (2016), principal agent of IPM for WA management in apple is parasitoid, *A. mali*. In New Zealand, application of mineral oil, or oil plus buprofenzino in IPM programme (CABI, 2018). The need for IPM to control pests ensued as result of shifting to organic agriculture where chemical use are restricted (Abu-romman and Ateyyat, 2014). More studies are advisable in the field of integrated fruit production (IFP) programme to curbed pesticide residue concern in food and also for sustainable production techniques (Shaw and Wallis, 2008).

Careful selection of pesticides and natural enemies are crucial to maintain minimal impact on natural enemies in integrated fruit production (Rogers *et al.,* 2011). Use of pesticides has impact on coccinellid population affecting fecundity and longevity, however they reappear quickly in the field after insecticide application if accessible to adequate prey (Obrycki and Kring, 1998). Reported low pest and high diversity of arthropods in integrated pest management orchards (Quarrell *et al.,* 2017). Nicholas *et al.* (2005) mentioned low level of WA infestations under IPM orchards due to elevated numbers of natural enemies. Aphids are constantly under threat during day and night when all natural enemy group prevailed (Gontijo *et al.,* 2015).

To obtain successful management of aphid, it is imperative to understand the population density of parasitoid to avoid pesticides use ensuing negative impacts

(Rocca and Greco, 2012). Plantation of alyssum flowers in the vicinity of trees might increase natural enemies of WA and improve biological control measures (Gontijo *et al.,* 2013). It is vital to gather accurate data and recordings in ecological studies for pest management purpose (Verghese and Jayanthi, 2002). Rogers *et al.* (2011) in their study suggested to use alternative crop thinners like carbaryl which are less disruptive to *A. mali* for sustainable control of WA. (Ateyyat and Al-Antary, 2010) suggested mass rearing and releasing of parasitoid to control WA as low abundance of *A. mali* has low parasitism level.

2.6.4 Entomopathogen nematode and fungi

Use of entomopathogenic nematodes (EPN) against WA control were reported ineffective but investigated to assess potential management in root infestations (CABI, 2018). Stokwe and Malan (2016) discussed and provided potential techniques of managing underground woolly aphid colonies with application of entomopathogenic nematode (EPN) and entomopathogenic fungi (EPF) as they also mentioned that enormous studies has been done for control of aerial WA infestation but not on roots. EPN and EPF are also regarded as natural enemies of soilborne insects for biological control measures. About 100 species of EPNs described across globe and 11 are commercialized. EPN have varying level of efficiency and thus biocontrol rely on species, host insects and environmental aspects (Stokwe and Malan, 2016). Nematode, *Steinernema carpocapsae* are reported to attack woolly aphid infestations.

Likewise about 200 species of EPF are pathogens of insect and mites and genera, Hypocreales (*Beauveria* and *Metarhizium*) used in biological control. These can be used in classical biological control, augmentation or conservation. Some of the commercially available EPF; *Beauveria, Metarhizium, Lecanicillium and Isaria.*

Suggested, feasibility study for management of WA with available commercial EPF (Stokwe and Malan, 2016).

2.6.5 Chemical Control

Chemical spray of chlorpyrifos and dimethoate are recommended when WA infestation reach unacceptable level (NPPC, 2017). Chemical control of woolly aphid are inadequate due to their white waxy filament characteristics, as such systemic insecticide, Vamidothion has been suggested although toxic to natural enemies (Monteiro *et al.,* 2004) as short residue chemicals does not provide efficient control (Stokwe and Malan, 2016). Moreover, Stokwe and Malan (2016) reported pesticides spray are sensitive to natural enemies and disrupted biological control. Furthermore, Wearing *et al.* (2010) described natural enemies of WA are prone to be killed by organophosphate insecticides.

WA are controlled with yellow mineral oil to kill hibernating aphids followed by Chlorpyrifos or demeton-methy spray in the growing season after flowering so as predators are undestroyed. Woolly aphid are difficult to control with contact pesticides (Bergh and Short, 2008). Diazinon and Chloropyrifos provided effective control but has toxic effects to parasitoid, *A. mali*. Pirimicarb are effective against WA without negative bearing on *A. mali* however, control of infestations on cankers or pruning wounds are ineffective. Nicholas et al. (2005a) reported growth of larvae in mummies/adults are not affected by fenoxycarb spray. Fenitrothion root-dip in nurseries is effective control measure. Treatment of subterranean population are with granules of dimethoate. Apple tree trunk banded with dimethoate checked the movement of 1st instar nymphs between aerial and subterranean habitats (CABI, 2018b). Soil application done with imidacloprid to manage WA in South Africa (Stokwe and Malan, 2016).

Chapter 3
Materials and Methods

3.1 Study method

3.1.1 Location

The field investigation of woolly aphid (WA) infestation and associated natural enemies was conducted from April-October 2018 (about 28 weeks). Two well-established apple orchards under Thimphu valley, Bhutan were randomly identified and evaluated over the growing season. The orchards were located in two different geographical locations approximately 47 kilometers apart (Fig.1). Orchard were assigned as Hongtsho-2 (N 27° 29' 0.4416") (E 89° 43' 46.6788") and Kausumche-3 (N 27° 19' 0.0876") (E 89° 33' 24.0876") respectively.

In both the orchards, mean temperature was recorded between Min-11.34°C to Max-23.20°C and received cumulative rainfall of 377.9 mm (March-October 2018) during the apple growing season. Data of daily climatic variables of maximum and minimum temperature (Degree Celsius) and rainfall (millimeter) was accessed from National Center for Hydrology and Meteorology, Thimphu, Bhutan. Orchard Hongtsho-2, is located at an altitude of 8880 ft. slightly higher elevation than orchard Kausumche-3 at 7096 ft.

3.1.2 Characteristics of orchard

Four apple orchards initially selected for assessment based on the history of aphid infestation in the previous cropping season with the support of agriculture extension personnel of the Gewog (Fig.2). However, two orchards were avoided for survey as season progressed, the aphid colonies are not distinctive for enumeration. The orchards comprised common apple variety, Royal Delicious (RD). In both the orchards, trees ranged between 5-40 years old plantations and followed line system with at least three meters between trees. Aphid colonies were found scattered in canopy of sampled trees. Orchard consist of 150 trees in Hongtsho-2 and 140 trees in orchard Kausumche-3.

Over the growing season, potato and chilli were cultivated as under crop in Hongtsho-2 and chilli in Kausumche-3 respectively. Farmers has been practicing such conventional agriculture production system for years. The orchards were under rain-fed irrigation management practices without any proper irrigation amenities. Both the orchards have record of pesticides application in the previous season with Carbendazim, TSO and Borax however, during the study period chemical spray schedule was absent. Both the orchards have timely weed management during the apple season. The orchards were evaluated for WA infestation and population fluctuations, parasitism and predation levels and diversity of potential natural enemies.

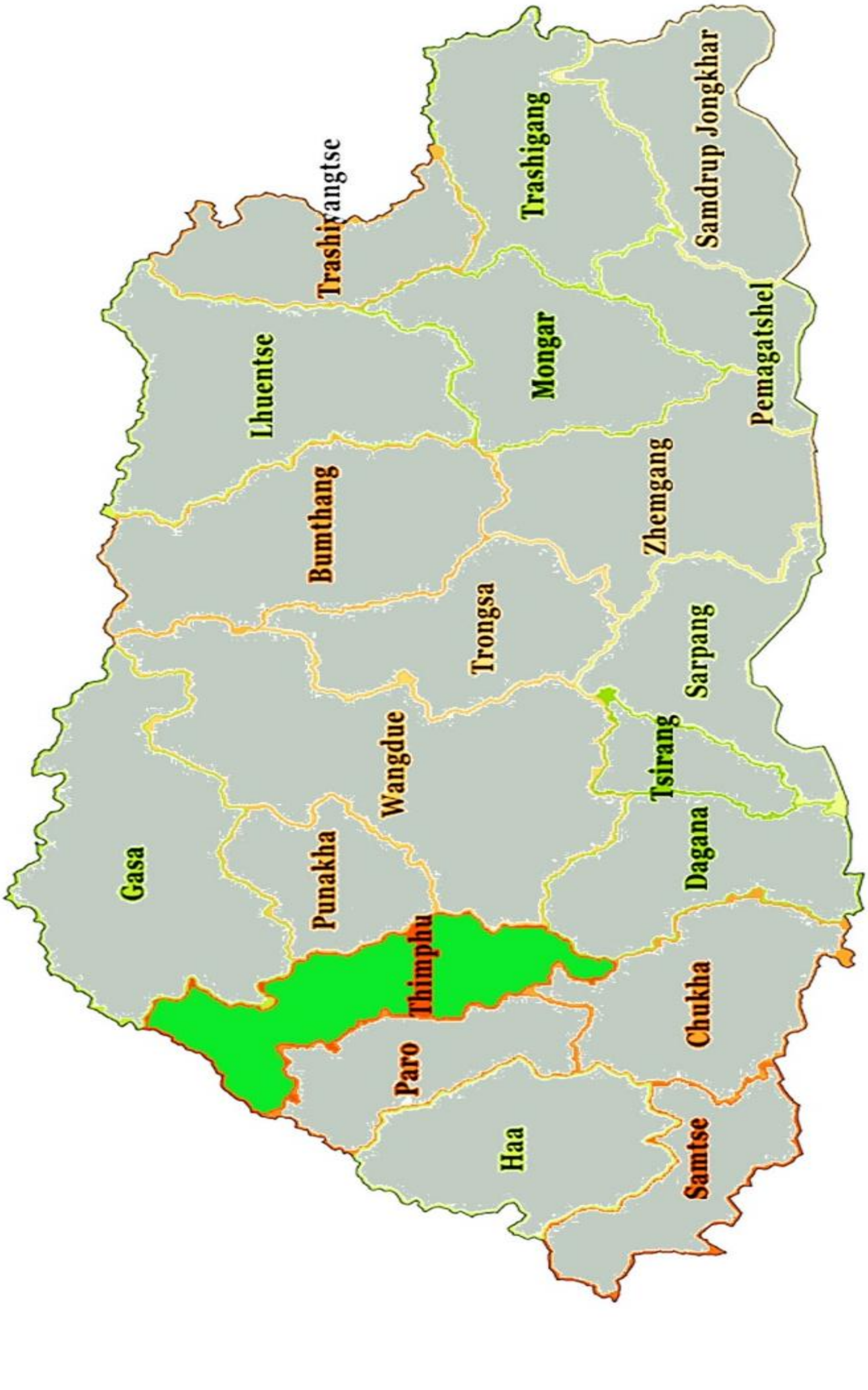

Figure 1 Survey site of woolly aphid (WA) and natural enemies from April-October, 2018 in Thimphu Valley, Bhutan.

Orchard Hongtsho-1

Orchard Hongtsho-2

Orchard Kausumche-3

Orchard Kausumche-4

Figure 2 Four orchards identified initially for survey of Woolly aphid (WA) from April-October, 2018

3.2 Population densities of woolly aphid

The aerial population of WA were assessed from April-October 2018 over the apple growing season. Selected five infested apple trees from each orchard using simple random sampling. Four infested terminal shoots (approximately 10cm) were sampled from four directions (shown in Fig.3) each tree reachable from the ground for aphid assessment. To facilitate insects count, the white filaments were rinsed with clean tap water. Colonies were visually examined bi-weekly and enumerated *in situ*.

Live aphid of all developmental stage were counted with the help of hand lens (10x) and insect counter by non-destructive means. The plant samples along with aphid colonies were collected for further observations. The methods were followed similar to Krebs (1989); Pringle and Heunis (2008); Gontijol, L. M. *et al.* (2012); and Lordan *et al*. (2014b).

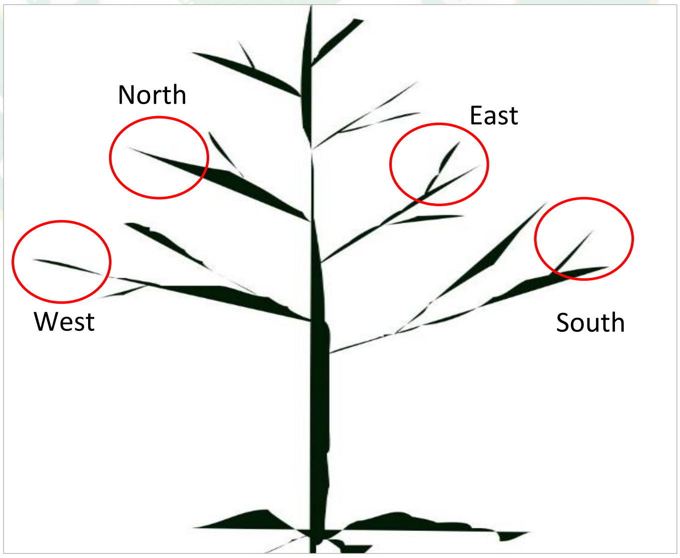

Figure 3 Schematic diagram of woolly aphid sampling on terminal shoots about 10cm from four directions during growing season from April-October 2018

3.3 Diversity of natural enemies associated with woolly aphid

Diversity of natural enemies associated with WA was investigated simultaneously while surveying aphid population. Bi-weekly inspection and enumeration of natural enemies were conducted on infested terminal shoots. Larva, predators and natural enemies feeding on WA colonies and vicinity were recorded. The canopy of the sampled trees were monitored and searched for adult predaceous insects associated with WA during the day between 9.00am to 4.00pm. The survey of flying beneficial insects in the designated orchard was monitored with sweep net and no record of trap every time the survey done. The composition and relative abundance of natural enemy diversity were measured with Shannon diversity index equation;

$$H' = -\sum p_i \, 1n \, p_i$$

During the sampling, adult predators including coccinellids, syrphids and lacewings were collected in vial containing 70% ethyl alcohol and brought to the laboratory. Adult insects were processed and pinned and preserved in insect box. Similarly, infested plant samples with aphid, black mummies and larva were collected in perforated plastic bags for rearing of parasitoids and adults. The emerged parasitoids and insects were preserved in 70% ethyl alcohol and later pinned for further observations. The insect specimens were transferred to the laboratory at Maejo University, Chiangmai and identified to species as appropriate. Collection, processing technique and method was similar to those followed by Aslan and Karaca (2005); Beltrán *et al.* (2017); Gontijol, L. M. *et al.* (2012).

3.4 Woolly aphid (WA) parasitism by *Aphelinus mali*

Potential parasitization of WA by parasitoid, *Aphelinus mali*, was assessed at the same time while monitoring the aphid population. Inspected and recorded black mummies and parasitized aphids (both intact and exited) bi-weekly. Parasitized aphid, black mummies and larvae samples were collected and reared. The emerged parasitoids were placed into the Eppendorf tubes containing 70% ethyl alcohol and preserved for identification as described similar to Beltrán *et al.* (2017). Insect specimens were transferred to Maejo University, Chiangmai, Thailand for identification and species confirmation. The insect specimens are deposited at NPPC, Semtokha, Thimphu for future references.

3.5 Data analysis

Data were computed for monthly comparison of the averages of woolly aphid abundance in the orchards and its parasitism by natural enemy parasitoid, *Aphelinus mali* over the season. All the data were log-transformed (*Log*10) before statistical analysis to achieve assumptions for normality test. The level of woolly aphid infestation and parasitism in two orchards were statistically analyzed with an independent sample t-test and determined an extremely significant difference in aphid population densities over the survey period. The diversity of natural enemies comparison in two orchards was determined by Shannon diversity index equation as mentioned above. All statistical comparisons were considered significant at $P<0.05$. Data were evaluated by Statistical Package of Social Sciences (SPSS) Version 22.

Chapter 4
Results and discussion

4.1 Population densities of woolly aphid

The apple orchards under study were visually monitored commencing 19 March 2018 for any signs of woolly aphid infestations. Trees were at dormant stage during the preliminary visit (Fig.2) and an overwinter aphid lesions are distinctively prevalent on tree canopies and root zones. White waxy and cottony powdery WA lesion was evident on twigs and branches, pruning cuts, and on remnants of plant parts that are pruned off in the fall. Evidence of galls formation and desiccation of plant parts were apparent on wherever heavy infestation existed. These observations are in congruent with Asante (1994) where both the roots and vegetative part of trees are damaged as a consequent of WA colonies formation.

Infested plant parts and debris that were clipped off during the pruning schedule were found scattered and unattended in and adjacent to the orchards. Such an inappropriate weed management practice and poor orchard sanitation might have served as habitat of overwintering aphids and re-emerged in spring (March) although evaluation was lacking. Thus, aphid re-infestation are thought to be from the overwintered infested plant debris, root zones and canopies which was in consistent with the reports of Lordan *et al.* (2014).

During the preliminary field inspection on 31 March 2018, in orchard Hongtsho-2, trees still remained at dormant stage without expression of any sign of bud break and fresh leaves. The initiation of aphid infestation was apparent on twigs and terminal buds however, the population were under progression and not prominent enough for enumeration. Meanwhile, the soils were found well-prepared and set for cultivating vegetable crops in-between trees in the coming summer. The orchards remained under rain-fed condition without any proper irrigation management facilities. Annual pruning schedule was followed.

On the same sampling day, flush of new leaves appeared on the trees and proper weed management and sanitation was visible in the orchard Kausumche-3. The variations of seasonal developmental stage of apple trees observed in two orchards could be due to different geographical locations. Orchard Hongtsho-2 and Kausumche-3 are situated at elevation of 8880 ft. and 7096 ft. respectively.

Woolly aphid density was evaluated over the growing season from April-October until fruit harvest and attempted to determine its population abundance and fluctuations. Aphid colonies were monitored from mid-March although there was no formation of distinct colonies hindering the count of insects. According to NPPC (2017) WA colonies commenced its emergence from March-April indicating appropriate commencement of this survey from April. WA colonies are rampant in the orchards round the season however, as mentioned by Ateyyat and Al-Antary (2010) that aphid population could be affected by severe low temperature. From the study, initial formation of WA colonies was first encountered on 14 April 2018 in orchard Kausumche-3 during the first sampling day. However, the aphid are speculated to emerged much earlier in the season (early March) before their colonization in April (Fig.4).

The previous studies reported that reproduction of overwintered aphids begin from March-April (CABI, 2018) which is in congruent with the finding as aphids were evaluated from mid-April. Nonetheless, WA colonies was recorded only two weeks later on 1 May 2018 in orchard Hongtsho-2. There was variations in temporal occurrence of WA colonies in two orchards supporting Asante (1994) that the reproductive life stages of WA is dependent on season. According to Gontijo *et al.* (2013) the densities of WA attained peak soon after buds outbreak.

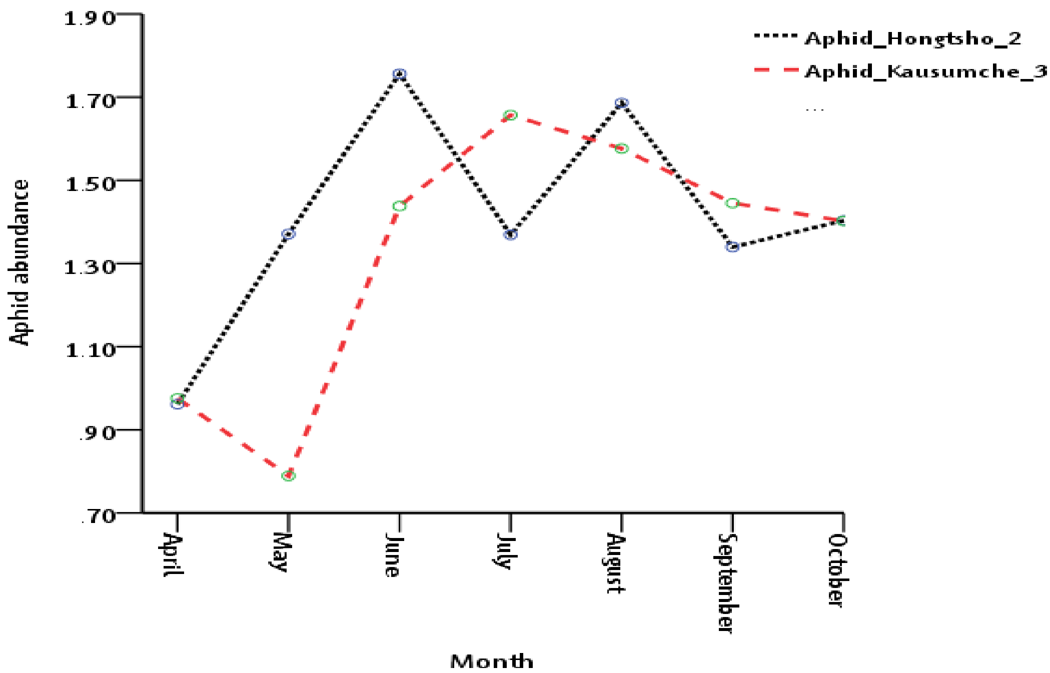

Figure 4 Mean comparison of woolly aphid abundance over the season (April-Oct. 2018) in two orchards

This pattern of WA abundance could be due to influence of the different environmental aspects in the two orchards as Beltrán *et al.* (2017) highlighting precipitation, climate and temperature might have influenced WA colonies. Pringle and Heunis (2008) also supported the study that WA emergence varied with orchards and season. Further, abetted by Lordan *et al.* (2014) that ecology of WA can be drastically influenced by climatic factor. Cultural management and chemical spray might have also influenced the WA populations in two orchards. (Stokwe and Malan, 2016) reported pesticides spray are sensitive to natural enemies and disrupt biological control.

Fresh flush of leaves and flowers are already blooming during the monitoring day on 1 May 2018 and assumed to provide food reserves for aphid development (Fig.5). Over the growing season, aphid infestations are found on the branches, twigs, shoots, leaf axils and spurs. Usually WA feed and multiply on tender parts of tree according to Verghese and Jayanthi (2002).

Figure 5 Overwinter woolly aphid lesions on trees in orchard Hongtsho-2 during monitoring survey on 31 March 2018

Figure 6 Woolly aphid colonies in the orchard Hongtsho-2 on 1 May 2018 survey

Similar to the temporal variations of aphid emergence, tree growth and developmental stages also differed in two orchards. Fruits attained marble size on 14 May 2018 in orchard Kausumche-3 and whereas same size was observed only on 30 May 2018 in Hongtsho-2. In general, leaves turned yellow and defoliated in the vicinity where heavy WA infestation occurred and presumed to cause injury fortunately, WA are not known to be important vector of apple virus diseases (CABI, 2018). In both the orchards, fruits were already harvested by end of September when WA colonies are predominant in the orchards. According to orchard owners, current yields are very low compared to previous season and WA infestations was regarded as the main cause of poor production (Kelzang Dolkar, 16.10.2018, personal communication).

Over the growing season, data pooled for analysis of an average monthly aphid abundance. Monthly mean aphid densities (Mean±SEM) per sample unit ranged between 9.15±0.97-57.00±4.24, and 6.15±0.58-45.35±3.09, in orchard Hongtsho-2 and Kausumche-3 respectively (Table.1). The comparison of mean aphid abundance in two orchards at different geographical locations varied. According to Pringle and Heunis (2008) aphid emergence vary in different orchards and season and also its infestation level varied with varieties (Ateyyat and Al-Antary, 2010) although, Royal Delicious (RD) was considered in the study. Cumulative average aphid density compared at (Mean±SE) Error bar: 95% Confidence Interval showed significant difference in WA infestation in two orchards evaluated over the apple season (Fig.7). The attributed reason for variations of aphid infestations in two orchards are thought to be influenced by biotic and abiotic factors which required further research.

Table 1 Average monthly (Mean ± SEM) of aphid population abundance in two orchards

Month (week)	Hongtsho-2 (Mean±SEM)	Kausumche-3 (Mean±SEM)
April (2-4)	9.15±0.97	9.45±0.99
May (6-8)	23.48±2.47	6.15±0.58
June (10-12)	57.00±4.24	27.40±2.44
July (14-16)	23.38±1.85	45.35±3.09
August (18-20)	48.55±3.20	37.68±2.78
September (22-24)	21.85±1.54	27.85±1.78
October (26-28)	25.30±1.29	25.25±2.00

Following the initial occurrence of WA colonies in orchard Hongtsho-2, woolly aphid density rapidly progressed. The aphid density remained relatively higher between June-August during the monitoring period as illustrated in figure 4. The peak of aphid population count (1481 numbers) was recorded in June. Aphid population suddenly dwindled from July however, population resurged and experienced another peak (1204 numbers) in August (Fig.4). The aphid infestation trend displayed two peak of aphid counts in the season which was identical to the bimodal peak of WA population reported by Beers *et al.* (2010; Lordan *et al.* (2014).

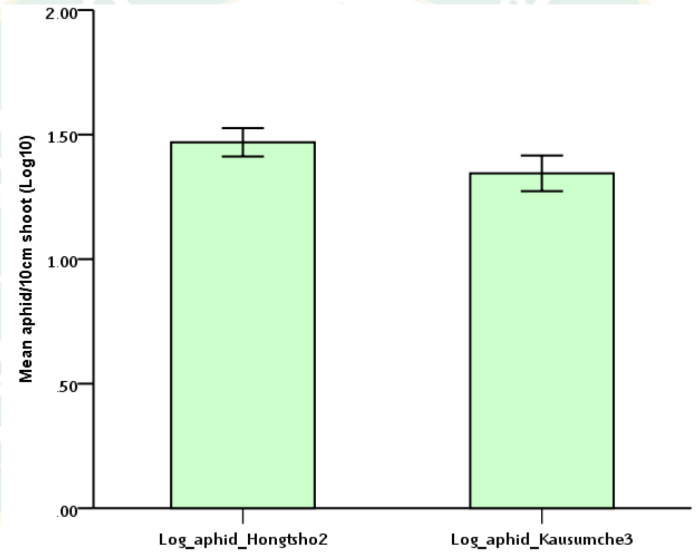

Figure 7 Density of cumulative mean aphid abundance compared at (Error Bars: 95%CI) in two orchards (April-October) over the apple season

In orchard Kasumche-3, first occurrence of aphid colonies was recorded on 14 April 2018, despite exhibiting single peak of aphid density over the entire season. The highest aphid counts (1028 numbers) was recorded in July, thereafter population plummeted erratically and remained more or less constant till end of sampling period in October (Fig.4). The observation of maximum level of aphid abundance in the growing season was presumed to attributions like abundance of tender plant resources and favorable environmental conditions which required further corroboration. Meanwhile, root infestation and rampant galls formation were noticeable on the exposed roots throughout the survey.

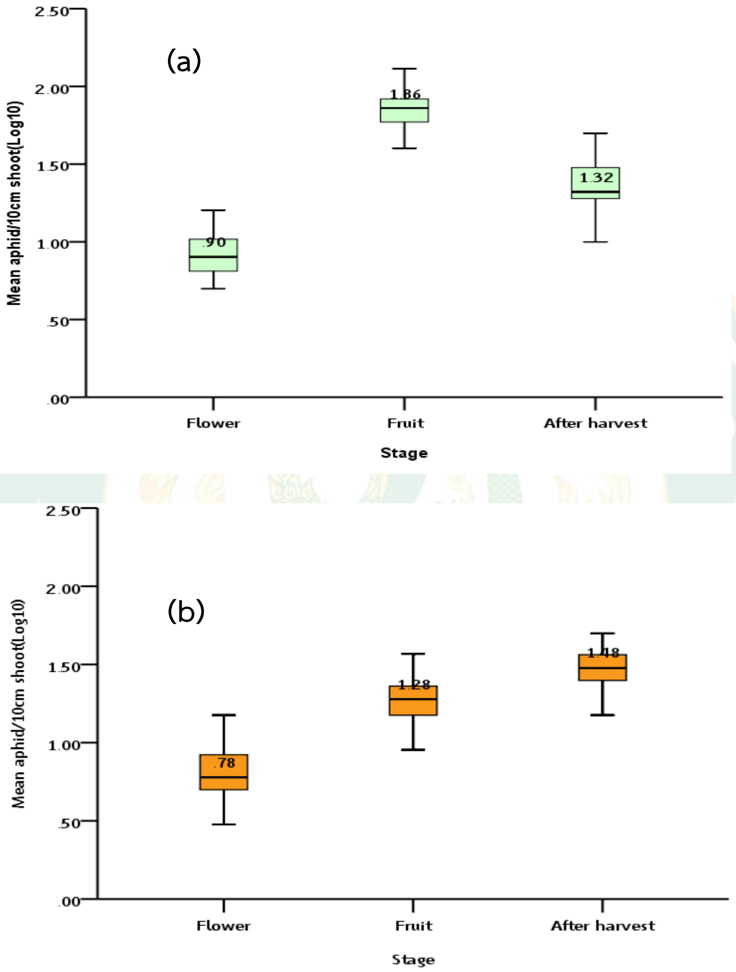

Figure 8 Boxplot distribution of aphid population at different stages in orchard a) Hongtsho2, and b) Kausumche3 from April-October 2018

Aphid densities examined at various phase of plant developmental stage, at flower stage, fruit stage and after harvest over the season. At the end of season, boxplot aphid distribution showed significant difference in aphid abundance at different stages within and between two orchards. In orchard Hongtsho-2, aphid density was observed higher during the fruit development stage whereas aphid density was relatively higher at end of season after harvest in orchard Kausumche-3 (Fig.8).

Generally, the health of apple trees deteriorated with yellowing of leaves and bearing fewer fruits. The orchard owners reported that current infestation level was higher and greatly impacted the yield compared to previous year. Actual impact and causality of chemical residues on WA population need additional study to validate. Gontijol *et al.* (2012) mentioned frequent outbreak of WA are due to change in pesticide program and disruption of biological control. Apple orchards has record of chemicals spray in the previous season.

There was substantial evidence that damages were perhaps result of WA galls injury which deterred the water conduction capacity of roots affecting shoot growth (Ateyyat and Al-Antary, 2009) and disrupts sap flow as a consequent of roots and limbs break (Quarrell *et al.,* 2017). Roots infestation caused major injury and difficult to control as augmented by Ateyyat and Al-Antary (2010).

An independent sample t-test statistically analyzed the cumulative mean population of aphid infestation in two orchards. A total of 533 randomly sampled infested shoots were analyzed. An unpaired t-test revealed orchard Hongtsho-2 (M=1.40, SD= 0.30) WA abundance in two orchards are found extremely statistically significant than orchard Kausumche-3 (M=1.27, SD= 0.38) over apple growing season, t=4.3410, ***P*=0.0001.** As mentioned earlier, aphid population level are presumed to be different due to various environmental factors. The orchards are located at two different geographical areas and elevations. Orchard Hongtsho-2 is situated at higher elevation and the cooler temperature might have discourage aphid growth which deemed more studies.

4.2 Diversity of natural enemies associated with woolly aphid

4.2.1 Diversity of natural enemies

The assessment for diversity and richness of potential natural enemies associated with WA was conducted simultaneously while monitoring aphid population. The study revealed seven species of insect natural enemies including one parasitic insects, *Aphelinus mali* (Hymenoptera:Aphelinidae); and six predaceous insects, including three coccinellids, *Coccinella transversoguttata, Harmonia sedecimnotata* and *Harmonia dimidiata* (Coleoptera:Coccinellidae); two syrphids, *Syrphus opinator* and *Heringia calcarata* (Diptera: Syrphidae); and a green lacewings, *Chrysoperla* sp. (carnea group) (Neuroptera:Chrysopidae) found associated with WA.

Table 2 Natural enemy presence (Yes/No) in apple host of two orchards associated with woolly aphid from April-October 2018

	Orchard	
Natural enemy	Hongtsho-2	Kausumche-3
Parasitoid (*A.mali*)	Yes	Yes
Coccinellid	Yes	Yes
Syrphid	Yes	Yes
Lacewing	No	Yes

Among these natural enemies, the only endo-parasitoid *A. mali* was predominantly abundant in the orchards found parasitizing woolly aphid colonies. It is native to USA and has widespread use in biological control of woolly aphid in apples (CABI, 2018). The presence of wasp parasitoid has been confirmed in the orchards of Thimphu valley which was never reported earlier. The study also recorded several potential predators and natural enemies associated with WA listed above which can greatly benefit as biological control agent (shown in Table 2) although lacewings was not recorded in orchard Hongtsho-2.

Apart from the presence of *A. mali*, the study recorded six predaceous insects comprising coccinellids, syrphids and lacewings which are useful insects that may have potential impact on suppression of WA in complementary with parasitoid. Gontijo *et al.* (2015) reported that maintaining diversity of predators and parasitoids are effective technique in regulating WA. Study suggest more assessment need on predaceous syrphid, *H. calcarata* and *A. mali* which are the key natural enemies responsible for regulating WA as the former has greater impact on aphid colonies (Bergh and Stallings, 2016). Specialist *H. calcarata* (Loew) (Diptera: Syrphidae) is native of US and it greatly underscored in the biological control of WA suppression (Gresham *et al.*, 2013).

Furthermore, diversity of two orchards computed with Shannon diversity equation showed species diversity indice of 0.083 in orchard Hongtsho-2, and 0.055 in Kausumche-3. It demonstrated that the diversity of species are higher in orchard Hongtsho-2 considering abudance and eveness of insect species in the community. Likewise, known literature by Lordan *et al.* (2014) enlisted some useful predators like ladybird beetles, lacewings and hoverflies used for WA management. Earwig, *Forficula auricularia* has also been recommended for supplementary biocontrol of WA (CABI, 2018) and are efficient predators of WA (Gontijo *et al.,* 2013).

They are potential biocontrol agent occurred in orchards regardless of WA presence. Bergh and Stallings (2016) gave account of several predators and natural enemies which can help reduce WA infestation but lacked adequate merits on their vital responsibilities. Limited information are available in Bhutan in regards to these natural enemies and the study established baseline for advanced research for natural biological control of WA.

Further investigation are worthwhile to explore additional predatory insects such as earwigs which are useful in WA management. In Europe, the supplementary biological control for WA is the earwig, *Forficula auricularia* (Dermaptera: Forticulidae). Earwigs are efficient nocturnal predators of woolly aphid (Gontijo *et al.,* 2013). During the survey, evidence of earwig was not recorded as it being nocturnal and sampling was carried out during the day time.

4.2.2 Abundance of natural enemies

During the investigation, presence of coccinellids, syrphids and lacewings are some of the natural enemies encountered in the apple orchards. *Apheinus mali* was the predominant parasitoid identified parasitizing woolly aphid colonies. As discussed earlier, there were other predaceous insects contributing to the suppression of WA in complementary interaction with the parasitoid *A. mali*. Gontijo *et al.* (2015) emphasized to maintaining diversity of predators and parasitoids considered effective technique in regulating WA. To reiterate, the study uncovered potential predators and natural enemies in the apple orchards of Thimphu valley such as ladybird beetles, hoverflies and lacewing.

Beside parasitism by *A. mali,* predaceous natural enemies such as ladybird beetles and syrphid was presumed to contributing WA population management. The mean density of natural enemies recorded bi-weekly in the orchards Hongtsho-2 (parasitoid 132.87±32.74, Ladybird 1.50±0.50, Syrphid 3±1.68), and (parasitoid 279.46±49.36, ladybird 3.8±0.97, syrphids 2.2±0.58) respectively over the apple season (data not shown). Ants and spiders (Araneae) were also spotted hovering in the vicinity of aphid colonies. Further evaluation is suggested to determine the level of parasitism and other natural enemies effective to maintain WA below threshold level.

Insect predators such as ladybird beetles, syrphids and lacewing are some of the natural enemies of WA encountered in the orchards. (Gontijol *et al.,* 2012) mentioned Syrphidae, Neuropterea and Coccinellidae are the groups of predators associated in WA colonies. The initial occurrence of predatory insects (coccinellids, syrphids) was recorded from June (Fig.9). Total count of coccinellids and syrphid flies was found between 22 and 23 numbers over the season. The occurrence of natural enemies varied with time although, there were no numerical difference in two orchards surveyed.

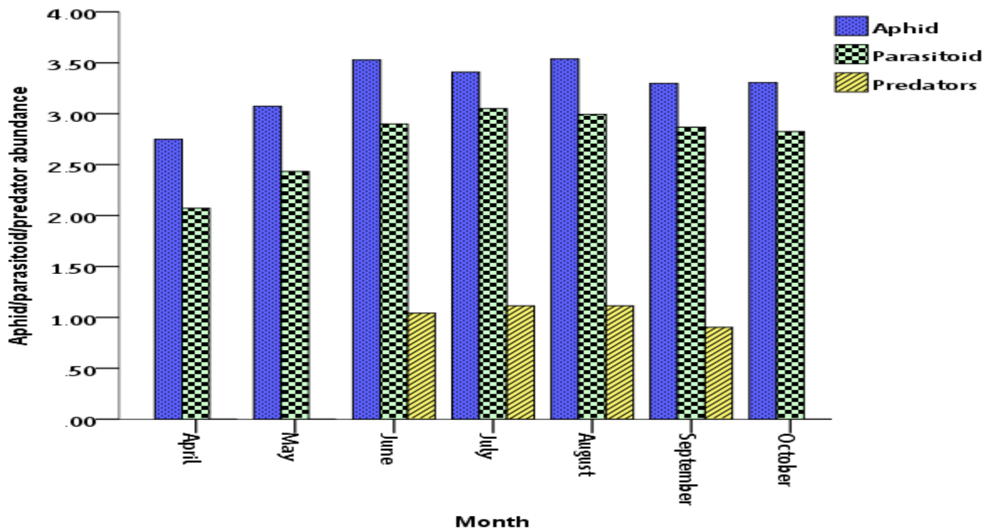

Figure 9 Mean monthly composition and distribution of natural enemies in two orchards from April-October

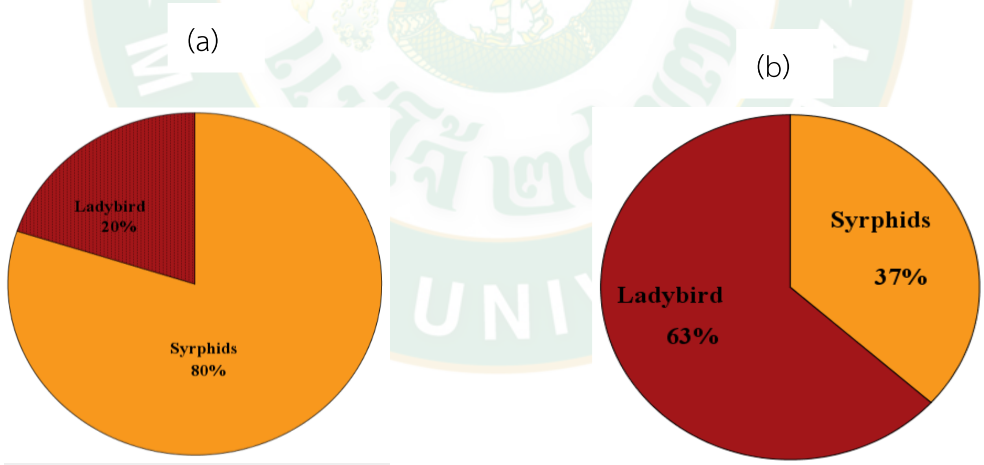

Figure 10 Proportion of natural enemies in two orchards (a) Hongtsho-2 and (b) Kausumche-3, compared over growing season (April-October, 2018)

Numerically, there was great difference in ladybird counts during the monitoring period. At the same time, proportion of natural enemies abundance in the orchards greatly differed comprising 20% ladybirds and 80% syrphids in Hongtsho-2 comparing 63% ladybirds and 37% syrphids in Kausumche-3 respectively (Fig.10). Their abundance and activity was recorded higher between June to September which also coincide with the occurrence of higher aphid counts during the apple season. Thus, these natural enemies can be considered for managing WA in the apple orchards.

In general, abundance of natural enemies remained higher between June-September (Fig.9.) which also coincide with higher densities of WA which has potential for considering biological control agent. Conversely, intensive use of chemicals in previous season might have disrupted abundance and diversity of natural enemies. As Beltrán *et al.* (2017) reported the occurrence of predators declined under broad-spectrum insecticide program and parasitism evidence was lacking. Likewise, Ateyyat and Al-Antary (2010) reported weak parasitism under heavy spray of pesticides as parasitoid population are inhibited. However, more research is suggested to obtain accurate information and enhance management of natural enemy guild such as management of predators and natural enemies.

4.3 Woolly aphid parasitism by *Aphelinus mali*

Parasitism by potential parasitoid, *Aphelinus mali* (Hymenoptera:Aphelinidae) was assessed for WA population suppression. A popular endo-parasitoid, *A. mali* is being deployed in the field of Integrated Pest Management (IPM) across the globe for managing WA. Currently, farmers are trying hard to manage woolly aphid with chemical spray. Due to dense white filaments covering and characteristics of WA colonies, chemical control has been reported inadequate. Moreover, chemical application has detrimental effects on natural enemies, environment and human health, thus demanding research quest to biological control technique with use of predators and parasitoids (Quarrell *et al.,* 2017).

This survey confirmed the presence of parasitoid *A. mali* in the apple orchards of Thimphu. It is widely adopted as principal agent in biological control of WA in apples. Ensuing the presence of wasp parasitoid, *A.mali* in the apple orchards of Thimphu valley, the level of WA parasitism information will underscore in considering conservative biocontrol measures.

Initial evidence of parasitism (black mummies) by *A. mali* was examined and recorded from May 2018 in both the orchards although WA colonies are already established from month of April. From the observations, it was derived that lack of natural enemies in the early season when overwinter aphid emerged in the orchards. Thus, aphid colonies soon established on apple trees affecting normal growth and development in the season. Stokwe and Malan (2016) also reported due to arrival of *A. mali* wasp in the late season, WA control was not efficient.

As illustrated in Figure 9b, in orchard Kausumche-3, parasitism abundance gradually dwindled from following monitoring in May however, regained parasitism level till attaining its peak of densities (586 numbers) in mid-August 2018. Parasitism remain higher during the month of June-October. Nevertheless, parasitism slowly diminished from following survey and remained more or less constant till end of sampling period in October 2018. Similarly, parasitism level in orchard Hongtsho-2, gradually accelerated up and recorded peak mummies count (333 numbers) in June. Disappointingly, parasitism (mummies) counts relatively declined from August and, thereafter never observed sign of parasitism (Fig.9b). Parasitism discontinued from September 2018 in orchard Hongtsho-2, although aphid infestations remained rampant in the orchard till end of monitoring survey.

The attributed reason for crashed of parasitism level was presumably numerous factors ranging from orchard management to environmental variations. The parasitism level compared in the two orchards at different geographical location exhibited extreme variations (Fig.9). CABI (2018) also reported having varying degree of WA management success depending on geographical regions. Ateyyat and Al-Antary (2010) mentioned parasitism of *A. mali* could be influenced by chemical orchard spray impeding parasitoid abundance. Although, chemical application was avoided during the study period but due to spray in the previous season, might have impacted the population of parasitoid and natural enemies.

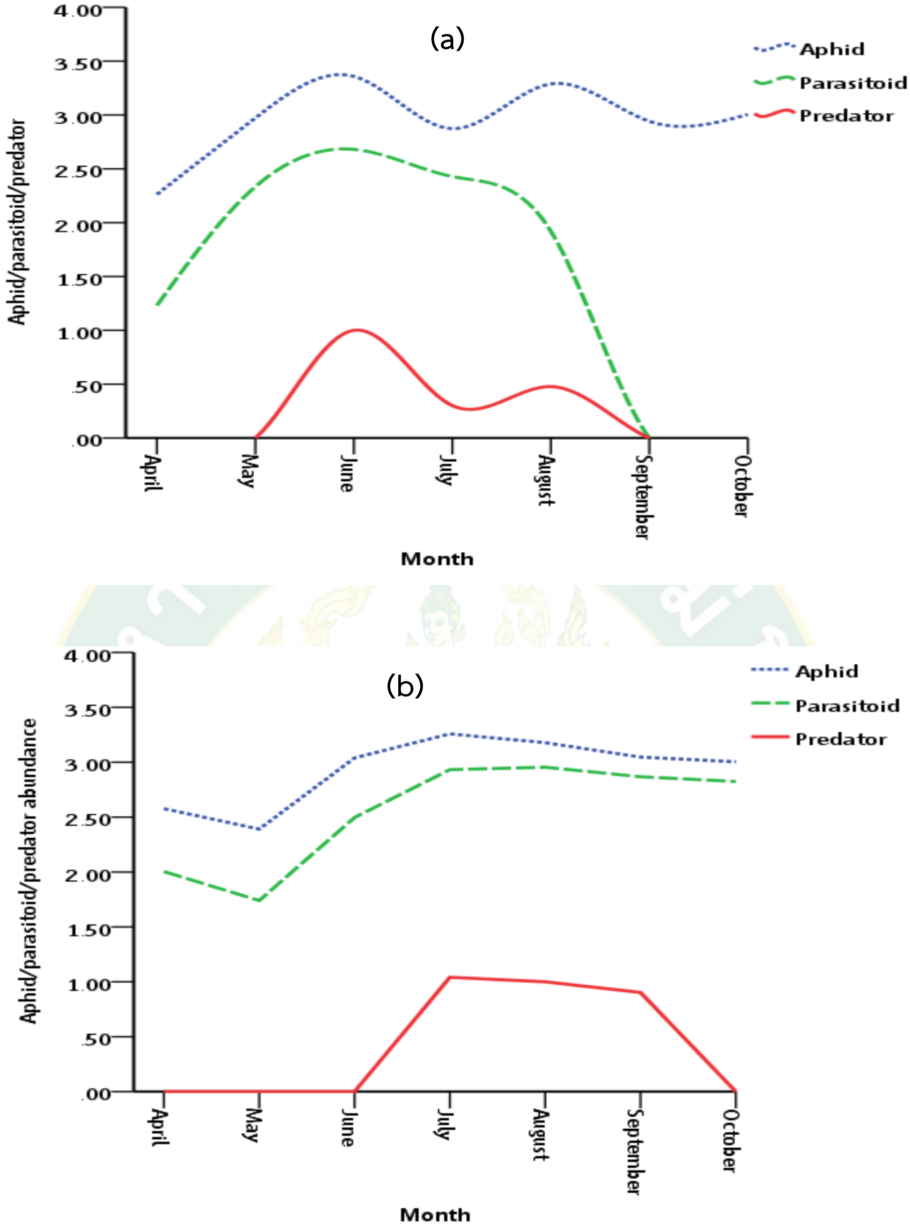

Figure 11 Mean monthly abundance of aphid, parasitoid and predators compared a) Hongtsho-2, and b) Kausumche-3, over apple season in two orchards

Moreover, due to heavy aphid infestation and colonization, parasitoid attack may be less effective as it deterred parasitoid to penetrate core of colonies thereby reducing parasitism (Bergh and Stallings, 2016). Unfortunately, factors involving abiotic and environmental influence was not considered in the investigation requiring in-depth studies to substantiate the postulation. It is known from the previous research that parasitoid *A. mali* are less efficient in controlling WA in cool climatic conditions (Wearing *et al.,* 2010).

In comparison, parasitism persisted in orchard Kausumche-3 from mid-April to October throughout study period, whereas parasitism terminated in mid-season in orchard Hongtsho-2. Therefore, it was viewed vital to explore alternatives biological control agents to manage the level of WA in the beginning of season before formation of colonies and at end of the season when parasitism by *A. mali* terminates. As discussed earlier, *A. mali* occur in the late season. Despite variations in the time of parasitism occurrence, mean parasitism was also different in the two orchards. Analyzed for statistical significance by an independent sample t-test. The results of Hongtsho-2 (M= .85, SD= .31) parasitism level significantly differed in Kausumche-3 (M=1.10, SD= .34 over the season, t= 6.5750, P= 0.0001. There was extreme statistical significant difference in the abundance of parasitism examined in two orchards.

At the same time, parasitism percentage in the two orchards showed comparatively different level of aphid suppression. General observation convinced that parasitism percentage was approximately 12 % in Hongtsho-2 and 34 % in orchard Kausumche-3 as presented in figure 10. The aphid parasitism was relatively higher in Kausumche-3 than in Hongtsho-2. The lower percentage of parasitoid abundance in orchard Hongtsho-2 could be due to low temperature which discouraged development of parasitoid.

In addition, there was heavy infestation colonies that deterred natural enemies to penetrate the core of colonies. Environmental factors and geographical difference could be contributing to such pattern as precipitation, climate and temperature are thought to influence WA colonies in apple (Beltrán *et al.,* 2017).

Ateyyat and Al-Antary (2010) suggested more rearing and release of parasitoids for effective control of WA. Zhou *et al.* (2014) also described techniques of maintaining overwinter aphid mummies with the use of hay mulch to have adequate parasitoid in early season. Such methods would be feasible in the orchards of Thimphu valley which require further exploration and evaluation studies.

(a) (b)

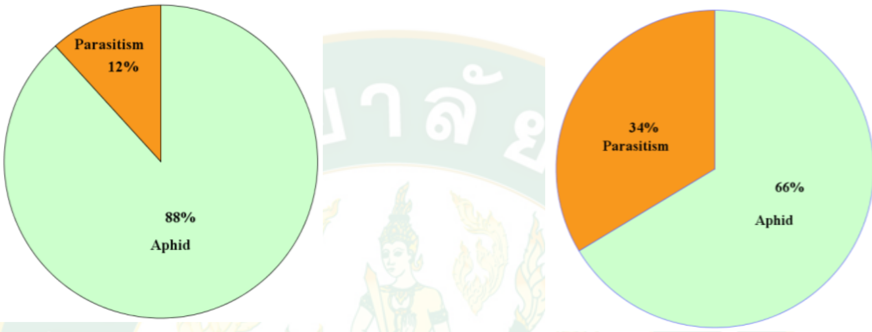

Figure 12 Parasitism percentage in two orchards (a) Hongtsho-2 and (b) Kausumche-3, compared over growing season

Therefore, research and tests for an alternative techniques apart from *A. mali* is worthwhile adopting resistant varieties as Abu-romman and Ateyyat (2014) cited it as potential technique for WA control. Wearing *et al.* (2010) recommended use of resistant varieties like M.793 and MM.106 against WA infestation in New Zealand which desrved to be tested. Ateyyat and Al-Antary (2010) discussed Golden Delicious are more resistant than Fuji and it is agreed WA infestation has drastic variations between apple cultivars (Nicholas *et al.,* 2005a). Besides, Stokwe and Malan (2016) mentioned the use of entomopathogenic nematodes (EPN) and entomopathogenic fungi (EPF) for WA management although reported varrying degree of efficiency.

Chapter 5
Conclusion

Woolly aphid commenced its colonies formation from mid-April although aphids are speculated to emerge much earlier (March) before colonization. The highest count of aphid density was recorded between June-August in orchards of Thimphu valley. Survey displayed variations in aphid abundance with single peak in Kasumche-3 and double peaks in Hongtsho-2. Cumulative average of WA population compared in two orchards illustrated significant difference. Both canopy and root colonies are rampant in the orchards during study period.

The natural endo-parasitoid, *A. mali* deployed in managing woolly aphid, *E. lanigerum* worldwide was recorded in orchards of Thimphu valley which was not reported earlier. Besides, six other predators associated with WA revealed consisting two coccinellids, two syrphid flies and a green lacewings. Relatively, the abundance of predators and natural enemies were higher during the monitoring survey of June-August when aphid infestation are also higher in the orchards. These predators could be useful in biological control program to manage WA in complement with parasitoid in apple orchards of Thimphu and avert use of insecticides.

Simultaneously, parasitism by parasitoid, *Aphelinus mali*, a popular biological control agent of WA was assessed while monitoring WA population. Parasitism was evident only from end of April when aphid colonies are already established on apple trees. Unfortunately, parasitism crashed in orchard Hongtsho-2 in the mid-season when WA infestations are rampant even after harvest. More studies on aspects of WA temporal changes and climatic effects are suggested to clearly understand the population intensities for effective interventions. In addition, it is worthwhile to explore alternative natural enemies to maintain aphid level in the beginning of growing season before the formation of colonies and at end of the season when parasitism by *A. mali* terminates.

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APPENDIX

Aphid Sampling Form **Annexure -1**

Grower: **Date of survey :**

Field location : **Comments**

	Stem or shoot	**Aphid per stem**	**Mummies/ Infected aphid**	**Ladybeetle larvae**	**Syrphid larvae**	**Adult Ladybeetle per Sweep**
Sample 1	1					
	2					
	3					
	4					
Sample 2	1					
	2					
	3					
	4					
Sample 3	1					
	2					
	3					
	4					
Sample 4	1					
	2					
	3					
	4					
Sample 5	1					
	2					
	3					
	4					
Total						
Average per stem						

N= North **E=East** **W= West** **S=South**

Annexure II. Cummulative data record form for woolly aphid and natural enemy

Week	Aphid (Hongtsho-2)	Aphd (Kausumche-3)	Parasitoid (Hongtsho-2)	Parasitoid (Kausumche-3)	Syrphid (Hongtsho-2)	Syrphid (Kausumche-3)	Ladybird beetle (Hongtsho-2)	Ladybird beetle (Kausumche-3)
week 2								
week 4								
week 6								
week 8								
week 10								
week 12								
week 14								
week 16								
week 18								
week 20								
week 22								
week 24								
Week 26								
Week 28								
Sum								

CURRICULUM VITAE

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DATE OF BIRTH	29 September 1974
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